Can Neutral Liposomes be Considered as Genetic Material Carriers for Human Gene Therapy?

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Abstract: Among the synthetic vectors exploitable in Human Gene Therapy (HGT), complexes of DNA with cationic liposomes (lipoplexes) are considered the best candidates. Some degree of cytotoxicity and a limited stability on serum are still limiting factors for applications *in vivo*. On the contrary the corresponding complexes of neutral liposomes are enough stable and non toxic. This review has the aim to offer a survey of existing data, namely structural properties, on neutral liposomes from the point of view of HGT; and to compare, whenever possible, the different features of the two classes of compounds. The outlook is that in near future neutral liposomes could behave as efficient and safe vectors of genetic material.

Keywords: Gene Therapy, DNA, Liposomes, Complexes, Metal ions, *X*-ray.

I. INTRODUCTION

Human Gene Therapy (HGT) is considered as the medicine of the future and may be defined as a treatment of diseases by transfer of genetic material into a specific cell of a patient [1]. The discovery that many diseases originate at the molecular level and the completion of the Human Genome Project have opened the way to the treatment of genetic diseases through replacement of defective or absent genes. However, gene therapy methods are also developing for treatment of cancer and many other diseases (cardiovascular, infectious, neurological and ocular). Depending on researchers' interest, HGT has been described as molecular surgery [2], molecular medicine [3] or nanomedicine [4]. HGT represents a therapeutic methodology with enormous potentialities and, since its appearance, has induced strong expectations of a rapid solution of many health problems both in the professional and the lay communities. Yet, very soon the cellular transfection turned out to be a complex process, involving many different steps, which has slowed down the development of efficient and safe clinical applications. In particular it was clear that, to solve the different aspects characterizing this medical technique, it was necessary to integrate medical expertise with additional skills: namely, biology, biochemistry, physics, chemistry, material science and genetic engineering. Roughly, the different steps of HGT include preparation of the carrier (viral or synthetic), its association with the therapeutic gene, and introduction of the resulting system into a definite tissue or in the blood stream. The gene-carrier system must then find the target tissues, enter the target cells and, after entering, traffic through the cytoplasm to reach and enter the nucleus. Here it will be transcribed to originate the therapeutic protein which, in turn, must act on its receptor to induce an appropriate therapeutic effect. It follows that successful HGT asks for suitable genes, appropriate delivery systems of simple and cheap manufacturing, efficacy and safety of the transfection process.

The question whether the transport of the genetic material and the infection of cells must be carried out by viruses or synthetic vectors is still unsolved. Most recent data on clinical trials worldwide* are in favour of viruses (roughly 66% *vs.* 7%) if one considers the whole period since the first trial approved in 1989 up to 2009. The high transfection efficiency *in vivo* is in favour of viruses, but there are even some significant limitations such as mutagenesis, carcinogenesis, immune response and the need of expensive techniques of genetic engineering to treat viruses. Many potential advantages, including non-immunogenicity and non-oncogenicity, lower toxicity, greater nucleic acid packaging capacity, easier and cheaper preparation, are instead in favour of synthetic vectors. Literature is rich in important developments in both areas, even if in current opinion synthetic carriers are considered as the future of a safe and efficient HGT [5]. Among the many synthetic carriers described so far, leading importance has been gained by cationic liposomes (CLs) which interact through their positive charge with the negative charged deoxyribonucleic acid (DNA), forming positively charged supramolecular structures where DNA is condensed. Such complexes (lipoplexes) have been found to be safe and rather effective in the *in vitro* experiments [6], but of limited efficiency in the *in vivo* applications: an overview of the relationships between physicochemical properties of lipoplexes and their way of interaction with cells has been recently published [7]. As the authors say "despite a lot of efforts, the precise mechanism of gene transfer with cationic vectors is still ill-defined". This review starts with a short discussion on the merits and limits of CLs as gene carriers, expecting that the huge amount of data collected in the last twenty years will also help in designing optimized neutral liposomes (NLs) for HGT. Structural and physicochemical properties of NLs, together with some application attempts, will then be described, with the aim of emphasizing their ability to play as synthetic carriers for the transport and internalization of DNA into cells and tissues.

II. CATIONIC LIPOSOMES AS GENE CARRIERS

The interest in CLs as gene transfer agents began with the pioneering work of Felgner and co-workers [8] who synthesized the first cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA). This compound was added to the neutral 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in water solution to give a liposome able to bind with DNA and form a stable complex which transfected DNA into some cultured cells, namely COS-7 (a derivative of the simian kidney cell line CV1) and TA1 (derived from the murine fibroblast cell line 10T½). As the authors noted "the technique is simple, highly reproducible and effective for both transient and stable expression of transfected DNA". The presence, as a helper of transfection, of the neutral liposome DOPE, characterized by an inverted hexagonal phase structure, was essential to obtain good efficiency, owing to its high fusogenic property [9] and to the effect of destabilization of the endosomal membrane [10] that favors the release of DNA into the cytosol. A second work by the

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* The Journal of Gene Medicine: Gene Therapy Clinical Trials Worldwide (*2009 edition*).

1570-193X/11 $58.00+.00

same author described new CLs, showing enhanced transfection properties [11]. After such a successful discovery, CLs have been the object of intensive investigation aiming at optimizing their performances and better understanding the way they work: an impressive number of new molecules and formulations have been designed and synthesized, in a continuous search for better overcoming the critical barriers encountered in gene delivery. Some recent reviews can help to orientate the reader in so great a bulk of information [12-14].

As already mentioned, the transfection efficiency of CLs is lowered by a certain degree of inherent cytotoxicity [15, 16], that causes some negative effects on cells, including shrinking and inhibition of the protein kinase C (PKC). Various strategies have been proposed to overcome the problem [17]. Besides, efficiency is negatively affected by a low stability on serum of lipoplexes [18], which compromises the *in vivo* experiments [19]. Recently, a comparison [20] of ten different commercial lipoplexes, while confirming the negative effect of toxicity, which increases with the increase of the lipid/DNA molar charge ratio, has confirmed that the transfection efficiency depends also on the lipid composition and the lipoplex size, as previously found [21, 22]. The structure of each cationic lipid is an assembly of three different moieties, namely a polar hydrophilic head and a hydrocarbon hydrophobic tail, bound together by a linker that can be made of some different functional groups (ether, ester, amide, carbammate, etc.). All three components take part in determining the whole toxicity. The hydrophilic head is responsible for the highest contribution: toxicity induced by a quaternary nitrogen is ten times the one induced by a ternary one [23]. This result is confirmed by the finding that a ternary amino head group is 4 to 20-fold less inhibitory of PKC than a quaternary one and that the highest transfection activity occurs with cationic derivatives of cholesterol with the lowest PKC inhibitory activity [24]. Results that agree with the considerable reduction of toxicity obtained when the positive charge is spread on a heterocyclic ring [25-27]. In this context it is important to point out that the absence of the positive charge in neutral liposomes entitles to foresee some kind of advantage over the corresponding cationic liposomes in the transfection procedures. The residual contribution to lipid toxicity is given by both the hydrophobic tails and the linkers. As for the first, conflicting results have been described: the single tailed cetyltrimethylammonium bromide was found to be more toxic and less efficient than the double tailed DOTMA [28], but in a different molecular context, the single tailed oleoyl ornithinate (OLON) is less toxic than the double tailed 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [29]. Finally, it has been observed that hydrolysable ester linkers reduce the toxicity of the corresponding CLs [30] and enhance their transfection efficiency and that better effects are obtained when the hydrophobic tails are linked to the cationic head by means of a carbammate linker [31, 32].

In addition to the cited obstacles, some others have been proved to affect the *in vivo* transfection via CLs vectors, beginning with the administration route [33]: in the intravenous gene delivery, cholesterol [34, 35] generally improves the transfection, while DOPE has sometimes the opposite effect [35]. Barriers of extracellular origin are caused by components of serum which can interfere with the travelling lipoplexes [36]: low density lipoproteins and globulins or negatively charged compounds as albumin destabilize the positively charged lipoplexes and some low molecular weight lipids present in serum can replace the original lipid. The consequence of these barriers is to destroy a certain amount of lipoplex, so only a fraction of it can reach the target cells. Such fraction is then exposed to intracellular obstacles acting at the different steps of the transfection process: fusion of the lipoplex with the cellular membrane, internalization by endocytosis, separation of the gene from the carrier to allow DNA enter the nucleus [37, 38]. The structure of the liposome itself can negatively affect the process: that happens when the liposome has limited loading capacity and/or low reproducibility of the formulation. Finally, the eventuality that single particles of a charged lipoplex aggregate one another giving rise to larger liposomes particles, unsuitable for transfection, must be considered [39]. All these barriers are responsible for the difficulty to realize standard delivery processes. Taking these limits of the CLs into account, a critical survey of the literature on NLs, examined under the perspective of the various features potentially useful in HGT, is presented in the next section. Two important elements however account *a priori* for studying NLs as DNA carriers: low inherent cytotoxicity [40, 41] and longer circulation lifetimes in serum with better clearance profiles [42].

III. NEUTRAL LIPOSOMES

Pioneering works on NLs go back to the end of the seventies and beginning of the eighties and aimed at understanding the role of Ca^{2+} and Mg^{2+} in inducing vesicles fusion [43, 44] and finding a reasonable mechanism for it [45-48]. On the basis of the results achieved some attempts to encapsulate and deliver genetic material were performed: poliovirus RNA [49] and Simian virus DNA [50, 51] were encapsulated with good yields in large unilamellar vesicles (LUV) of phosphatydilserine (PS) and delivered to cells. A few significant papers followed these preliminary studies: a plasmid DNA was encapsulated with high efficiency in a neutral lipid made of egg phosphatidylcholine (PC) and cholesterol [52]; lecithin liposomes [53] were used to deliver a model oligodeoxyribonucleotide (F-DNA) into nuclei of cultured human keratinocytes. In the meantime, strategies to design liposomes that could circulate in serum for many hours before removal by the reticuloendothelial system or phagocytosis by macrophages were suggested: in the former case polyethylene glycol-coated liposomes (PEG) were experimented successfully [54-56], in the latter it was found that gangliosides and sphingomielyn act synergistically to reduce the rate and extent of uptake of liposomes by macrophages [57] and that an analogous effect is obtained using phosphatidylinositol [58, 59]. Furthermore, the problem of the low unloading efficiency of internalized liposomes started being considered: the idea was to convert a highly stable circulating liposome into an unstable liposome once entering the endosome, by exploiting the decrease of pH within this compartment [60, 61]. The biophysical mechanism of action of pH sensitive liposomes involves a transition of the liposome from the lamellar to the more fusogenic hexagonal phase [62]. In an experiment, folic acid was used [63] to prepare folate targeted liposomes that were able to release 20-25% of their load in tumour cells. Interesting works were published in the same years, based on different techniques, dealing with: (i) the measurement of forces acting between PC and phosphatidylethanolamine (PE) bilayers in aqueous electrolyte solutions [64]; (ii) the differential scanning calorimetric determination [65] of structural transitions operated on lecithin by DNA in aqueous solution of Ca^{2+} ions; (iii) the analysis of the structural organization and the phase behavior [66] of the DNA-Ca^{2+}-DPPC complex, studied by means of freeze-fracture technique. While studies on NLs as helpers of cationic liposomes in transfection experiments [67] were going on, a new interest in their use as autonomous carriers of genetic material arose. The use of complexes of DNA and NLs in the presence of metal ions as vectors of transfection *in vitro* and *in vivo* was tested [68, 69] and a new method for entrapping plasmid DNA (pDNA) into NLs, by simply adding $CaCl_2$ and ethanol to aqueous mixtures of the corresponding lipids and pDNA lead to higher efficiency than previously known procedures. Encapsulations of pDNA in DOPC, DOPC-DOPE, DOPC-DOPE-cholesterol [70] and DPPC [71] were realized and targeted transfections performed.

The structure of the complexes of NLs with DNA started being considered: it was demonstrated that neutral (zwitterionic) liposomes condense with polynucleotides in aqueous solutions of divalent metal cations through a self assembly process which leads

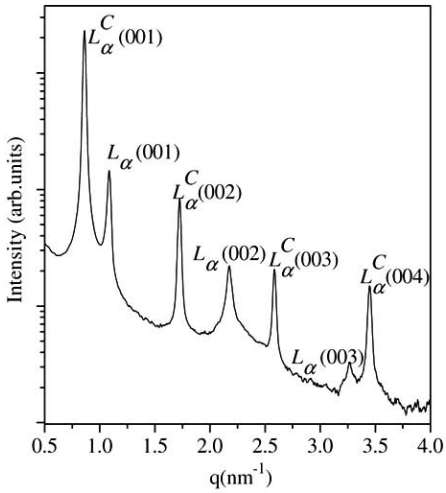

Fig. (1). Synchrotron SAXS pattern of DOPC-DNA-Mn^{2+} complex at molar ratio 3:4:12.

Table 1. The Lamellar Repeat Distance of the L^c_α Complexed Phase (d_1) and of the L_α Uncomplexed Phase (d_2) as a Function of Metal Concentration

(Reprinted from *Phys. Rev. E*, **2003**, *67*, 11904-6, http://pre.aps.org)

DOPC	DNA	Mn^{2+}	d_1(nm)	d_2 (nm)
3	4	1		6.38
3	4	4	7.33	6.33
3	4	6	7.34	6.24
3	4	12	7.34	6.04
3	4	18	7.35	6.00
3	4	24	7.31	5.88

to stable ternary complex NLs-DNA-M^{2+}. DOPC [72] was converted in the corresponding liposome and mixed with calf thymus DNA in hepes buffered aqueous solutions of different divalent cations (Mn^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}). Simultaneous small (SAXS) and wide angle *X*-ray scattering (WAXS) measurements were carried out on the ternary complexes obtained, using different mole ratios of the components: in a typical experiment on DOPC-DNA-Mn^{2+} the mole ratio was 3:4:12 and the corresponding synchrotron *X*-ray diffraction (XRD) pattern (Fig. **1**) at 298 K is reported. The spectrum is characterized by two intense sharp peaks at d_1 = 7.34 nm and d_2 = 6.04 nm*:* similar peaks were observed in all the samples investigated, which ranged from 3:4.1 to 3:4:24 molar ratios of the three components (Table **1**). The most significant XRD data reported as a function of the relative concentration of Mn^{2+} at a fixed DOPC/DNA ratio show a constancy of the d_1 spacing and an appreciable continuous decrease of d_2 from 6.38 to 5.88 nm, the latter value being very close to the one measured in a DOPC/Mn^{2+} 3:24 mixture (d = 5.84 nm). Both series of data reveal the liquid-crystalline nature of lamellar phases [73]: the d_1 spacing was associated with the L^c_α phase of the ternary complex DOPC-DNA-Mn^{2+}, the d_2 with the L_α phase of DOPC-Mn^{2+} following a commonly accepted nomenclature [74, 75]. The first structure is similar to that found in CLs-DNA complexes [76, 77] made of stacks of alternating lipid bilayers and DNA monolayers. A schematic picture of the L^c_α phase of the ternary complex DOPC-DNA-Mn^{2+} was proposed (Fig. **2a**): the DNA strands are sandwiched between the lipid bilayers and bound together through the hydrated metal ions. The simultaneous presence of two lamellar structures, confirmed by an analogous XRD study [78], was interpreted by plotting (Fig. **3**) the integrated intensities of the first order peaks of the ternary complex and of the uncomplexed DOPC against the ratio of the metal ion concentration versus the one of the DNA phosphate groups. An increase of the Mn^{2+} concentration favours the formation of the ternary complex with a complementary reduction of the DOPC-Mn^{2+}: the saturation is reached at a ratio $[Mn^{2+}] / [PO_4^{2-}] \cong 6$, corresponding to a constant volume fraction of the two structures (~ 70% to ~ 30% respectively). The formation of the ternary complex is promoted by the positively charged metal ions that bind the polar heads of DOPC with the negatively charged phosphate of DNA. This suggests that NLs behave like cationic lipids in the presence of metal ions and that, in the case of neutrals, the driving force for the complexation is caused by the release of the counterions.

A similar study was made using DPPC as a neutral liposome [79, 80]: the complex DPPC-DNA-Ca^{2+} was prepared either from multilamellar (MLV) or unilamellar (ULV) vesicles of DPPC. In both cases two distinct lamellar phases were observed, the one cor-

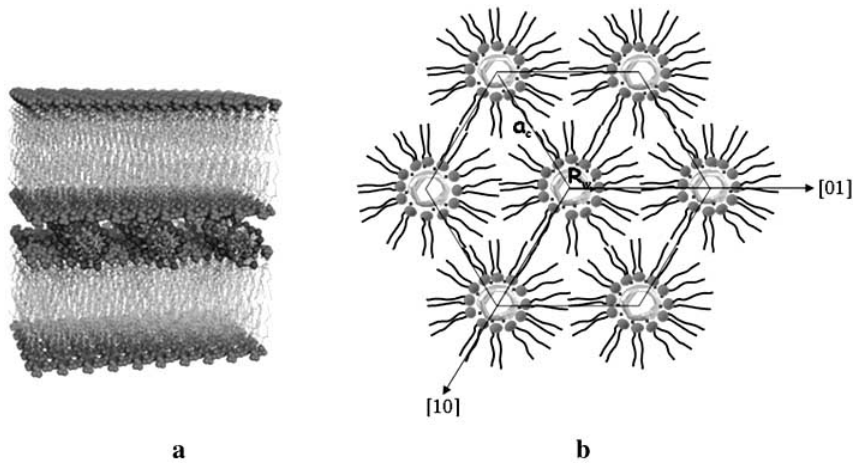

Fig. (2). **a**: proposed model for $L_\alpha{}^c$ phase of the ternary complex DOPC-DNA-Mn^{2+}; **b**: schematic picture of structure of the H^c_{II} phase of DOPE-DNA-M^{2+} complexes. The DNA strands are collocated in the inner of the structural elements.- (Reprinted from *Europhys. Lett.*, **2004**, *67*, 671, with permission of the editor, www.epljournal.org).

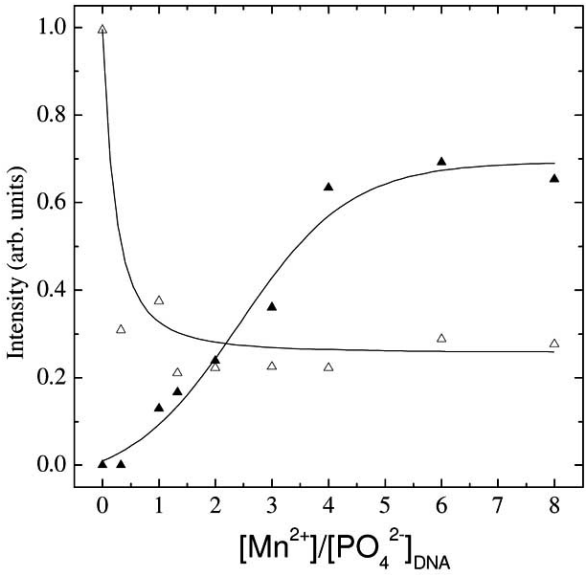

Fig. (3). The integrated intensities of the first order diffraction peaks of the complexed (▲) and the uncomplexed DOPC liposome (Δ), as a function of molar ratio metal cation/DNA. (Reprinted from *Phys. Rev. E*, **2003**, *67*, 11904-8, http://pre.aps.org)

responding to DPPC bound to DNA, the other to the unbound DPPC. Both lamellar phases are present in the precipitate fraction of the sample prepared with MLVs, while only the complexed phase is present in the precipitate of the sample prepared with ULVs, the uncomplexed lipid being in the supernatant. As for DOPC, the complex has a lamellar structure where DNA layers are embedded in the DPPC layers. The two lamellar phases are present in both the gel (298K) and the liquid crystalline (328 K) thermotropic phases: a value of d = 7.84 nm has been observed for the ternary complex. The difference from the value found in the ternary complex with DOPC (7.34 nm) was cleared up by considering that the presence of a double bond on both hydrocarbon chains in DOPC induces some rigidity of the tails. This rigidity is absent in the fully saturated chains of DPPC, which gives rise to a higher freedom in the membrane fluctuation of the latter. It must also be noticed that the lamellar repeat distance in complexes with MLVs at 298 K increases slightly from 7.85 to 7.88 nm with Ca^{2+} concentration moving from 1 to 4 mM. Surprisingly it drops to a minimum of 7.86 nm when Ca^{2+} concentration is equal to 5 mM and slowly rises back at increasing values of Ca^{2+} concentrations. The experimental evidence suggests that with 5 mM concentration of Ca^{2+} a special compact structural arrangement is obtained, indicative of increased order. To strengthen such interpretation the authors observed that the 5 mM concentration is the only one where a DNA-DNA in plane correlation is present on the *X*-ray spectrum. Starting from these data and from the number of estimated lipid molecules per DNA phosphate in the complex, already determined as 4.5-5 [71], and considering that Ca^{2+} ions bind preferably to complexed than to uncomplexed DPPC molecules [81], the authors concluded that the value of 5 mM of Ca^{2+} roughly corresponds to one $CaCl_2$ for every two DPPC molecules. On the basis of these results a model was proposed where every Ca^{2+} bridges two adjacent DPPC molecules, making the net positive charge on the trimethylammonium portions of the two DPPC molecules bind with the phosphates of DNA. A different interpretation was previously suggested [82] while studying the interactions of bivalent metal cations with DNA (and double-stranded polynucleotides) and ULV of PC. The results of the Scatchard plots of PC-DNA-Mn^{2+} and DNA-Mn^{2+} complexes, together with data of elemental analysis, lead to propose an arrangement where each Mn^{2+} bridges two DNA phosphates with three PC molecules.

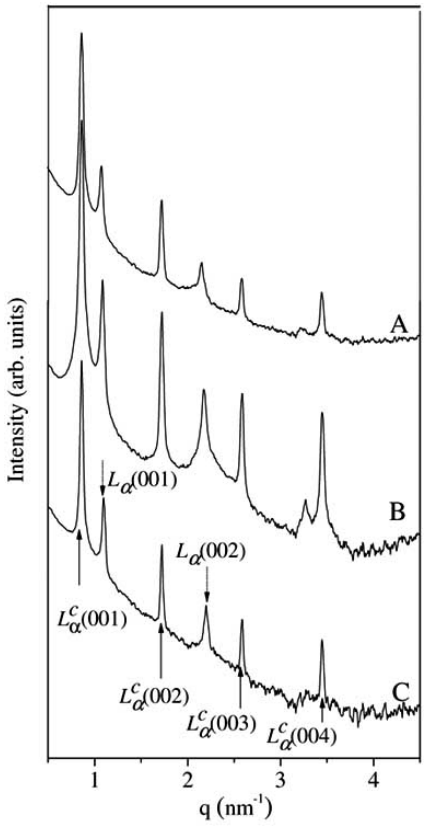

Fig. (4). Synchrotron SAXS pattern of DOPC-DNA-Mn^{2+} at different metal concentration *vs* fixed DOPC/DNA molar ratio (3:4). A) 3:4:6; B) 3:4:12; C) 3:4:18.

After the publication of the structural works on DOPC and DPPC an extended systematic approach to NLs based complexes has been undertaken and some important insights on their structure and morphology obtained. Ternary complexes NLs-DNA-M^{2+} (M = Ca, Mg, Mn, Fe) were prepared, using liposomes bearing unsaturated (DOPC, DLPC and DOPE) or saturated (DPPC) hydrocarbon tails. Microscopic structures of lipids and their corresponding ternary complexes were investigated, having in mind a twofold goal: to test (i) whether different metal cations are equally active in promoting the DNA condensation into the ternary complexes with different lipids and (ii) to what extent structure and phase symmetry of the original lipids affect the structure of the self-assembled complexes. These aspects are of great general interest, not only for fundamental implications, but mainly in view of an approach to gene delivery based on a correct knowledge of the relationships between structure and transfection efficiency. Complexes of calf thymus DNA with DOPC [83, 84], DLPC [84], and DPPC [85] exhibit the already discussed multilamellar liquid-crystalline L_α^c phase, consisting of ordered assemblies where hydrated DNA helices are sandwiched between the lipid bilayers, and the metal cations mediate the binding of the phosphate groups of DNA with the lipid polar heads. In all cases the L_α^c phase coexists with the uncomplexed L_α phase of the parent lipid. SAXS patterns of the system DOPC-DNA-Mn^{2+} at different Mn^{2+} concentrations (Fig. **4**) confirm the presence of two sets of peaks (each one including the fundamental and higher-order harmonics). Layer spacings, $d_1 \sim 7.52$ nm and $d_2 \sim 5.9$ nm respectively for the complex and the uncomplexed pure lipid, were found: these values are substantially analogous to those determined in slightly different experimental conditions [72]. Similar values ($d_1 \sim 7.06$ and $d_2 \sim 5.86$) were found for the DLPC-DNA-

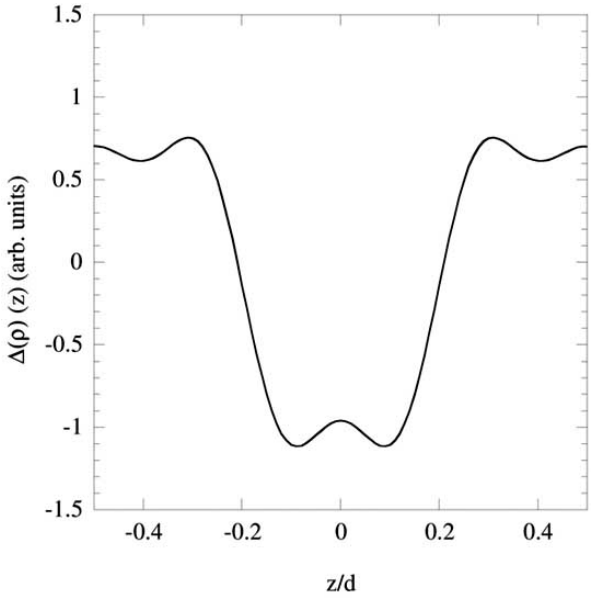

Fig. (5). Electron-density profile along the normal to the bilayer in the $L_\alpha{}^c$ phase of the triple complex DOPC-DNA-Mn^{2+} (3: 4:12).

M^{2+} complexes and for DLPC-M^{2+}. The electron density profile (Fig. **5**) along the normal to the bilayers in the L_α^c phase of the complex DOPC-DNA-Mn^{2+} shows two density maxima corresponding to phospholipid headgroups and a minimum in the middle corresponding to the terminal hydrocarbon chain. The distance between the centres of the density maxima gives the phosphate-phosphate group separation d_{PP} ~ 4.51 nm and allow to calculate the water layer thickness $d_W = d_1 - d_{PP}$ ~ 3.01 nm. This value is sufficient to accommodate double stranded DNA helices surrounded by one hydration layer, plus two thin layers of hydrated metal ions: these results confirm the structure previously reported (Fig. **2**). Likewise, in the complex with DLPC were calculated water layer thicknesses d_W in the range of 2.8-3.0 nm, depending on metal cation. Analogous results were found [85] in the ternary complexes of DPPC and DNA with Ca^{2+}, Mg^{2+} and Mn^{2+}: layer spacings of the L_α^c phases (d_1 ~ 7.9 nm for $Ca^{2+}$and Mg^{2+} and 8 nm for Mn^{2+}) are in a range similar to the ones found in complexes with DOPC and DLPC. The SAXS pattern of the DPPC-DNA-Ca^{2+} (Fig. **6**) shows a correlation peak, marked as DNA in the figure, corresponding to the DNA-DNA interaction already reported [79], and indicative of a higher organization of the DNA chains between the liposome layers. In this case the thermotropic phase behaviors of the complexes in a temperature range between 30 an 55 °C, well above the main transition temperature of the pure lipid (T_m = 41.3 °C) were studied: the results show the situation for the complexes with Ca^{2+} (Fig. **7**), quite similar to the one with Mg^{2+}, and Mn^{2+} (Fig. **8**). An important conclusion arises from the analysis of such patterns: coexistence of complexed and uncomplexed phases persists over the whole explored thermal range but, as temperature increases, the relative concentrations of the lamellar phases of pure lipids change in favor of one of the ternary complex and such effect is higher in case of Mn^{2+}. An increase of temperature was found to increase the rate of complex formation in the fluid L_α phase [86] and this effect is amplified when temperature produces a transition from the gel to the fluid phases (between 45 and 50 °C in this case).

The change of the basic geometrical shape of the lipid molecule from cylindrical (DOPC, DLPC, DPPC) to wedge-like (DOPE) induces a structural transformation of the corresponding complexes**.** The equilibrium phase of pure DOPE in excess water consists of an

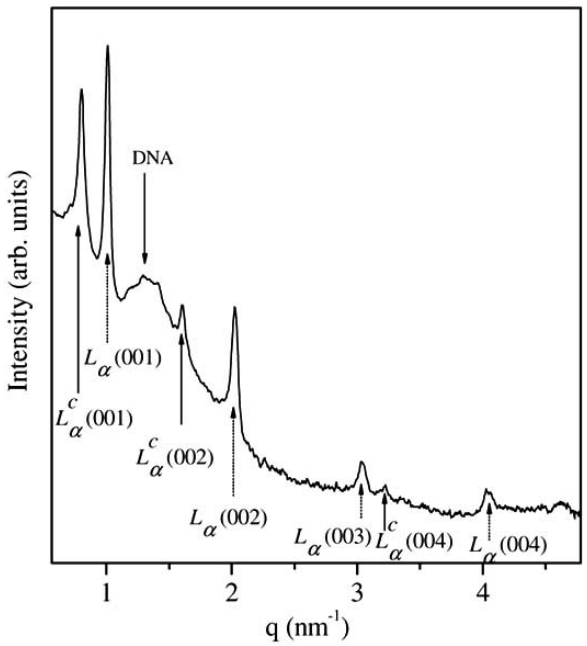

Fig. (6). SAXS pattern of DPPC-DNA-Ca^{2+} complex at molar ratio 3:4:24.

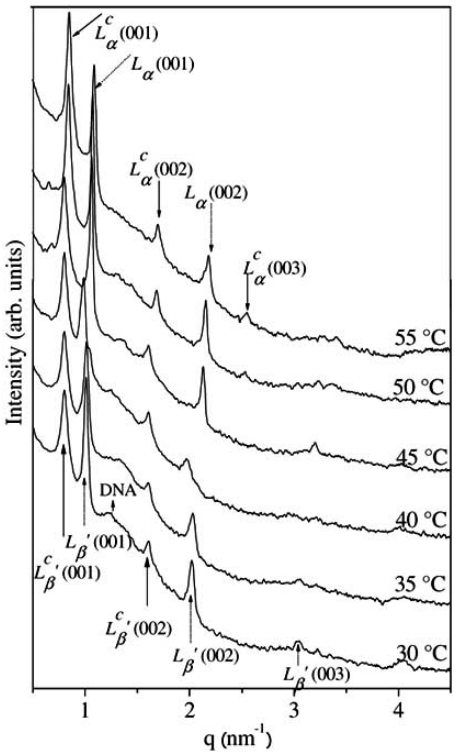

Fig. (7). Synchrotron XRD patterns of the DPPC-DNA-Ca^{2+} complex at 3:4:24 molar ratio as a function of temperature.

inverted hexagonal H_{II} lattice [87], whose structure elements are infinitely long rigid rods, all identical and cristallographically equivalent. They are made of cylinders filled with water and dispersed in the continuous medium of the hydrocarbon chains, whereas the polar groups are located at the water-hydrocarbon chain interface. A unit cell spacing a = 7.44 nm, for DOPE in excess water was calculated from SAXS data [88] (Fig. **9**). The electron density profile (Fig. **10**) along the *[10]* direction, allowed to calculate a steric lipid bilayer thickness d_L = 4.42 nm and hence an

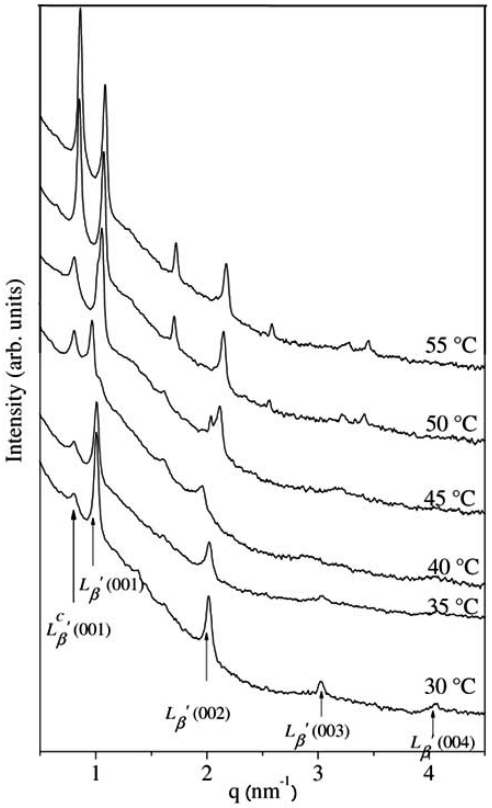

Fig. (8). Synchrotron XRD patterns of the DPPC-DNA-Mn^{2+} complex at a 3:4:24 molar ratio as a function of temperature.

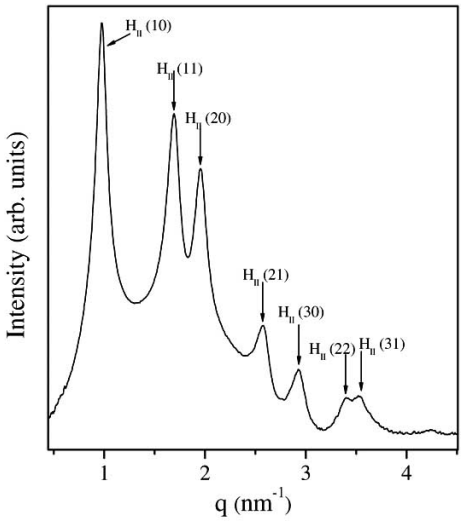

Fig. (9). SAXS pattern of pure DOPE. (Reprinted from *Europhys. Lett.*, **2004**, *67*, 671, with permission of the editor, www.epljournal.org)

average diameter of the water core d_W =3.02 nm. It was demonstrated [84, 88] that DOPE and divalent metal cations Mn^{2+}, Mg^{2+}, Co^{2+} and Fe^{2+} in water solution are able to condense DNA into a new inverted-hexagonal phase H_{II}^C: as an example the SAXS pattern for the ternary complex DOPE-DNA-Fe^{2+} at 3:4:12 molar ratio is reported (Fig. **11**). As it was observed for DOPC, DLPC and DPPC, two different sets of peaks are identified also in this spectrum and labelled as H_{II} and H_{II}^C with different unit cell spacings, namely a = 7.45 nm and a_c = 6.87 nm, respectively. The first set of peaks corresponds to the phase H_{II} of pure DOPE, whereas the second set is consistent with the 2D columnar inverted hexagonal phase H_{II}^C of the DOPE-DNA-Fe^{2+} complex. In this structure DNA strands are supposed to fill the water gap inside the cylinders of pure DOPE, as supported by the electron density profiles (Fig. **12**) calculated along the [10] direction. The two shoulders at z/d ~ 0.26 and 0.73 respectively correspond to phosphate groups and are used to localize the centres of the polar headgroups. From the structural data, values of d_{PP} = 3.26 nm, d_L = 4.36 nm were calculated, leading to a water layer thickness d_W = 2.51 nm, large enough to accommodate a double-stranded DNA molecule surrounded by a hydration layer [77]. A schematic representation of the structure of the complex was proposed (Fig. **2b**). Noteworthy, the ratio between H_{II} and H_{II}^C depends also on the incubation time: the SAXS pattern registered after 48 hours shows the total disappearing of phase H_{II} wholly transformed into the ternary complex. This result asks for an accurate determination of the correlations between incubation times and the yields of ternary complexes.

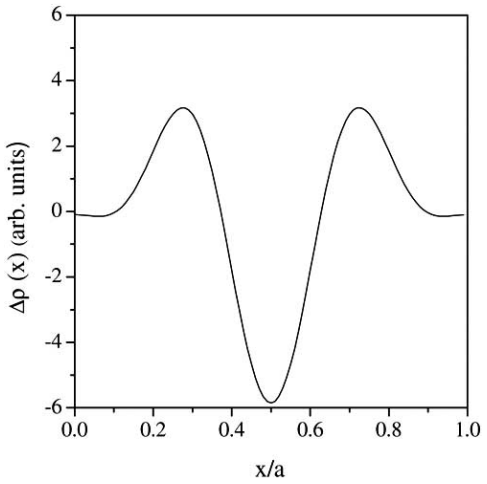

Fig. (10). Electron density profile of the pure DOPE along the [10] direction of the unit cell. The origin corresponds to the center of water core. (Reprinted from *Europhys. Lett.*, **2004**, *67*, 671, with permission of the editor, www.epljournal.org)

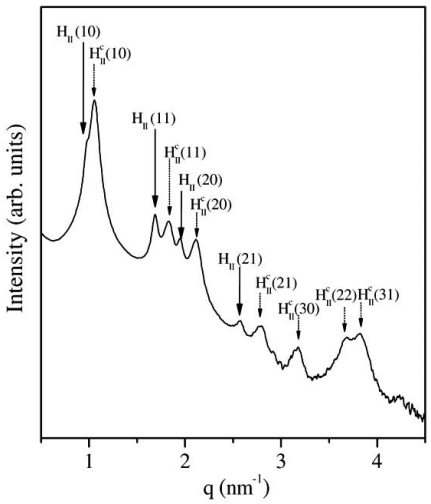

Fig. (11). SAXS pattern of DOPE-DNA-Fe^{2+} complex at 3: 4:12 molar ratio. (Reprinted from *Europhys. Lett.*, **2004**, *67*, 672, with permission of the editor, www.epljournal.org)

In all cases the process of self-aggregation is controlled by the release of counter-ion entropy upon neutralization of DNA phos-

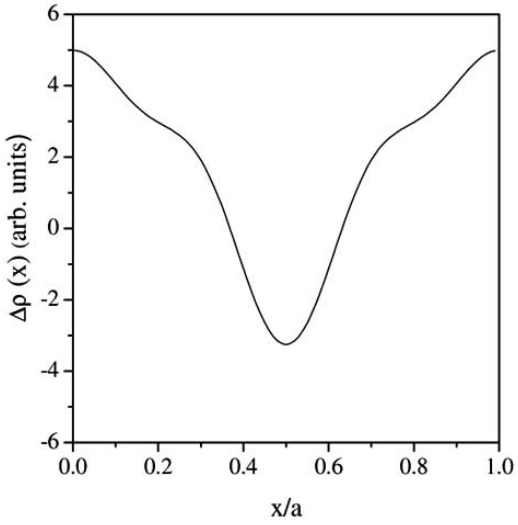

Fig. (12). Electron density profile of the DOPE-DNA-Fe^{2+} complex (3:4:12) along the [10] direction of the unit cell. The origin corresponds to the center of the water core. (Reprinted from *Europhys. Lett*., **2004**, *67*, 672, with permission of the editor, www.epljournal.org)

phate groups by metal cations. Generally pDNA has been preferred to linear DNA, considering that the native supercoiled conformation of the former is physiologically active and hence more promising for therapeutic applications. A study on solid supported ternary complexes of DOPC-DNA-Mn^{2+} [89] can be considered useful in such perspective: using, the Energy Dispersion *X*-ray Diffraction (EDXD) and an original experimental procedure [90, 91], it was possible to examine their structure, which is essentially identical to its counterpart in aqueous solution. Solid supported complexes can emerge as an interesting state in order to obtain high resolution structural information on ternary complexes of NLs. Using the same technique, with the aim of increasing the knowledge on NLs, a study [92] was made to explore the effect of hydration on the structural features of these multilamellar systems, starting from the consideration that adsorbed water plays a major role in the effectiveness of lipid drug delivery systems where lipid-cell interactions are involved. The hydration kinetics of oriented lipids DOTAP and DOPC, bearing different headgroups and both able to entrap DNA, was studied: results show that the long-range order in multilamellar lipid systems strictly depends on the hydration level, but in a non-monotic way. First, adsorbed water molecules promote the gain of spatial coherence along the normal to the lipid bilayers reaching a maximum: then, water molecules penetrate the interbilayer region and behave as bulk water, producing disorder. This confirms the existence of a correlation between the degree of hydration of lipid bilayers and the structure of interbilayer water [93, 94].

The whole of these achievements asks of course for an evaluation of the attitude of the complexes of NLs to behave as carriers of DNA. Complexes DOPC-DNA (pGreenLantern plasmid)-M^{2+}, with M = Ca or Mn, were used [95, 96] to transfect mouse fibroblast NIH 3T3 cell lines. The expression of the green fluorescent protein (GFP), analyzed by means of fluorescence microscopy, demonstrates the capability of complexes with neutral liposomes to transfect DNA, even though the efficiency observed is rather low, if compared with the corresponding complexes of CLs. A higher efficiency was obtained with an analogous complex where DOPE was added to DOPC to get a final formulation 1 to 4 (authors' unpublished data), which encourages to continue research in the field and shows that the lipid formulation influences the transfection efficiency also in NLs, a behaviour well known in CLs. As recently outlined [97], a mixture of the four components DOTAP/DOPC/DOPE/DC-Chol shows higher transfection efficiency with respect to the binary DOTAP/DOPC and DOPE/DC-Chol

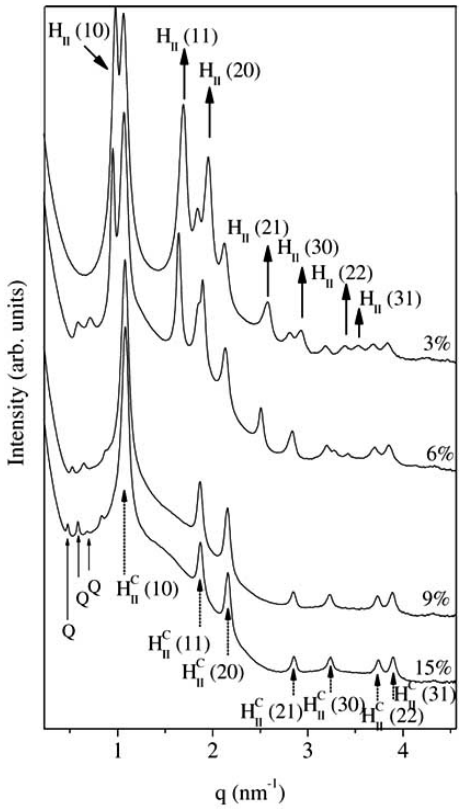

Fig. (13). Synchrotron XRD patterns of the DOPE-DOPE/PEG(350)-DNA-Mn^{2+} complex as a function of different concentrations of the DOPE/PEG component in the lipid mixture.

[98]. A result confirmed by the finding [99] that the transfection pattern increases monotonically with the number of lipid components. In addition it has been found [100] that the transfection efficiency on C6 rat glioma cells by DC-Chol/DOPE/DNA lipoplexes and the toxicity of lipoplexes to cells are dramatically affected by cationic liposome/DNA weight ratio. Finally [101], a mixture of dilauroyl (12 carbon medium-chain) and dioleoyl (18 carbon long-chain) homologues of O-ethylphosphatidylcholine synergically enhances transfection. This mixture transfects 30-fold more efficiently than either compound separately.

Existing data at the moment are too limited to predict analogous correlations in NLs, which could be of great importance in order to address the synthesis of new liposomes. An interesting approach could be represented by a pegylated NL [102], since pegylation effects on CLs are well known. Positive effects are a prolonged circulation lifetime of lipoplexes on serum and a reduction of the formation of aggregates [56, 103, 104], a property used for better tumour gene expression [105, 106]. Negative effects may come from the steric barrier of PEG, which might inhibit the transfection activity of lipoplexes by reducing the particle-membrane contact. Under definite experimental conditions [107] PEG-lipids had a minimal effect on the binding and subsequent endocytosis of the lipoplexes, but severely inhibited active gene transfer into the cytoplasm. In a different context, PEG chains are reported to decrease both the interaction and cellular internalization of DNA complex [108].

In order to check the effect of pegylation on NLs, the structures of mixed complexes DOPE-DOPE/PEG(350)-DNA-M^{2+} (M = Ca, Mg, Mn) were therefore studied**.** The result of the XRD investigation on the complex with Mn^{2+} (Fig. **13**) shows that, with an amount of 3% of DOPE-PEG, the two phases H_{II} and H_{II}^{C}, indexed on 2D hexagonal lattice with different unit cells (a =7.42 nm and a_C

=6.82 nm respectively) coexist as usual. As for DOPE, the former set of peaks is attributed to DOPE-DOPE/PEG(350)-Mn^{2+}, while the latter is consistent with the 2D columnar inverted hexagonal phase DOPE-DOPE/PEG(350)-DNA-Mn^{2+}. It is worth noticing that a new phase, indexed in the SAXS pattern as Q (Fig. **13**), appears at higher concentrations of DOPE-PEG (6, 9 and 15%): the corresponding peaks are spaced in the ratios $\sqrt{2};\sqrt{3};\sqrt{4};\sqrt{6};\sqrt{8};\sqrt{9};\sqrt{10}$ consistent with a cubic Q^{224} phase [109], with the space group *Pn3m*. A transition $H_{II} \rightarrow Q_{II}$ has been found in different contexts [110, 111] and a review describing phases and phase transitions of the phosphatidylcholines [112] has been published. In this case the transition takes on a particular importance in view of an extensive use of lipid based cubic phase materials for drug delivery applications; in addition bicontinuous cubic crystalline phases in mixture of lipids seem to play an important biological role in membrane fusion [113]. This ability, together with the well known fusogenic property of DOPE and its destabilizing effect on targeted endosomal membranes makes the complexes DOPE-DOPE/PEG(350)-DNA-M^{2+} extremely interesting for application in HGT. In addition, the possibility of inducing structural transitions to the cubic phase, *via* a proper control of the metal cation concentration, represents a significant progress toward a design-based approach to drug delivery.

Beside the development of the research aiming at better understanding structure and function of the NLs-DNA-M^{2+} complexes for HGT application, other scientists followed different approaches: there has been recently proposed [114] a way to reduce the cytotoxic effect of cationic vectors by encapsulating the positively charged lipoplexes into an outer leaflet made of NLs. The authors decided to prepare what they call asymmetric liposomes, to distinguish such new particles from the normal vectors, considered to be symmetric: in these ones, the inner and outer lipid layers have the same composition, while in the others the inner and outer lipid layers are different. Briefly they have hypothesized to keep the strongly encapsulated lipoplex DNA-DOTAP in the inner leaflet of a second liposome (DMPC; DOPC) made of neutral lipids. The inverse emulsion technique [115, 116] used for such preparation has resulted in low yields so far.

CONCLUSIONS AND FUTURE DEVELOPMENTS

For the moment the use of NLs as autonomous carriers of genetic material in human gene therapy can be considered no more than an opportunity to be explored as an alternative to CLs. Considering that these are still regarded as the most promising synthetic vectors, despite the many limits they still encounter, particularly in the *in vivo* applications, and that NLs behave as important helpers in transfection experiments with cationic liposomes, a future for NLs as autonomous carriers appears feasible. Lack of toxicity and high stability in serum are added values in their favour. Some of the results outlined in this review are worth interesting developments. It has been found that complexes reflect the structure and symmetry of the parent lipids and that the different bivalent metal cations are equally active in promoting the DNA condensation into the ternary complexes. These achievements provide an important structure-composition correlation, that may be used in designing at the best these materials as non-viral DNA carriers in HGT. Additional developments of the research in the field, currently investigated in our laboratories, concern the use of pegylated NLs in the management of brain related diseases, where CLs have started being experimented [117-121], also introducing new protocols [122] for a more efficient encapsulation. Better results could be obtained with NLs, thanks to their ability to reduce opsonization. The recent interest on the so-called intelligent carriers which is developing on CLs could also represent an interesting opportunity for NLs [123-125].

The structural aspects of NLs in determining transfection efficiency don't exhaust the discussion on the feasibility of HGT with NLs. Many other aspects, such as Z-potential values, liposome size, type and extension of DNA association are also very important, but data are still lacking. As for Z-potential, preliminary measures in our laboratories show values close to zero, which means that the interaction between the complex DNA-vector and the cell membrane must be classified as adhesion-fusion, a mechanism requiring however deeper investigations. The entry of NLs in the world of HGT and the consequent opportunity to compare properties and activity with the ones of cationic and anionic liposomes could lead to a better understanding of this process [126, 127].

Apart from these perspectives directly concerning HGT, the many interesting research opportunities NLs present, from structural studies to synthesis of modified or new original liposomes, from evaluation of structure-property relationships to application in alternative fields such as drug delivery, are important reasons to keep on research in this field.

ACKNOWLEDGEMENTS

The authors thank the Polytechnic University of Marche (Italy) and the Ministry of Education, University and Science (Rome, Italy) for funding some of the studies that have been reviewed in this work. The University Spin-off BINT is kindly acknowledged for its generous financial support. The Polytechnic University of Marche is also acknowledged for funding the manuscript preparation.

ABBREVIATIONS

HGT	=	Human Gene Therapy
DOTAP	=	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	=	*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
OLON	=	oleoyl ornithinate
PC	=	phosphatidylcholine
PE	=	phosphatidylethanolamine
PS	=	phosphatidylserine
DOPC	=	1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine
DLPC	=	1,2-dilinoleoyl-*sn*-glycero-3-phosphatidylcholine
DPPC	=	1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
DC-Chol	=	N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
ULV	=	unilamellar vesicles
MLV	=	multilamellar vesicles
DOPE	=	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
PEG	=	poly-ethyleneglycol
CLs	=	cationic liposomes
NLs	=	neutral liposomes
DNA	=	deoxyribonucleic acid
pDNA	=	plasmid deoxyribonucleic acid
PKC	=	protein kinase C
XRD	=	Synchrotron *X*-ray Diffraction
SAXS	=	Small Angle X-ray Scattering
WAXS	=	Wide Angle *X*-ray Scattering

EDXD = Energy Dispersion *X*-ray Diffraction

GFP = green fluorescent protein

Note: Patents have not been considered in this review.

REFERENCES

[1] Mulligan, R.C. The basic science of gene therapy. *Science*, **1993**, *260*, 926-932.

[2] Behr, P. Synthetic gene transfer vectors. *Acc. Chem. Res.*, **1993**, *26*, 274-278.

[3] Villar, J.; Méndez-Alvarez, S. Cells, diseases and genotypes: the revolution of molecular medicine. *Int. Microbiol*., **2001**, *4*, 57-58.

[4] Berger, F. Nanomedicine and oncology. In *Cancer Nanotech, 2006*. Paris, May, 17-18, **2006**.

[5] Kostarelos, K.; Miller, A.D. Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for synthetic non-viral vectors. *Chem. Soc. Rev*., **2005**, *34*, 970-994.

[6] Ewert, K.; Slack, N.L.; Ahmad, A.; Evans, H.M.; Lin, A.J.; Samuel, C.E.; Safinya, C.R. Cationic lipid-DNA complexes for gene therapy: understanding the relationship between complexes structure and gene delivery pathways at the molecular level. *Curr. Med. Chem.*, **2004**, *11*, 133-149.

[7] Barteau, B.; Chèvre, R.; Letrou-Bonneval, E.; Labas, R.; Lambert, O.; Pitard, B. Physicochemical parameters of non-viral vectors that govern transfection efficiency. *Curr. Gene Ther.*, **2008**, *8*, 313-323.

[8] Felgner, P.L.; Gadek, T.R.; Holm, M.; Roman, R.; Chan, H.W.; Wenz, M.; Northrop, J.P.; Ringold, G.M.; Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Nat. Acad. Sci. USA*, **1987**, *84*, 7413-7417.

[9] Koltover, I.; Salditt, T.; Rädler, J.O.; Safinya,. C.R. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science*, **1998**, *281*, 78-81.

[10] Farhood, H.; Serbina, N.; Huang, L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta*, **1995**, *1235*, 289-295.

[11] Felgner, J.H.; Kumar, R.; Sridhar, C.N.; Wheeler, C.J.; Tsai, Y.J.; Border, R.; Ramsey, P.; Martin, M.; Felgner, P.L. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem*., **1994**, *269*(4), 2550-2561.

[12] Montier, T.; Benvegnu, T.; Jaffres, P.A.; Yaouanc, J.J.; Lehn, P. Progress in cationic lipid-mediated gene transfection: a series of bio-inspired lipids as an example. *Curr. Gene Ther.*, **2008**, *8*, 296-312.

[13] Safinya, C.R.; Ewert, K.; Ayesha, A.; Evans, H.M.; Raviv, U.; Needleman, D.J.; Lin, A.L.; Slack, N.L.; George, C.; Samuel, C.E. Cationic liposome-DNA complexes: from liquid crystal science to gene delivery applications. *Philos. Trans. R. Soc., A.*, **2006**, *364* (1847), 2573-2596.

[14] Karmali, P.P.; Chaudhuri, A. Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions and future promises. *Med. Res. Rev*., **2007**, *27*, 696-722.

[15] Lv, H.; Zhang, S.; Wang, B.; Cui, S.; Hyan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release*, **2006**, *114*, 100-109.

[16] Dass, C.R. Lipoplex-mediated delivery of nucleic acids: factors affecting *in vivo* transfection. *J. Mol. Med*., **2004**, *82*, 579-591.

[17] Tan, Y.; Huang, L. J. Overcoming the inflammatory toxicity of cationic gene vectors. *Drug Target*, **2002**, *10*, 153-160.

[18] Foradada, M.; Pujol, M.D.; Bermudez, J.; Esterlich, J. Chemical degradation of liposomes by serum components detected by NMR. *Chem. Phys. Lipids*, **2000**, *104*, 133-148.

[19] Chesnoy, S.; Huang, L. Structure and function of lipid-DNA complexes for gene delivery. *Annu. Rev. Biophys. Biomol. Struct*., **2000**, *29*, 27-47.

[20] Masotti, A.; Mossa, G.; Cametti, C.; Ortaggi, G.; Bianco, A.; Grosso, N.D.; Malizia, D.; Esposito, C. Comparison of different commercially available cationic liposome-DNA lipoplexes: parameters influencing toxicity and transfection efficiency. *Colloids Surf. B: Biointerfaces*, **2009**, *68*, 136-44.

[21] Liu, F.; Qi, H.; Huang, L.; Liu, D. Factors controlling the efficiency of cationic lipid-mediated transfection *in vivo* via intravenous administration. *Gene Ther.*, **1997**, *4*, 517-523.

[22] Liu, Y.; Mounkes, L.C.; Liggitt, H.D.; Brown, C.S.; Solodin, I.; Heath, T.D.; Debs, R.J. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol*., **1997**, *15*, 167-173.

[23] Bottega, R.; Epand, R.M. Inhibition of protein kinase by cationic amphiphiles. *Biochemistry*, **1992**, *31*, 9025-9030.

[24] Farhood, H.; Bottega, R.; Epand, R.M.; Huang, L. Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. *Biochim. Biophys. Acta*, **1992**, *1111*, 239-246.

[25] Meekel, A.A.P.; Wagenaar, A.; Smisterova, J.; Kroeze, J.E.; Haadsma, P.; Bosgraaf, B.; Stuart, M.C.A.; Brisson, A.; Ruiters, M.H.J.; Hoextra, D.; Engberts, J.B.F.N. Synthesis of Pyridinium amphiphiles used for transfection and some characteristics of amphiphile/DNA complex formation. *Eur. J. Org. Chem*., **2000**, *4*, 665-673.

[26] Roosjen, A.; Smisterova, J.; Driessen, C.; Anders, J.T.; Wagenaar, A.; Hoextra, D.; Hulst, R.; Engberts, J.B.F.N. Synthesis and characteristics of biodegradable pyridinium amphiphiles used for *in vitro* DNA delivery. *Eur. J. Org. Chem*., **2002**, *7*, 1271-1277.

[27] Ilies, M.A.; Seitz, W.A.; Caproiu, M.T.; Wentz, M.; Garfield, R.E.; Balaban, A.T. Pyridinium-based lipids as gene-transfer agents. *Eur. J. Org. Chem*., **2003**, *14*, 2645-2655.

[28] Pinnaduwage, P.; Schmitt, L.; Huang, L. Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells. *Biochim. Biophys. Acta*, **1989**, *985*, 33-37.

[29] Tang, F.X.; Hughes, J.A. Synthesis of a single-tailed cationic lipid and investigation of its transfection. *J. Control. Release*, **1999**, *62*, 345-358.

[30] Leventis, R.; Silvius, J.R. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta*, **1990**, *1023*, 124-132.

[31] Choi, J.S.; Lee, E.J.; Jang, H.S.; Park, J.S. New cationic liposomes for gene transfer into mammalian cells with high efficiency and low toxicity. *Bioconjug. Chem*., **2001**, *12*, 108-113.

[32] Liu, D.; Hu, J.; Qiao, W.; Li, Z.; Zhang, S.; Cheng, L. Synthesis of carbammate-linked lipids for gene delivery. *Bioorg. Med. Chem. Lett.*, **2005**, *15*, 3147-3150.

[33] Li, S.; Rizzo, M.A.; Bhattacharya, S.; Huang, L. Characterization of cationic lipid-protamine-DNA (LPD) complexes for intravenous gene delivery. *Gene Ther.*, **1998**, *5*, 930-937.

[34] Templeton, N.S.; Lasic, D.D.; Frederik, P.M.; Strey, H.H.; Roberts, D.D.; Pavlakis, G.M. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat. Biotechnol*., **1997**, *15*, 647-652.

[35] Li, S.; Tseng, W.C.; Stolz, D.B.; Wu, S.P.; Watkins, S.C.; Huang, L. Dynamic changes in the characteristics of cationic lipid vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Ther*., **1999**, *6*, 585-594.

[36] Zelphati, O.; Uyechi, L.S.; Barron, L.G.; Szoka Jr, F.C. Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim. Biophys, Acta*, **1998**, *1390*, 119-133.

[37] Miller, A.D. Cationic liposomes for gene therapy. *Angew. Chem. Int. Ed*., **1998**, *37*, 1768-1785.

[38] Rubanyi, G.R. The future of human gene therapy. *Mol. Aspects Med*., **2001**, *22*, 113-142.

[39] Zhang, Y.; Anchordoquy, T. J. The role of lipid charge density in the serum stability of cationic lipid/DNA complexes. *Biochim. Biophys. Acta*, **2004**, *1663*, 143-157.

[40] Koiv, A.; Palvimo, J.; Kinnunen, P.K. Evidence for ternary complex by histone H1, DNA and liposomes. *Biochemistry*, **1995**, *34*, 8018-8027.

[41] Chonn, A.; Cullic, P.R.; Devine, D.V. The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol*., **1991**, *146*, 4234-4241.

[42] Tardi, P.G.; Boman, N.L.; Cullis, P.R. Liposomal doxorubicin. *J. Drug Target*., **1996**, *4*, 129-140.

[43] Papahadjopoulos, D.; Vail, W.J.; Pangborn, W.A.; Poste, G. Studies on membrane fusion. II: Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. *Biochim. Biophys. Acta*, **1976**, *448*, 265-83.

[44] Newton, C.; Pangborn, W.; Nir, S.; Papahadjopoulos, D. Specificity of Ca^{2+} and Mg^{2+} binding to phosphatidylserine vesicles and resultant phase changes of bilayer membrane structure. *Biochim. Biophys. Acta*, **1978**, *506*, 281-287.

[45] Portis, A.; Newton, C.; Pangborn, W.; Papahadjopoulos, D. Studies on the mechanism of membrane fusion: evidence for an intermembrane Ca^{2+}-phospholipid complex, synergism with Mg^{2+} and inhibition by spectrin. *Biochemistry*, **1979**, *18*, 780-790.

[46] Wilschut, J.; Düzgünes, N.; Fraley, R.; Papahadjopoulos, D. Studies on the mechanism of membrane fusion: kinetics of calcium ion induced fusion of phosphatidylserine vesicles followed by a new assay for mixing of aqueous vesicles contents. *Biochemistry*, **1980**, *19*, 6011-6021.

[47] Düzgünes, N.; Wilschut, J.; Fraley, R.; Papahadjopoulos, D. Studies on the mechanism of membrane fusion. Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochim. Biophys. Acta*, **1981**, *642*, 182-195.

[48] Düzgünes, N.; Nir, S.; Wilschut, J.; Bentz, J.; Newton, C.; Portis, A.; Papahadjopoulos, D. Calcium- and Magnesium-induced fusion of mixed phosphatidylcholine vesicles: effect of ion binding. *J. Membr. Biol*., **1981**, *59*(2), 115-25.

[49] Wilson, T.; Papahadjopoulos, D.; Taber, R. The induction of poliovirus RNA into cells via lipid vesicles (liposomes). *Cell*, **1979**, *17*, 77-84.

[50] Fraley, R.; Subramani, S.; Berg, P.; Papahadjopoulos, D. Introduction of liposome-encapsulated SV40 DNA into cells. *J. Biol. Chem*., **1980**, *255*, 10431-10435.

[51] Fraley, R.; Straubinger, R.M.; Rule, G.; Springer, E.L.; Papahadjopoulos, D. Liposome- mediated delivery of deoxyribonucleic acid to cells: enhanced efficiency of delivery related to lipid composition and incubation conditions. *Biochemistry*, **1981**, *20*, 6978-6987.

[52] Alino, S.F.; Garcia-Sanz, M.; Irruarrizaga, A.; Alfaro, J.; Hernandez, J. High encapsulation efficiencies in sized liposomes produced by extrusion of dehydration-rehydration vesicles. *J. Microencapsulation* **1990**, *7*, 497-503.

[53] Koizumi, K.; Fujii, M.; Ito, A.; Fujita, H.; Sasaki, M. A basic study of photo-antisense regulation: protective role of liposomes in the intracellular delivery process of exogenous oligonucleotides. *Photomed. Photobiol*., **1999**, *21*, 85-87.

[54] Litzinger, D.C.; Huang, L. Amphipatic poly(ethylene glycol) 5000-stabilized

dioleoylphosphatidylethanolamine liposomes accumulate in spleen. *Biochim. Biophys. Acta*, **1992**, *1127*(3), 249-254.

[55] Gabizon, A.; Catane, R.; Uziely, B.; Kaufman, B.; Safra, T.; Cohen, R.; Martin, F.; Huang, A.; Barenholz, Y. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res.*, **1994**, *54*, 987-992.

[56] Papahadjopoulos, D.; Allen, T.M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S.K.; Lee, K.D.; Woodle, M.C.; Lasic, D.D.; Redemann, C.; Martin, F.J. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA*, **1991**, *88*, 11460-11464.

[57] Allen, T.M.; Chonn, A. Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett.*, **1987**, *223*(1), 42-46.

[58] Allen, T.M.; Hansen, C.; Rutledge, J. Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta*, **1989**, *981*(1), 27-35.

[59] Gabizon, A.; Papahadjopoulos, D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA*, **1988**, *85*, 6949-6953.

[60] Tycko, B.; Keith, C.H.; Maxfield, F.R. Rapid acidification of endocytic vesicles containing asialoglycoprotein in cells of a human hepatoma line. *J. Cell Biol.*, **1983**, *97*, 1762-1776.

[61] Tycko, B.; Maxfield, F.R. Rapid acidification of endocytic vesicles containing α_2-macroglobulin. *Cell*, **1982**, *28 (3)*, 643-651.

[62] Chu, C-J.; Szoka Jr, F.C. pH sensitive liposomes. *J. Liposome Res.*, **1994**, *4*(1), 361-395.

[63] Vogel, K.; Wang, S.; Lee, R.J.; Chmielewski, J.; Low, P.S. Peptide-mediated release of folate-targeted liposome contents from endosomal compartments. *J. Am. Chem. Soc.*, **1996**, *118*, 1581-1586.

[64] Marra, J.; Israelachvili, J. Direct measurements of forces between phosphatidylcholine and phosphatidylethanolamine bilayers in aqueous electrolyte solutions. *Biochemistry*, **1985**, *24*, 4608-4618.

[65] Tarahovsky, Y.S.; Khusainova, R.S.; Gorelov, A.V.; Nicolaeva, T.I.; Deev, A.A.; Dawson, K.A.; Ivanitsky, G.R. DNA initiates polymorphic structural transitions in lecithin. *FEBS Lett.*, **1996**, *390*, 133-136.

[66] Tarahovsky, Y.S.; Deev, A.A.; Masulis, I.S.; Ivanitsky, G.R. Structural organisation and phase behaviour of DNA-calcium-dipalmitoylphosphatidylcholine complex. *Biochemistry (Moskow)*, **1998**, *63*(10), 1126-1131.

[67] Hui, S.W.; Langner, M.; Zhao, Y.Li.; Ross, P.; Hurley, E.; Chan, K. The role of helpers in cationic liposome-mediated gene transfer. *Biophys. J.*, **1996**, *71*, 590-599.

[68] Kovalenko D.V.; Shafei, R.A.; Zelenina, I.A.; Semenova, M.L.; Samuilova, O.V.; Zhdanov, R.I. Metallonucleoliposome complexes as a vehicle for gene delivery to mouse skeletal muscles *in vivo*. *Genetika*, **1996**, *32*, 1299-1301.

[69] Zhdanov, R.I.; Buneeva, O.A.; Podabed, O.V.; Kutsenko, N.G.; Tsvektova, T.A.; Lavrenova, T.P. Transfer of the functional genes into eukaryotic cells by neutral phospholipid liposomes. *Vopr. Med. Him.*, **1997**, *43*, 212-216.

[70] Bailey, A.L.; Sullivan, S.M. Efficient encapsulation of DNA plasmids in small neutral liposomes induced by ethanol and calcium. *Biochim. Biophys. Acta*, **2000**, *1468*, 239-252.

[71] Kharakoz, D.P.; Khusainova, R.S.; Gorelov, A.V.; Dawson, K.A. Stoichiometry of dipalmitoylphosphatidylcholine-DNA interaction in the presence of Ca^{2+}: a temperature-scanning ultrasonic study. *FEBS Lett.*, **1999**, *446*, 27-29.

[72] Francescangeli, O.; Stanic, V.; Gobbi, L.; Bruni, P.; Iacussi, M.; Tosi, G.; Bernstorff, S. Structure of self-assembled liposome-DNA-metal complexes. *Phys. Rev. E*, **2003**, *67*, 11904 (1-11).

[73] Luzzati, V. In *Biological Membranes*, Chapman, D.; Wallah, F.H. Eds.; Academic Press: New York, London, **1968**, pp. 21-23.

[74] Koltover, I.; Salditt, T.; Rädler, J.O.; Safinya, C.R. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science*, **1998**, *281*, 78-81.

[75] Lasic, D.D.; Strey, H.; Stuart, M.C.A.; Podgornik, R.; Frederick, P.M. The structure of DNA-Liposome complexes. *J. Am. Chem. Soc.*, **1997**, *119*, 832-833.

[76] Rädler, J.O.; Koltover, I.; Salditt, T.; Safinya, C.R. Structure of DNA-Cationic liposome complexes: DNA intercalation in multilateral membranes in distinct interhelical packing regimes. *Science*, **1997**, *275*, 810-814.

[77] Podgornik, R.; Rau, D.C.; Parsegian, V.A. The action of interhelical forces on the organisation of DNA double helices: fluctuation-enhanced decay of electrostatic double-layer and hydration forces. *Macromolecules*, **1989**, *22*, 1780-1786.

[78] Uhrikova, D.; Hanulova, M.; Funari, S.S.; Khusainova, R.S.; Sersen, F.; Balgavy, P. The structure of DNA-DOPC aggregates formed in presence of calcium and magnesium ions: a small-angle synchrotron X-ray diffraction study. *Biochim. Biophys. Acta*, **2005**, *1713*, 15-28.

[79] Mc Manus, J.J.; Rädler, J.O.; Dowson, K.A. Does calcium turn a zwitterionic lipid cationic? *J. Phys. Chem B*, **2003**, *107*, 9869-9875.

[80] McManus, J.J.; Rädler, J.O.; Dawson, K.A. Phase behaviour of DPPC in a DNA-calcium-zwitterionic lipid complex studied by small angle X-ray scattering. *Langmuir*, **2003**, *19*, 9630-9637.

[81] Huster, D.; Arnold, K. Ca^{2+}-medaiated interaction between dextran sulfate and dimyristoyl-*sn*-glycero-3-phosphocholine surfaces studied by 2H nuclear magnetic resonance. *Biophys. J.*, **1998**, *75*, 909-916.

[82] Bruni, P.; Gobbi, G.; Moranti, G.; Iacussi, M.; Maurelli, E.; Tosi, G. Use and activity of metals in biological systems. I. The interaction of bivalent metal cations with double-stranded polynucleotides and phospholipids. *Gazz. Chim. Ital.*, **1997**, *127*, 513-517.

[83] Francescangeli, O.; Stanic, V.; Lucchetta, D.E.; Bruni, P.; Iacussi. M.; Cingolani, F. X-ray diffraction study of dioleoylphosphatidylcholine-DNA-Mn^{2+} complexes. *Mol. Cryst. Liq. Cryst.*, **2003**, *398*, 259-267.

[84] Pisani, M.; Bruni, P.; Conti, C.; Giorgini, E.; Francescangeli, O. Self-assembled liposome-DNA-metal complexes related to DNA delivery. *Mol. Cryst. Liq. Cryst.*, **2005**, *434*, 643-651.

[85] Pisani, M.; Bruni, P.; Caracciolo, G.; Caminiti, R.; Francescangeli, O. Structure and Phase Behavior of Self-Assembled DPPC-DNA-Metal Cation Complexes. *J. Phys. Chem. B*, **2006**, *110*, 13203-13211.

[86] Francescangeli, O.; Pisani, M.; Stanic, V.; Bruni, P.; Iacussi, M. Supramolecular ordering of self-assembled liposome-DNA-metal complexes. *Recent Res. Dev. Macromol.*, **2003**, *7*, 247-63.

[87] Turner, D.C.; Gruner, S.M. X-ray diffraction reconstruction of the inverted hexagonal (H_{II}) phase in lipid-water systems. *Biochemistry*, **1992**, *31*, 1340-1355.

[88] Francescangeli, O.; Pisani, M.; Stanic, V.; Bruni, P.; Weiss, T.M. Evidence of an inverted hexagonal phase in self-assembled phospholipid-DNA-metal complexes. *Europhys. Lett.*, **2004**, *67*(4), 669-675.

[89] Caracciolo, G.; Sadun, C.; Caminiti, R.; Pisani, M.; Bruni, P.; Francescangeli, O. Structure of solid-supported lipid-DNA-metal complexes investigated by energy dispersive X-ray diffraction. *Chem. Phys. Lett.*, **2004**, *397*, 138-143.

[90] Caminiti, R.; Rossi Albertini, V. The kinetics of phase transitions observed by energy-dispersive X-ray diffraction. *Int. Rev. Phys. Chem.*, **1999**, *18*, 263-299.

[91] Caracciolo, G.; Caminiti, R.; Pozzi, D.; Friello, M.; Boffi, F.; Congiu Castellano A. Self-assembly of cationic liposomes-DNA complexes: a structural and thermodynamic study by EDXD. *Chem. Phys. Lett.*, **2002**, *351*, 222-228.

[92] Caminiti, R.; Caracciolo, G.; Pisani, M.; Bruni, P. Effect of hydration on the long-range order of lipid multilayers investigated by in situ time-resolved energy dispersive X-ray diffraction. *Chem. Phys. Lett.*, **2005**, *409*, 331-336.

[93] Ge, M.; Freed, J.H. Hydration, structure and molecular interactions in the headgroup region of dioleoylphosphatidylcholine bilayers: an electron spin resonance study. *Biophys. J.*, **2003**, *85*, 4023-4040.

[94] Zhou, Z.; Sayer, B.G.; Hughes, D.W.; Stark, R.E.; Epand, R.M. Studies of phospholipid hydration by high-resolution magic-angle spinning nuclear magnetic resonance. *Biophys. J.*, **1999**, *76*, 387-399.

[95] Bruni, P.; Pisani, M.; Amici, A.; Marchini, C.; Montani, M.; Francescangeli, O. Self-assembled ternary complexes of neutral liposomes, deoxyribonucleic acid and bivalent metal cations. Promising vectors for gene transfer? *Appl. Phys. Lett.*, **2006**, *88*, 73901.

[96] Bruni, P.; Pisani, M.; Amici, A.; Marchini, C.; Montani, M.; Francescangeli, O. Study and function of self-assembled liposome-DNA metal complexes for gene transfer. *ESRF Highlights*, **2006**, 35-37.

[97] Gaziola de la Torre, L.; Rosada R.S.; Trombone, A.P.F.; Frantz, F.G.; Coelho-Castelo, A.A.M.; Silva, C.L.; Santana, M.H.A. The synergy between structural stability and DNA-binding controls the antibody production in EPC/DOTAP/DOPE liposomes and DOTAP/DOPE lipoplexes. *Colloids Surf B: Biointerfaces*, **2009**, *73*, 175-184.

[98] Caracciolo, G.; Pozzi, D.; Caminiti, R.; Marchini, C.; Montani, M.; Amici, A.; Amenitsch, H. DNA release from cationic liposome/DNA complexes by anionic lipids. *Appl. Phys. Lett.*, **2006**, *89*, 2339031-2339033.

[99] Caracciolo, G.; Pozzi, D.; Caminiti, R.; Marchini, C.; Montani, M.; Amici, A.; Amenitsch, H. Transfection efficiency boost by designer multicomponent lipoplexes. *Biochim. Biophys. Acta-Biomembr.*, **2007**, *1768*, 2280-2292.

[100] Congiu, A.; Pozzi, D.; Esposito, C.; Castellano, C.; Mossa, G. Correlation between structure and transfection efficiency: a study of DC-Chol-DOPE/DNAcomplexes. *Colloids Surf. B-Biointerfaces*, **2004**, *36*, 43-48.

[101] Wang, L.C. New strategy for transfection: mixtures of medium-chain and long-chain cationic lipids synergistically enhance transfection. *Gene Ther.*, **2004**, *11*, 1358-1362.

[102] Pisani, M.; Fino, V.; Bruni, P.; Francescangeli, O. DNA condensation into inverted hexagonal phase in aqueous dispersion of poly(ethylene)-functionalized dioleoylphosphatidylethanolamine and metal cations. *Mol. Cryst. Liq. Cryst.*, **2009**, *500*, 132-143.

[103] Meyer, O.; Kirpotin, D.; Hong, K.; Sternberg, B.; Park, J.W.; Woodlei, M.C.; Papahadjopoulos, D. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. *J. Biol. Chem.*, **1998**, *273*(25), 15621-15627.

[104] Klibanov, A.L.; Maruyama, K.; Torchilin, V.P.; Hunag, L. Amphipathic polyethylenglycols effectively prolong the circulation time of liposomes. *FEBS Lett.*, **1990**, *268*, 235-237.

[105] Xu, L.; Tang, W.H.; Huang, C.C.; Alexander, W.; Xiang, L.M.; Pirollo, K.F.; Rait, A.; Chang, E.H. Systemic p53 Gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. *Mol. Med.*, **2001**, 7(10), 723-734.

[106] Ambegia, E.; Ansell, S.; Cullis, P.; Heyes, J.; Palmer, L.; MacLachlan, I. Stabilized plasmid–lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression. *Biochim. Biophys. Acta*, **2005**, *1669*, 155-163.

[107] Song, L.Y.; Ahkong, Q.F.; Rong, Q.; Wang, Z.; Ansell, S.; Hope, M.J.; Mui,

B. Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes. *Biochim. Biophys. Acta*, **2002**, *1558*, 1-13.

[108] Deshpande, M.C.; Davies, M.C.; Garnett, M.C.; Williams, P.M.; Armitage, D.; Bailey, L.; Vamvakaki, M.; Armes, S.P.; Stolnik, S. The effect of poly(ethylene glycol) molecular architecture on cellular interaction and uptake of DNA complexes. *J. Control. Release*, **2004**, *97*, 143-156.

[109] Pisani, M.; Fino, V.; Bruni, P.; Di Cola, E.; Francescangeli, O. Metal induced cubic phase in poly(ethyleneglycol)-functionalized dioleoylphosphatidylethanolamine aqueous dispersions. *J. Phys. Chem. Lett. B*, **2008**, *112*, 5276-5278.

[110] So, P.T.C.; Gruner, S.M.; Erramilli, S. Pressure-induced topological phase transitions in membranes. *Phys. Rev. Lett.*, **1993**, *70*, 3455-3458.

[111] Koynova, R.; Tenchov, B.; Rapp, G. Low amounts of PEG-lipid induce cubic phase in phosphatidylethanolamine dispersions. *Biochim. Biophys. Acta*, **1997**, *1326*, 167-170.

[112] Koynova, R.; Caffrey, M. Phases and phase transitions of the phosphatidylcholines. *Biochim. Biophys. Acta*, **1998**, *1376*, 91-145.

[113] Lin, A.J.; Slack, N.L.; Ahmad, A.; George, C.X.; Samuel, C.E.; Safynia, C.R. Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behaviour in lamellar cationic liposome-DNA complexes. *Biophys. J.*, **2003**, *84*, 3307-3316.

[114] Whittenton, J.; Harendra, S.; Pitchumani, R.; Mohanty, K.; Vipulanandan, C.; Thevananther, S. Evaluation of asymmetric liposomal nanoparticles for encapsulation of polynucleotides. *Langmuir*, **2008**, *24*, 8533-8540.

[115] Pautot, S.; Frisken, B.J.; Weitz, D.A. Production of unilamellar vesicles using an inverted emulsion. *Langmuir*, **2003**, *19*, 2870-2879.

[116] Yamada, A.; Yamanaka, T.; Hamada, T.; Hase, M.; Yoshikawa, K.; Baigl, D. Spontaneous transfer of phospholipid-coated oil-in-oil and water-in-oil micro-droplets through an oil/water interface. *Langmuir*, **2006**, *22*, 9824-9828.

[117] Zhang, Y.; Zhu, C.; Pardridge W.M. Antisense gene therapy of brain cancer with an artificial virus gene delivery system. *Mol. Ther.*, **2002**, *6*(1), 67-72.

[118] Pardridge, W.M. shRNA and siRNA delivery to the brain. *Adv. Drug Deliv. Rev.*, **2007**, *59* 141-152.

[119] Ningya, S.; Zhang, Y.; Zhu, C.; Boado, R.J.; Pardridge, W.M. Brain specific expression of an exogenous gene after i.v. administration. *Proc. Nat. Acad. Sci. USA*, **2001**, *98*(22), 12754-12759.

[120] Fricker, G.; Miller, D. S. Modulation of drug transporters at the blood-brain barrier. *Pharmacology*, **2004**, *70*(4), 169-176.

[121] Boado R.J. Blood-brain barrier transport of non-viral gene and RNAi therapeutics. *Pharm. Res.*, **2007**, *24*, 1772-1787.

[122] Skiørringe, T.; Gjetting, T.; Jensen, T.G. A modified protocol for efficient DNA encapsulation into pegylated immunoliposomes (PILs). *J. Control. Release*, **2009**, *139*, 140-145.

[123] Voinea, M.; Simionescu, M. Designing of intelligent liposomes for efficient delivery of drugs. *J. Cell. Mol. Med.*, **2002**, *6*(4), 465-474.

[124] Shi, G.; Guo, W.; Stephenson, S.M.; Lee, R.J. Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations. *J. Control Release*, **2002**, *80*, 309-319.

[125] Alvarez-Lorenzo, C.; Bromberg, L.; Concheiro, A. Light-sensitive intelligent drug delivery systems. *Photochem. Photobiol.*, **2009**, *85*, 848-860.

[126] Volodkin, D.V.; Ball, V.; Voegel, J.C.; Mohwald, H.; Dimova, R.; Marchi-Artzner, V. Control of the interaction between membranes or vesicles: adhesion, fusion and release of dyes. *Colloids Surf. A: Physicochem. Eng. Aspects*, **2007**, *303*, 89-96.

[127] Mingming, M.; Gong, Y.; Bong, D. Lipid membrane adhesion and fusion driven by designed minimally multivalent hydrogen-bonding lipids. *J. Am. Chem. Soc.*, **2009**, *131*, 16919-16926.

Received: November 23, 2009 Revised: March 24, 2010 Accepted: September 02, 2010