

# **A nanometric DNA-zwitterionic phospholipid unilamellar vesicle formulation compacted for gene delivery: Adiabatic differential scanning microcalorimetric study**

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## **Abstract:**

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Complexes between nucleic acids and phospholipid vesicles have been developed as stable non-viral gene delivery vehicles. Currently employed approach uses positively charged lipid species and a helper zwitterionic lipid, the latter being applied for the stabilization of the whole complex. However, besides problematic steps during their preparation, cationic lipids are toxic for cells. Present work describes some energetics issues pertinent to preparation and use of neutral lipid-DNA self-assemblies, thus avoiding toxicity of lipoplexes. Differential scanning calorimetry data showed stabilization of polynucleotide helix upon its interaction with liposomes in the presence of divalent metal cations. It is thus possible to suggest this self-assembly as an improved formulation for use in gene delivery.

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**Key words :** DNA-liposome self-assembly, thermodynamics, divalent metal cation, differential scanning microcalorimetry, non-viral gene delivery

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Nucleic acids-membrane associations comprise the least functionally studied macromolecular assembly, yet attract the attention of researchers due to their potential in the field of gene therapy (Leckband & Israelashvili, 2001). The design of novel nucleic acid delivery formulations proceeds mainly as searches of alternatives to highly efficient but risky viral based vehicles (Templeton, 2001; Liu & Huang, 2002; Miller, 2003). The main objective is to achieve compaction of genetic material within highly restricted compartments, while decreasing its cytotoxicity. In the light of well-established potential of liposomes as gene carriers (Templeton, 2001), the current work concerns mainly the stability and physical properties of DNA within the lipid surrounding. Such particles, referred to as lipoplexes are composed of positively charged lipid species and a helper neutral lipid, used for the stabilization of the liposome complex. Despite the considerable efforts that had been made to characterize the structure of these complexes, the origin of molecular forces responsible for self-assembly formation, determination of their charge, colloidal properties, stability against dissociations, cytotoxicity issues, and unravelling characteristics related to efficient intracellular delivery and gene expression remain unclear. A possible alternative to the toxic cationic lipids is the employment of zwitterionic lipid species, which are much safer for target cells (Kharakoz, 1999). Neutral liposome-DNA self-organization is mediated by various inorganic cations, acting as condensing agents. In the light of recent strong evidence that divalent cations enhance the efficacy of plasmid DNA-cationic lipid formulations (Lam & Cullis, 2000), it is of particular interest to study the effect of different divalent cations on the transfection potency of lipid-DNA complexes. In this context, a preliminary results of promising ternary DNA-dipalmitoylphosphatidylcholine (DPPC) -  $Mg^{2+}$  complex preparation and its thermodynamic properties are presented herein.

## Materials and Methods

### Materials

Synthetic DPPC and calf thymus DNA were kind gifts of Prof. P. Balgavy (J. A. Comenius University, Bratislava, Slovakia). EDTA was purchased from Sigma Chemical Co., St. Louis, MO, USA.  $MgCl_2 \cdot 6H_2O$ ,  $NaHPO_4$  and NaCl were obtained from Merck, Darmstadt, Germany. The presented nucleic acid concentrations and the molar ratios are based on the average nucleotide molecular weight of 308 calculated from the known DNA composition employed (Uhrikova, *et al.*, 1998).

### Methods

#### Preparation of vesicles

1.2 mM lipid in standard SSC buffer, pH = 7.2 was used in all experiments and was stored at 4°C. The formation of a thin layer of lipids of a 15 ml round-bottomed flask was achieved by a hand-shaking and hydration in SSC buffer (20 x concentrated aqueous solution, composed of Na-chloride 175.32 g/L (3 M) and  $Na_3$ -citrate x 2  $H_2O$  88.23 g/L (0.3 M)) at around 70°C. Vortexing of the lipid with the desired aqueous solution above the gel-to-liquid crystalline phase transition of the lipid ( $T_m$ ) for around 30 min resulted in multilamellar vesicles. The DNA concentration used throughout all experiments was 1.8 mM based on the abovementioned assumption. Unilamellar vesicles were obtained by extrusion of multilamellar vesicle 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 homogeneous vesicle suspension. This allowed the preparation of vesicles with a mean diameter of 90 nm and a trap volume in the range of 1.5-2.0 l/mole.

### Preparation of liposome-nucleic acid mixtures

DPPC-DNA formulations were obtained by mixing appropriate volumes of unilamellar vesicles dispersion, calf thymus DNA solution and  $MgCl_2$  solution in SSC buffer to obtain the desired molar ratios.

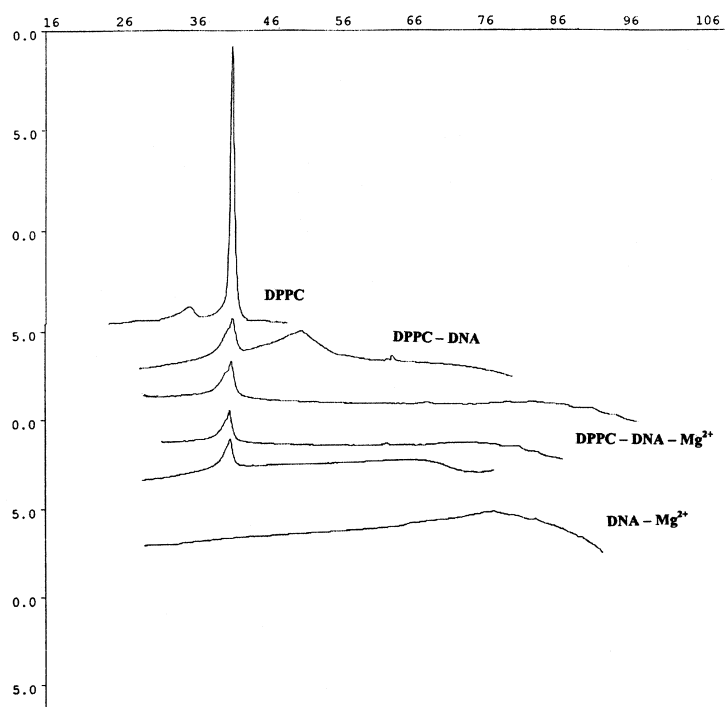
### Differential Scanning Calorimetry

Differential scanning microcalorimetric measurements were performed employing Privalov type DASM-4 adiabatic differential scanning microcalorimeter (Russian Academy of Sciences-Puschino, Moscow Region) with a scan rate of  $0.5^\circ K.min^{-1}$ . Instrumental base line calibration mark was obtained by scanning at  $50 \mu W$ ,  $T=4$ , as described (Ivanov, 1988).

### Results and Discussion

Although double-stranded DNA (dsDNA) has been shown to bind to zwitterionic lipids (Malghani & Yang, 1998), its thermodynamic stability features remain to be elucidated. Only results obtained with unilamellar DPPC vesicles are presented, in the light of recent evidence for their better performance in gene delivery studies with respect to internalization mechanisms (Templeton, 2001). The interaction of calf thymus DNA with phosphatidylcholine liposomes in the presence of  $Mg^{2+}$  ions was studied using the adiabatic differential scanning microcalorimetry.

Figure 1. depicts representative thermograms of DPPC liposomes and their complexes with DNA and  $Mg^{2+}$ . The first curve on the top is



**Figure 1.** DSC heating scans of the main phase transition of DPPC multilayers and unilamellar vesicles obtained from them, upon their associations with various amounts of calf thymus DNA and  $0.5 \text{ mM } Mg^{2+}$ . Each complex is denoted in the abbreviated form. Data is presented as excess apparent heat capacity vs. temperature curves. Details of sample preparation and measurements are outlined in Materials and Methods.

a calibration and shows typical DPPC multilayer phase transitions, with pre-transition at 36°C with a  $H_{cal} = 3.9$  kJ/mol and the gel-liquid crystal, or main phase transition ( $T_m$ ), at 41.9°C. The subsequent lower curves show the phase transitions upon interactions of DPPC with various quantities of DNA and  $Mg^{2+}$ . DPPC unilamellar vesicles' thermogram peak appeared broader with a decreased maximum and the pre-transition peak disappears (data not shown). The curve denoted for nucleic acid-phosphatidylcholine mixture DPPC-DNA) indicates that phase change occurs with unilamellar lipids (41.9°C) and their complexes with nucleic acid. The latter lipid-DNA phase showed its peak further at around 51.3°C when mixed in equimolar amounts (1:1). In this type of complex formation, the measured value of  $T_m = 41.9$  and  $H_{cal} = 31.9$  kJ/mol were determined. The interaction with liposomes resulted in the significant decrease of excess apparent specific heat capacity.

The next three lower curves, marked as DPPC-DNA- $Mg^{2+}$ , show the phase change behaviour of ternary mixtures of DPPC:DNA: $Mg^{2+}$  in 1:1:1, 1:3:1 and 1:5:1 ratio, respectively. The curve of the equimolar preparation possessed a narrower unchanged (41.7°C) main signal, compared to DPPC vesicles' peak, with a further change to the DNA phase transition. The  $T_m$  value remained, however  $H_{cal}$  diminished to 9.7 kJ/mol. Interestingly the DNA phase peak moved to 89°C. The first peak was at the main phase transition temperature corresponding to the melting of lipid. The second one is at 89°C and corresponded to the DPPC-DNA complex. This latter peak is attributed to the stabilization of the DNA secondary structure by tight packing of DNA molecules in unilamellar vesicles, bridged by  $Mg^{2+}$ -ions. This is a particular case of liposome surface induced nucleic acid condensation of the "spaghetti and meatballs" structure (Templeton, 2001). The effect is driven by surface cationization of vesicles, sensed by a conformational change in the choline group of DPPC. It tilts towards the bilayer surface plane since its positively charged quaternary nitrogen is

attracted by the opposite charge of the nucleic acid polyanion. The main phase peak sharpened upon increasing the DNA amount threefold, as shown in the next lower curve. Interestingly, the DNA phase peak shifted to a lower value of 71°C. This trend was maintained upon increasing the DNA amount further (ternary molar ration of 1:5:1). The triple complex of DNA-metal ion-phosphatidylcholine vesicle remained stable at different incubation times, which is in agreement with small- and wide-angle X-ray scattering measurements (Uhríkova *et al.*, 1998). Apparently,  $Mg^{2+}$  decreases the DNA effective radii and creates groove narrowing, by ligand binding to six or eight water molecules, or alternatively to nucleic acid phosphate in the minor groove in a fully hydrated state (Hud & Polak, 2001).

The bottom curve denoted as DNA- $Mg^{2+}$  represents equimolar mixture of DNA and  $Mg^{2+}$ , which had a major signal at around 85°C, which indicates a divalent cation triggered high temperature DNA stabilization. Unilamellar vesicles treated with the same concentration of  $Mg^{2+}$  did not produce such a shift. Obviously, divalent metal cations do not contribute essentially in the stabilizing of zwitterionic lipid structure. Therefore, DNA contributes to the stabilization of ternary complex towards higher temperatures. Apparently,  $Mg^{2+}$ -DNA creates polymorphic phase transitions in phosphatidylcholine moiety.

It is well-established that positively charged particles deliver DNA into cultured cells by electrostatic mechanisms of binding to the negatively charged membranes. Liposomes enter cells by various routes, such as through endocytic pathway and direct membrane fusion. Gene delivery designs involving receptor mediated transfer face problems, since endosomes fuse rapidly with lysosomes, thus degrading the nucleic acids. The ternary complex between nucleic acid, divalent inorganic cation and extruded liposome formulated from zwitterionic lipid, described in this work, can deliver genes into cells via direct fusion with the cell membrane. This model is in accordance with a recent proposal (Templeton,

2001). The major advantage of such non-viral nanocondensate formulation is the ability to act across tight barriers *in vivo*.

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