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Formation of Supramolecular Structures by Negatively Charged Liposomes in the Presence of Nucleic Acids and Divalent Cations

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Cationic liposomes are being increasingly studied as delivery vehicles for bioactive agents such as DNA and other polynucleotides. The mechanism of interaction of DNA with liposomes and the organization of these interacting structures during and after the interaction are still poorly understood. Nucleic acids are known to induce aggregation and size enlargement of liposomes. In the case of phosphatidylcholine (PC) vesicles, these processes depend on the presence and concentration of divalent metal cations and the amount of cholesterol in the liposomes. In this study, anionic small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) composed of dicetylphosphate (DCP):PC:cholesterol at 2:7:1 molar ratios were prepared and incubated with the DNA (from wheat) and Ca^{2+} (50 mM) at 25°C with the aim of transferring the genetic material into the liposomes by inducing fusion of liposome–liposome aggregates created in the presence, and with the help, of DNA. The organization and the nature of the resultant liposome–DNA–Ca^{2+} complexes were investigated by scanning tunneling microscopy (STM) and fluorescence microscopy. Observations of complexes with similar appearances with both SUV and MLV, as shown by two quite different microscopic approaches, prove that the resultant forms are real and not artifacts of the methodology used. At this stage it is not clear whether the detected complexes represent an intermediate state before fusion of liposomes which will lead to engulfing of the genomic material by the fused liposomes, or the final form. In either case the structures consisting of some adhered or semifused liposomes bearing the nucleic acid seem to be candidates as vehicles for in-vitro and in-vivo transfection.

Keywords Anionic Liposome, Fluorescence Microscopy, Multilamellar Vesicles (MLV), Scanning Tunneling Microscopy (STM), Small Unilamellar Vesicles (SUV)

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Nucleic acids are increasingly being considered for therapeutic applications, either to alter the function of numerous proteins or to interfere with the function of specific nucleic acids. Aptamers (Sullenger et al. 1990; Ess, Hutton, and Aronow 1994), antigene nucleic acids (Volkmann, Dannull, and Moeling 1993; Grigoriev et al. 1993), ribozymes (Koizumi, Kamiya, and Ohtsuka 1992; Kashani-Sabet et al. 1994), and antisense oligonucleotides (Stein and Cheng 1993; Li and Huang 1997) are among the nucleic acid drugs with successful in-vitro and in-vivo applications. On the other hand, liposomes are considered an important class of vehicles with potential for use in the protection and delivery of nucleic acids to cells and tissues. Recently, complexes of phospholipid vesicles and DNA have been tested for gene transfer into a variety of target cells (Hofland and Huang 1995; Li et al. 1996). Most liposomes used for this purpose are constructed of cationic lipids which are reported to have high levels of transfection in vitro (Felgner 1991) and a low to modest transfection in animal experiments (Zhu et al. 1993). However, the potential for toxicity (Raz et al. 1994; Filion and Phillips 1996), serum instability, and reduced bioavailability (Litzinger 1997) of cationic liposomes limit the usefulness of their complexes in vivo. To alleviate the disadvantages of the cationic liposomes, anionic liposome formulations were developed in this laboratory with the principal aim of DNA delivery. The occurrence of aggregation of such liposomes in the presence of nucleic acids and divalent cations was indirectly shown earlier by turbidity measurements and light scattering (Mozafari and Hasirci 1998). The presence of an aggregate implies that the polyelectrolyte (DNA) and the divalent cation can induce interaction of liposomes which could eventually end up fusing with and possibly engulfing the DNA located at the interphase. This could then mean that anionic liposomes containing a genomic material could be fused with cells through the action of DNA and divalent cations in the medium, leading to transfection.

The current study is devoted to provide more direct evidence of interaction between anionic liposomes and the formation of such supramolecular structures, using scanning tunneling microscopy (STM) and fluorescence microscopy.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine) and $CaCl_2$ were purchased from Merck AG (Germany). Cholesterol (CHOL), dicetylphosphate (DCP, dihexadecylphosphate), oil red O, and calcein were all from Sigma Chemical Co. (USA).

Lambda DNA/Hind III Marker for gel electrophoresis was the product of MBI Fermentas (Lithuania).

All the chemicals were used without further treatment or purification.

Preparation of Nucleic Acid Fragments

Since an expression vector DNA and a recipient organism were not available, genomic DNA was used. DNA extracted from wheat seedlings (*Triticum aestivum* L. Gerek) following the methods of Saghai-Maroof et al. (1984) was a gift from Prof. M. Yücel (Middle East Technical University, Ankara, Turkey). The concentration and purity of the DNA samples were evaluated with a double-beam UV/VIS spectrophotometer (Shimadzu UV-2100S, Japan) at 260 and 280 nm (Allen et al. 1972; Müller, Ziegler, and Schweizer 1993). In order to reduce the size, the genomic DNA sample was fragmented by probe sonication at a setting of 20 W by a sonic dismembrator (ARTEK Systems Co., USA), in a cooling bath (3–5°C), for 4 min, with 30 s on/30 s off cycles. The size range of the fragmented DNA molecules were determined through gel electrophoresis (Maniatis, Fritsch, and Sambrook 1982) by comparison with the λ DNA size marker. For this, λ DNA/Hind III was run along the samples as a size marker. Using the size scale on the information sheet of the λ DNA marker, the digested DNA molecule size was determined. The highest-molecular-weight fragment in λ DNA was 23,130 base pairs. Based on this calibration, the digested DNA molecules were found to be in the 250–100 bp (85–340 nm) range. This analysis was carried out with sonication and electrophoresis with the DNA concentrations of 160, 320, 800, and 1600 μg/mL with almost identical results and were thus considered reproducible.

Liposome Preparation

DCP (0.2 mL, 50 mM in $CHCl_3$: methanol, 1:1), PC (0.7 mL, 50 mM in $CHCl_3$), and CHOL (0.1 mL, 50 mM in $CHCl_3$), were evaporated to dryness under a stream of nitrogen by a rotary evaporator (Bibby Sterilin, RE-100, England). The resultant lipid film was suspended in 2 mL μQ deionized H_2O (obtained through Sation 9000 ultrapure water system, Spain) to form multilamellar vesicles (MLV).

In order to prepare small unilamellar vesicles (SUV), the MLV suspension was probe sonicated at 20 W for 15 min under a nitrogen atmosphere.

Interaction of DNA and Calcium Ions with Liposomes

An aqueous solution of fragmented DNA (1 mL, 100 μg/mL) was introduced to the MLV or SUV medium (2 mL) and incubated with the liposomes in the presence of Ca^{2+} (50 mM) for 30 min at 25°C. Separation of liposome-associated DNA from the free DNA was carried out by centrifugation (Sorvall RC-5B Refrigerated Superspeed Centrifuge, USA) at 40,000*g* for 60 min at 3°C.

Scanning Tunneling Microscopy (STM)

The STM used in the present study was employed successfully earlier in the imaging of biopolymers (Zareie et al. 1996a, 1996b), and liposomes (Zareie et al. 1996c). It consisted of two main modules: a scanner and a coarse positioner. These modules could be separated to perform distinct measurements, performance tests, and experiments.

For STM studies, aqueous solutions of fragmented DNA (10 μL, 100 μg/mL), suspensions of MLV, or SUV–DNA–Ca^{2+} complexes (20 μL, 0.1 mg/mL) were deposited on the Au (111), which was grown epitaxially on mica (De Rose, Lampner, and Lindsay 1993) separately. The samples were then dried in a clean room at room temperature under atmospheric pressure with a relatively slow drying rate. The STM was operated in air at atmospheric pressure with a tip-to-substrate bias of 1–1.5 V (sample positive) and tunneling currents of 10–20 pA.

Fluorescence Microscopy

For fluorescence microscopic studies, oil red O (0.1 mL, 25 mM in $CHCl_3$) was added to the lipid solution before the solvent-removal step. Free oil red O molecules were then removed by centrifugation at 40,000*g* for 60 min at 3°C. Oil red O containing MLV were incubated with DNA molecules (1 mL, 100 μg/mL) and Ca^{2+} (50 mM) as described above. Before examination, calcein (5 mM) was added to the suspension to view calcium-containing complexes. Samples were viewed with an Olympus BX5O fluorescence microscope at room temperature, and micrographs were obtained with a PM2O-type camera through a B (Blue) filter.

RESULTS AND DISCUSSION

Recently it was shown in our laboratory that DNA alone does not induce an interaction between anionic liposomes (Mozafari and Hasirci, 1998). Calcium ions alone were shown to induce interaction between liposomes, and this interaction was amplified when DNA fragments were also introduced to the aqueous liposome–Ca^{2+} suspension. The presence of at least 10 mM Ca^{2+} was essential to bring about an interaction between them, and the increase in the absorbance (the indication of interaction)

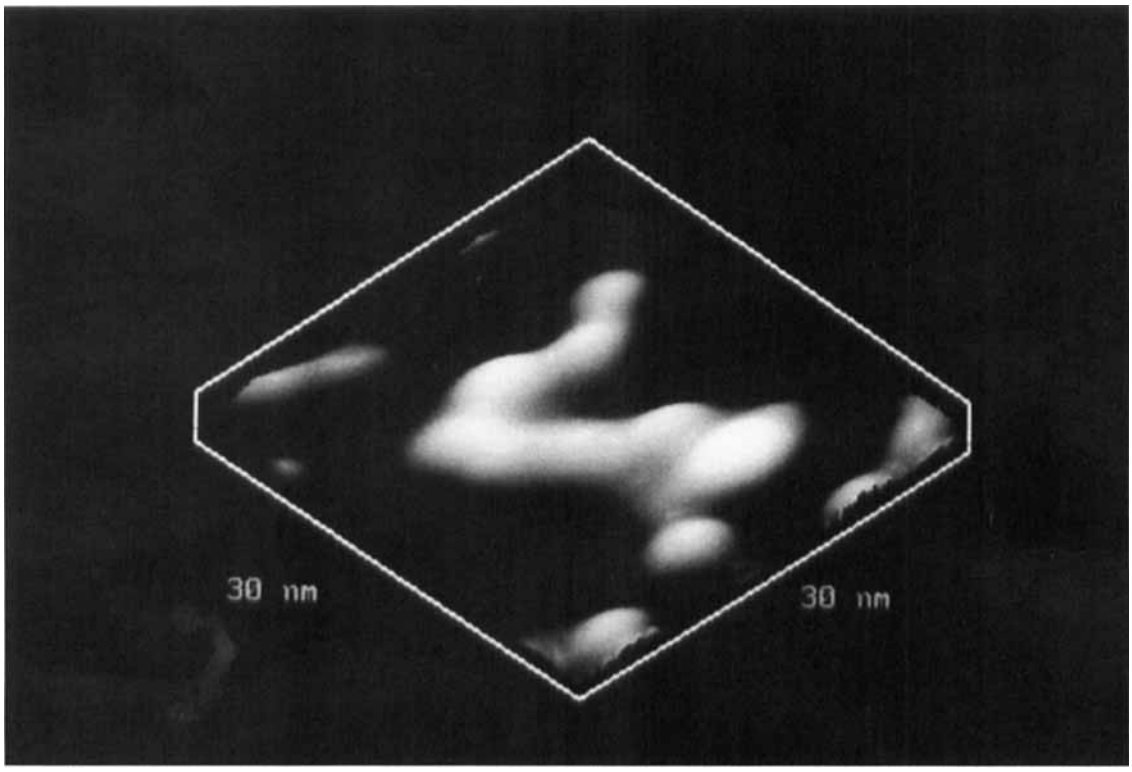

FIG. 1. Scanning tunneling micrograph of a DNA fragment (from wheat) on gold. Micrograph dimensions: 30 × 30 nm.

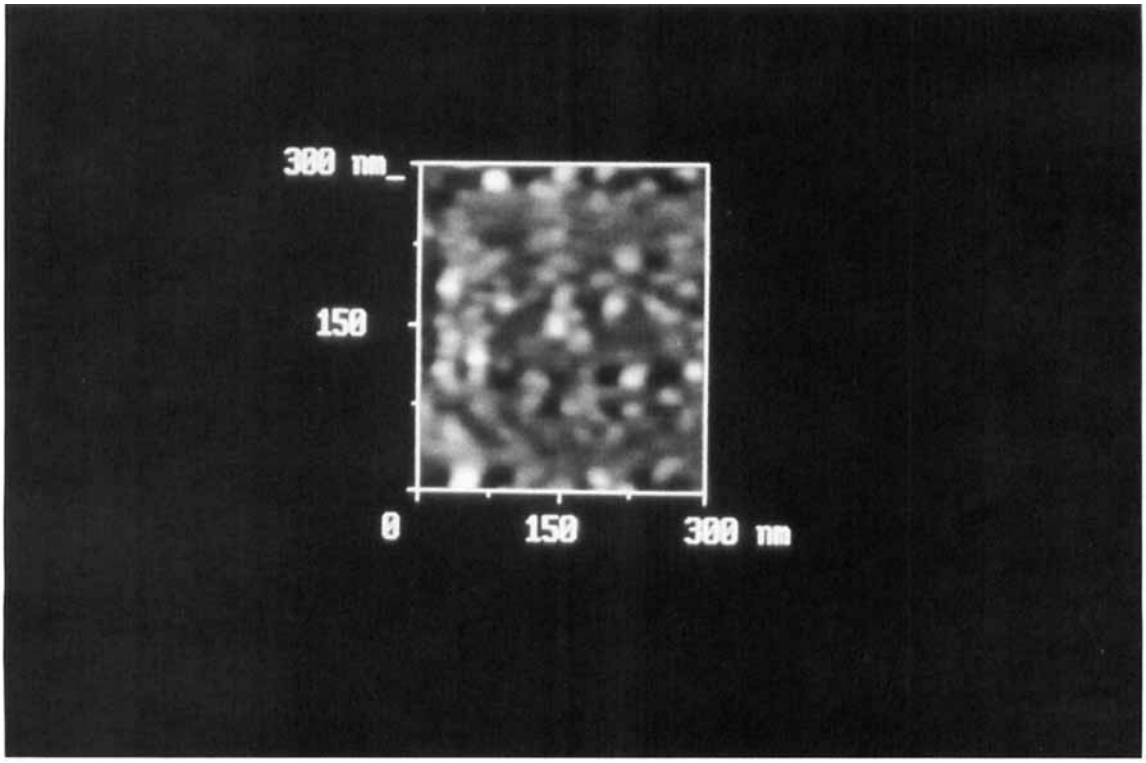

FIG. 2. Scanning tunneling micrograph of MLV (DCP:PC:CHOL 2:7:1) on gold. Micrograph dimensions: 300 × 300 nm.

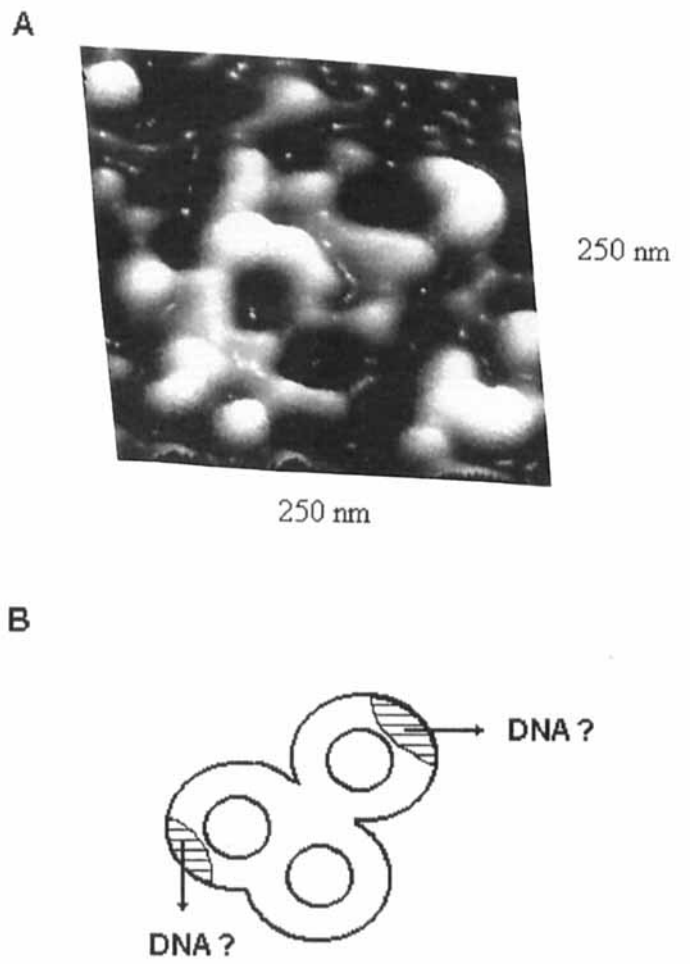

FIG. 3. Supramolecular complex formed by the interaction of SUV, DNA, and Ca^{2+}. (A) STM of complex on gold. Micrograph dimensions: 250 × 250 nm. (B) possible organization of the triple complex.

was almost exponential with Ca^{2+} concentration. When DNA and anionic liposomes were brought in contact, no change in medium turbidity was observed, stressing the necessity of Ca^{2+} presence. In addition, it was observed that MLV containing PC undergo interactions with DNA and Ca^{2+} (50 mM) even in the absence of negatively charged lipids (e.g., DCP). The tendency of vesicles to aggregate in the presence of DNA and Ca^{2+} were found to depend also on their cholesterol contents. Upon increasing the ratio of cholesterol in the liposomes to 40%, the interactions were inhibited.

Scanning Tunneling Microscopy

Figure 1 shows a STM image of a double-stranded DNA fragment used in this study. As stated in the Materials and Methods section, sonication was performed in order to reduce the size of the chromosomal DNA, and its size was found to be in the range of 85–340 nm by electrophoresis. In no other stage of the procedure was sonication performed. Thus, the dimensions of the DNA fragments were not expected to change any further. The length of the DNA fragment shown in Figure 1 was measured to be 41 nm. This value is much smaller than the size range obtained by gel electrophoresis and could possibly be a result of supercoiling or simple chain bending and folding during sample preparation for STM and was also encountered in our earlier studies (Zareie 1995; Zareie et al. 1996a). The gel electrophoresis was carried out in an aqueous medium in which the DNA fragments are in a fully hydrated state, while during STM sample preparation a room-temperature dehydration step is involved, which definitely leads to dehydrated and therefore more compact chains. The diameter of the DNA chain in Figure 1 is between 2.5 and 7 nm, significantly higher than 2.0 nm, the diameter of double-stranded B-DNA (Lehninger, Nelson, and Cox 1993), and further supports the occurrence of nucleic acid supercoiling.

A scanning tunneling micrograph of the MLV (DCP:PC:CHOL, 2:7:1) is shown in Figure 2. The periodicity of lipid bilayers is clearly observed in this figure. The bilayer thickness of the MLV is about 110 nm and the outermost radius of the vesicle is more than 300 nm, much higher than the value of 59.4 nm obtained by light-scattering measurements (which is carried out in aqueous medium) (Mozafari and Hasirci 1998). Here again STM appears to have led to spreading of the samples (in this case, MLV).

The STM in Figure 3A demonstrates formation of a supramolecular structure possibly consisting of SUV–DNA–Ca^{2+}. The presence of three liposomes in the center is seen, appearing aggregated and with "bulkier than the rest" (lighter) regions at certain locations of the circular shapes. The sizes of these regions are quite compatible with the lengths of DNA fragments as determined by electrophoresis. It is plausible that DNA fragments are adsorbed on the outside surface of the liposome before inducing interaction of liposomes with the possible result of liposome fusion. Although DNA is among the reactants, it is not clearly distinguishable in the micrograph. A schematic interpretation of the aggregation mechanism explained above is presented here (Figure 3B).

Fluorescence Microscopy

Fluorescence microscopy revealed images of MLV–DNA–Ca^{2+} complexes (two complexes with three liposomes each in Figure 4A, and one complex with two liposomes in Figure 4B).

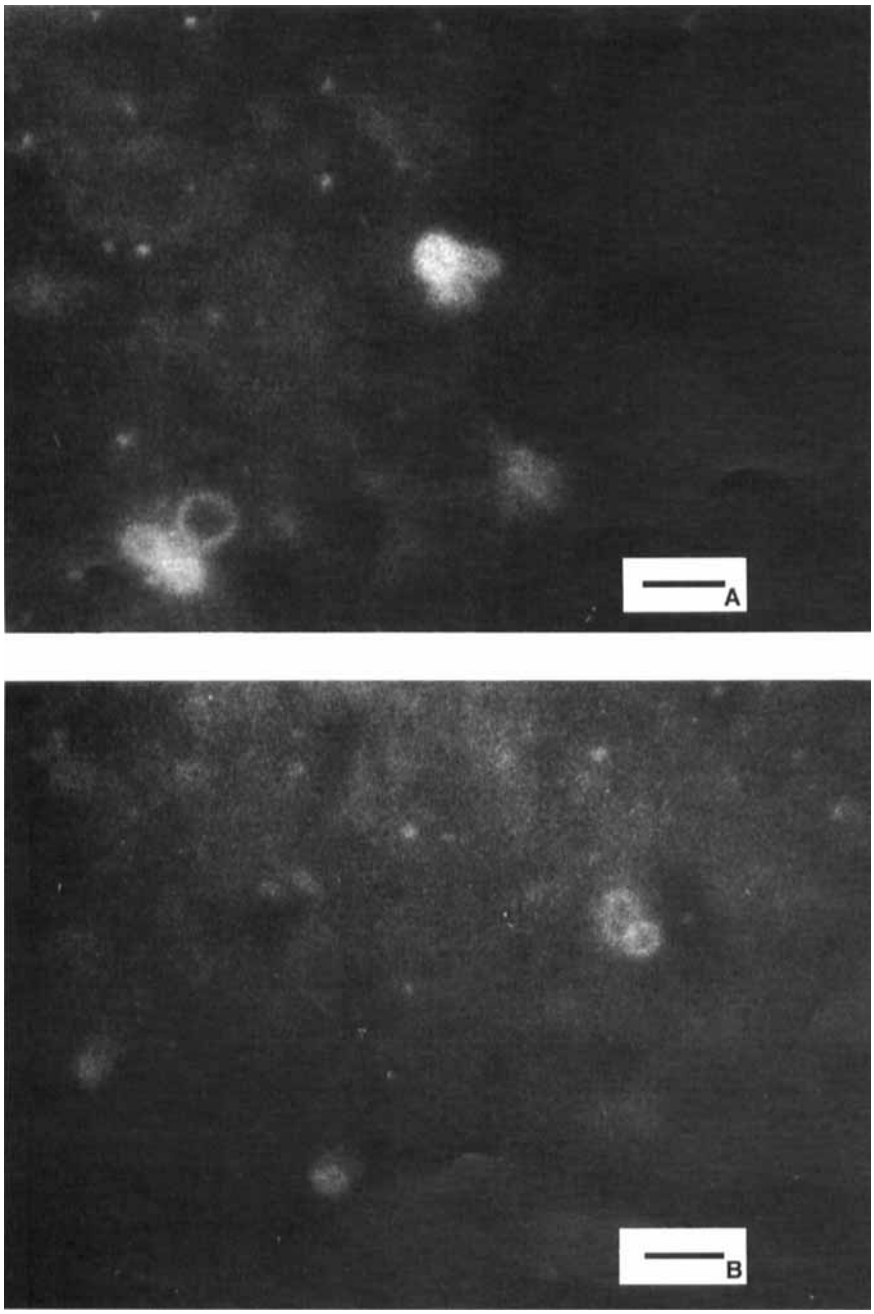

FIG. 4. (A) and (B) fluorescence microscopy of DNA and Ca^{2+}-induced liposomal interaction. MLV (DCP:PC:CHOL 2:7:1) containing 25 mM oil red O incubated with DNA (100 μg/mL) and 50 mM Ca^{2+} at 25°C (bars represent 1 μm each).

Liposomal aggregates have an average diameter of 1,200 nm. This value is also higher than the value of 165.4 nm obtained earlier by our group with the light-scattering method for similar complexes (Mozafari and Hasirci 1998). These differences are expected not just because the two methods have different approaches and sensitivities but also because the complexes selected for imaging and, therefore, for size measurement were the largest ones (for ease of recording) in the population.

The striking thing, however, is that two or three MLV are photographed in their aggregated/complexed form (as they were in STM), which could be the interim state before fusion. The STM of the complexes could have been regarded as an artifact of drying, but since fluorescence microscopy is carried out in aqueous solution, the authenticity of the complexes is proven. Whether the MLV fuse or stay in complex form with the DNA will still have to be resolved.

Observation of aggregates both for SUV and MLV indicate that this state is common to both and is expected when the similarity between the SUV and MLV membrane compositions are considered. What has to be elucidated, however, is whether the complexes eventually turn into fused liposomes or not, because transfection capacity will be strongly dependent on fusion capability.

CONCLUSION

In order to overcome the disadvantages of using cationic liposomes as vehicles of efficient nucleic acid delivery, nontoxic and thus biocompatible anionic liposomes were used to study the interaction between liposomes and DNA. Since our earlier studies implied complex formation (as judged by size increase in light scattering upon interaction), study of structure and organization of anionic liposome–DNA–Ca^{2+} complexes was also considered. Thus, the main aim of the study was to develop a method for transferring DNA to target cells and to test this on a model system via the use of anionic liposomes and to show conclusively (via fluoresence microscopy and STM) that this is achieved. Induction of fusion of liposomes through the catalytic action of DNA and Ca^{2+} would mean that genetic material carrying liposomes and target cells, when incubated with a polyelectrolyte like DNA and with Ca^{2+}, would fuse and transfer the contents of the liposome into the target cell. Or, in another approach, DNA could be introduced to a medium containing target cells and Ca^{2+}, and through DNA's localization between two to three liposomes and fusion, the DNA would be taken into the fused liposomes. Whichever would be the future approach, the success of induction of liposome fusion by DNA and Ca^{2+} would have great potential in this direction. The absence of fused liposomes with only the formation of complexes is an inconclusive result, though a positive step in the right direction because fusion cannot take place without first establishing a contact. An important requirement in this complex formation was the presence of Ca^{2+}. It thus appears that Ca^{2+} binds to the liposome surface, creating a positive surface. The negatively charged DNA then binds to these surfaces, and since it is a polyelectrolyte it can bind two or more liposomes simultaneously, leading to complex formation. Both SUV and MLV were shown, using two significantly different methods, to go through formation of similar structures. Hence the observations were real and not artifacts of the method of measurement. It was therefore possible to bring DNA and liposomes into a very intimate contact which led eventually to the formation of anionic liposome–DNA–Ca^{2+} complexes.

In conclusion, it can be stated that by protecting the nucleic acids from inactivation or degradation, and by facilitating DNA delivery to a variety of target cells, these supramolecular structures might have a great potential for use as an efficient transfection system.

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