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Divalent Cation Induced DNA–Zwitterionic Vesicle Formulation Compacted for Gene Delivery: Thermodynamic Aspects

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Divalent Cation Induced DNA–Zwitterionic Vesicle Formulation Compacted for Gene Delivery: Thermodynamic Aspects[#]

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Abstract

Motivation. 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. The present work describes some energetic issues pertinent to preparation and use of neutral lipid–DNA self–assemblies, thus avoiding toxicity of lipoplexes. Thermodynamic measurements 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.

Method. Thermotropic phase transitions of zwitterionic liposomes and their complexes with DNA in the presence of Mg^{2+} is investigated and compared to the available data for neutral/cationic lipid binary nanocondensates with nucleic acids. Adiabatic differential scanning calorimetric measurements of synthetic phosphatidylcholine vesicles and calf thymus DNA and their ternary complexes with Mg^{2+} were used to deduce the thermodynamic model describing their structural transitions.

Results. The increased DNA–mediated ternary complex thermal stability is achieved by affecting the melting transition temperature of the lipids by nucleic acid induced electrostatic screening of the phospholipid bilayers.

Conclusions. Although the pharmacodynamical features of the zwitterionic lipid–Mg^{2+}–DNA nanocondensates remain to be tested in transfection experiments, at least from physicochemical viewpoint, their stability data is encouraging to approach them as a novel DNA–based delivery formulation.

Keywords. Neutral lipid–DNA self–assemblies; Mg^{2+}; adiabatic differential scanning calorimetry; phase transitions; non–viral gene delivery.

Abbreviations and notations

CL, cationic lipid	ULV, unilamellar vesicles
DPPC, dipalmitoylphosphatidylcholine	ΔC_p, heat capacity
DSC, differential scanning calorimetry	ΔH_{cal}, enthalpy
MLV, multilamellar vesicles	

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1 INTRODUCTION

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 [1]. The design of novel nucleic acid delivery formulations proceeds mainly as searches of alternatives to highly efficient but risky viral based vehicles [2–4]. 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 [2,5], 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 structural factors affecting their subsequent transfection efficiency, the origin of molecular forces responsible for this 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 remains unclear.

A possible alternative to the toxic cationic lipids is the employment of zwitterionic lipid species, which are much safer for target cells [6]. 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 [7], 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 model ternary DNA–phosphatidylcholine–Mg^{2+} complex preparation and its thermodynamic properties are presented herein.

2 MATERIALS AND METHODS

2.1 Materials

Synthetic dipalmitoylphosphatidylcholine (DPPC) and calf thymus DNA were a kind gift of Prof. P. Balgavy (J. A. Comenius University – Bratislava, Slovakia). EDTA was purchased from Sigma Chemical Co., St. Louis, MO, USA. $MgCl_2.6H_2O$, $NaHPO_4$ 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 [8].

2.2 Methods

2.2.1 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 at around 70°C. Vortexing of the lipid with the desired aqueous solution above the gel–to–liquid crystalline phase transition temperature of the lipid (T_m) for around 30 min resulted in multilamellar vesicles (MLV). The DNA concentration used troughout all experiments was 1.8 mM based on the abovementioned assumption. Unilamellar vesicles (ULV) 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) creates homogeneous vesicle suspension. This allows 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.

2.2.2 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.

2.2.3 Differential Scanning Calorimetry

Differential scanning calorimetric (DSC) measurements were performed employing Privalov type DASM–4 adiabatic differential scanning calorimeter (Russian Academy of Sciences–Puschino, Moscow Region), with a scan rate of 0.5 $K.min^{-1}$. Instrumental base line calibration mark was obtained by scanning at 50 ΔW, $\Delta T = 4$, as described [9].

3 RESULTS AND DISCUSSION

Although double–stranded DNA has been shown to bind to zwitterionic lipids [10], 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 [2]. The interaction of calf thymus DNA with phosphatidylcholine liposomes in the presence of Mg^{2+} ions was studied using the adiabatic differential scanning calorimetry. Figure 1 depicts representative thermograms of DPPC liposomes and their complexes with DNA and Mg^{2+}. The first curve on the top is calibration mark and shows a typical DPPC multilayer phase transitions, with pre–transition 36°C with a ΔH_{cal} = 3.9 kJ/mol and the gel–liquid crystal, or main phase transition (T_m) at 41.9°C. The subsequently marked curves to the bottom represent the change of phase transitions upon interactions with various quantities of

DNA and Mg^{2+}. DPPC unilamellar vesicles' thermogram peak appears broader with a decreased maximum. The pre–transition peak disappears. The curve denoted for nucleic acid – phosphatidylcholine mixture (DPPC – DNA) indicates that phase separation occurs between lipids and the nucleic acid. In this type of complex formation, the measured value of T_m = 41.9°C and ΔH_{cal} = 31.9 kJ/mol were determined.

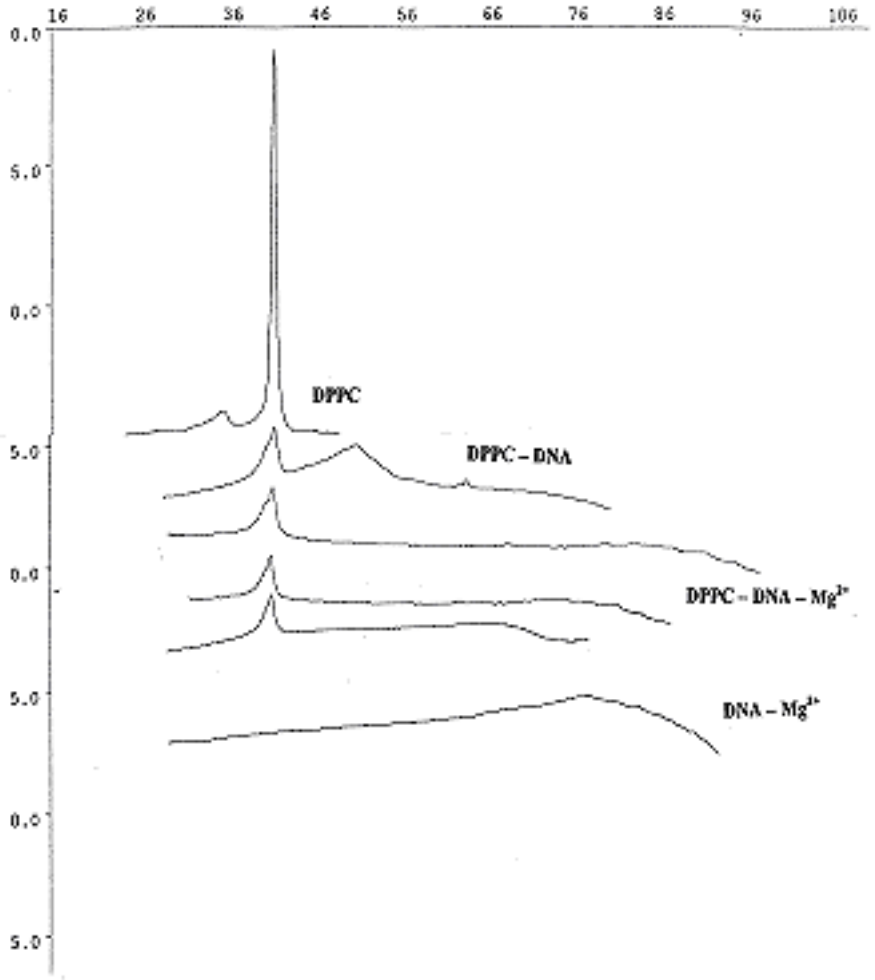

Figure 1. DSC heating scans of the main phase transition of DPPC multilayers and unilamellar vesicles obtained from them, upon their associations with various amounts of calf thymus DNA and 0.5 mM Mg^{2+}. Each complex is denoted in the abbreviated form. Data is presented as excess apparent heat capacity (ΔC_p) vs. temperature (T°C) curves. The thermograms are separated from each other at suitable distances for better representation. Details of sample preparation and measurements are outlined in Materials and Methods.

The interaction with liposomes results in the significant decrease of excess apparent specific heat capacity. Three separated phases are observed. The first one is the unilamellar vesicles' (ULV) phase peak, followed by DNA–liposome mixture phase having a peak at around 51.3°C, when mixed in equimolar amounts (1:1). The observed third peak belongs to nucleic acid melting and differs from that reported for plasmids. Thus, when plasmid DNA alone is measured as a thermogram, it possesses a peak at 60°C belonging to linear and open–circle plasmid DNA.

Another peak of such plasmid DNA phase usually is much larger, observed above 80°C, corresponding to supercoiled form of the plasmid. The peak at 60°C is either due to deformation of liquid crystalline phase of supercoiled plasmid, or due to heat–induced transition from supercoiled to circular DNA, indicating unwinding and topological changes [11]. As oppose to irreversible plasmid melting behaviour, certain synthetic nucleic acids permit performance of hysteresis experiments. The observed thermal behaviour of complexes formed between zwitterionic phospholipids and calf thymus DNA (Figure 1) is similar to those reported for cationic lipid–DNA mixtures (CL–DNA) [12]. In both cases, the gel–to–liquid crystalline transition of the lipids in the lipoplexes is shifted to higher temperature values, compared to the transition temperature of the lipid alone. This indicates similar self–assembling features of cationic and neutral lipids with DNA, which is a function of charge ratio of the complexes. The next three curves to the bottom marked as DPPC – DNA – Mg^{2+} show the phase behaviour of this ternary mixtures in equimolar 1:1:1, or in 1:3:1 and 1:5:1 ratios with increased DNA amount, respectively. The equimolar peak possesses narrower signal, compared to DPPC ULV's peak, with further DNA phase separation. The T_m value remained, however ΔH_{cal} diminished to 9.7 kJ/mol. The specific heat capacity is maintained upon complexation with divalent cation. The main phase transitions shifts somehow to 41.7°C. Interestingly the DNA phase peak moves further to 89°C. At the 1:1:1 molar ratio of the triple complexation, the self–assemblies display two peaks. The first one corresponds to the main phase transition temperature corresponding to the lipid melting. The second one is at 86°C and corresponds to the melting of the DPPC–Mg^{2+}–DNA complex. The latter peak is attributed to the stabilization of the DNA secondary structure by a tight packing of DNA molecules with several 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 [2]. 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. Aggregation of adsorbed DNA on the ULV surfaces creates a local topological tension, followed by temperature–, concentration–, pH– and cell line–dependent nucleic acid intercalation on the membrane. The phospholipid–DNA complexation results in a shift in neutral lipid–DNA (NL–DNA) nanocondensate transitions toward higher temperatures Moreover, demixing gaps appear for small mole lipid fractions. Recent biophysical treatment [13] explains the observed peak broadening by this nucleic acid–induced demixing. In this respect, further electrostatic free energy change, ΔG_{el}, due to change in surface area related with gel–to–liquid crystalline–phase transition diminishes the transition temperature, ΔT_m. Maximum electrostatic shift due to introduction of surface charge and entropic effect of counteions become main effects. Such a Gouy–Chapman approach treats the adsorbed DNA as a condensed two–dimensional layer of rodlike counterion contributing to the change of free energy by its electrostatic pressure [13]. The main phase peak sharpens upon increasing the DNA amount twice, as shown in

the next curve beneath (Figure 1). Interestingly, the DNA phase peak shifts to lower values of 71°C. This trend is maintained upon increasing the DNA amount more (ternary molar ration of 1:5.1). In both cases ΔC_p slightly decreases compared to ULV heat capacity value. It is not clear at this stage which lipid/DNA ratio is optimal for achieving maximal transfection. The triple complex of DNA–Mg^{2+}–phosphatidylcholine vesicle remains stable at different incubation times, which is in agreement with small– and wide–angle X–ray scattering measurements (SAXS and WAXS) [8]. 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 [14]. The last curve denoted as DNA – Mg^{2+} is a liposome–free control measurement and represents equimolar mixture of DNA and Mg^{2+}, which brings about a major signal at arround 90°C. The Mg^{2+} ions at the equimolar ratio of Mg^{2+}:DNA increase the T_m value by 33.7°C, reaching a maximum at 85°C, due to Mg^{2+}–induced phase separation with an increased gel – liquid crystal phase transition temperature, which indicates the divalent cation triggered high temperature DNA stabilization. ULV treated with the same concentration of Mg^{2+} did not produce such a shift, which is normally detected spectroscopically [21]. Obviously, divalent metal cation does not contribute essentially in stabilizing of the zwitterionic lipid structure. Therefore, DNA contributes to stabilization of ternary complex towards higher temperatures. More calorimetric measurements combined with spectroscopic studies are needed to deduce whether this is the case.

The lipid–based delivery of DNA polyelectrolyte into cultured cells by electrostatic mechanisms of binding to their negatively charged membranes is well established [2,3,5,15]. 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 the work herein, could deliver genes into cells via direct fusion with the cell membrane. This model is accordance with recent proposal [2]. The major advantage of such non–viral nanocondensate formulation is the ability to act across tight barriers *in vivo*. Only few purely neutral phospholipid–DNA mixtures have been studied and presented in the literature. Thus, our findings would be valuable to compare with those reported for CL–DNA lipoplexes. The current study represents initial calorimetric study of the melting behaviour of a model neutral lipid–Mg^{2+}–DNA (NL–Mg^{2+}–DNA) complexes. Similar thermotropic phase transitions are expected for other amphiphiles complexed with DNA. Saturated lipids are not currently employed for transfection purposes, mainly due to the presence of lipid phase transitions [13]. However, this view could be reconsidered after optimization of lipid/DNA ratio in terms of polyelectrolyte theory, beside the other relevant factors influencing transfection efficiency mentioned above. More specifically, the electrostatic bridging potential of metal ions is not considered enough in terms of their ability for improved cell–surface

interactions.

Theoretically, by bridging together the membrane and DNA polyanions, inorganic metal cations can induce local membrane invaginations, destabilizations and fusion, thus facilitating the translocation of therapeutic nucleic acid sequences through the formed cell membrane phospholipid–liposome fusions. This could open new insights into designing novel constructs for an endocytosis–independent cellular internalization mechanisms. Such a construct potentially can be further improved by linking the gene of interest to a nuclear localization signal (NLS) for subsequent nuclear import. Currently, both polyplexes and lypoplexes are being tested in transfection experiments as promissing substitutes of the problematic viral vectors for gene transfer in human gene therapy trials. Many new polymers and cationic lipids are synthesized and their transfection potential is regularly reported [16–19]. However, the molecular details of transfection remain to be undestood. Thus, different cell lines give different transfection potency profiles and these are affected by the particular experimental design [15]. Despite all these hurdles, it is now clear that the structure of the lipids and their formulation stability are crucial factors in the constructions of gene delivery vehicles. Having considered the prevailing research interests in lipid–based systems [16,17; http://www.wiley.co.uk/genetherapy/clinical/], the initial design of lipoplexes with improved properties becomes important. Therefore, biophysical data on the structural–activity relationships of DNA–liposome formulation is needed to evaluate their potential to deliver nucleic acids to target cell. As justified recently [20], this difficulty requires the preliminary evaluation of newly designed formulations by physicochemical methods.

The present work is an attempt to provide new thermodynamic data of divalent metal cation–induced zwitterionic liposome–DNA mesophase aggregate formation. Comparison of our results with those obtained for CL–DNA complexes was done, trying to obtain new insights from changes in the lipid–phase behaviour after association with DNA. More specifically, the substitution of toxic cationic/neutral lipid constructs with neutral amphiphiles and their complexation with Mg^{2+} is investigated.

Our results demonstrate that zwitterionic liposome–DNA formulations exhibit a separate thermotropic phase profile. This effect is enhanced, when Mg^{2+} is added, cationizing the vesicles and complexing them with DNA polyanion. Because of the screened charges, we believe these particles to be less metastable, with a diminished tendency to form large aggregates, compared with CL–DNA formulations. This supports further the recent experimental results on divalent metal cation–enhanced transfection levels [7]. Even though, formation of such large and homogeneous particles may possess improved *in vitro* transfection properties, their employment *in vivo* seems to be problematic. Serum usually destabilizes CL–DNA structures and affects negatively transfection [20]. However, the ability to construct small nanoformulations, such as those presented in the present study, that remain stable and non–aggregating in *in vivo* surroundings will open new routes

for gene therapeutics.

4 CONCLUSIONS

NL–Mg^{2+}–DNA formulation described herein are highly dynamic structures, thus frequently changing their pharmacological features. On the other hand, the ternary complex appears to be reproducible liposome–DNA formulation, but its serum stability is not yet well–defined. More immunological experiments are needed for their *in vivo* evaluation. Nevertheless, this preliminary thermodynamic study on the stability of neutral liposome–polynucleotide nanostructures should contribute further to design and characterization of novel lipoplexes with improved transfection properties and thus for the development of better gene transfer systems for use in human gene therapy.

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