Nucleotide Insertion in Cationic Bilayers

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The interaction between 2′-deoxyadenosine 5′-monophosphate (DMP) and cationic liposomes made up of dioctadecyldimethylammonium bromide (DODAB) in water is described. At maximal adsorption, the molar ratio DODAB/DMP is 2:1 and electrophoretic mobility for the liposomes attains a minimum at a positive value. At 5 mM ionic strength, maximal DMP adsorption on the liposome becomes close to zero, demonstrating that the electrostatic attraction essentially drives the DODAB/DMP complexation. Over the millimolar range of DMP concentrations (0.4−1.5 mM), upon nucleotide addition, turbidity of the liposome dispersion (0.08 mM DODAB) steeply increases as a function of time in contrast with the much smaller flocculation rates upon NaCl addition over a much higher range of NaCl concentrations (40−210 mM NaCl). The nucleotide behaves as a hydrophobic anion with an affinity for the membrane that is much higher than that exhibited by a simple anion as chloride. In water, liposome electrophoretic mobility decreases as a function of DMP concentration but the liposome/DMP complex remains positively charged, even at the highest DMP concentrations tested. DMP-induced rupture of liposomes containing [^{14}C]sucrose was evaluated from dialysis of DMP/liposomes mixtures. In water, DMP-induced leakage of radioactive liposomal contents suggests that the DMP/bilayer interaction is not superficial. Although the interaction preserves the positive liposome charge, it does not preserve its integrity. At maximal adsorption, DMP insertion in the cationic bilayer is the most reasonable explanation for the remaining positive charge on the vesicle, the 2:1 DODAB:DMP molar ratio, and leakage of internal contents from the liposome.

Introduction

The entrance of exogenous DNA in the nucleus of transfected cells is not well understood.[1−8] Several cloned genes linked to their own or heterologous promoter−enhancer sequences are now available. Their deliverance to cells by processes such as calcium phosphate precipitation, electroporation and lipofection has allowed their expression in a large number of cell types.[9−17] These methods somehow mediate cytoplasmic delivery of a small portion of DNA with its subsequent incorporation into the nucleus. Among the variety of cellular transfection techniques commonly used, only lipofection using cationic liposomes sucessfully introduced a glucocorticoid receptor derivative, overexpressed and purified from *Escherichia coli*, into mammalian cells.[18] Despite its importance, the establishment of more general physicochemical principles driving the interaction between cationic liposomes and DNA is still in its infancy.

In the DNA−cationic liposome complex, the nucleic acids or short, single-strand antisense oligonucleotides are believed to be simply complexed (instead of encapsulated) with cationic unilamellar vesicles by electrostatic interactions. Intricate topological rearrangements may occur, including DNA condensation, liposome aggregation, and fusion.[19−21] Recently, an unexpected topological transition from liposomes to optically birefringent liquid-crystalline condensed globules was revealed by X-ray diffraction and optical microscopy: a novel multilamellar structure with alternating lipid bilayer and DNA monolayers.[22]

In this work, we use a monomer of the DNA biopolymer to further probe the interaction between a nucleotide and dioctadecyldimethylammonium bromide (DODAB) large liposomes[23,24] from a physicochemical point of view. The results shed new light on the mode of interaction between DNA and cationic liposomes: although the positive sign of the liposome charge is kept, at maximal nucleotide adsorption, the hydrophobic attraction between the nucleotide and the bilayer core leads to nucleotide insertion in the cationic bilayer with formation of a 2:1(molar ratio) lipid /adsorbed nucleotide complex, maintenance of liposome positive charge, and leakage of liposome internal contents. Energy minimization by molecular modeling also validated the hypothesis of nucleotide insertion in the cationic bilayer, suggesting absence of physical restrictions to the structure proposed.

Material and Methods

Chemicals. DODAB 99.9% pure was obtained from Fluka Chemie AG (Switzerland) and used as such without further purification. 2′-Deoxyadenosine 5′-monophosphate (DMP) 99% pure was purchased from Sigma. All other reagents were analytical grade and were used without further purification. Water was Milli-Q quality.

Liposome Preparation. DODAB vesicle dispersions were prepared by two different methods: (a) by heating the DODAB powder in water at 56 °C;[25] and (b) by chloroform vaporization of a DODAB chloroformic solution injected in water at 70 °C.[26] Both methods yield large unilamellar vesicles with a mean zeta-average diameter of 345−711 nm depending on ionic strength (0−5 mM monovalent salt).[31] DODAB concentration was analytically determined by microtitration.[27]

Determination of DMP Adsorption in DODAB Liposomes. The DMP/liposome interaction was promoted by mixing 0.5

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mL of a 1.0 mM DODAB dispersion with 0.5 mL of a DMP solution ranging from 0 up to 2.0 mM DMP. Thereafter, the mixture was incubated (30 min at 37 °C) and centrifuged (150 00 g for 1h at 4 ºC) to separate liposomes from free DMP in solution. The supernatant was filtered through polycarbonate membranes (0.4 μm cutoff). Liposome retention by the filtering membranes was evaluated from determination of turbidity at 400 nm in the filtered solution. Routinely, the large vesicles were retained by the filter, turbidity being equal to or very close to zero. DMP adsorption in the liposomes was obtained from inorganic phosphorus determination[28] in the filtered solutions where liposomes were completely absent. At 0.5 mM DODAB, adsorbed DMP concentration (in millimoles per liter) is plotted as a function of final DMP concentration in the mixture (in millimoles per liter). Maximal adsorption is depicted from the plateau on the curve or from linearization of the adsorption isotherms in accordance to the Langmuir model.[29] Linearization of adsorption isotherms allows determination of an affinity constant, K (in reciprocal moles), and maximal adsorption, $(x/m)_{max}$, expressed as the molar ratio adsorbed DMP/DODAB at maximal adsorption.[29] The effect of 5 mM NaBr on formation of the DODAB/DMP complex was evaluated from DMP adsorption isotherms obtained as described above but using DMP solutions and DODAB dispersions previously prepared and kept in 5 mM NaBr.

Instead of adding DMP to previously prepared vesicles, as a control for an eventual increase in DMP adsorption due to a DMP offer both to the inner and outer vesicle compartments, 0.5 mM DODAB liposomes were prepared by both methods in 0.8 mM DMP solutions (excess DMP) and incubated at two different incubation temperatures (1 h, above or below the phase transition temperature) before determining the DODAB:DMP molar proportion. All controls were done in quadruplicate for each vesicle preparation method and incubation temperature. Molar proportions thus obtained were equal to those obtained by mixing previously prepared vesicles with excess DMP.

Assay for DMP-Induced Liposome Flocculation. A 0.5 mM DODAB dispersion (0.2 mL) was rapidly mixed with 1 mL of a DMP solution (0.2−2.0 mM DMP) in a glass cuvette placed in the cuvette holder of a Hitachi U-2000 spectrophotometer at 25 °C. Turbidity at 400 nm was continuously recorded as a function of time after mixing over a time scale of hours. A similar experiment was performed to evaluate the NaCl-induced liposome flocculation. In this case, DODAB liposomes were prepared in a 0.511 M D-glucose solution of 0.522 Os/kg to avoid the liposome shrinkage that would occur because of mixing hyperosmotic solutions of increasing NaCl concentrations with liposomes prepared in water. All NaCl solutions added to the liposomes to induce flocculation were isoosmotic to the inner aqueous compartment. Isoosmotic solutions contained NaCl and D-glucose in different proportions so that NaCl concentration could be increased in the solutions without changing the total osmolarity of the solution added to the liposomes. To compare liposome flocculation induced by DMP and NaCl, respectively, kinetic data for NaCl-induced flocculation were also obtained. In this case, turbidity at 400 nm was recorded over a time scale of days because flocculation took place at much slower rates than those induced by DMP.

Microelectrophoresis of DMP/DODAB Complex. DODAB dispersions in water obtained as previously described were diluted to obtain 0.10, 0.20, and 0.40 mM DODAB, mixed v/v with DMP solutions over a range of DMP concentrations (0−1.0 mM), and incubated (1 h at 40 °C) before performing the electrophoretic mobility (EM) measurements as a function of DODAB concentration in the mixtures. Mobilities were measured using a Rank Brothers microelectrophoresis apparatus with a flat cell at 25 °C. The sample to be measured was placed into the electrophoresis cell, electrodes were connected, and a voltage of 60 V was applied across the cell. Velocities of individual vesicles over a given tracking distance were recorded, as was direction of vesicle movement. Average velocities were calculated from data on at least 20 individual vesicles. EM was calculated according to the equation EM = cm(μm/V)(1/t), where μm is the distance over which the vesicle is tracked (micrometers), cm is the interelectrode distance (7.27 cm), V is the voltage applied (±60 V), and t is the average time in seconds required to track one vesicle a given distance u. At a given DODAB concentration, EM was determined as a function of DMP concentration.

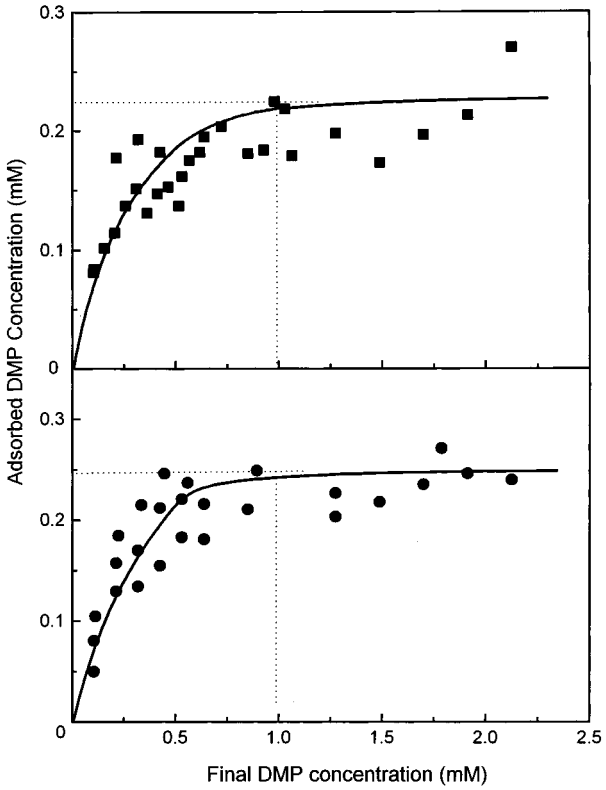

Figure 1. Adsorption isotherm for the adsorption of DMP from water solution onto large DODAB vesicles. Vesicles were obtained by Snippe's (■) or by the chloroform vaporization method (●). One should notice that maximal adsorption is achieved approximately at the point indicated by dashed lines. The DODAB:DMP molar proportion at this point is ca. 2:1, as also depicted from linearization of the isotherms in Table 1.

Determination of Liposome Rupture upon Interaction with DMP. Entrapment efficiency for DODAB liposomes was obtained from dialysis and radioactive labeling of the intraliposomal aqueous compartment.[24] Equal volumes of liposomes prepared in water (1 mM DODAB) containing [^{14}C]sucrose (LS) and a dialysis control of water containing [^{14}C]sucrose (S) were dialyzed in two separate bags against 2 L of water (changed three times), respectively, overnight with vigorous stirring. Before dialysis, aliquots of LS and S were reserved for the determination of [^{14}C]sucrose entrapment efficiency of the liposomes (ENT). After dialysis, the radioactivity, in counts per minute (cpm), was determined for the dialysates and for the reserved aliquots. Entrapment can be taken as:[24]

$$\mathrm{ENT} = (1/\mathrm{C})(\mathrm{cpm}_2/\mathrm{cpm}_1 - \mathrm{cpm}_{2c}/\mathrm{cpm}_{1c})$$

where 1 and 2 subscripts refer to counts of LS before and after

TABLE 1: Nucleotide Adsorption Parameters for DMP Adsorption onto DODAB Liposomes Prepared by Two Different Methods[a]

vesicle preparation method	affinity constant, K (M^{-1})	limiting adsorption $(x/m)_{max}$	molar proportion DODAB:DMP at limiting adsorption
Snippe	8.8 ± 1.1	0.48 ± 0.03	2.1:1.0
chloroform vaporization	20.9 ± 5.7	0.42 ± 0.10	2.5:1.0

[a] Affinity constants (K) and limiting adsorption $(x/m)_{max}$ were obtained by linearization of adsorption isotherms using a fitting to the Langmuir model.[29] Maximum adsorption is given as a molar proportion DMP:DODAB. Mean values were obtained from linearization of three to five different adsorption isotherms for each vesicle preparation.

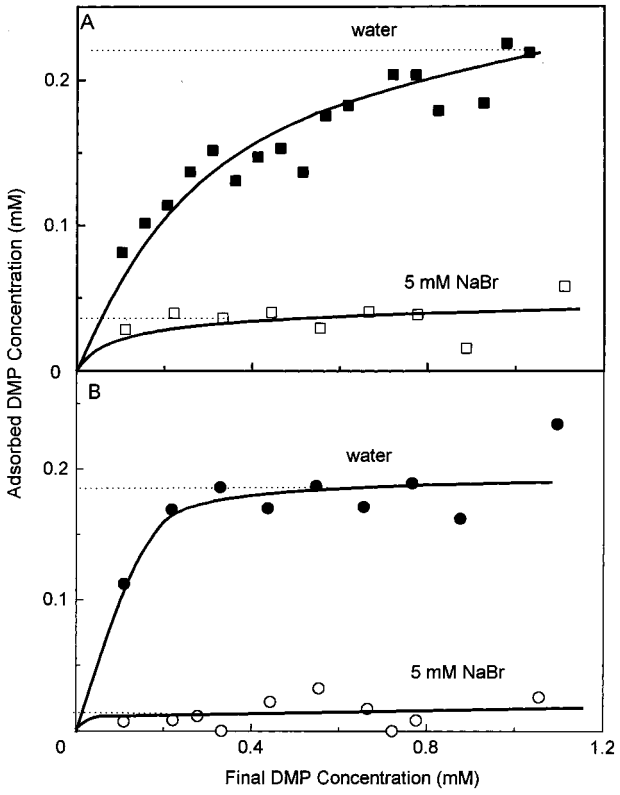

Figure 2. The effect of 5 mM NaBr on DMP adsorption onto large DODAB vesicles either obtained by Snippe's method in water (■) or 5 mM NaBr (□) or obtained by the chloroform vaporization in water (●) or 5 mM NaBr (O). The final DODAB concentration in each mixture is 0.5 mM.

dialysis, respectively; 1c and 2c subscripts are counts of S before and after dialysis, respectively; and C is the molar DODAB concentration. Thus, ENT is expressed in liters per mole ENT determined for both vesicle preparations were very similar, 15.5 ± 5.7 M^{-1}, and in agreement with ENT previously reported for DODAB vesicles obtained by chloroform vaporization.[24,26]

DMP-induced vesicle disruption was evaluated from two different assays: (a) the LS dialysate containing a total radioactivity equal to cpm_{total} was previously added to the DMP solution, incubated (0.5 h at 37 °C), and placed inside hemichamber *a* of an equilibrium dialysis chamber so that leakage was inferred from total radioactivity in hemichamber *b* (cpm_b) once the equilibrium was attained between *a* and *b* compartments, which were separated by a cellulose dialysis membrane; (b) the LS dialysate was added to DMP solutions and incubated (0.5 h at 37 °C), and each mixture was placed inside a dialysis bag and submitted to 6 h of dialysis against water (2 L, 3×),

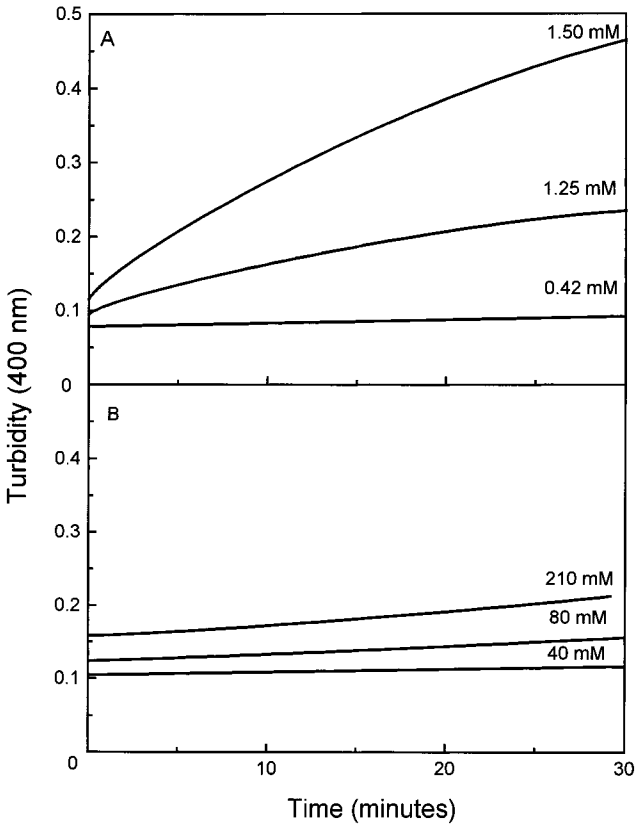

Figure 3. DMP effect on turbidity of large vesicles (0.08 mM DODAB) prepared by Snippe's method as a function of time after addition of DMP for the final DMP concentration indicated in *A*. The NaCl effect on turbidity kinetics of large vesicles (0.08 mM DODAB) similarly prepared upon addition of three different NaCl solutions is shown in *B* for comparison. One should notice that in *B*, vesicles are in a 0.511 M D-glucose solution so that NaCl addition could be kept isotonic to the environment where the vesicles were prepared.

radioactivity being counted before (cpm_1) and after the dialysis procedure (cpm_2).

The percentiles of liposome rupture from the equilibrium and the conventional dialysis procedures described above were calculated respectively as:

$$\%R_e = 100\ [cpm_b/(cpm_{total}/2)]$$

$$\%R_d = 100\ [(cpm_1 - cpm_2)/cpm_1]$$

Final DODAB and DMP concentration in the mixtures submitted to dialysis or to equilibrium dialysis were 0.5 and 1.0 mM, respectively.

Geometry Optimization of Nucleotide Insertion in the Bilayer. Energy minimizations were carried out using the DISCOVER module of the INSIGHT II package (Molecular Simulations Inc., San Diego) on an IBM RS 6000−3AT workstation. A dielectric constant $\epsilon = 3$ was used to mimic the lipidic bilayer environment, minimization convergency being achieved for energy derivatives smaller than 0.05 kcal/(mol*A).

Results and Discussion

Molar Proportion of 2:1 for DODAB/Nucleotide Complex and Importance of Electrostatic Attraction. Figure 1 shows two adsorption isotherms for DMP adsorption onto large DODAB liposomes in pure water. Both dispersion methods used for preparing the liposomes, which produce large unilamellar vesicles,[23,24] yielded similar adsorption profiles for the nucleotide

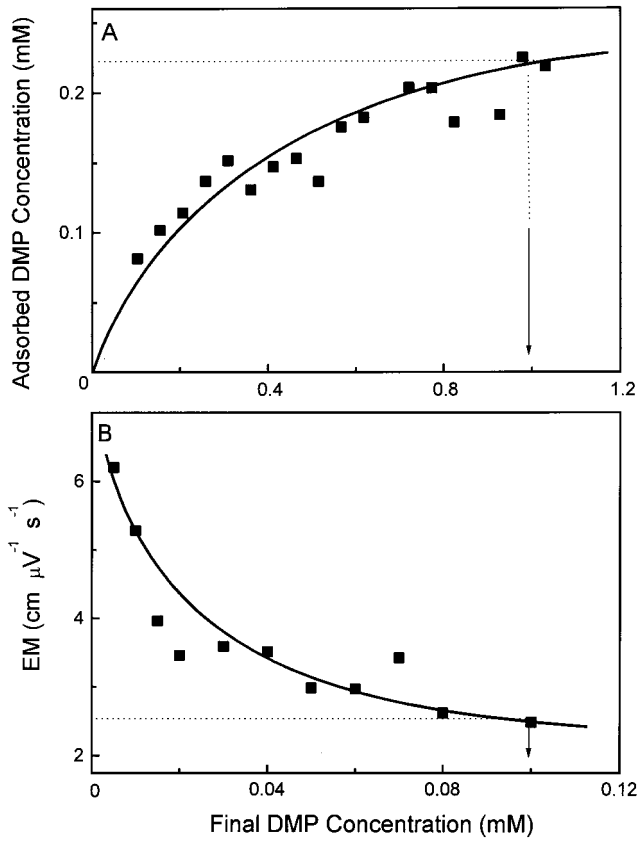

Figure 4. Correlation between minimal electrophoretic mobility (EM) and maximal DMP adsorption onto large DODAB vesicles prepared by Snippe's method. In *A*, final DODAB concentration is 0.5 mM; in *B*, it is 0.05 mM. Whereas the plateau for maximal adsorption at 0.5 mM DODAB is attained at 1.0 mM DMP in the mixture, the plateau for minimal electrophoretic mobility at 0.05 mM DODAB is attained at 0.1 mM DMP. There is a correlation between maximal DMP adsorption and minimal electrophoretic mobility.

onto the liposome as depicted from the comparison between Figure 1A and 1B. At a fixed DODAB concentration (0.5 mM), there is a langmuirian increase in DMP adsorption as a function of final nucleotide concentration with limiting adsorption being attained at ca. 1.0 mM DMP. Limiting DMP adsorption can be determined by two different procedures: (a) from linearization of the adsorption isotherms using the Langmuir model (Table 1); (b) from attainment of the plateau level at limiting adsorption as determined directly from the adsorption curve (Figure 1). At limiting adsorption, the molar ratio cationic lipid:nucleotide in the complex is ca. 2:1, both from the linearization procedure (Table 1) and from the adsorption curves (Figure 1, point defined by the dashed lines). A final DMP concentration of 1.0 mM in the DODAB/DMP mixture results in ca. 0.25 mM of adsorbed DMP onto 0.50 mM DODAB (dashed lines in Figure 1), figures that illustrate a molar ratio for the DODAB:DMP complex equal to 2:1, in pure water.

In 5 mM NaBr, nucleotide adsorption on the cationic bilayer becomes close to zero (Figure 2). This shows the importance of the electrostatic attraction between the negatively charged nucleotide and the positively charged membrane in determining complexation. Screening of the electrostatic attraction by 5 mM ionic strength substantially affects complexation. The zeta-potential for large DODAB vesicles prepared in water is 58 mV, whereas for the same vesicles prepared in 5 mM NaBr, it is 6 mV.[30] This explains the substantial effect of a relatively small ionic strength (as is 5 mM NaBr) on nucleotide adsorption (Figure 2). The electrostatic attraction between nucleotide and cationic bilayer becomes quite small for a zeta-potential of +6 mV on the membrane. This result points out the importance of the electrostatic attraction in driving formation of the cationic liposome /nucleotide complex in water.

Nucleotide as A Hydrophobic Anion of High Affinity for Cationic Bilayers in Water. From the 2:1 DODAB/DMP molar proportion obtained at limiting adsorption, the sign of the DODAB/DMP complex should be positive. This prediction was

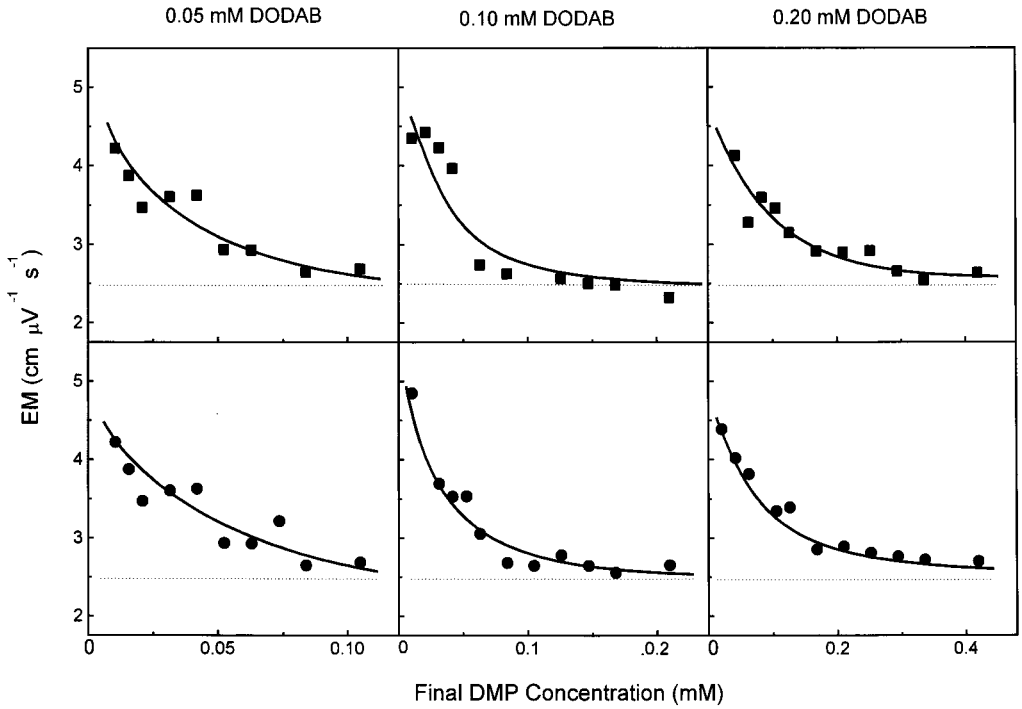

Figure 5. Electrophoretic mobilities (EM) of large DODAB vesicles as a function of final DMP concentrations in the mixtures for three different DODAB concentrations and two different vesicle preparation methods: Snippe's (■) or chloroform vaporization (●). Dashed lines indicate the leveling-off of EM (at ca. 2.5 of mobility) taking place at a final DMP concentration equal to approximately twice the DODAB concentration in each subfigure.

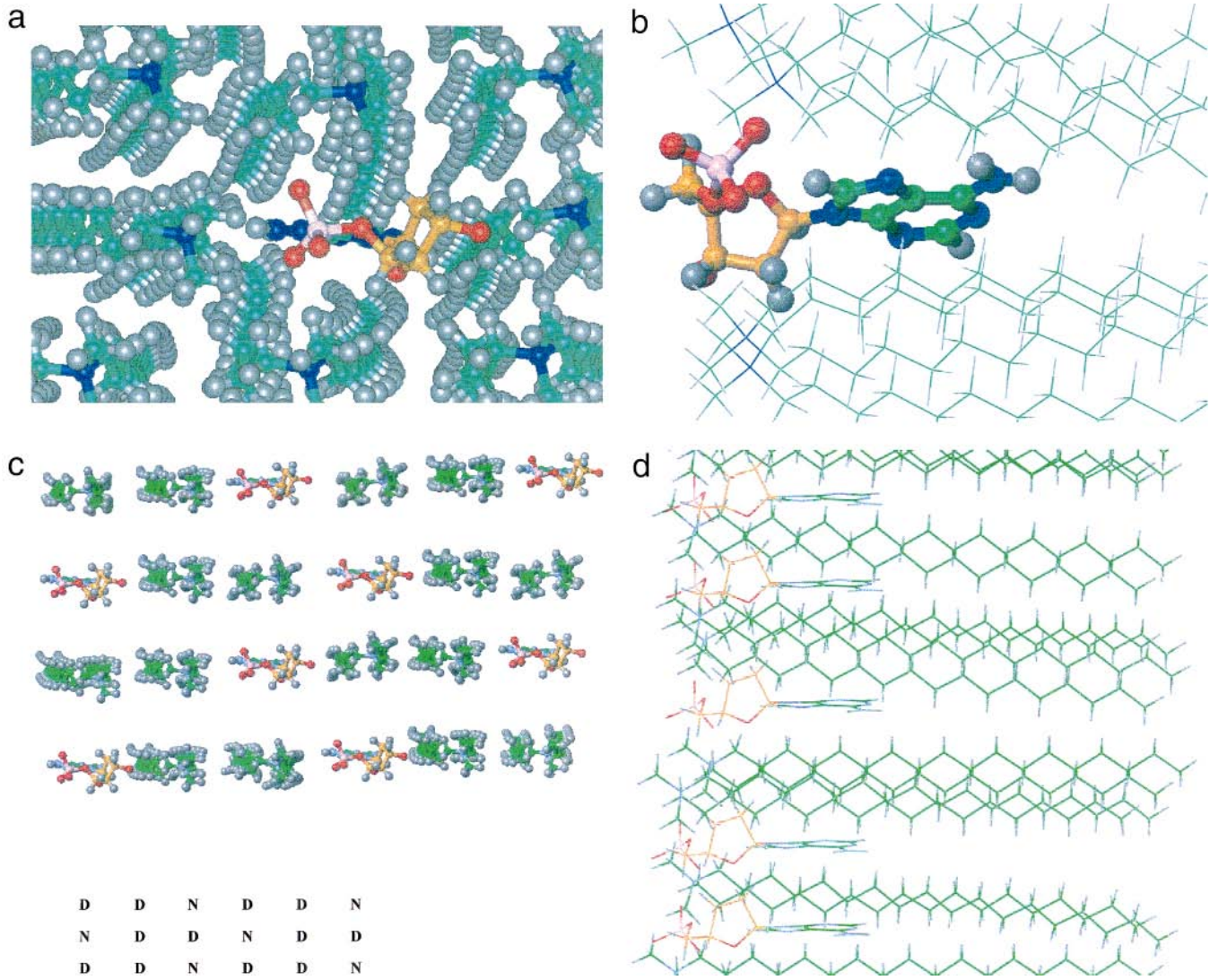

Figure 6. Three-dimensional representation of the energy-minimized nucleotide/DODAB complex: (*a*) top view below maximum adsorption; (*b*) cross-section below maximum adsorption; (*c*) top view at maximum nucleotide adsorption (notice the molar ratio DODAB:DMP equal to 2:1); (*d*) cross-section at maximum nucleotide adsorption. For the sake of clarity, carbon atoms of riboses are shown in orange. In (*c*), D represents DODAB and N, the nucleotide.

indeed confirmed from determination of electrophoretic mobilities for liposomes as a function of nucleotide concentration (Figures 4 and 5). However, when the DMP concentration (0.4−2.0 mM) in the mixture becomes much higher than the DODAB concentration (0.08 mM), there is a DMP-induced flocculation for the large DODAB vesicles (Figure 3 A) that is very rapid in comparison with NaCl-induced flocculation over a much higher range of NaCl concentrations (40−210 mM NaCl) (Figure 3 B). This result consistently illustrates an important property of the nucleotide: it behaves as a hydrophobic anion that has a much higher affinity for the cationic membrane than the one exhibited by a small inorganic anion such as Cl^-. The hydrophobic component of the interaction indeed causes a very high affinity between nucleotide and bilayer that leads to charge neutralization on the liposome and rapid flocculation over a range of very low nucleotide concentrations (Figure 3). To avoid flocculation, the DODAB:DMP molar ratio should be kept at 2:1 so that the positive charge of the complex can be maintained. An excess of nucleotide relative to DODAB definitely causes flocculation (Figure 3).

Correlation between Minimal Electrophoretic Mobility for Cationic Liposomes and Maximal Nucleotide Adsorption. The technique for analytical determination of adsorption isotherms requires DODAB and DMP concentrations inside the millimolar range of concentrations, whereas vesicle microelectrophoresis requires DODAB concentrations that are at least 100 times smaller than those used for the adsorption isotherms. The requirements of infinite dilution and one single vesicle submitted to the electric field (without interference of other charged vesicles on mobility) do not allow reliable determination of electrophoretic mobilities for a concentrated dispersion of charged vesicles.[31] Hence electrophoretic mobilities for the large DODAB vesicles as a function of the nucleotide concentration in the mixtures were obtained at 0.05 mM DODAB, a concentration where only a few, sufficiently far apart and noninteracting vesicles are visible in the dark microscope field. At limiting adsorption, the DODAB/adsorbed DMP molar ratio is 2:1 (Figure 4 A), whereas the electrophoretic mobility for the complex is equal to +2.5 (Figure 4 B). Although final DODAB or DMP concentrations in the mixtures for the adsorption isotherm (Figure 4 A) are 10 times larger than those for the EM curve as a function of DMP concentration (Figure 4 B), at a DODAB:DMP ratio equal to 2:1 in both curves (see dashed lines in Figure 4), maximal adsorption for the nucleotide on the liposomes occurs simultaneously to minimal EM.

TABLE 2: DMP-Induced Leakage of Intravesicle Contents from Conventional ($\%R_d$) and Equilibrium ($\%R_e$) Dialysis Parameters (See Material and Methods)

final DODAB concentration (mM)	final DMP concentration (mM)	R_d (%)	R_e (%)
0.5	0	57	-
0.5	1.0	85	-
2.0	0	82	-
2.0	3.2	100	-
0.5	0	-	74
0.5	1.0	-	93

[a] DODAB vesicles were prepared by Snippe's method.[25]

In Figure 5, the electrophoretic mobility for large DODAB vesicles at three different DODAB concentrations decreases as a function of DMP concentration in the mixtures, attaining a minimal positive mobility of 2.5 cm μm V^{-1} s^{-1} at a DMP concentration in the mixture that is ca. twice the DODAB concentration in each mixture. One should recall that the adsorption isotherms point out a molar proportion of 2:1 (DODAB/adsorbed DMP) precisely when a molar proportion of 1:2 (DODAB/added DMP) occurs (Figures 1 and 4 A). Again, a leveling-off for mobilities at a positive mobility value is consistent with the 2:1 molar ratio (DODAB/adsorbed DMP).

Hydrophobic Insertion for the Nucleotide in the Cationic Bilayer. A puzzling question that arises at this point is: why at maximal adsorption would there be only one DMP molecule per two DODAB molecules in the complex? If the complexation were only electrostatically driven by electrostatic attraction the molar proportion should be 1:1 and the DMP limiting adsorption should be much higher. A plausible explanation for the obtained molar ratio would be occupancy of two DODAB molecules by one inserted nucleotide in the bilayer with the phosphate polar head neutralizing only one cationic DODAB polar head and the rest of the nucleotide molecule hydrophobically interacting with the DODAB hydrocarbon chains. This interaction mode would consistently explain all the results described above, including those that describe the nucleotide behavior as the one expected for a hydrophobic anion.

From vesicle leakage and disruption experiments, there is a DMP-induced rupture of the large DODAB vesicles equal to 85−100%, in contrast to a liposome spontaneous rupture (without DMP addition) of 57−82% (Table 2). These experiments offer one more piece of evidence in favor of the nucleotide insertion hypothesis at maximal adsorption. In addition, DMP adsorption was determined for vesicles directly prepared in a DMP solution under conditions of maximal adsorption (not shown). The DODAB:DMP molar ratio thus obtained was 2:1, reconfirming the nucleotide insertion hypothesis. In fact, if nucleotide insertion does occur, it does not matter whether the insertion took place from the inside or from the outside of the vesicle: the final result will be the same.

DMP insertion into the cationic bilayer is illustrated in Figure 6. At a low DMP:DODAB molar ratio, Figure 6a shows a top view of the ionic interaction between the phosphate polar head of DMP and the cationic polar head of DODAB, with the aromatic moiety of the former nicely fitting into the gap between adjacent hydrocarbon tails. The cross-section view (Figure 6 b) shows that the gap width is not completely fulfilled along all extension of the hydrocarbon tails, generating a defect in the bilayer structure. At limiting adsorption, when the 2:1 DMP:DODAB ratio for the complex is achieved, nucleotide insertion in the bilayer could yield a regular array such as the one shown in Figure 6c and 6d. In this representation, at the membrane surface level, one should notice the rather similar space occupation for lipidic and nucleotide polar heads. At the inner level of the monolayer, the cross-section view illustrates the vacancies in the bilayer that result from insertion of each DMP molecule (Figure 6d). This could account for the rapid leakage of liposome internal contents obtained in the liposome rupture assays (Table 2). One should notice that the nucleotide insertion hypothesis successfully generated molecular configurations of minimal energy that did not offend the basic law that two molecules cannot occupy the same place in the physical space. From this point of view, the inserted nucleotide configuration successfully underwent the energy minimization procedure that yielded the structures in Figure 6.

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