*Original Article*

# Surface Recognition and Complexations Between Synthetic Poly(ribo)nucleotides and Neutral Phospholipids and Their Implications in Lipofection

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## Abstract

Thermodynamic features related to preparation and use of self-assemblies formed between multilamellar and unilamellar zwitterionic liposomes and polynucleotides with various conformation and sizes are presented. The divalent metal cation or surfactant-induced adsorption, aggregation and adhesion between single- and double-stranded polyribonucleotides and phosphatidylcholine vesicles was followed by differential adiabatic scanning microcalorimetry. Nucleic acid condensation and compaction mediated by $Mg^{2+}$ and *N*-alkyl-*N,N,N*-trimetylammonium ions (CnTMA, n=12), regarding to interfacial interaction with unilamellar vesicles. Microcalorimetric measurements of synthetic phospholipid vesicles and poly(ribo)nucleotides and their ternary complexes with inorganic cations were used to build the thermodynamic model of their structural transitions. The increased thermal stability of the phospholipid bilayers is achieved by affecting their melting transition temperature by nucleic acid induced electrostatic charge screening. Measurements give evidence for the stabilization of polynucleotide helices upon their association with liposomes in the presence of divalent metal cations. Such an induced aggregation of vesicles either leads to heterogeneous multilamellar DNA-lipid arrangements, or to DNA-induced bilayer destabilization and lipid fusion. In contrast, stable monodispersed complexes are formed after compaction of DNA with surfactant, followed by the addition of vesicles. Surfactants bind to DNA in a cooperative manner and increased number of nucleic acid-bound $C_{12}TMA$ leads to a rise in the size of the resulting DNA-surfactant complexes, due to their aggregation. The formation of these bundles is governed by both elctrostatic and hydrophobic interactions of surfactant chains, the reaction being mediated by condensed counterions, steric hindrance or by intrinsic chain flexibility. İn here, further employment of these polyelectrolyte nanostructures as an improved formulation in therapeutic gene delivery trials, as well as in DNA chromatography is discussed..

**Keywords:** Polyelectrolyte phospholipid-polynucleotide nanostructures; Differential scanning microcalorimetry; Phase behaviour; Non-viral gene delivery; DNA chromatography**.**

## Introduction

Therapeutic gene delivery is achieved by utilizing viral or non-viral, synthetic or physical methods(1-6). Despite their well-established cell penetration properties, viral-based delivery vectors (1-5) possess immunotoxic side effects (1, 4-9). In this context, non-viral gene therapy has been proposed as a suitable alternative (4, 8-11). Non-viral nucleic acid therapy includes complexes obtained from DNA-synthetic

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polycations (polyplexes) (12-15), or DNA-lipid (lipoplexes) nanomixtures (2, 5, 9-11, 16). Whatever the approach is, in both polyplex and lipoplex systems, the aim is to increase the transgene expression, while improving their bioavailability and decreasing their toxicity. Therefore, the desired nucleic acid packaging becomes an objective of physical pharmacy, requiring major contributions from physicochemically oriented groups.

Achievement of stable nucleic acid-lipid formulation with controllable features is a prerequisite before starting *in vitro* transfection assays. Parameters of potential interests to be followed are phase behaviour, size and morphology, structural transitions of nucleic acids studied, induced by various condensing agents, such as various detergents with different electrostatic and hydrophobic nature, charged and neutral polymers, metal ions, as well as mixtures of cationic and anionic macromolecules, and thermodynamically stable lipid vesicles. Despite extensive research reports on nucleic acid aggregation with liposomes of various lipid composition, the colloidal factors and forces governing their complex formation remain to be understood. Additional requirements of size homogeneity, stability, ability to keep the entrapped therapeutic gene sequence in sufficient concentration and reproducible manufacturing issues render the development of such gene carriers difficult.

Following the interesting recent results of using cationic, small membrane-permeant molecules (17), we have focused on designs involving such small cations as rapidly moving through model cellular membranes. These could then bind nuclear DNA with high affinity, affecting the topology of bound DNA after associating with it and favoring penetration of model cell membranes by this bound nucleic acid via hydrophobic ion pair formation.

Since understanding the energetics of DNA-lipid recognition and complexation is of major importance in this context, we have focused on the thermodynamics of lipid and amphiphile-resembling ligand binding to DNA. Attempt was done for comparison of phase behaviour of ligands and their binding modes and how this affects the energy of DNA-lipid complex formation. Assuming that alkylammonium ion and related structures form a membrane-relevant structure, their phase paramaters were compared with 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), concerning affinity for DNA binding, in order to find more clues from the correlation between nucleic acid compaction and transfection efficiency obtained from previous studies of DNA associations with lipid dispersions and polycations with different chain lengths. In our opinion, deducing from both theoretical and experimental studies will improve the current knowledge of surface molecular interactions of these promising formulations in terms of designing improved gene delivery systems, as well as developing novel DNA chromatographic stationary phases.

## Experimental

### Materials

Polyethylene glycol (PEG-20,000), $MgCl_2.2H_2O$, NaCl, synthetic polyriboadenylic acid:polyribouridilic acid (poly$(A:U)_n$), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), and SSC ($1.5 \times 10^{-4}$ mol/l Na-citrate, $1.5 \times 10^{-3}$ mol/l NaCl, pH=7.2) were all purchased from Sigma (St. Louis, MO, USA) and used without further purification. 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and FM 4-64 were a product of Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade. Alkylamine aqueous solutions were stored in tightly sealed containers, to prevent their reaction with athmospheric CO2, as suggested (19). Lipid and $C_{12}$TMA solutions were mixed in polyethylene vials in desired ratios. Following solvent evaporation under nitrogen gas flow, the samples were evacuated at room temperature for 2 h. The cationic detergent was dissolved in duble distilled water and added to the dry lipid, prior to measurement.

### Methods

*Preparation of Polynucleotide Solutions and Concentration Determinations*
Polynucleotides and calf thymus DNA (SIGMA) were dissolved in SSC, Tris-HCl and HEPES buffer solutions. The concentration of

single- and double-stranded polynucleotides was determined spectrophotometrically, by using the molar extinction coefficients per base pair. During all kinetic and calorimetric experiments, polynucleotide concentration was 0.14 mg/ml of 10 mM buffer used/10 mM NaCl (pH=7.22). Calf thymus DNA, with a MW of 8.6 MDa (= 13 kb) (Sigma, D4764), specificity of 42% GC; $T_m = 87°C$, and ~20 $A_{260}$ units per mg DNA was used. The presented nucleic acid concentration and the molar ratios were based on the average nucleotide molecular weight of 308 calculated from the known DNA composition, as employed previously(18).

*Preparation of liposomes*

Chromatographic tests for purity of the lipids were not performed, however the purity of the lipid preparation was assured from the half-widths of their main phase transitions. 1.2 mM of lipid in a standard SSC buffer, pH=7.2, was used in all experiments and stored at 4°C. Formation of a thin layer of lipid on a 15 ml round-bottomed flask was achieved by hand-shaking and hydration in the specified buffer at above its' main phase transition temperature. Unilamellar vesicles (ULV) were obtained by extrusion of the multilamellar vesicle (MLV) suspension through two stacked polycarbonate filters (Nucleopore, Inc.) of 100 nm pore size at around 60°C. Repeated extrusion (10 times) through the extruder (Lipex Biomembranes, Inc., Vancouver, B. C., Canada), created a homogeneous vesicle suspension.

*Preparation of liposome-nucleic acid mixtures*

Nucleic acid-lipid mixtures were prepared 1 h before microcalorimetric measurements, by mixing of either phosphatidylcholine MLV or ULV dispersions and solvent, varying nucleic acid concentration and keeping DPPC concentration fixed. Control experiments of DNA-lipids in the absence of detergent or divalent cations, were performed in parallel. Lipid vesicles' concentration was 0.3 mg/ml. The preparation of phosphatidylcholine ULV-calf thymus DNA complexes, was the same as in the case of MLVs, i.e. by mixing DNA

solution with aqueous DPPC dispersion in the presence of cationic surfactant or $Mg^{2+}$. The DNA concentration used throughout all experiments was 1.8 mM, based on the above mentioned assumption. A freeze-thaw protocol was followed to ensure equal distribution of solutes between lamellae and adequate hydration of the lipids. Their comparison with liposomes prepared the case of without employing freeze-thaw procedure showed no difference in terms of homogeneity of the suspension. This was done by placing the sample in a cryo-tube and freezing it in liquid nitrogen for around 30 sec. The cryo-tube was subsequently removed and plunged into a warm water bath (~60°C). When the sample was thawed, the whole cycle of freeze-thawing was repeated 6 times.

*Estimation of the amount of bound DNA*

For this purpose, the well-established protocol of Monnard P.-A. *et al* (1997) (20) was followed. All the complexes formed could be vizualized by the fluorescence microscopy, through staining the DNA with DAPI and staining the lipids with FM 4-64.

*UV/VIS Spectrophotometry*

The concentration of DNA was controlled by ultraviolet (UV) absorption at 260 nm, based on the fact that 1.0 absorbance unit (A)=50 µg/ml nucleic acid. The spectra of phospholipids and polynucleotides alone, or their combination in the presence of detergent or Mg2+ were recorded with a Shimadzu A160 double beam spectrophotometer (Schimadzu Co. Ltd., Japan) using 3 ml quartz cuvettes thermostatized within ±0.3˚C by a circulating water bath connected to the cuvette holder. The absorption spectra of polynucleotides and lipids were separated from each other by simultaneously performing the measurements at their corresponding wavelengths, respectively (18).

*Differential Scanning Calorimetry*

Calorimetric measurements were performed using a Privalov type high sensitivity differential adiabatic scanning microcalorimeter DASM-4 (Biopribor, Pushchino, Russian Federation),

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with a sensitivity higher than $4.10^{-6}$ cal $K^{-1}$ and a noise level less than $5.10^{-7}$ W. Heating runs were performed with a scan rate of 0.5 K/min. The temperature at the maximum level of the excess heat capacity curve was taken as the transition temperature ($T_m$) and the transition width ($\Delta T_{1/2}$) was determined at the transition half-height. The calorimetric enthalpy ($\Delta H_{cal}$) of transition was determined as the area under the excess heat capacity curve (18). Care was taken to ensure the reproducibility of the obtained results in terms of the instrumental drift. For this purpose, microcalorimetric measurements of the melting behaviour of lipids in complex with poly(A: U)n and calf thymus DNA in various lipid/DNA ratios were carried out using another instrument (SETARAM® DSC microcalorimeter), equipped with Hewlett-Packard PC and an accompanying company supplied computer program. A scanning rate of 0.5 or 1.0°C/min and a scanning range between 17°C and 95°C was used throughout the measurements. the amplification range was 0.250 mV, with 1500 points.

### Fluorescence Microscopy

Our recent protocol for fluorescent visualization of DNA was applied(21). Briefly, the model nucleic acid was visualized with an Olympus BH-2 fluorescence microscope at room temperature. The microscope was equipped with a 100x oil-immersion objective Princeton™ charge-coupled device (CCD) camera. The observed images of the DAPI-treated DNA were quantified by using a computer image software Object Image™. DNA and lipid particles were observed after treatment with DAPI and FM 4-64, respectively in various concentrations until the best visualization was achieved. The quantified images were tranfered to Adobe Photoshop 5.0™ or Canvas™ and printed on a high quality dye printer.

### Results and Discussion

Since, the stability of the DNA-liposome formulations is a detrimental factor for subsequent cellular studies, the first contacts between nuclic acids and lipids, namely, adhesion, aggregation of DNA onto liposomes, and energetics of cation-induced complex formation between them, are emphasized. The present work describes the preliminary measurements on poly(ribo)nucleotide-zwitterionic liposome self-assembly formation as a possible alternative for the currently employed problematic cationic lipids in gene delivery, as well as for further use in nucleic acid chromatography.

### Phase behaviour of neutral liposome-DNA structures

The potential of complexes formed between polynucleotides and oppositely charged cosolutes for use in nucleic acid separation, purification and gene transfection is now well-established. The objectives are two-fold: to employ them as a controlled pharmaceutical formulation for gene delivery trials, and their use in DNA chromatography for analyses. In both cases, the energetics of nucleic acid-phospholipid associations becomes important for deducing the resultant structures. Thus, measuring their phase behaviour, followed by their further morphological and structural characterization would provide further clues for creating thermodynamically stable lipid-DNA nanostructures.

To mimic the route of action of more effective viruses and having considered the current cytotoxicity problems of cationic lipids, our current research has been focused on complexes formed between neutral liposomes and DNA, induced by inorganic and surfactant cations acting as condensing agents. The motivation for such design has come from relevant reports on stimulatory effects of $Ca^{2+}$ and $Mg^{2+}$ on transfection efficiency (22,23).

Figure 1. depicts thermotropic phase transitions of DPPC multilamellar dispersions in the presence of poly(A:U)$_n$ and calf thymus DNA, used here to show the effect of various length, size and conformation of nucleic acids on complexation with zwitterionic lipid, in the presence of $Mg^{2+}$. $Mg^{2+}$ ions could slightly affect the apparent specific heat capacity ($\Delta C_p$). Interestingly, the phase separation between DPPC-MLV and poly(A:U)$_n$ indicates that interactions and stabilization of polyribonucleotide chains occur even in the absence of metal cations. The effect of chain length and conformation is more apparent. Specific heat capacity is higher for this polyribonucleotide, in comparison with the

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calf thymus DNA. It is still not clear whether under these experimental conditions this effect is dependent on base composition, sequence or size(18,19), since in this model nucleic acid binds to lipid surfaces in a sequence-independent manner.

Figure 2 depicts DSC heating scans of DPPC vesicles and their ternary complexes with calf thymus DNA in the presence of either $Mg^{2+}$ or $C_{12}TMA$. The observed peak distribution indicates that the fraction of liposome-free DNA is less encountered than the bound DNA in the lipoplex. The liposome-DNA association results in a decrease in $\Delta C_p$. Interestingly, no DSC signal was detected for the $Mg^{2+}$-DPPC mixture (data not shown), previously observed turbidimetrically (18). Addition of C12TMA to the DPPC-ULV-DNA mixture results in broadening of the thermograms, consequently leading to difficulties in estimation of their onset point, peak top, and hence the endothermic effect. For these reasons, the quantitative evaluation of the surfactant effects should be conducted with precaution. The comparison of their properties (reported here) with respect to DNA-liposome condensation to that of $Mg^{2+}$ has been performed in here, in order to test the electrostatics and hydrophobicity reasoning with regarding to the thermodynamics of phospholipid binding to DNA followed by its compaction. DSC is a very sensitive way of studying certain changes in bilayer packing, with the presence of units interfering with chain packing in the bilayer causing a decrease in the temperature of the main transition (24). The incorporation of a substance in a liposome bilayer has a more profound effect on the lipid phase transitions peaks. The shift or disappearance of pretransition or main phase transition can thus be used as an indication of the existance and level of included materials in the leaflets. Thus, the entrapment of molecules in liposomes could be quantified by determining the change in temperature of the transition onset, as well as measuring the peak temperature as shifts in the transition temperature.

### DPPC-detergent-DNA complexes

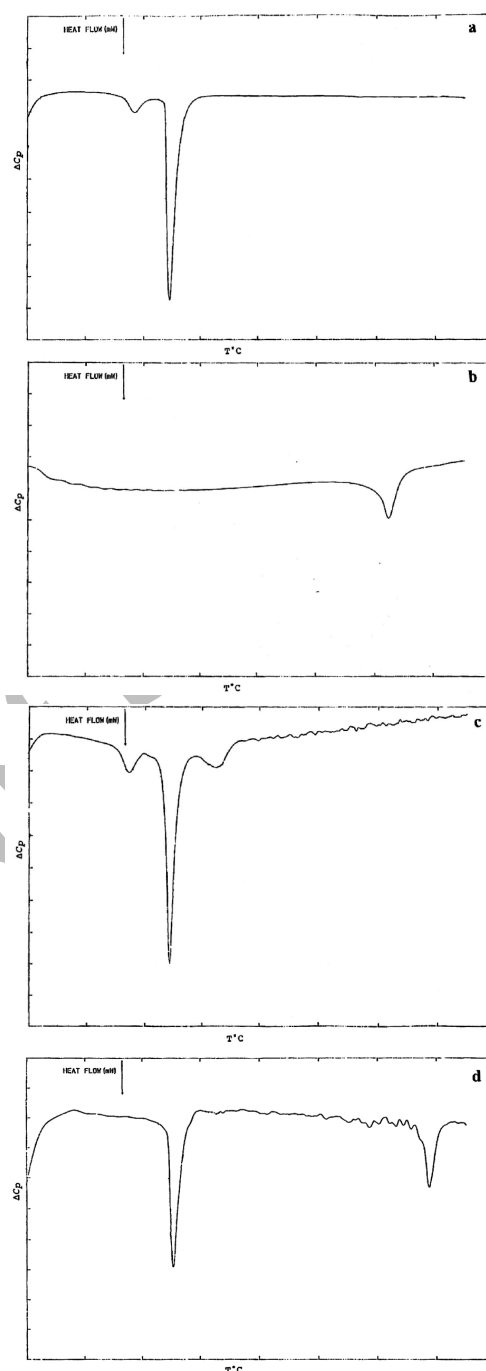As surfactants are used for protein extractions from phospholipid membranes (25), specifically



**Figure 1.** Microcalorimetric phase behaviour of DPPC multilayers in the presence of poly(ribo)nucleotides and $Mg^{2+}$. $\Delta Cp$ bar is 0.5 $J.K^{-1}$. Concentration of the synthetic biopolymers were: 1 mM of (A):(U) bp of poly(A:U) duplex (Sigma), and 1 mM of DPPC (assuming average molecular mass of dimensionless 770) and DNA (assuming average molecular mass of base pair of 643 and molecular weight of calf thymus DNA of 1,800 kDa) in 10 mM HEPES/10 mM NaCl; pH=7.2, cell volume 1.5 ml. Differential scanning microcalorimetric measurements were performed using SETARAM DSC microcalorimeter, equiped with Hewlett-Packard PC and company supplied software. (a): DPPC-MLV+MgCl$_2$; (b): poly(A:U)n+MgCl$_2$; (c): DPPC-MLV+poly(A:U)+MgCl$_2$; (d): DPPC-MLV+calf thymus DNA.
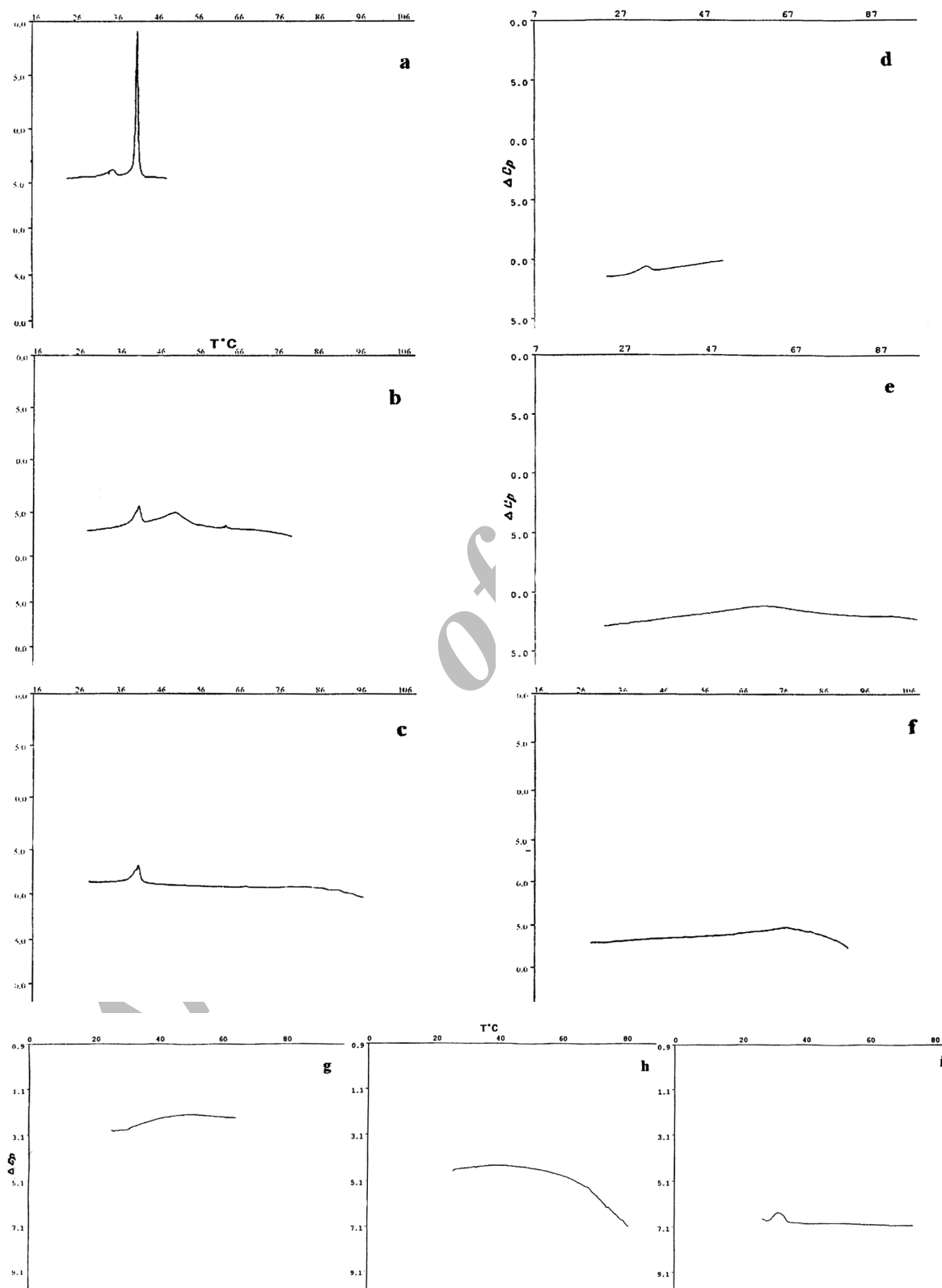
**Figure 2.** Phase behaviour of the mixtures of DPPC-ULV with calf thymus DNA in the presence of $Mg^{2+}$ and $C_{12}TMA$, respectively. (a) DPPC-MLV; (b) binary DPPC-ULV- DNA mixture; (c) equimolar ternary DPPC-ULV- $Mg^{2+}$-DNA complex; (d) DPPC-ULV-DNA complex in the presence of $C_{12}TMA$; (e) DPPC-ULV-DNA complex in the presence of $C_{12}TMA$ following heating of the sample; (f) DNA- $Mg^{2+}$ binary mixture. (g) DNA- $C_{12}TMA$ binary mixture; (h) DNA; (i) DPPC-ULV- $C_{12}TMA$ binary mixture.

or non-specifically attached to cell membrane fractions, it is worth examining these complexes. Hence, it is needed to study their binding characteristics, as drugs form complexes with proteins, membrane phospholipids and nucleic acids (DNA and RNA), and their subsequent release depends on the strength of binding and the reversibility of these interactions. These interactions, could be examined by thermodynamic measurements. The DSC scan (d) represents a thermogram of a complex formed between DPPC and DNA in the presence of C12TMA ions. Evaluation of the physicochemical stability of samples with respect to temperature variation is crucial due to their thermodynamic profiles in terms of manufacturing issues. Since the enthalpy of binding is a temperature dependent event (16), the effect of heating the sample is emphasized [Figure 2 (e) vs. (d)]. The single melting peak corresponding to the ternary complex formed between DPPC-C12TMA-DNA appears at approximatly 33˚C. The melting behaviour of the control sample DNA-C12TMA binary mixture (Fig. 2 (e)) is composed of two separate phases. The first situated at 47˚C belongs to DNA phase, the separate melting behaviour of which is shown with two different concentrations in figure 2 (h). The second phase is a structural transition observed after 85˚C and continues until 100˚C, corresponding to the complex of DNA bound to C12TMA. This type of melting behaviour indicates the occurance of an aggregation reaction between the latter molecules. At the end, the maximal membrane deformation causes its breakage. While, large enthalpy variations with temperature are compensated by the hydrophobic component of entropy, it is possible to estimate electrostatic and hydrophobic contributions to the enthalpy at various temperatures by applying additivity(19). Figure 2 (g) is a DNA sample, prepared as described in the experimental section, showing a typical melting behaviour of DNA and its synthetic models, which appears as a single peak. This highly cooperative process represents unwinding of the double stranded structure into two polynucleotide strands, which fold into separate chaotic globules. The equality of the heat capacities of the native and denatured DNA represents a typical feature of this process, which

makes quantitative determination of thermal effects of melting, as well as free energy $\Delta G$ of stabilization of the native DNA structure easier. The prediction is that the position of the binding peak would be shifted upon changing the DNA concentrations. The various DNA concentration in lipoplexes, result in a prolonged second peak, compared with that of the higher DNA amount, as also indicated by the experimental enthalpies. However, the relationship ofthis effect to that of transfection efficiency of these lipoplexes still remains to be elucidated. In this case, the effect of heating and aggregation is more profound. Such dependance of the electrostatic component of binding Gibbs free energy and entropy on the reactant concentration suggests the existance of an aggregation reaction, which was also observed in DNA-lipid complexes (18,19).

### DNA-Mg$^{2+}$ binary mixture

The Mg$^{2+}$ions at equimolar amounts with DNA, increase the $T_m$ value by 33.7ºC, due to Mg$^{2+}$-induced duplex stabilization (Fig. 2 (f)). ULVs treated with the same Mg$^{2+}$ concentration did not produce such a shift, which is normally detected spectroscopically (18). Mg$^{2+}$ induces the formation of substantial amounts of circular DNA, suggesting that Mg$^{2+}$ cations stabilize the interaction of polynucleotide cohesive ends, the effect being dependent on the concentration of MgCl$_2$ and possibly being a sequence-specific event (26). The formed circular molecules are stabilized by Mg$^{2+}$, but they are not covalently closed. Although, Mg$^{2+}$ stabilizes end-to-end interactions, it is likely that a dynamic equilibrium exists between the linear and circular fragments.

### DNA-surfactant complexes

The major problem with the use of surfacatnts is that the data are difficult to analyze. The amount of surfactant adsorbed at the surface is also not known and has to be either measured or inferred from thermodynamic models (27). Moreover, DNA alone has no surface activity and thus needs a "helper" molecule. DNA backbone is quite hydrophilic and may not have enough affinity with the surfactant chain to produce a significant overcharging, unless salt is added. Data show that surfactant

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binds to nucleic acid with a high degree of cooperativity. This kind of interaction results in stabilization of polynucleotide chains, due to electrostatic binding of cationic surfactants to DNA polyanions, followed by hydrophobically driven complexation of new surfactants resulting in phase separation of unbound and DNA-bound surfactant molecules (28). In the bulk, the majority of surfactants are free and complexation with DNA occurs at the surface. Above the critical concentration surfactant molecules cooperatively bind with DNA, leading to a substantial concentration of hydrophobic surface-active aggregates within the solution until the point of surface saturation has been reached. The binding of a greater number of surfactants is favored by the decrease in hydrophobic energy of the CH-parts. In lower amounts, binding of $C_{12}$TMA to DNA is not favored thermodynamically, as the system lowers its free energy by keeping the surfactant in the bulk, thus gaining entropy. Binding occurs by increasing the density of the system, which gives rise to electrostatic gain and compensations of the entropy loss.

The described mode of DNA complexation with the cationic surfactant is highly dependent on its' molecular structure (29) and concentration (27-30]). The results could be considered of interest for gene delivery designs. DNA compaction has been achieved using high and toxic amounts of lipids and surfactants. This could be also performed by careful selection of surface active compounds, with controllable electrostatic and hydrophobic properties. Addition of surfactants results in the cooperative binding seen as a phase transition, which can undergo charge reversal of its' complexes with DNA, when added in higher quantities. Thermodynamic stability features can be exploited for designing new stationary phases for conformation-specific DNA purification protocols. Such an induced association of cationic surfactant to DNA decreases the effective negative charge of the polynucleotide, favoring the DNA-surfactant complexes to approach a negatively charged target cell membrane. The mechanism related to the interaction of DNA with surfactants, is expressed briefly as: free surfactant+DNA→

DNA-surfactant aggregates, seems to be also valid for other systems, such as the interesting dissymetric gemini surfactant binding to DNA (31). Interestingly these structures with different chain lengths can transfect DNA better than that the individuals could achieve alone.

**Surfactant-lipid binary mixture**

$C_{12}$TMA shifted the main phase transition temperature ($T_m$) towards lower degrees and increased the transition enthalpy. This indicates that this surfactant somehow destabilizes the gel phase of DPPC vesicles, showing a fluidizing effect on them. While, both monoalkyl chain- and gemini surfactants are active at membraneous surfaces, the DSC heating curve depicts the important differences between them. Thus, they bind differently to liposomes, as deduced from the observed shifts of $T_m$. Apparently, $C_{12}$TMA binds to interfacial regions, while gemini surfactants form partially associated micellar structures in bulk solution prior to liposome recognition (32).

**The structure of DNA-neutral liposome aggregates**

Structures similar to cationic-DNA complexes are likely to occur (33). Lipid-DNA arrangements are probably formed as overlaying layers of DNA adsorbed onto lipid bilayers, following charge neutralization, governed by the adsorbed cations ($Me^{2+}$) on the surface of the cationic lipids. The formation of alternating lipid-DNA assemblies is due to the arrangements of DNA as parallel condensates between the lipid bilayers. A similar structure is reported for more biologically significant virus-membrane arrangements (34). This is expected, due to 3-D correlation forces between the DNA-covered lipid layers, after nucleic acid-induced formation of MLVs from ULVs.

The process of DNA compaction was followed by fluorescent microscopy (data not shown). The compaction process could be achieved by treating nucleic acids with cationic surfactants, PEG, metal cations and liposomes. The structures are highly dependent on size and conformation of nucleic acids. Therefore, deducing such arrangements for plasmid DNA and higher order nucleic acids should be carred

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out with precaution. Under the employed conditions, the initially relaxed DNA in solution is complexed by surfactant molecules, which adsorbs on the nucleic acid surface forming micelle-like domains. C12TMA molecules also partition into lipid bilayers, forming swollen mixed bilayers. Afterwards, the DNA in unfolded form is apparently adsorbed on to the surface of surfactant-DPPC vesicles, as also suggested by a fluorescent study on neutral lipids, employing cationic surfactants (35). Since surfactant molecules become incorporated into the liposome bilayers, due to the partition equilibrium between bilayer and the aqueous phase, normally the binding of C12TMA–DNA complexes to the vesicle surface through hydrophobic forces results in opening of the micelle-like domains and partitioning of C12TMA ions in the lipid bilayer. Hence, employment of cationic surfactants tend to form a fully relaxed DNA, which is bound with significant stability to plasma membranes, resulting in its'difficulty to internalize within the cell structure by endocytosis. In contrast, unilamellar phosphatidylcholine vesicles interact with DNA via a mechanism of nucleic acid helix-induced liposome aggregation and subsequent lipid fusion. A liposome with higher negative curvature could be formed, which is advantageous for further increasing the gene transfection efficacy. Thermotropic phase behaviour reported in figure 1 and 2, probably represent several intermediary DNA-lipid structures (9). Usually, several MLV aggregates around a $Mg^{2+}$-induced compacted DNA as grapes, determined by thermodynamic preference factors. ULVs follow DNA-induced fusion, resulting in generation of liposomes with preferable higher curvature. Even though the suggested structure remains to be confirmed by further structural analyses, such a local DNA unwinding is likely to occur, because of the spectroscopic evidence for significant disruption of the planar interactions between the bases (35,36).

In general, lipoplex should be positively charged and cause membrane perturbation and local defects resulting in size instabilities, in order to achieve an efficient *in vitro* transfection(9,10). Whether the electrostatics of the formed DNA-liposome complexes is relevant to efficacy of transgene expression is still unclear, but it plays a major role in genosome formation and at least at several stages of the transfection process. Thus, lipoplexes are formed by electrostatic neutralization of the employed lipids and nucleic acids(DNA, poly(ribo)- or oligodeoxynucleotides and RNAs). The process is governed by the removal of their small counterions, depending on lipid and medium composition, and on conformation and concentration of nucleic acids. $Mg^{2+}$ binds to lipid headgroups, causing their condensation, increasing vesicle size and inducing defects promoting curvature changes on the surface of liposomes. These perturbations lead to interfacial exposure of the phospholipid bilayer, resulting in aggregation and fusion of the lipid vesicles.

The process is hydrophobically and electrostatically controlled, since liposomes aggregate and fuse in the presence of oppositely charged particles (37). In the currently presented system, zwitterionic liposomes fuse in the presence of $Mg^{2+}$. Hence, polyanions like DNA play an active role in adhesion, aggregation and fusion, by bridging two liposomes in close contact, with surface adsorbed metal cation-induced fusion. Since, a variety of possibilities exist regarding the structure of liposome-DNA formulations, it seems that, besides contributions due to charge neutralization or relative lipid/DNA ratios, the absolute concentration of the engaged system components play an additive role in thermodynamically preferred lipoplex structure formation.

The mechanisms underlying the delivery of therapeutic genes, using lipid-based gene carriers, is still controversial. Besides all other factors, the conformation of nucleic acids appears to play an essential role. In order to make progress one of the possible mechanisms, the involvement of induced conformational changes in the resulting phases and influence on lipid-DNA structure formation with their further relevance for transfection efficacy, was studied. The physicochemical approach presented here was performed to test the ability of single- and double stranded polynucleotide chains to promote membrane fluidity changes, vesicle surface destabilization, changed sensitivity to
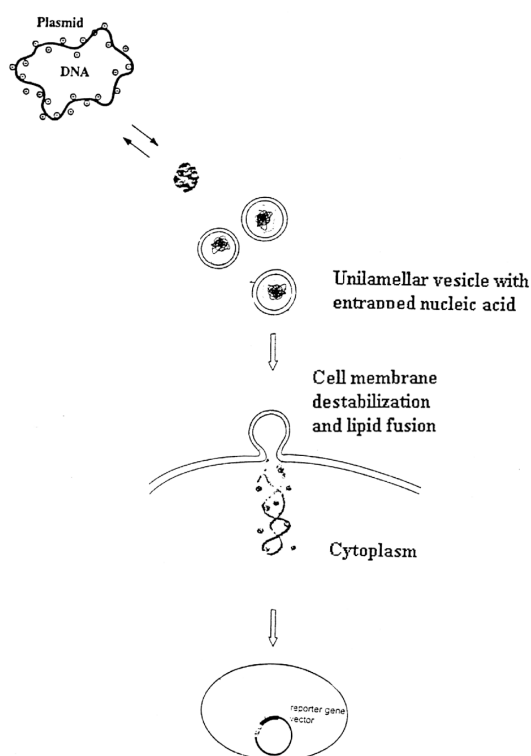
**Figure 3.** Possible mechanism for liposome-cell fusion and further internalization of lipoplexes mediated by nucleic acid.

ions and to deduce how these features could be related to nucleic acid-$Me^{2+}$-mediated translocation of DNA through biomembranes. Given the structural information available from the literature and based on our own data, a model for the membrane translocation can be proposed (Figure 3), based on the emerging relationships between transfecting cationized phospholipids and the cellular anionic lipid phases.

The first step is the formation of nucleic acid-liposome (lipoplex) complex in either MLV or ULV formand its' approaching of the target cell surface via suraface forces. DNA first dissociates from lipoplexes due to neutraliztion by anionic lipids of the target cell surface through fusion between them. As a result nucleic acid expands, as seen by fluorescence microscopy in a difficult to measure rate. The effect is reversible and highly depends on lipid phases. Thus, the measurable parameters such as hydration, temperature, pressure, surface tension, etc can be used to predict the particular vesicle curvature. Formation of non-lamellar phases is highly probable, with further effects on transfection. The rate of fusion and DNA

release is governed by the obtained higher negative lipid curvature. It is not clear at this stage whether both interfacial electrostatics and hydrophobicity are engaged in this process. Despite the previous data supporting the role of both these parameters, recent results from confocal imaging and fluorescence correlation spectroscopy study indicates that at least for single-stranded oligodeoxynucleotides their transfer across membranes of giant vesicles used to mimic the cell surface is similar for both negatively charged and neutral lipids and that the transfection efficiency of the lipid-DNA complexes is independent of their charge density (38). Apparently, the lipid composition of the cell membrane is more important in this type of recognition. Subsequently, the uptake of the lipoplex occurs at the aqueous membrane surface of the cellular surface, during which unfolding of the hydrophobic core of the nucleic acid is probable. The third step is composed of insertion and translocation of the polynucleotide chain into the membraneous phase, governed by lipid-lipid, lipid-protein and DNA-integral membrane protein associations. In this respect, the mechanism differs from the pore formation route of cell-penetrating peptides(39,40). The exact number of forces engaged in this structure remains to be studied. The last step corresponds to the cellular internalization of the DNA into the cytoplasm through diffusion, which is the next hurdle as it is a poor solvent reservoir for polynucleotides. Such a model is similar to the model proposed for cationic lipids.

**Relevance of the structures to gene transfection**

The proposal is in accordance with generally accepted principles of action of lipoplexes (1-11), being DNA condensation, compaction and complexation with cations, endocytosis, and nuclear trafficking. Polyanionic DNA is apparently condensed with cationic transfection reagents such as surfactants, or complexed with cationic or neutral liposomes, as described prior to cellular targeting. These are taken up by cells, mostly through endocytosis. Electrostatics governing the recognition of anionic lipids at cell surface is difficult to control. In addition, the use of $Mg^{2+}$ leads to a heterogeneous size

distribution of lipid-DNA assemblies. Frequently occuring MLVs represent other hurdles for uptake. Therefore, recent efforts are devoted to non-electrostatic vesicle design (43), relying on weaker H-bond formation. The occurence of any of the four mechanisms known so far for internalization of liposomal contents into the cells, liposome adsorption on the cell surface; adsorption of liposomes followed by selective transport of their lipophilic compounds from vesicle bilayer to plasma membrane; endocytosis of liposomes and subsequent degradation of its content and lipid fusion of vesicles with cellular surface, depends on lipid composition, charge, size, lamelarity, nucleic acid conformation and concentration, as well as the presence of blood or serum. Due to the resemblance of the viral entry route, liposome-cell fusion using fusion-inducing agents is preferrable.

Avoiding degradation of the internalized DNA by endocytic events and by cytoplasmic nucleases is a great goal. Usually, too few cells receive and express foreign DNA and even survived nucleic acids within the cytoplasm must dissociate either before or after entry into the nucleus, for further gene transcription to occur. Despite the current desire for employment of lipid-based gene delivery tools, lack of targeting, uncertainities concerning the structure of DNA-lipid complexes, and heterogeneity are problems awaiting their solution. In addition, if employed for systematic delivery, the DNA is subjected to blood clearance occuring as opsonization, which removes arround 90% of the hydrophobic particles in blood (1) and constitutes the major limitation for using lipid vesicles (1-11, 44, 45). Usually neutral multilamellar and unilamellar vesicles follow a slower clearance rate than negatively and positively charged MLVs. ULVs are characterized with longer residence times than MLVs (7, 9-11). The model based on data obtained with isolated macromolecules does not necessarily correspond to that existing *in vivo*. Deductions drawn from experiments with cell cultures used for studying liposome-cell interactions should be handeled with precaution, since the situation often looks different *in situ*. The event seems to be cell type-specific and shows general dependence

on experimental protocol, presence of serum, or other undesired complexating agents and temperature. The success of transfection assays frequently relies on the particular gene reporter system employed.

To keep the model simple, we focused on relatively simple lipid phase transitions. It is clear that the mechanism proposed is oversimplified in terms of unclarified implications of this sort of delivery in intracellular trafficking and gene expression. However, data presented here, as well as that taken from the literature, give further support for generating new insights and hypotheses on non-viral gene transfer vectors. Thus, such an approach helps the identification of further details concerning cellular uptake of lipoplexes, DNA escape from endosomes, nucleocytoplasmic delivery and nuclear uptake. The liposome-DNA structures proposed still have to be confirmed by other methods. Transfection assays report a limited efficiency with neutral lipids (38,46). Moreover, in contrast to our suggestion, T. Stegmann and J.-Y. Legendre (47) observed that the transfection efficiency is not determined by efficiency methods such as the of membrane fusion or lipid mixing.

Animal species differences, as well as hurdles regarding *in vivo* neutral liposome-cell interactions, uncertainities of their kinetics and tissue and intra-organ distributions are issues remaining to be elucidated (44,45-47). However, recent reports on employment of neutral liposomes (48,49) are encouraging, and studies should be continued to examine them as alternative therapeutic gene formulations.

The concept of metal-based pharmaceuticals, undertaken here, will open new insights for studying whether these supramolecular complexes follow the similar principles of binding to cellular receptors and will further define the issue of nucleic acid receptors on cell surfaces.

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