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ARTICLE

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DNA-induced endocytosis upon local microinjection to giant unilamellar cationic vesicles

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Abstract We suggest a novel approach for direct optical microscopy observation of DNA interaction with the bilayers of giant cationic liposomes. Giant unilamellar vesicles, about 100 µm in diameter, made of phosphatidylcholines and up to 33 mol% of the natural bioactive cationic amphiphile sphingosine, were obtained by electroformation. "Short" DNAs (oligonucleotide 21b and calf thymus 250 bp) were locally injected by micropipette to a part of the giant unilamellar vesicle (GUV) membrane. DNAs were injected native, as well as marked with a fluorescent dye. The resulting membrane topology transformations were monitored in phase contrast, while DNA distribution was followed in fluorescence. We observed DNA-induced endocytosis due to the DNA/lipid membrane local interactions and complex formation. A characteristic minimum concentration (C_{endo}) of D-*erythro*-sphingosine (Sph^+) in the GUV membrane was necessary for the endocytic phenomenon to occur. Below C_{endo}, only lateral adhesions between neighboring vesicles were observed upon DNA local addition. C_{endo} depends on the type of zwitterionic (phosphocholine) lipid used, being about 10 mol% for DPhPC/Sph^+ GUVs and about 20 mol% for SOPC/Sph^+ or eggPC/Sph^+ GUVs. The characteristic sizes and shapes of the resulting endosomes depend on the kind of DNA, and initial GUV membrane tension. When the fluorescent DNA marker dye was injected after the DNA/lipid local interaction and complex formation, no fluorescence was detected. This observation could be explained if one assumes that the DNA is protected by lipids in the DNA/lipid complex, thereby inaccessible for the dye molecules. We suggest a possible mechanism for DNA/lipid membrane interaction involving DNA encapsulation within an inverted micelle included in the lipid membrane. Our model observations could help in understanding events associated with the interaction of DNA with biological membranes, as well as cationic liposomes/DNA complex formation in gene transfer processes.

Key words DNA interactions · Cationic giant unilamellar vesicle · Microinjection · Sphingosine · Endocytosis

Abbreviations *DNA 21b* Oligonucleotide DNA ss 21b · *DNA 250 bp* Calf thymus DNA ds 250 bp · *GUV(s)* Giant unilamellar vesicle(s) · *DPhPC* 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine · *DMPC* 1,2-Dimyrisoyl-*sn*-glycero-3-phosphocholine · *SOPC* β-Stearoylγ-oleoyl-L-α-phosphocholine · *eggPC* Egg phosphocholine · *PC* Phosphocholine · *Sph^+* D-*erythro*-Sphingosine · *PEG* Poly(ethylene glycol)

Introduction

Associaton of DNA with membranes and membrane/DNA complex formation play important roles in biological processes such as DNA replication and segregation (Firshein 1989). Moreover, sphingolipids have been found in nuclear membranes and chromatin fractions, and their amount seems to vary between active and repressed chromatin (Alessenko et al. 1982). The transfer of sphingolipids from sites of synthesis to the plasma membrane is blocked during mitosis, whereas that of phosphatidylethanolamine remains unchanged (Kobayashi and Pagano 1989). Sphingosine, a breakdown product of cellular sphingomyelin, is a bioactive molecule which regulates the transcription and replication processes, cell growth, differentiation, and apoptosis by protein kinase C-independent pathways (Hong et al. 1990; Zhang et al. 1990; Sakakura et al. 1996).

The binding of DNA to liposomes which contain sphingosine has been systematically investigated by differential scanning calorimetry (DSC) and fluorimetry (resonance energy transfer, RET) in liposome suspensions, and using the monolayer technique (Kinunnen et al. 1993; Kõiv and Kinnunen 1994; Kõiv et al. 1994, 1995). These experiments showed that the interaction between DNA and

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sphingosine-containing liposomes is electrostatic in nature. More specifically, this interaction should involve the protonated amino group of D-*erythro*-sphingosine (Sph^+) and the negatively charged phosphates of DNA. Binding of DNA to DMPC vesicles containing Sph^+ was dependent on the pH: the decrease of fluorescence intensity, due to RET between marked DNA and lipids, was 80% at pH 5.9, 70% at pH 7.4, and 40% at pH 8.5. DNA binding to membranes containing Sph^+ was stronger at acidic pH but was present at basic pH as well. Measurements of pK_a for Sph^+ in mixed micelles with Triton X-100 have yielded different values, i.e., 6.7 and 7.7, but the exact value in liposomes is not known. It is generally assumed that Sph^+ is at least partially protonated at physiological pH. DNA binding to liposomes containing Sph^+ could be reversed by including phosphatidic acid in the lipid membrane. Sphingosine phase separation and the formation of domains enriched in sphingosine, caused by the attached DNA, were evident from DSC and monolayer experiments.

Liposomes containing synthetic cationic lipids are currently used as carriers of antisense oligonucleotides and plasmid DNA, which regulate specific gene transfer (Felgner et al. 1987; Singhal and Huang 1994; Zelphati and Szoka 1996). Recently, liposomes containing sphingosine were used for DNA transfection with high efficiency and low toxicity (Paukku et al. 1997). We have recently shown, by using the patch clamp method of bilayers, that the electrotransfer of plasmid DNA through the membrane is dependent upon the presence of sphingosine (Hristova et al. 1997).

Considerable experimental and theoretical efforts have been focused on characterizing the structure of DNA/cationic liposome complexes (Gershon et al. 1993; Sternberg et al. 1994; Dan 1996; May and Ben-Shaul 1997). Despite the extensive studies, the mechanism of DNA ineraction with positively charged liposomes and the structure of the resulting complexes are still poorly understood. Surprisingly, until now, all studies on DNA/liposome interactions have been done on liposome suspensions. Adding DNA to such suspensions leads to liposome aggregate formation (Gershon et al. 1993; Kinunnen et al. 1993; Jääskeläinen et al. 1994; Sternberg et al. 1994; Rädler et al. 1997). No information about the effects of DNA interacting locally, as a result of local and temporal delivery of DNA to a part of the membrane of an individual vesicle, is available. This type of study would be of particular interest in modeling biological events in living cells.

Currently, we are developing a novel approach for direct optical microscopy visualization and studying the interactions of individual vesicles with colloidal particles (Angelova et al. 1994; Dietrich et al. 1997), as well as the effects of active substances injected locally by a micropipette to a part of the vesicle membrane (Wick et al. 1996). The giant unilamellar vesicles, big enough (50–150 µm in diameter) for the membrane shape and morphology transformation to be clearly seen under an optical microscope, were prepared by the liposome electroformation method. Electroformation can quickly supply a large number of giant unilamellar vesicles (GUVs), and can be used efficiently in studies involving individual vesicles, micromanipulation, and microinjection (Guedeau-Boudeville et al. 1995; Mathivet et al. 1996; Wick and Luisi 1996; Menger and Angelova 1998).

In this work we present experiments from viewing with an optical microscope the effects of short DNA microinjected locally to GUVs containing sphingosine. We observed DNA-induced endocytosis due to the local DNA/lipid membrane interactions and complex formation. We are suggesting a possible mechanism for DNA/lipid membrane interaction, complex formation, and membrane shape and morphology transformations.

Materials and methods

GUVs were prepared from 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC). β-stearoyl-γ-oleoyl-L-α-phosphocholine (SOPC), egg phosphocholine (eggPC), and their mixtures with sphingosine at PC/Sph^+ ratios of 97:3, 93:7, 85:15, 75:25, and 67:33 mol%. (Synthetic DPhPC, Avanti Polar Lipids; synthetic SOPC, Sigma Sph^+, from bovine brain sphingomyelin, Sigma). The initial lipid deposit and the resulting GUVs become unstable for Sph^+ concentrations higher than 33 mol%. GUVs were formed by the liposome electroformation method (Angelova and Dimitrov 1986; Dimitrov and Angelova 1988).

The particular electroformation protocol established in this work was as follows: SOPC, SOPC/Sph^+, eggPC, eggPC/Sph^+ solutions were prepared in diethyl ether/methanol (9:1) at 0.3 mg/ml of total lipid; DPhPC and DPhPC/Sph^+ solutions, in chloroform/diethyl ether/methanol (2:7:1) at 0.9 mg/ml of total lipid. A droplet of lipid solution (1 µl) was deposited (avoiding sliding) on each of the two parallel platinum wires (diameter 0.8 mm, distance between axes 3 mm) and dried under nitrogen for 30 min. An a.c. electrical field, 10 Hz, 0.3 V pp, was applied to the electrodes. Distilled water (1.2 ml, pH 5.5–6.0, conductivity 3.3 µS cm^{-1}) was added (avoiding agitation) to the working chamber. The voltage was gradually increased (over 15 min) up to 2.5 V pp. The GUVs were ready in 2 h for further utilization. In each preparation at least 10 GUVs of diameter 100–150 µm were available.

The "short" DNAs used were: (1) oligonucleotide 21b (ssDNA; 21 bases: 5′-CAACCATATCTACACAGGGTC-3′) MW = 6.23×10^3 (kindly supplied by the Institute of Molecular Biology, BAS, Sofia) and (2) calf thymus 250 bp DNA (dsDNA 250 bp), MW = 1.65×10^5, prepared by ultrasonication from calf thymus highly polymerized DNA (Sigma). Molecular size was checked by 3% agarose gel electrophoresis. The DNA for local GUV microinjection was in a distilled water solution at a concentration of 0.01 mg/ml. DNA/GUV membrane interaction at a single microinjection occurred under conditions of large lipid excess with respect to the DNA (about 10^3 lipid molecules/nucleotide). DNAs were marked for fluorescent microscopy visualization with Hoechst 33258 dye (Molecular Probes, Ex/Em = 352/461 nm) at a dye/nucleotide ratio of 1:10. The Hoechst dye molecules in water solution

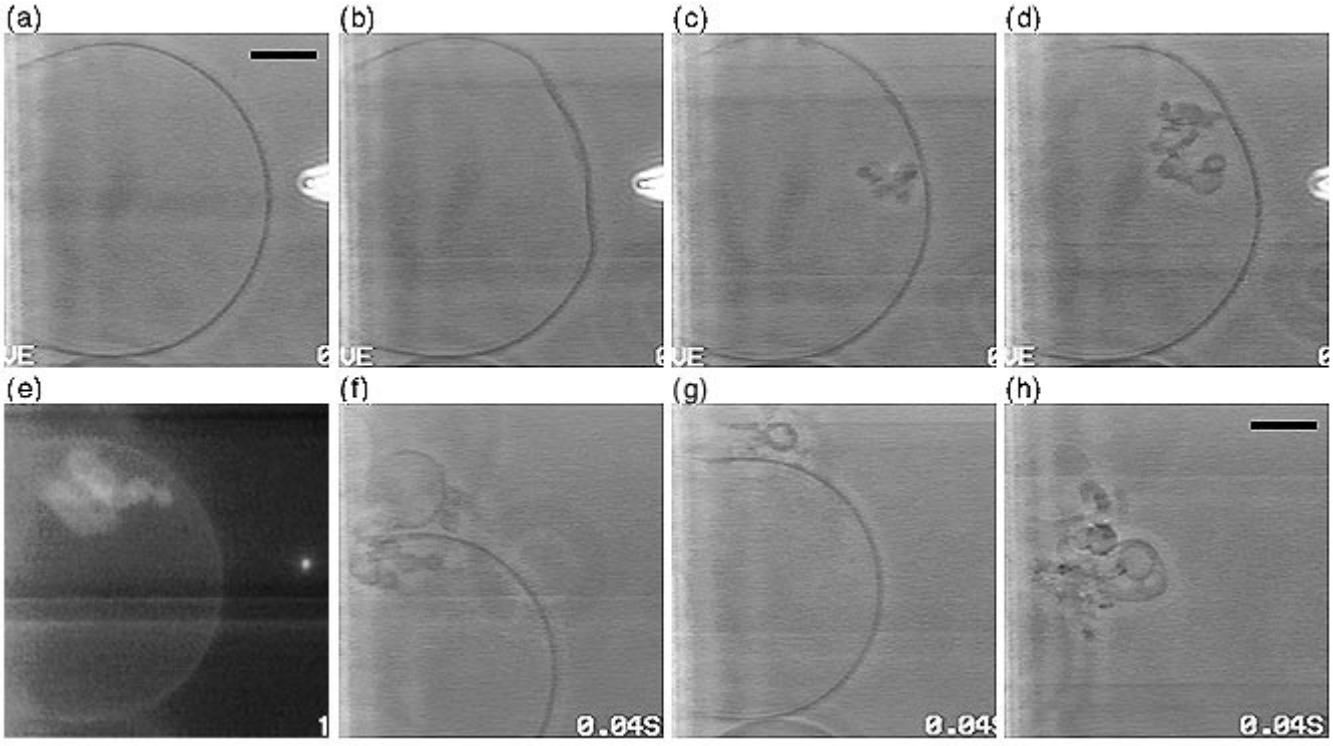


Fig. 1a–h Kinetics of membrane topology transformations of an initially quasi-spherical giant unilamellar vesicle (GUV) (DPhPC/Sph^+, 67 : 33 mol%) as a result of three microinjections of DNA 21b: (**a**) initial GUV; (**b**) first injection destabilizes a part of the membrane and makes it highly fluctuating; (**c**) an eruption of vesicles toward the inner vesicle space is produced within 1 s; (**d**) subsequent injections destabilizes the membrane again, and series of endocytic vesicle eruptions follow in intervals of a few seconds; (**e**) distribution of marked DNA fluorescence within the affected GUV; (**f**) internal vesicles going out of the GUV, mimicking exocytosis; (**g**) "mother" GUV re-heals with a smaller diameter; (**h**) the initial GUV is finally transformed into a multilayered lipid/DNA aggregate of irregular shape. *Bar* = 30 µm

have three positive charges, strongly bind to the DNA helix, and become fluorescent only after binding to DNA (Loontiens et al. 1990).

The poly(ethylene glycol) solution (PEG, MW 4000, Merck for microinjection was at a concentration up to 30% w/w.

Microinjection was carried out using an Eppendorf Transjector. The microcapillary inner diameter for performing local microinjection onto a GUV was 0.5–1 µm in diameter. Injected volumes were on the order of picoliters ($1–10\times10^{-12}$ l). The injection was performed from a distance of about 5 µm from the GUV surface, taking care to avoid any contact with the cationic lipid membrane (which would immediately seal the capillary with a lipid patch). The injected solution covers about 10% of the injected GUV surface (for a GUV of about 100 µm diameter).

The kinetics for membrane morphology transformations after local microinjection of DNA to the GUV were followed by phase contrast microscopy. DNA fluorescence distribution (labelled with Hoechst dye) was monitored by Zeiss filter set (Ex/Em = 365/>420 nm).

We used a Zeiss Axiovert microscope, equipped with a Narishige micromanipulator, and a Hamamatsu chilled charge-coupled device camera connected to an image recording and processing system.

Results

The membrane tension that electroformed GUVs have at their "birth" is dependent on the kinetics of their formation. It can be controlled to a certain extent by the parameters of the external a.c. field, which determine the amplitude of the electroosmotically induced mechanical vibrations of growing membranes, applied during GUV formation. Larger electroosmotic vibrations during GUV growth result in the formation of a flaccid, highly fluctuating ellipsoidal GUV. This is due to the fact that the entrapped volume is smaller than that of the corresponding spherical shape vesicle. Minimum electroosmotic vibrations during GUV growth result in the formation of a stressed, spherical vesicle. Our estimation of the mechanical condition of a GUV is qualitative. One can have an idea about it from the shape and thermal fluctuations of the GUV membrane. Therefore, in our study we could qualitatively distinguish between flaccid (with large excess area), quasi-spherical (low excess area), and stressed (no excess area) GUVs. As a result, we could qualitatively test the DNA/GUV interaction with GUVs having initially different membrane tensions.

The image sequence shown in Fig. 1 presents the kinetics of membrane topology transformations of an initially quasi-spherical GUV (Fig. 1a), made of DPhPC/Sph^+

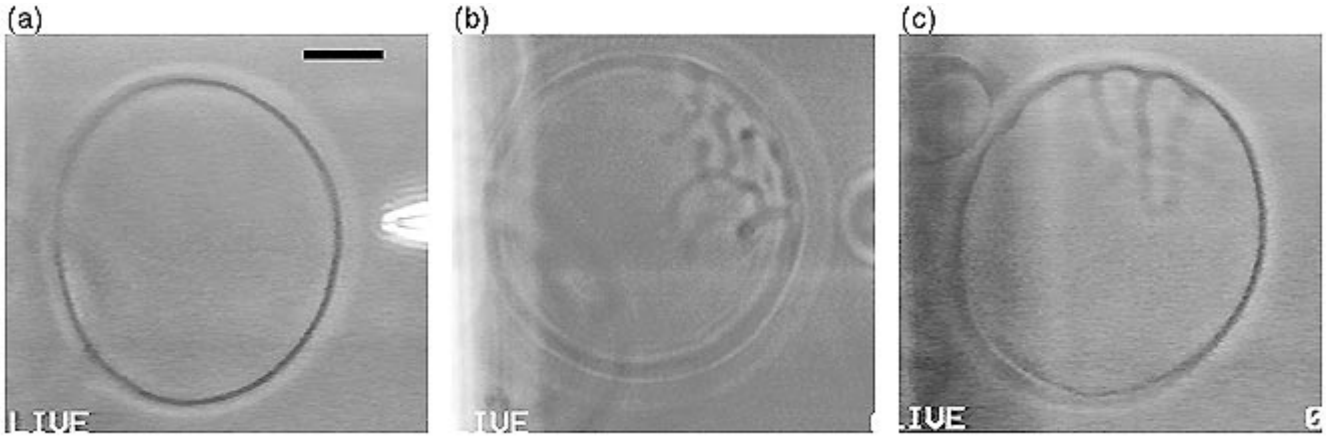


Fig. 2a–c Effects of DNA 21b local microinjection into an initially flaccid GUV (DPhPC/Sph^+, 67:33 mol%): (**a**) initial GUV; (**b**) membrane endocytic tube-like, branched, and flexible liposomes start growing in a few places after the injection; (**c**) some of them close and detach from the GUV membrane, but the others stay like open funnels for at least 30 min. *Bar* = 30 µm

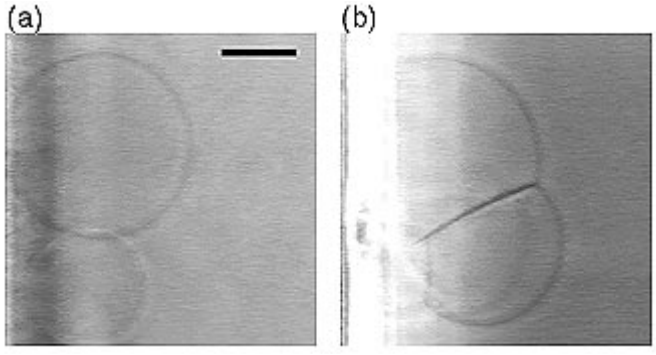


Fig. 3 **a** Neighboring GUVs made up of DPhPC/Sph^+ (97:3 mol%). **b** DNA 21b microinjection caused strong irreversible adhesion between vesicles, but no endocytosis. *Bar* = 30 µm

(67:33 mol%) as a result of the DNA 21b (marked with Hoechst dye) microinjection. The contact of the DNA molecules with the membrane upon the first injection destabilizes it, making the affected membrane area highly fluctuating (Fig. 1b). An eruption of vesicles toward the inner vesicle space (endosomes of about 5 µm diameter) follows in about 1 s (Fig. 1c). Subsequent injections destabilize the membrane again, and a series of endocytic vesicle eruptions (protuberances) follow in intervals of a few seconds (Fig. 1d). Figure 1e presents the distribution of DNA fluorescence within the affected GUV. It is seen that some of the DNA is associated with the endosomes, and part of it is spread entirely over the GUV membrane. We observed endosomes going out of the "mother" GUV (mimicking exocytosis) (Fig. 1f). The "mother" GUV reheals with a smaller diameter (Fig. 1g). As a result of three microinjections the initial GUV is finally transformed into a multilayered lipid/DNA aggregate of irregular shape, as shown in Fig. 1h.

The results of the DNA 21b local microinjection to an initially flaccid GUV are shown in Fig. 2. Membrane endocytic tube-like liposomes start growing in a few places after the microinjection (Fig. 2b). These endosomes (about 5 µm in diameter and a few tenths of µm in length) are quite branched and flexible. Some of them close and detach the GUV membrane, but the others stay like open funnels (Fig. 2c) for at least 30 min. Under fluorescence, one can see that both kinds of endosomes, as well as the "mother" GUV membrane, are associated with DNA (images not shown).

The effects of the DNA 21b local microinjection to the GUV were dependent on: (1) the Sph^+ concentration in the GUV membrane, and (2) the type of zwitterionic PC membrane component used. A characteristic minimum concentration of Sph^+ (C_{endo}) in the GUV membrane is necessary for the typical endocytic phenomenon to occur. C_{endo} depends on the type of zwitterionic PC lipid used, being about 10 mol% for DPhPC/Sph^+ GUVs, and about 20 mol% for SOPC/Sph^+ or eggPC/Sph^+ GUVs. Below C_{endo}, only lateral adhesions between neighboring vesicles were observed after DNA local microinjection, as shown in Fig. 3, owing to the too low concentration of Sph^+.

The effects of DNA 250 bp locally injected to a DPhPC/Sph^+ (67:33 mol%) GUV are presented in Figs. 4 and 5. Again, endocytic vesicles were formed. Typical sizes were from about 1–2 µm for initially stressed vesicles (Fig. 4), to about 8 µm for initially quasi-spherical vesicles (Fig. 5). On average, the typical endosome sizes induced by DNA 250 bp were smaller than those in the case of DNA 21b. The DNA fluorescence in the case of DNA 250 bp is predominantly associated with the endocytic vesicles and is less dispersed over the GUV membrane far away from the spot of initial microinjection: see Fig. 4c and Fig. 5c in comparison with Fig. 1e.

Similar to the DNA 21b, the effects of DNA 250 bp local injection to GUVs were dependent on the Sph^+ concentration and the type of zwitterionic PC lipid used.

The capacity of DNA 250 bp to induce endocytosis was reduced when it was fluorescently marked with the Hoechst dye. For instance, the native DNA 250 bp induces endocytosis in both DPhPC/Sph^+ (67:33 mol%) and SOPC/Sph^+ (67:33 mol%). GUVs. Hoechst dye-marked DNA

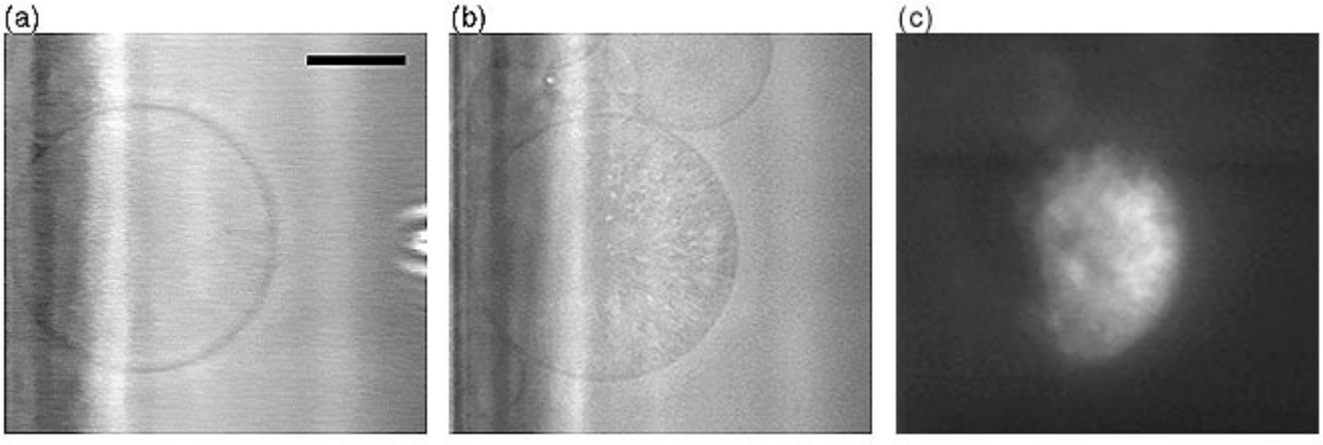


Fig. 4a–c Effects of DNA 250 bp locally microinjected onto an initially stressed GUV (DPhPC/Sph$^+$, 67:33 mol%): (**a**) initial vesicle; (**b**) endocytic vesicles formed were of sizes about 1 µm, and highly Brownian; (**c**) marked DNA fluorescence. *Bar* = 30 µm

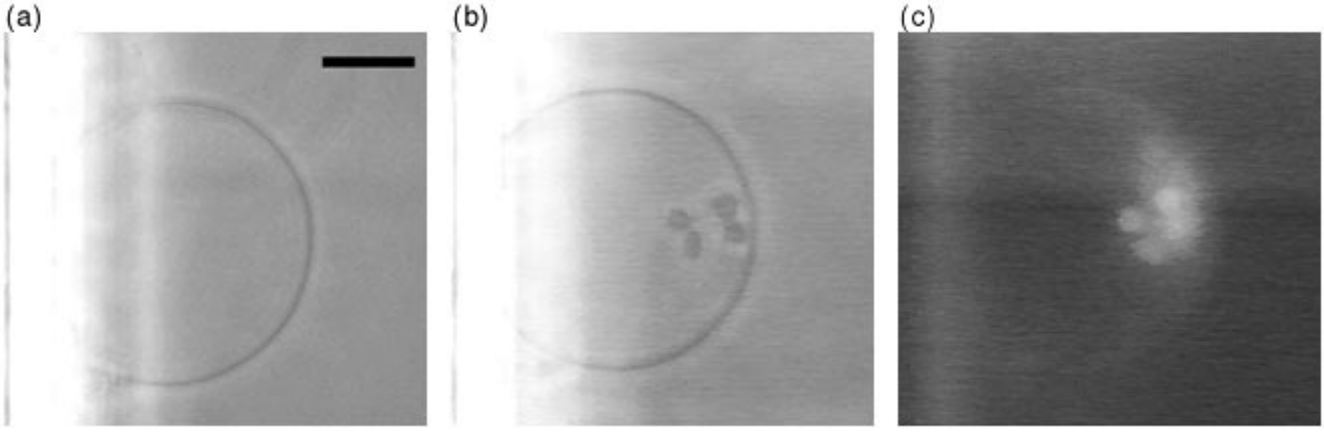
250 bp induces endocytosis on DPhPC/Sph$^+$ (67:33 mol%) GUVs but not on SOPC/Sph$^+$ (67:33 mol%). Only strong irreversible adhesions between neighboring vesicles (but no endocytosis) were observed in the latter case (see Fig. 6), probably owing to the decreased charge density of the DNA by the bound Hoechst dye.

In order to clarify the structure of the DNA/cationic membrane complex, we performed the following experiment: first, native DNA (21b or 250 bp) was microinjected onto a GUV, and endosome formation observed. Thus, the DNA/cationic membrane complex was formed. As already noted, in this complex the DNA is associated with the endosomes, as well as with the "mother" vesicle membrane. Then Hoechst dye solution was injected onto the same GUV. No fluorescence was detected. It shows that DNA molecules, once associated with the cationic membrane, become inaccessible for the dye molecules. One can assume that the DNA is protected by lipids in this DNA/cationic GUV complex over the entire GUV, in the endosomes as well as on the "mother" vesicle membrane. No DNA remains simply adhered to the GUV membrane (i.e., partially exposed to the water medium) in this case.

The fact that polymer solution is microinjected locally outside, at the GUV membrane, might cause osmotic effects. Therefore, we checked for eventual nonspecific osmotic effects. PEG has been shown neither to interact directly with phospholipids nor to induce the formation of nonlamellar structures by phosphatidylcholine. PEG binds water efficiently and is excluded from the surface of liposome membranes. Some GUVs were locally injected for up to 2 min with PEG solutions, with concentrations up to 30% w/w, at different pressures, using a micropipette with an inner diameter of 0.6 µm. PEG solution flow around the GUV was clearly visible in phase contrast owing to the different refractive index. No GUV topology transformations were observed. PEG was just diffusing into the bulk with no visible consequences for the GUV (images not shown). This result is not surprising bearing in mind that the injected PEG solution volume, in total not more than 5 µl, was freely diffusing into the 1.2 ml bulk of our working chamber.

We carried out the following control experiments: (1) DNA solutions injected onto GUVs containing no Sph$^+$ (made just of the PC zwitterionic lipids); (2) local injec-

Fig. 5a–c Effects of DNA 250 bp locally microinjected to an initially quasi-spherical GUV (DPhPC/Sph$^+$, 67:33 mol%): (**a**) initial vesicle; (**b**) endocytic vesicles formed were of sizes about 8 µm; (**c**) marked DNA fluorescence distribution. *Bar* = 30 µm



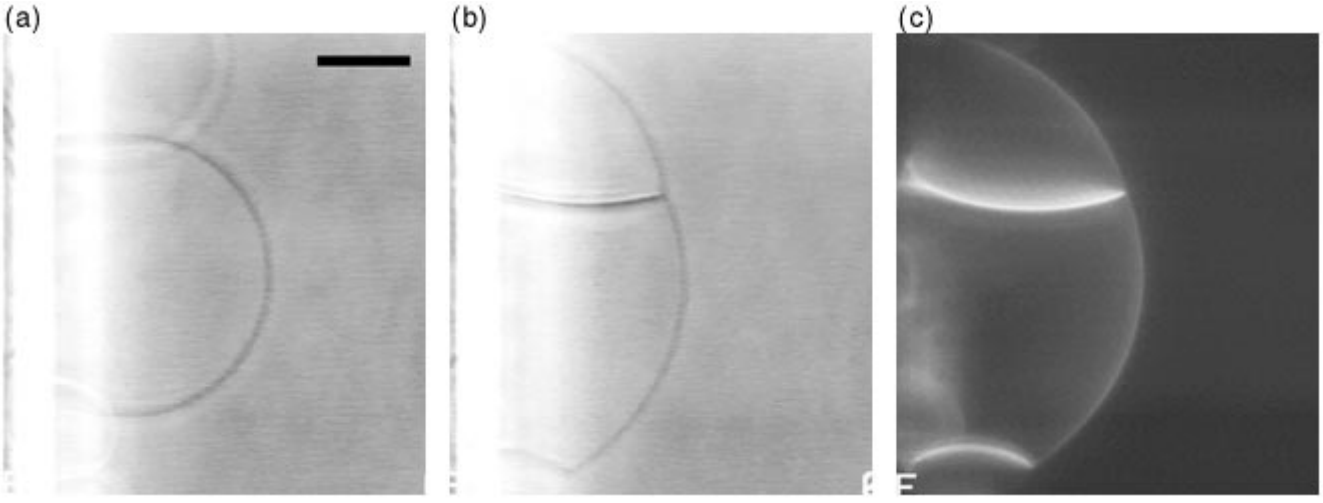


Fig. 6a–c Effects of DNA 250 bp (marked with Hoechst dye) locally microinjected onto SOPC/Sph^+ (67:33 mol%) GUVs: (**a**) initially quasi-spherical neighboring vesicles; (**b**) strong irreversible adhesion between neighboring vesicles; no endocytosis; (**c**) marked DNA fluorescence distribution. *Bar* = 30 µm

tion of water at different pressures onto GUVs containing Sph^+; (3) pouring DNA solutions with a large micropipette onto GUVs, in order to have homogeneous DNA concentration distribution and interaction with the entire GUV membrane (in contrast to the locally and temporary created DNA concentration at a spot on the membrane). No membrane morphology transformations were observed in cases (1) and (2). In case (3), the GUVs burst and transformed into multilamellar aggregates, like those in Fig. 1h. Neither fluorescence nor membrane topology transformations were observed when Hoechst dye solutions were injected directly onto GUVs (whether containing Sph^+ or not). The observations presented are an average of at least five experiments of the same kind.

The studies presented in this work were performed in distilled water (pH 5.5–6.0). The addition of 2 µg of lipid and up to 0.1 µg of DNA to 1.2 ml distilled water did not cause significant change of pH. The presence of buffers or salts suppresses GUV formation. The resulting vesicles are smaller in size, many of them are multilamellar, and therefore are not usable. In all cases, including previously mentioned references, as well as our experiments, make us believe that the triggering interaction between the DNA and Sph^+/PC GUV membrane is electrostatic. We assume the presence of salts, or pH variations between 5.9 and 7.4, could modify but would not eliminate the results of electrostatic interaction.

Discussion

Comprehensive theoretical consideration of the molecular structure of DNA/cationic liposome complexes was presented by May and Ben-Shaul (1997). A molecular theory is suggested for the thermodynamic stability of two (similar) types of structural complexes formed between DNA (rod-like, either single stranded or double stranded) and cationic liposomes. Both types involve a lipid monolayer-coated DNA as the central structural unit. This central structural unit can be surrounded by another, oppositely curved, monolayer, thus forming a bilayer mantle, or a bundle of hexagonally packed DNA-monolayer units can be surrounded by a common outer monolayer. The formation free energy of these complexes, starting from a planar cationic/neutral lipid bilayer and bare DNA, is expressed as the sum of electrostatic, bending, mixing, and chain frustration contributions. The interplay between these factors is dependent on the cationic/neutral lipid composition. The authors found that the most stable monolayer-coated DNA units are formed when the charged/neutral lipid composition corresponds (nearly) to charge neutralization. The optimal monolayer radius corresponds to close DNA-monolayer contact. The uncharged lipid appears to play an important role in the formation of a monolayer-coated DNA complex: first, it enables the adjustment of the charge on the monolayer surface to ensure charge neutrality; second, if properly chosen, it lowers the cost of elastic energy associated with bending the monolayer (imposing high "negative" curvature) around the DNA. The calculated stabilization energies in this case were typically of the order of 1 $k_B T$/Å of DNA length. Suppose the DNA-lipid system contains a large excess of lipid molecules. In such a case, the complexes formed may adjust their lipid composition and radii so as to minimize the elastic energy contribution, with the excess bilayer serving as a lipid reservoir. As a general conclusion, to stabilize the lipid enveloped-DNA complex the lipid should have as low as possible bending rigidity, as high as possible fluidity, and favour the formation of hexagonal structures.

The DNA-lipid monolayer coated unit can be considered as a kind of inverted cylindrical micelle entrapping a DNA molecule in the enclosed water pool. Experimental study of the solubilization and structural properties of nucleic acids in reverse micelles is presented by Battistel et al. (1989). They showed that, surprisingly, even large

(250 kDa) DNA molecules can be hosted in small reverse micelles (with radii of 20–50 Å) composed of cationic, neutral, or anionic surfactants.

A theoretical consideration of the interactions between DNA molecules adsorbed on fluid membranes is presented by Dan (1996). The interaction between adsorbing DNA and the lipid heads changes the equilibrium density, i.e., area per molecule, in the affected region of the outer monolayer. Assuming that membrane fluidity implies that the two monolayers composing the bilayer can be decoupled, and that the DNA adsorption energy has a high enough value, Dan (1996) calculated a rise in the membrane-induced, attractive interactions between the adsorbed DNA molecules. These balance the direct repulsive interactions between DNA molecules. As a result, DNA adsorbed on membranes is predicted to form ordered domains characterized by finite spacing, which varies with the membrane characteristics and the solution Debye screening length.

Using the optical trapping and optical manipulation technique, the "physical endocytosis" of hydrophilic spherical particles by GUVs was studied (Angelova et al. 1994; Pouligny et al. 1995). Theoretical considerations showed that in the case of strong adhesion between the particle and the GUV membrane, the two monolayers constituting the lipid bilayer can be decoupled. Only the outer monolayer "rolls up" and encapsulates the particle by a topology transformation of the GUV membrane. As a result, a large asymmetry ($S_{ext} < S_{int}$) between the two GUV monolayers is produced.

In the present work we are studying the effects of delivering DNA locally to a part of the cationic GUV membrane. Both kinds of DNA used, DNA 21b and DNA 250 bp, can be considered as rigid rods, 22 Å in diameter and 72 Å and 862 Å in length, respectively. Persistence length of single stranded oligonucleotide DNA, 14 to 22 bases, at different buffer conditions, is of the order of 53–88 Å (Porschke and Jung 1985); persistence length of double stranded DNA is about 500 Å at physiological conditions, increasing up to 872 Å at low salt concentrations (Porschke 1991). The GUV membrane was, in all cases, in the liquid-crystalline phase. One of the neutral lipids used, DPhPC, has particularly high steric asymmetry – two branched saturated hydrocarbon chains 16 : 0 [$(CH_3)_4$] – indicating potential for formation of inverted hexagonal structures. It is known that sphingosine favors the formation of hexagonal structures (Kõiv et al. 1994). Most of the sphingosine molecules should be protonated under the conditions of our experiment.

We observed DNA-induced adhesion and endocytosis due to the DNA/lipid membrane local interactions and complex formation. A possible mechanism should explain the following findings:

1. Both factors, the Sph^+ in the GUV membrane and DNA in the microinjected solution, are necessary for the phenomena under consideration to occur.
2. A characteristic minimum concentration of Sph^+ (C_{endo}) in the GUV membrane is necessary for the endocytic phenomenon to occur.
3. Below C_{endo}, only lateral adhesions between neighboring vesicles were observed upon DNA local addition.
4. C_{endo} depends on the type of zwitterionic PC lipid used, being about 10 mol% for DPhPC/Sph^+ GUVs and about 20 mol% for SOPC/Sph^+ or eggPC/Sph^+ GUVs.
5. The characteristic sizes and shapes of the resulting endosomes depend on the kind of DNA, and the initial GUV membrane tension.
6. When the fluorescent DNA Hoechst marker dye was added after the DNA/lipid local interaction and complex formation, no fluorescence was detected, although the DNA in the DNA/GUV complex had been previously associated with the endosomes, as well as with the "mother" vesicle membrane.

The reason for the vesicles sticking to each other in cases like those shown in Figs. 3 and 6 is probably simple membrane adhesion stabilized by electrostatic interactions of the DNA molecule with each of the two GUV membranes. Lamellar packing (sandwiching the DNA helixes arranged within the contact zone between the two GUV membranes) like those proposed by Rädler et al. (1997) could be suggested (illustration not shown). The findings 1 to 5 could be, more or less, explained by this simple model of DNA adhesion to the membrane.

Finding 6 however, makes us think about something more sophisticated. This observation could be explained if one assumes that the DNA is protected by lipids in the DNA/lipid complex, and is thereby inaccessible to the Hoechst dye molecules. Figure 7 presents an illustration of a possible mechanism we suggest for understanding all the observed phenomena (1 to 6). Figure 7a presents the stage of initial, electrostatically driven, DNA adsorption to the cationic membrane. In Fig. 7b the adhesion energy density between the DNA and outer monolayer increases owing to lateral diffusion of Sph^+ furnished by the excess lipid phase of the GUV. The outer monolayer decouples from the inner one and "rolls" up the DNA rod, forming cylindrical defects resembling germs of inverted hexagonal structures. Whenever and wherever the electrostatic attraction, membrane bending rigidity, lipid geometry, and component demixing factors reach a favorable combination, the GUV membrane undergoes the topology transformation presented in Figure 7c. The theoretical background for the possibility of such topology transformation and for the resulting complex to be stable has been presented at the beginning of this discussion. The peculiar membrane inclusion, inverted cylindrical micelle entrapping a DNA molecule (and probably some water), is formed consuming only the outer GUV monolayer. The resulting membrane asymmetry, in this case ($S_{ext} < S_{int}$), is much larger than the one possibly induced in the case of simple DNA adsorption to cationic membranes. Furthermore, domains of different structural and mechanical properties should appear on the scale of the GUV membrane affected by the local DNA microinjection. The creation of large membrane surface asymmetry and lateral gradients of the mechanical properties of the GUV membrane result in membrane invaginations as shown in Fig. 7d, and, eventually, in endo-

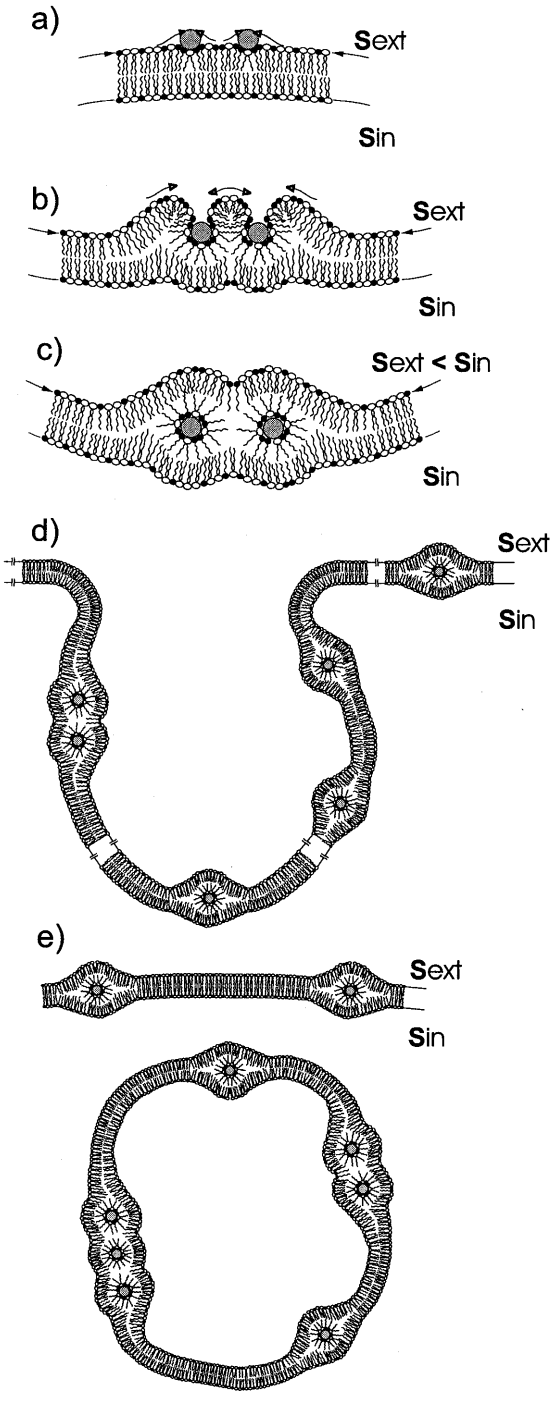


Fig. 7a–e A sketch of the suggested mechanisms of "short" DNA/membrane complex formation, the induced GUV membrane topology transformations, and the resulting endosome: (**a**) DNA adsorption to the GUV (planar) membrane (the *dashed circles* represent transverse sections of the DNA molecules); (**b**) lateral diffusion and rise of the Sph^+ concentration, decoupling of the two monolayers composing the bilayer membrane, and external monolayer "rolling" up on the DNA molecule; (**c**) the external lipid monolayer undergoes topology transformation and encapsulates the DNA molecule within a cylindrical inverted micellar structure; membrane asymmetry, $S_{ext} < S_{int}$, is created; (**d**) membrane invaginates at the scale of a few micrometers; (**e**) endosome forms

some formation; see Fig. 7e. Characteristic sizes and shapes of membrane invaginations and resulting endosomes should depend on local membrane composition, the initial GUV condition (presence or not of excess area), and hydrodynamics of the DNA flux upon the local microinjection.

We suggest a possible "nontrivial" mechanism of DNA/-lipid membrane interaction involving DNA molecule encapsulation within an inverted micelle included in the lipid membrane. Currently we do not have any absolute evidence to prove it. We do not claim either that this is the only possibility. However, we found some serious experimental indications and theoretical considerations for proposing it as a possibility. X-ray studies (or other structural methods) on our lipid/DNA systems would be of particular interest for elucidating the possibility for the proposed structures to exist as equilibrium structures. Our model observations could help in understanding the association of DNA with biological membranes, as well as cationic liposomes/DNA complex formation in gene transfer processes.

In conclusion, we would like to point out that in this paper we are effectively suggesting a general approach and method for the direct study of DNA/membrane interactions which could be easily applied to testing the effects of a particular kind of DNA interacting locally with a model or biological membrane.

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