RESUBMITTED – JUNE 19, 2000

BIOPHYSICAL AND STRUCTURAL PROPERTIES OF DNA/DIC₁₄-AMIDINE COMPLEXES: INFLUENCE OF THE DNA/LIPID RATIO.

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ABSTRACT

Cationic liposomes are used as vectors for gene delivery both in vitro and in vivo. Comprehension of both DNA/liposome interactions on a molecular level and a description of structural modifications involved, are prerequisites to an optimization of the transfection protocol and thus successful application in therapy.

Formation, and stability of a DNA/cationic liposome complex, were investigated here at different DNA:lipid molar ratio (ρ). Isothermal titration calorimetry (ITC) of cationic liposomes with plasmid DNA was used to characterize the DNA-lipid interaction. Two processes were shown to be involved in the complex formation. A fast exothermic process was attributed to the electrostatic binding of DNA to the liposome surface. A subsequent slower endothermic reaction is likely to be caused by the fusion of the two components and their rearrangement into a new structure. Fluorescence, and differential scanning calorimetry (DSC) confirmed this interpretation. A kinetic model analyzes the ITC profile in terms of DNA/cationic liposome interactions.
INTRODUCTION

Transfer of nucleic acids into cells requires the use of transfection agents such as virus-based vectors (1;2) or cationic liposomes (3-10). Cationic liposomes offer several advantages over viral vectors including the low immunogenic and inflammatory responses, the potential transfer of unlimited-size expression units, and the possibility for engineered cell-specific targeting. *In vitro* studies have established the efficiency of such DNA/cationic liposome complexes (4;6;8;10;11); *in vivo*, these systems are in most cases less effective for gene transfer than viral vectors and unstable in serum (12-14).

Transfection efficiency depends to a great extent on the cell lines or and the lipid composition (15;16). Reasons for these discrepancies are largely unknown. One of them is our poor knowledge of the structure of the DNA/lipid complex and of its mode of entry into the cell. Extensive efforts have been made to characterize the DNA/cationic lipid complex (17) leading to different structural models (18;19). Gershon et al. (1993) (20) suggested that DNA is encapsulated inside large unilamellar liposomes. On the other hand, in the so-called “sandwich-like” structure, observed by X-ray (21), freeze-fracture electron microscopy (22) or cryo-transmission electron microscopy (23), DNA is adsorbed between liposome bilayers in an alternating flat lamellar packing (24). More recently, another model suggested that the liposomes are broken, resulting in DNA coated by a cylindrical bilayer, as shown by freeze-fracture electron microscopy (25).

These structural studies provide however no information about the nature of the interactions involved in the complex formation. The interactions between cationic liposomes of diC_{14}-amidine (Fig. 1) and a plasmid DNA as well as the structural modifications involved in the complex formation, were investigated here at different DNA:lipid molar ratios (ρ). This complex
has been used successfully to transfect cells in vitro (10). Isothermal titration calorimetry (ITC) was used to characterize the interactions involved in the complex formation. Fluorescence spectroscopy, and differential scanning calorimetry (DSC) provided detailed information about the rearrangement of the two components during the complex formation.

A kinetic model is proposed to analyze the ITC profile in terms of DNA and cationic liposome interactions.
MATERIALS AND METHODS

Materials
N-t-butyl-N’-tetradecylamino-propionamide or diC\textsubscript{14}-amide (Vectamidine\textsuperscript{TM}) (Fig. 1) was purchased from BiotechTools (Brussels, Belgium). The pc-DNA 3.1 plasmid (5.4 kbp) was obtained from Invitrogen (Leek, The Netherlands) and amplified in \textit{E.coli}. The circular plasmid was isolated and purified using a Qiafilter Plasmid Kit (QIAGEN, Westburg, The Netherlands). Hepes and FITC-dextran (fluoresceine isothiocyanate) were purchased from Sigma-Aldrich (Bornem, Belgium). Pyrene-PC (or 1 hexadecanoyl-2-1-pyrenedecanoyl-sn-glycero 3 phosphocholine) was provided by Molecular Probes, Leiden, The Netherlands.

Preparation of liposomes
diC\textsubscript{14}-amide was dissolved in chloroform, dried under a nitrogen stream and stored overnight in a desiccator under vacuum. Liposomes were formed by addition of 10 mM Hepes, pH 7.3, buffer to the lipid film and mechanical mixing above the transition temperature. Prior to each experiment, the liposomal suspension was degassed under vacuum and vortexed for ten minutes.

Isothermal Titration Calorimetry (ITC) measurements
ITC experiments were performed using an OMEGA titration microcalorimeter (MicroCal Inc., Northhampton, MA; (26)). All samples (DNA and liposomes) were dialyzed overnight against 10 mM Hepes (pH 7.3) and degassed for 10 minutes before measurements. At constant time intervals (every 7 or 6 minutes respectively for the high and low lipid concentration), aliquots of DNA plasmid solution were injected, via a 100 µl rotating stirrer-
syringe, into the sample cell (volume = 1.33 ml) containing cationic liposomes of diC14-amidine in 10 mM Hepes (pH 7.3).

In control experiments, the DNA solution was injected into pure buffer. The heat of dilution was subtracted from the experimental curve in the final analysis.

**Fusion studies**

DNA induced fusion of diC14-amidine liposomes was monitored using pyrene-PC (1 hexadecanoyl-2-1-pyrenedecanoyl-sn-glycero 3 phosphocholine). Association of pyrene monomers ($\lambda_{ex} = 346$ nm; $\lambda_{em} = 376$ nm) leads to the formation of so-called pyrene excimers ($\lambda_{ex} = 330$ nm; $\lambda_{em} = 477$ nm). The excimer formation is proportional to the density of pyrene-PC (10% in mole) on the liposome surface. Therefore, any lipid mixing between labelled and unlabelled liposomes (present on a twenty fold excess) results in dilution of pyrene-PC, reflected by decrease of the excimer fluorescence and concomitant increase of the monomer fluorescence. Fluorescence was continuously recorded at the excimer maximum emission (477 nm). Labelled and unlabelled liposomes were mixed under stirring in a quartz fluorescence cuvette to a final diC14-amidine concentration of 177 $\mu$M.

The liposome fusion triggered by DNA was quantified by considering the 0 % fusion level as corresponding to the initial excimer fluorescence (without DNA) and the 100 % level, to the fluorescence measured after addition of an excess of Triton X-100 (0.2 % v/v final) in order to maximize pyrene-PC dispersion. Each sample was incubated for 5 minutes at the experimental temperature (28°C) before starting the experiment. DNA was added every 100 seconds to allow the stabilization of the fluorescence signal.
**Differential Scanning Calorimetry (DSC) measurements**

DSC measurements were performed on a MC-2 ultrasensitive differential scanning calorimeter (MicroCal Inc., Northhampton, MA) using twin 1.2 ml total-fill cells. The samples were scanned from 10°C to 40°C at 1 degree/min. All samples were degassed before measurements. The experimental data were processed using Origin software from MicroCal.

**FITC-Dextran release**

FITC-dextrans (40 kDa fluoresceine isothiocyanate-dextran) were diluted into 10 mM Hepes/150 mM NaCl buffer (pH 7.3) to a final concentration of 2 mM. The labelled liposomes (18.7 $\times$ 10^{-3} M) were prepared by injection above 23°C (50-55°C) of an ethanol diC_{14}-amidine solution containing ^{14}C-DPPC (6 $\times$ 10^{-3} µCi / µmole of lipid) into the vortexed dextran solution. Free FITC-dextrans were separated from encapsulated ones by passage through a Sepharose CL- 6B (0.5 ml/min). Labelled DPPC was used to determine the liposomal concentration.

The fluorescence experiments were carried out on an SLM-8000 spectrofluorimeter, at 37°C, using an excitation wavelength of 492 nm and an emission wavelength of 520 nm. Each measurement was performed at least in duplicate with both liposomes and plasmid DNA from different batches. A liposome solution diluted in Hepes/NaCl buffer (pH 7.3) (final sample volume: 1 ml) was used as "zero" fluorescence ($I_0$). Maximal release ($I_f$) was obtained after addition of 10 µl TX-100. The release measured at each ratio was calculated as $= [ (I_t - I_0) / (I_f - I_0)] \times 100$, where $I_t$ is the fluorescence measured at time $t$ at the plateau for each ratio.
RESULTS

The overall interaction process between plasmid DNA and cationic diC₁₄-amidine liposomes was investigated using high sensitivity isothermal titration calorimetry (ITC) (26). Plasmid DNA was injected into the cell containing unilamellar cationic liposomes of diC₁₄-amidine (Fig. 2-A and -B). The lipid concentration was always kept well above the critical micellar concentration (C.M.C. = 3.9 \times 10^{-7} \text{ M}) during the whole titration, making the contribution of free lipid negligible.

At high lipid concentration (above 10 mM), a fast exothermic process (Fig. 2-A, ▲) and a concomitant endothermic one (Fig. 2-A, ●) were observed after DNA addition. The fast exothermic process (equilibrium was reached within one minute) reflects the electrostatic binding of the plasmid DNA to the positive liposome surface as observed in most binding processes between charged molecules (27;28). The accompanying endothermic process, is about 6 times slower (see insert in fig 2-A) than the exothermic one, and suggests a rearrangement of the two components. Curve 2-A clearly shows that this endothermic process is cooperative as reflected by the exponential increase of the signal. In a non-cooperative binding mechanism, the enthalpy peak would be constant (28;29).

At low lipid concentrations, the exothermic process could not be detected anymore but the endothermic one was still observed (Fig. 2-B). No heat effect was observed above \( \rho \geq 0.6 \) (Fig. 2 (A-B)). At this ratio, DNA complexation is maximal. At higher DNA:lipid ratios, free DNA was indeed detected in the supernatant of centrifuged complexes (data not shown). Although formation of DNA-lipid complex is, classically, a one step process, transfection experiments demonstrated that the transfection efficiency associated to the complex was not
significantly different when it was formed, as in the ITC experiment, by a stepwise addition (data not shown).

The ITC data provided, however, little insight about the endothermic reaction that involves probably major internal reorganization of the DNA/lipid complex. It has been reported that divalent cations such as Ca^{++} (30) and polycationic amino acids (31) induce the fusion of anionic liposomes. The mechanism whereby these cations can induce fusion arise primarily from neutralization of the surface charge of the anionic lipids. Similarly, multivalent anions such as oligonucleotides or DNA, can trigger the fusion of cationic liposomes (20;32;33). The endothermic reaction here observed could therefore be assigned to a DNA-induced lipid fusion.

A fluorescent lipid (pyrene-DPPC) was inserted into the cationic liposomes to detect a possible lipid mixing at different DNA:cationic lipid molar ratios (34). Insertion of pyrene-DPPC did not affect significantly their transfection efficiency (data not shown). As illustrated in Fig. 3-A, DNA caused a substantial decrease of the excimer fluorescence within ten seconds after DNA injection, reflecting a rapid and accelerating fusion (Fig. 3- B). Flocculation was observed above a 0.6 molar ratio (ρ ≥ 0.6). A similar phenomenon has been reported previously (35-37). DNA induces fusion of cationic liposomes and leads to larger structures with increasing ρ. The final DNA/lipid ratio at which cationic liposome fusion was observed, did not depend on the addition protocol (stepwise addition or one single addition) (data not shown).

DNA ability to induce lipid mixing is probably related to its capacity of destabilizing the lipid bilayer organization (38). Differential scanning calorimetry (DSC) revealed a gel-liquid transition at 23.0° ± 0.1°C and an enthalpy of 7548 ± 293 kcal / mole of lipid for diC_{14}-amidine cationic liposomes in 10 mM Hepes. At low DNA:lipid molar ratio (e.g.: ρ = 0.03; ρ = 0.21), neither the transition temperature nor the enthalpy were affected (Fig. 4 (A-B)). At higher
DNA:lipid molar ratio, DNA destabilizes the liposomal bilayer in a way reminiscent of that described for cholesterol: no significant shift of the transition temperature and a concomitant decrease of the enthalpy. Destabilization was completed at a 1.0 molar ratio (fig. 4-B) suggesting a new organization of the lipid structure.

Release of fluorescent dextran-FITC (fluoresceine isothiocyanate) with a Stokes diameter of 60 Å and encapsulated into the diC14-amidine liposomes confirms the DNA-induced destabilization of the liposomal bilayers (Fig. 5-A). A 80 % release was recorded at a DNA:lipid molar ratio of 0.6 (Fig. 5-B), supporting the hypothesis that DNA triggers the disruption of the liposomal membrane.
DISCUSSION

Our data suggest that the DNA/diC\textsubscript{14}-amidine liposome complex formation proceeds in several steps:

\[ D + L \xrightarrow{k_1} C \xrightarrow{k_3} F \]

where \( D \) is DNA, \( L \) diC\textsubscript{14}-amidine liposomes, \( C \) a soluble DNA-lipid complex, and finally, \( F \) represents the fused complex.

**STEP 1:** The primary driving force of the complex (\( C \)) formation is the electrostatic interaction between the negative phosphate groups of DNA (\( D \)) and the positive charged groups of the diC\textsubscript{14}-amidine liposomes (\( L \)). A single plasmid DNA (5.4 kbp) binds several positively charged diC\textsubscript{14}-amidine liposomes (60 nm diameter, as determined by laser light scattering) (data not shown). This process is rapid and exothermic as verified by isothermal titration calorimetry at high total lipid concentration. It is assumed that the complex dissociates with a \( k_2 \) rate constant.

**STEP 2:** Charge neutralization abolishes repulsion between cationic liposomes (32). In addition, the bilayer organization is strongly destabilized (as illustrated in FITC-dextran release analysis, fig.5 and in DSC profiles, fig.4) undergoing a slow entropy-driven endothermic fusion process. Maximal fusion is reached at \( \rho > 0.6 \).
These two steps can be described by the following set of differential equations:

\[
\begin{align*}
\frac{d[D]}{dt} &= \frac{d[L]}{dt} = k_2 [C] - k_1 [D][L] \\
\frac{d[C]}{dt} &= k_1 [D][L] - (k_2 + k_3) [C] \\
\frac{d[F]}{dt} &= k_3 [C]
\end{align*}
\]

with [D], [L], [C], and [F] as molar concentrations of D, L, C, F, and k₁, k₂, and k₃ the corresponding phenomenological (macroscopic) rate constants. k₁ described a second order process; k₂ and k₃, a first order mechanism.

Numerical solutions of the concentration profiles D(t), L(t), C(t) and F(t) were calculated for a set of different values of rate constants, applying the Runge Kutta method for differential equations. Using the initial concentrations and time scale described in fig. 2-A and 2-B, a realistic set of estimated rate constants was derived: \( k_1 = 35000 \text{ s}^{-1} \cdot \text{M}^{-1} \), \( k_2 = 8.5 \times 10^{-3} \text{ s}^{-1} \) and \( k_3 = 1.7 \times 10^{-1} \text{ s}^{-1} \).

Figures 6 (A and B) illustrate the concentration changes of reactant (D) and products (C and F), resulting from the first DNA injection in the ITC cell:

- high concentration (figure 6-A): \([D]_{\text{start}} = 7.7 \times 10^{-7} \text{ M (nucleotide)}\) and \([L]_{\text{start}} = 3.8 \times 10^{-6} \text{ M (lipid)}\);

- low concentration (figure 6-B): \([D]_{\text{start}} = 1.5 \times 10^{-7} \text{ M (nucleotide)}\) and \([L]_{\text{start}} = 7.7 \times 10^{-7} \text{ M (lipid)}\).

L concentration is much higher than that of D and products (C and F) and was thus considered as constant and not represented. At low lipid concentration, formation of the complex C is the limiting factor (\( k_1 \) is rate limiting). As soon as it is formed, C is further converted into larger fused particles (F); both processes occur at almost the same rate (Fig. 6-B). C does not accumulate. This explains why only the endothermic contribution was detectable on the
calorimetric pattern (Fig. 2-B). At high lipid concentration (Fig. 6-A), the reaction proceeds similarly but faster due to high concentration, but interestingly, C accumulates during the first minute of the process. It is precisely within this period of time that the fast exothermic process was observed experimentally (Fig. 2-A) in the ITC pattern. In this situation, the $k_3$ is rate limiting.

To summarize, this model allows to analyze the structural changes observed around a critical DNA:lipid ratio in terms of interactions and complex formation. The kinetic model does not take into account the transformation of the fused complex into larger structures (flocculation). The main reason is that above a 0.6 ratio, no heat changes were detectable in the titration calorimetry profile. DSC (Fig. 4) and dextran-FITC release experiments (Fig. 5) demonstrated however a complete destabilization of the lipid bilayer above 0.6 ratio. Close to a 0.6 DNA:lipid molar ratio, further addition of DNA fully destabilizes the lattice due to compensation of the lipid positive charges. As suggested by Düzgünès et al. (1989) (32) to explain polyanion-induced fusion of dioleyloxypropyltrimethylammonium (DOTMA) liposomes, water molecules are expelled from the liposomal surface during the complex formation, making it more hydrophobic. Consecutively the intervesicular electrostatic repulsions are reduced, leading to a collapse of the complex into larger macroscopic aggregates (37;39). Those large particles start to sediment, as reflected by the white flocculating aspect of the solution.

Those results could corroborate the “rod-shaped” structural model (25) with a breakup of the liposomes and the coating of the DNA by cylindrical bilayers. However, they are not in agreement neither with the Gershon model (20) nor with the “sandwich-like” model (22) both of them supporting persistence of liposomal structures after complex formation.
In order to relate different biophysical characterization of DNA/diC_{14}-amidine complexes with their transfection properties, we have compared the transfection efficiency on CHO cells at various DNA:lipid molar ratio. The resulting profile (fig. 7) shows clearly that transfection increases strongly near the critical 0.6 molar DNA:lipid molar ratio, is maximal around 0.8 and then decreases at 1.0. This profile is reminiscent of the modification of the biophysical parameters at various DNA:lipid ratios. There is an obvious parallel between the results of our biophysical experiments and the transfection properties of the DNA/diC_{14}-amidine complexes, showing that maximal transfection activity occurs in a range where liposome destabilization through DNA is close to maximal.
ACKNOWLEDGMENTS

We would like to thank Dr. El Ouahabi A. for helpful discussions and suggestions. We also thank the F.R.I.A. (Fonds pour la Formation à la Recherche dans l’Industrie et dans l’Agriculture, Belgium) and V.I.B. (Vlaams Interuniversitair Instituut voor Biotechnologie, Belgium) for supporting this work. Dominique Maes is a Research Associate of the Belgian National Science Foundation (Fonds Wetenschappelijk Onderzoek).
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FIGURE LEGENDS

Figure 1.
Structure of N-t-butyl-N’-tetradecyl-3-tetradecylamino-propionamidine or diC14-amidine.

Figure 2.
Titration calorimetry (ITC) of unilamellar diC14-amidine liposomes with plasmid DNA (pcDNA 3.1) in a 10 mM Hepes buffer (pH 7.3) at 28°C.

(A) **High lipid concentration.** The upper part of the figure shows the calorimetric trace as a function of time. Each peak corresponds to the injection of 10 µl of a DNA solution (76.2 mM in nucleotides) in a 1.33 ml. cell filled with the liposomal suspension (10.1 mM diC14-amidine total). Insert represents an enlargement of the first peak of titration.

The lower part of fig. 1-A. shows the binding isotherm resulting from integration with respect to time: reaction enthalpy (kcal/mole of injectant) is plotted as a function of the DNA:lipid molar ratio; (●) exothermal component of the titration curve; (▲) endothermal component of the titration curve.

(B) **Low lipid concentration.** The upper part of the figure shows the heat capacity tracings as a function of time. Each peak corresponds to the injection of 10 µl of the DNA solution (8.8 mM in nucleotides) in a liposomal suspension (0.93 mM diC14-amidine total).

The lower part of fig. 1-B. shows the binding isotherm resulting from integration with respect to time: reaction enthalpy (kcal/mole of injectant) is plotted as a function of the DNA:lipid molar ratio.
Figure 3.
DNA-induced lipid mixing of cationic lipids using pyrene PC.

(A) Kinetics of DNA-induced lipid mixing of diC\textsubscript{14}-amidine liposomes. Fluorescence cell contains 177 μM of diC\textsubscript{14}-amidine liposomes. Appropriate volumes of the DNA solution (20 mM in nucleotides) were injected into the fluorescence cell each 100 sec (total time scan: 1000 sec) and under mixing. TX-100 (0.2 % final) was injected at the end of the reaction and corresponds to total lipid mixing. The DNA injections are indicated by arrows.

(B) Final extent of lipid mixing as a function of the DNA:lipid molar ratio. Error bars represent standard errors of the mean (triplicates). Fluorescence value at DNA:lipid ratios higher than 0.6 was recorded one second after DNA injection and thus, before flocculation.

Figure 4.
Differential scanning calorimetry (DSC) of the DNA/lipid complex at different molar ratios:

Concentration of the lipid in the calorimetric cell: 0.93 mM. Buffer: Hepes 10 mM, pH 7.3.

(A) Calorimetric profile at different DNA:lipid molar ratios versus the temperature.

Scan rate: 1 degree/minute.

(B) Excess heat capacity peak area (kcal/mole) versus the DNA:lipid molar ratio.

Figure 5.
Kinetics and final extent of FITC-dextran (40 kDa) release from diC\textsubscript{14}-amidine liposomes after DNA (pcDNA 3.1) addition:

(A) Kinetics of release of FITC-dextran (2 mM) encapsulated into diC\textsubscript{14}-amidine liposomes. Samples contained 4.43 μM of lipid. Appropriate volumes of DNA solution (3.7 μM) were
injected at \( t = 100 \) sec to reach the desired DNA:lipid molar ratio. 10 \( \mu l \) of TX-100 was added at 650 sec to induce the complete FITC-dextran release.

(B) Final extent of FITC-dextran release as a function of the DNA:lipid molar ratio.

Figure 6.
Simulated concentration changes at high (fig 6-A) and low (fig 6-B) reactant levels versus time (\( \text{DNA} = D; \text{Complex} = C; \text{Fused complex} = F \)), resulting from the first DNA injection into diC\(_{14}\)-amidine liposome suspension. Concentrations were calculated using the set of equations described in discussion. The concentration changes of reactant and products are representative of those resulting from the first injection of DNA into the ITC cell containing the diC\(_{14}\)-amidine liposomes. The lipid curve was not represented here because lipids were in large excess in comparison with the other concentrations.

Figure 7.
Transfection efficiency of CHO cells as a function of DNA:lipid molar ratio. DiC\(_{14}\)-amidine vesicles were mixed with 5 \( \mu g \) DNA. Cells and cationic vesicles were incubated 3h. The CAT activity is expressed as the ratio of acetylated chloramphenicol to the total chloramphenicol activity.
Biophysical and structural properties of DNA-cationic liposome complexes: Influence of the DNA/lipid ratio
Veronique Pector, Jan Backmann, Dominique Maes, Michel Vandenbranden and Jean-Marie Ruysschaert

J. Biol. Chem. published online July 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909996199

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