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SHORT COMMUNICATION

DNA-Induced Aggregation and Fusion of Phosphatidylcholine Liposomes in the Presence of Multivalent Cations Observed by the Cryo-TEM Technique

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Abstract By means of cryoelectron transmission microscopy (cryo-TEM), we were able to demonstrate the formation of ternary complexes (TC): DNA–phosphatidylcholine liposome–divalent metal cations. Addition of Ba^{2+} to TC led to visualization of DNA compacting on the liposome surface. Staining the TC by Tb^{3+} cations revealed the changed secondary structure of DNA located between fused liposomes. Cryo-TEM and liposome turbidity data were analyzed during TC formation. Liposome aggregation and the liposome fusion induced by DNA in TC were observed. Because TC displayed the property of DNA cationic liposome complexes as well as their own unique properties, we were able to consider cationic lipoplexes as a particular case of TC. The involvement of TC and direct DNA–lipid interactions in the formation nuclear pore complexes were assumed.

Keywords DNA · Phosphatidylcholine liposome · Metal cation · Liposome fusion · Cryo-TEM

Introduction

At present, in the area of research on lipid–nucleic acid interactions, complexes of DNA and zwitterionic phospholipids are considered trivial supplements to complexes of DNA–cationic lipids (lipoplexes) (Safinya 2001). Hundreds of papers are published every year about cationic lipoplexes (Wasungu and Hoekstra 2006; Ewert et al. 2005), but only one-tenth of them are focused on DNA–zwitterionic lipid interactions (Uhrikova et al. 2005; McManus et al. 2003). Cationic lipids have to be considered as exotics for living systems because only one cationic lipid, sphingosine, in minor quantity, is present in a cell. The majority of cell lipids are zwitterionic or negatively charged. Although cationic surfactants are alien and may exhibit toxic effects in the cell, they are used as a basis for preparation of highly active carriers of DNA into a cell.

DNA–zwitterionic lipid complexes have been studied for more than 30 years in an attempt to understand the role of lipids in the attachment of DNA to membrane and the lipid's influence on the function of enzymes of template synthesis (DNA and RNA polymerases) (Manzoli et al. 1982). Infrared spectroscopic analysis of DNA–lipid complexes (total fraction of rat liver lipids) has shown DNA denaturation in the presence of these lipids; therefore, DNA compaction during its coiling on liposomes has been suggested (Shabarshina et al. 1979). Calorimetric study of the mixture of polyA*polyU–phosphatidylcholine liposomes in the absence of divalent cations has shown minor changes in the melting temperatures of lipid bilayers and polynucleotides (Kuvichkin et al. 1999). On the basis of studies of DNA–lipid complexes, their participation in the formation of nucleoid structure as well as in the replication and transcription processes was proposed (Sukhorukov et al. 1980). Later, a hypothesis about the role of DNA–membrane interactions in nuclear pore formation was suggested (Kuvichkin 1983).

By studying DNA–lipid interactions in vitro, Budker et al. (1980) have revealed the existence of specific

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interactions between zwitterionic phospholipids and polynucleotides in the presence of divalent metal cations. These complexes, hereafter referred to as ternary complexes (TC), are proposed to play an important role in the cell function. Intensive study of TC have been carried out since the 1980s (Viktorov et al. 1984; Kuvichkin and Sukhomudrenko 1987; Kuvichkin et al. 1989; Kuvichkin 1990; Hayes et al. 2001; McManus et al. 2003; Uhrikova et al. 2005).

Briefly, the results of the study of TC are as follows:

(1) DNA forms complexes with three main lipids: phosphatidylcholine (PC), phosphatidylethanolamine, and sphingomyeline. The addition of other lipids alters the parameters of complex formation (Kuvichkin and Sukhomudrenko 1987).
(2) The ability of divalent cations to form complexes with DNA and PC correlates with an ability of these cations to bind to PC (Kuvichkin and Sukhomudrenko 1987; McLaughlin et al. 1981).
(3) The liposomes fuse partially (hemifusion) or completely (full fusion) in the presence of divalent cations and DNA. DNA serves as a fusogene (Kuvichkin et al. 1989; Hayes et al. 2001).
(4) DNA partially unwinds in TC because it becomes more accessible to the digestion of S1-endonuclease (Budker et al. 1980), and its secondary structure resembles partially denatured DNA (Kuvichkin and Sukhomudrenko 1987).
(5) SAXS experiments showed a similarity of DNA–cationic liposome complexes to TC (Uhrikova et al. 2005; McManus et al. 2003).

The necessity of divalent cations for TC formation is a basic difference from the DNA–cationic lipoplexes. Therefore, for the analysis of TC structure, it is necessary to separately consider the interaction of divalent cations with both DNA and PC lipid bilayers.

It is known that divalent cations weakly interact with PC lipid bilayer (Viktorov et al. 1984). The association constant of the divalent cation with PC is $K_a = 2.7\ M^{-1}$ for Ca^{2+} and $1.8\ M^{-1}$ for Mg^{2+} (McLaughlin et al. 1981). Mean association constant of PC for Ca^{2+} and Mg^{2+} was estimated at $10–30\ M^{-1}$ (Seelig 1995). Nevertheless, divalent cations caused appreciable changes in the lipid bilayer structure (Inoko et al. 1975).

The interaction between DNA and divalent cations and trivalent cations is complex. The metal–base bindings were direct for the Mg with $K = 3.20 \times 10^5\ M^{-1}$ and indirect for the Ca cation with $K = 3.0 \times 10^4\ M^{-1}$. Both major and minor groove bindings were observed with no alteration of the B-DNA conformation (Ahmad et al. 2003). At low ionic strength, Tb^{3+} binding alters the secondary structure of DNA. Native and sonicated DNA undergo a transition from the B to psi form, the latter being a compact structure characteristic of aggregated DNA (Gersanovski et al. 1985). In light of the foregoing data, the analysis of interactions between DNA and PC liposomes in the presence of polyvalent cations seems complicated. In fact, the interaction of DNA with large liposomes ($\geq$100 nm) seems to be different from that of small unilamellar vesicles ($\leq$50 nm). In the former case, liposome fusion was not observed; in the latter, it was (Hayes et al. 2001).

In the present work, we attempted to keep TC in their native form by use of cryoelectron microscopy. This technique has been selected because TC staining with uranyl acetate, phosphotungstic acid, and ammonium molibdate resulted in drastic changes of the experimental conditions of TC formation, leading to unexpected novel structures. One of the most reliable methods is considered to be the quick freezing technique (freeze-fracture, freeze-etching) and the cryoelectron microscopy (cryo-TEM) (Nagayama and Danev 2008), with Zernike phase plates used for better image contrast (Danev and Nagayama 2001).

There have been many articles published in which cationic lipid–DNA interactions were studied with cryo-TEM (Huebner et al. 1999; Sennato et al. 2005; Almgren et al. 2000; Schmutz et al. 1999). DNA-coated unilamellar vesicles as well as multilamellar DNA–lipid complexes were observed. DNA-induced fusion of vesicles was found. However, to date, to our knowledge, there have been no cryo-TEM studies of TC.

Here, we report a macroscopic property of TC: DNA–PC liposome–Me^{n+}, where Me^{n+} is the divalent or trivalent metal cation observed via spectroscopy. Microscopic traits of TC were observed with phase-contrast cryo-TEM, which was used to prove in situ native morphology of TC at various stages of DNA compaction, liposome aggregation, and fusion.

Materials and Methods

Chemicals

Calf thymus DNA was purchased from Sigma-Aldrich. Egg yolk 3-sn-phosphatidylcholine, $MgCl_2$, $BaCl_2$, terbium (III) acetate, and NaCl were purchased from Wako (Japan). HEPES (2-(4-)2-hydroxyethyl)-1-piperazinylethenesulfonic acid) was purchased from Dojindo (Japan).

The DNA solution and the liposomes were prepared in 0.01 M HEPES buffer, pH 7.2. DNA segments 400 bp long were prepared by ultrasonic treatment of DNA solution during 20 min in an ultrasonic bath (Uhrikova et al. 2005).

A liposome suspension was prepared by the extrusion technique, as previously described (Frisken et al. 2000).

Liposome suspensions were extruded after performing a freeze-thawing procedure 10 times through a sequence of two filters with pore diameters of 800, 400, 200, and 100 nm and one filter of 50 nm. Liposomes with diameters of 200, 100, or 50 nm and a combination of 200- and 50-nm liposomes were used. The turbidity of the liposome suspension was measured by a spectrophotometer (Hitachi U-3300). Immediately after preparation of TC (20 min incubation at room temperature), they and control PC liposomes were quickly frozen in liquid gas.

Quick Freezing

A drop of the sample was put on a copper grid and coated with a carbon film that had holes in it. Most of the liquid was removed with blotting paper, leaving a thin film stretched over the holes. The specimen was instantly shock-frozen by plunging into liquid ethane, which was cooled to 90 K by liquid nitrogen into a temperature-controlled freezing unit (Zeiss, Oberkochen, Germany). The remaining ethane was removed with blotting paper, and the specimen was transferred to the electron microscope (Danino et al. 2001).

Zernike Phase Contrast Electron Microscopy (ZPC TEM)

Phase Plate Preparation

The phase plate was prepared from amorphous carbon films. The films were deposited by vacuum evaporation (JEOL JEE-400) on a freshly cleaved mica surface. For observation at 300-kV acceleration voltage, the film thickness corresponding to the $\pi/2$-phase plate was approximately 32 nm. At that thickness, the transparency of 300-kV acceleration electrons was estimated to be 70% (Danev and Nagayama 2001).

After preparation, the films were floated on the water's surface, then transferred to a molybdenum aperture with several holes 50 μm in diameter, which resulted in a cutoff frequency for special resolution of 0.5 nm. A hole approximately 0.5 μm in diameter in the center of the carbon film was used by a focused ion beam machine (JEOL JFIB-2000) (Schmutz et al. 1999).

300 Kv TEM Observation

The experiments were carried out on a JEOL JEM-3100FFC electron microscope operated at 300-kV acceleration voltages with or without the Hilbert Differential Contrast phase plate. The microscope was equipped with an field-emission gun and omega-type energy filter. Objective lens parameters were as follows: spherical aberration coefficient 5 nm, chromatic aberration coefficient 4.7 nm.

All observations were performed with a nominal magnification of ×40,000 and a final magnification with a CCD camera of ×60,000 and an electron dose of $200^{e-}/nm^2$ in zero-loss filtering mode. The energy window width was set at 10 eV. A special heated aperture holder was used to support the phase plates. To avoid contamination, the phase plates were kept at approximately 200°C at all times. All images were recorded with a Gatan Mega Scan 795 2 K × 2 K CCD camera (Danev and Nagayama 2001).

Results

Kinetics of TC Formation

TC were formed during interaction of DNA with PC–liposome–Me^{2+} (Mg^{2+}, Ba^{2+}, or Tb^{3+}); the kinetics of their formation were studied by turbidity. Typical kinetics of TC formation in the presence of Mg^{2+} consisted of two phases: rapid (0–10 sec) and slow (1–10 min), which continued until the complex turbidity curve plateaued (Fig. 1a).

In the case of Tb^{3+}-TC, the picture was far different. The slow phase seemed to be suppressed or developed very slowly (Fig. 1b). When Tb^{3+} ions were added after the formation of TC with Mg^{2+}, an additional rapid phase emerged (Fig. 1a). If Mg^{2+} was added after the formation of TC with Tb^{3+}, a small increase in turbidity was observed (Fig. 1b). Unlike Mg^{2+} and Tb^{3+} cations, Ba^{2+} ions had little influence on TC formation (Fig. 2a). The successive addition of Mg^{2+} ions stimulated TC formation, which led to a similar turbidity curve (Fig. 2b). The

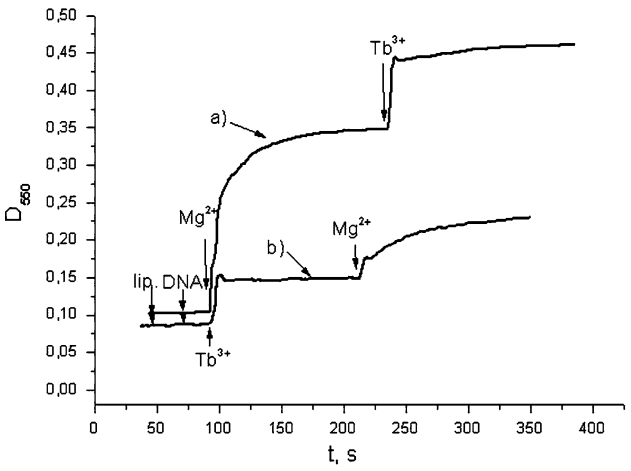

Fig. 1 Turbidity kinetics during TC formation. **a** DNA–PC liposome–Mg^{2+}. **b** DNA–PC liposome–Tb^{3+}. Concentrations: DNA, 20 μg/ml; PC liposome, 0.2 mg/ml; $[Mg^{2+}] = [Tb^{3+}]$, 5 mM

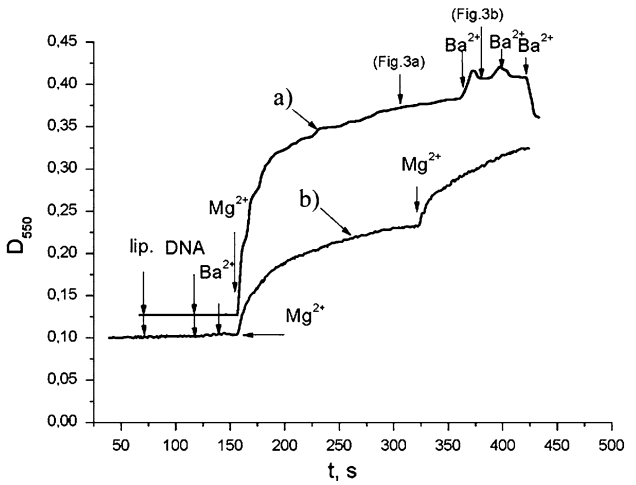

Fig. 2 Effect of Ba^{2+} on TC formation. **a** Ba^{2+} weakly inhibits assembly of TC with Mg^{2+}. **b** Ba^{2+} has little influence on TC formed with Mg^{2+}. Concentrations: DNA, 20 μg/ml; PC liposomes, 0.2 mg/ml; $[Mg^{2+}] = [Ba^{2+}]$, 5 mM

addition of Ba^{2+} ions to TC after the complex formation with Mg^{2+} had little effect on turbidity (Fig. 2a). This allowed us to use Ba^{2+} as TC nondestructive dyes, which allowed us to observe DNA condensation by a cryo-TEM technique.

The experimental results on the turbidimetry for TC have shown a certain degree of additivity in the cation actions as inducers of TC formation and successive aggregation or fusion of the complexes (Figs. 1 and 2). This intriguing macroscopic behavior will be detailed next, along with the microscopic methods.

ZPC TEM Study of TC

ZPC TEM was used for detailed morphological characterization of TC. An obvious question while studying these complexes is whether DNA condensation is observed on the PC liposome surface, as had been previously shown for cationic lipoplexes (Sennato et al. 2005). Figure 3 shows TC formed with Mg^{2+} in the absence of Ba^{2+} (Fig. 3a) or with subsequent addition of Ba^{2+} (Fig. 3b). Irregularly dispersed threads, the thickness of which correspond to DNA molecules, can be observed on the liposome surface. We found the DNA threads in the PC liposome surface and in solution (Fig. 3b).

The number of DNA threads on an equal area of the liposome surface and in solution was counted by a rough estimation of the thread distribution. The quantity of DNA threads on the liposome surface was three times more than that in solution, indicating that the DNA molecules are condensed on the surface of zwitterionic liposomes, similar to cationic liposome–DNA complexes (Clausen-Schaumann and Gaub 1999). Taking into account the data that

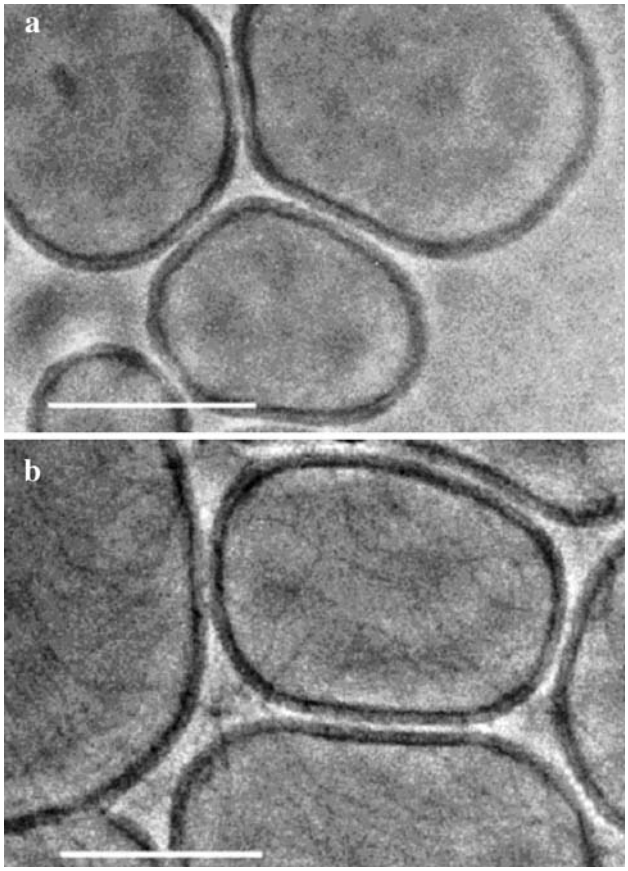

Fig. 3 DNA condensation on liposome surface. **a** Condensation of DNA produced with Mg^{2+} ions added. **b** Ba^{3+} ions added after TC formed with Mg^{2+}. Bar = 100 nm. PC liposome size, 100 nm. Concentrations: $[Mg^{2+}] = [Ba^{2+}]$, 5 mM; DNA, 20 μg/ml; PC liposome, 1.0 mg/ml

show that liposomes <50 nm in diameter fuse during TC formation—unlike large liposomes ≥100 nm in diameter (Hayes et al. 2001)—both types of liposomes were used, and the following results were obtained. Large liposomes formed contacts that were frequently extended but that lacked attributes of fusion (Fig. 3a). Further analysis revealed bridges between the large liposomes formed by fibrils of different diameters (Figs. 3b and 4a, b). We think that these fibrils are DNA because, as shown in Fig. 3, in the presence of Ba^{2+} ions, it was possible to discern threads of DNA stretching from one liposome to another. The diameter of the threads may increase as a result of interactions between lipids and DNA adhered to liposomes (Fig. 4b).

To demonstrate the presence of single-stranded DNA (ssDNA) between the liposomes, we used a Tb^{3+} ion, which can serve as an ssDNA dye during electron microscopy. Indeed, a dark belt was observed in the extensive contacts between the large liposomes of TC formed with Mg^{2+} with the addition of Tb^{3+}, even at concentrations of <0.2 mM (Fig. 4d). This indicated that

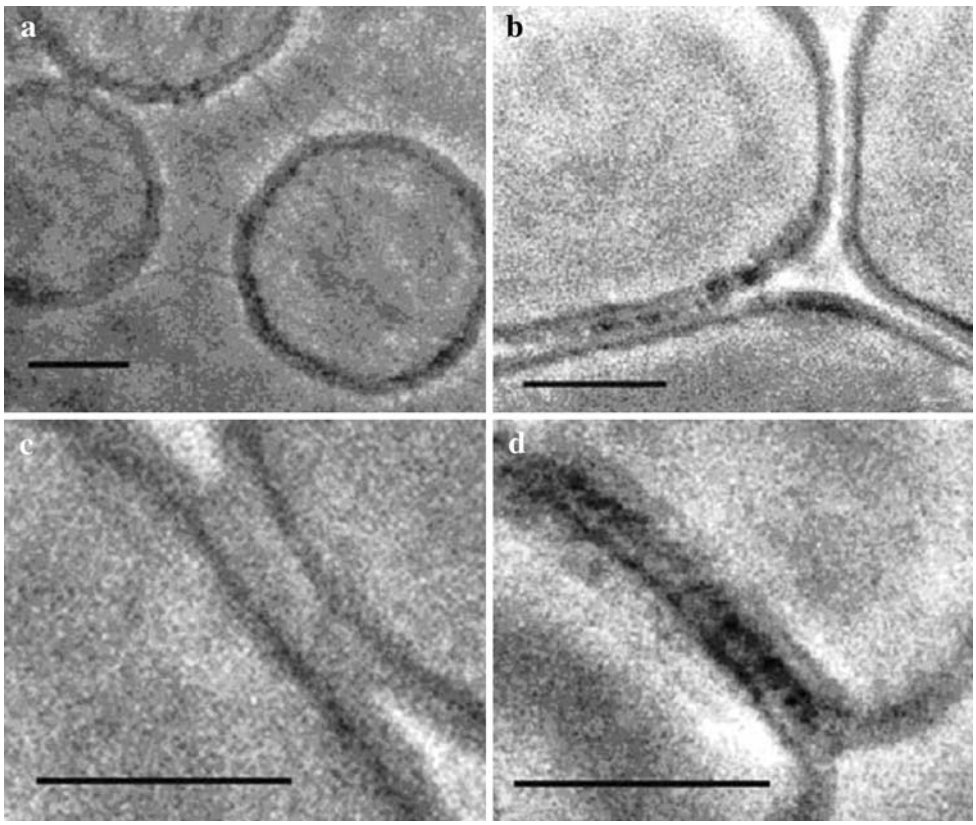

Fig. 4 Fine structure of contacts between PC liposomes in TC with Mg^{2+} (**a**, **b**) and after their staining by Tb^{3+} (**c**, **d**). **a** Fibrils of DNA-binding PC liposomes. **b** The same fibrils at tight aggregation-type contacts between liposomes. **c** Extended contacts between large liposomes formed with Mg^{2+} with subsequent Tb^{3+} ion addition. **d** Fine structure of TC stained by Tb^{3+}. Concentrations: sonicated DNA, 20 μg/ml; PC liposome, 1.0 mg/ml; Mg^{2+}, 10 mM; [Tb^{3+}], 0.2 mM. Bar = 50 nm

ssDNA was located between the liposomes. Such ssDNA sites or DNA sites with single-stranded structure preferably bound Tb^{3+} (Topal and Fresco 1980), resulting in the appearance of black bands between liposomes.

Thus, the zone of contact between liposomes had a broad area of half bilayers in contact with each other (Fig. 4c). It resembled bilayer image blur caused by the interaction of lipids with DNA-like liposome during hemifusion (Fig. 4c). The fine structure of the same contacts after Tb^{3+} staining is shown in Fig. 4d. The absence of the dark bands between the touching liposomes in TC with Mg^{2+} occurred because of the weak contrast of images without Tb^{3+} staining. Contact zones between large liposomes thereby have a complex structure composed of DNA that has a changed secondary structure and lipids that actively interact with such DNA.

In the case of small liposomes (diameter <50 nm), interaction between only one DNA molecule and two liposomes resulted in DNA unwinding and liposomes fusion (Fig. 5c). Thus, interaction between liposomes and DNA depended on the size of the contacting liposomes or their curvature. Liposomes prepared from egg phosphatidylcholine by extrusion-trough 50-nm filters displayed a fairly narrow distribution by size, and they looked like single-layered vesicles with an average diameter of 40 nm (Fig. 6a). Multilayered liposomes were occasionally observed, but they were few in number.

The addition of Mg^{2+} ions had no effect on the size and the shape of PC liposomes (data not shown), a finding in good agreement with earlier biophysical data on weak interaction between Mg^{2+} and PC liposomes (McLaughlin et al. 1978). An addition of DNA to PC liposomes also did not change the liposome size (Fig. 5b). However, a successive addition of Mg^{2+} significantly altered liposome size and shape (Fig. 5c, d). Only a few liposomes had diameters of ≤50 nm; most of the liposomes were ≥100 nm in diameter. Analysis of the distribution of liposome sizes before and after TC formation (Fig. 6) indicated that small liposomes completely fused to create larger ones.

Discussion

The comparison of DNA condensation on the cationic lipid bilayer surface with DNA condensation on the zwitterionic lipid bilayer allowed us to draw conclusions about the differences between these two systems.

The DNA condensation on the cationic liposomes is characterized by very thick DNA packaging with a regular spacing of d = 6.5 nm between adjacent DNA strands. The mixing of cationic and zwitterionic lipids (1:1) reduces the degree of DNA condensation. Spacing between adjacent strands of DNA doubles to 13.0 nm (Clausen-Schaumann and Gaub 1999).

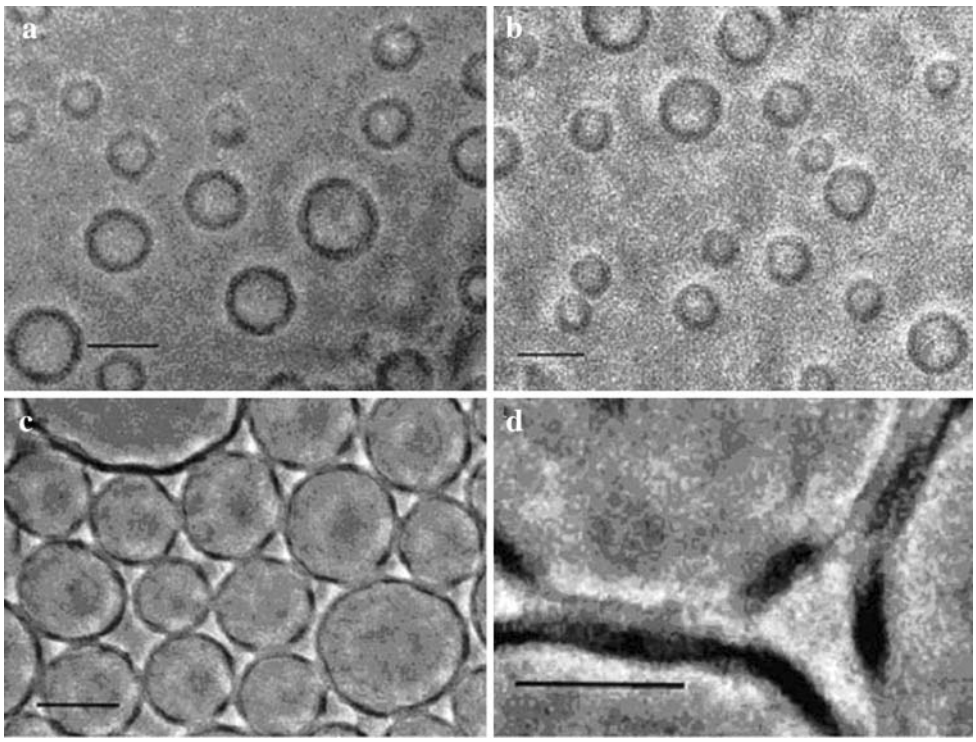

Fig. 5 Fusion of small PC liposomes with formation of large liposomes. **a** Small PC liposomes, 50 nm in diameter, prepared by extrusion technique. **b** Mg^{2+} and DNA separately have no effect on the shape and size of these liposomes. **c** TC formed from liposomes 50 nm in diameter. **d** Fine structure of TC

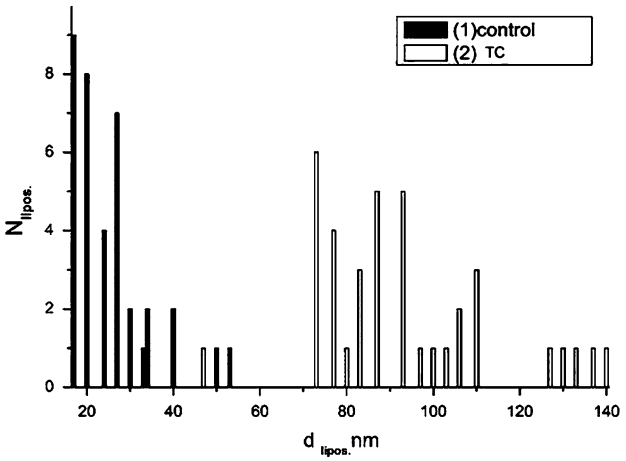

Fig. 6 Diagram of PC liposome size distribution. (1) Small PC liposomes (diameter <50 nm). (2) Large liposomes resulting from fusion after TC formation

By means of atomic force microscopy techniques, it has been shown that DNA threads on the surface of PC lipid bilayer in the presence of magnesium ions are distributed more chaotically than on cationic lipid bilayer surfaces (Malghani and Yang 1999). The liposome with the condensed DNA can easily interact with liposomes in the close proximity. This fact can explain the fast stages of liposome aggregation and fusion, which both caused an increase in TC turbidity (Figs. 1 and 2). The slow phase of turbidity change of liposomes in TC with Mg^{2+} can be explained by the increase in distance between fused liposomes or their aggregates formed after a fast phase.

The slow phase of the kinetics curve is almost absent when TC formed with Tb^{3+} (Fig. 1b) or when Tb^{3+} was added to TC after their formation with Mg^{2+} (Fig. 1a). In our opinion, it is caused as a result of DNA condensation by Tb^{3+} ions in solution and on the liposome surface because interaction of Tb^{3+} with DNA is much stronger than DNA or Tb^{3+} with PC bilayer. Full fusion was observed in liposomes $\leq$50 nm in diameter in TC (Fig. 5). Each large liposome represents the result of the fusion of approximately 30 initial liposomes.

What, then, represents the zones of contacts between them? This is most likely the result of incomplete fusion of liposomes that cannot fuse completely because of formation of a great number of contacts between the liposomes, which hampers their full fusion. However, indirect traces may help us visually define how many liposomes have fused to form one large liposome.

If we assume that fusion of small liposomes with the participation of DNA and Mg^{2+} fit into a model proposed earlier (Kuvichkin 2002), with DNA unwinding in a fusion zone (Fig. 8a), then after liposomal full fusion, ssDNA in a complex with lipids will be visible on the surface of the large liposome, thus outlining fusion boundaries between fused liposomes. Moreover, DNA threads condensed on the liposome surface during fusion will concentrate by moving to the fusion boundaries, thus making them visible (Fig. 7b). These boundaries can be viewed on some images

with sufficient magnification. A dark ring may be seen in the center of each large liposome (Fig. 7c–e). After small liposomes fuse with large liposomes, a ring is formed around the large liposome and the boundaries between them. However, this ring around the central liposome is not continuous. It consists of several sectors that indicate that small liposomes are fusing with central liposome and with each other. The central liposome has a compound structure that might be a result of DNA-induced fusion of several initial small liposomes (Fig. 7e). These data were obtained at the resolution limit of the cryo-TEM we used in our experiment; however, heterogeneity of the surface of fused liposomes is obvious (Figs. 5 and 7), in contrast to surface homogeneity of initial liposomes (Fig. 3a).

In cationic liposomes, as a result of the high degree of DNA condensation on the surface of the liposome, the liposomes have no possibility of interaction except by winding one liposome around another. The giant forces of interaction between liposome bilayers disrupt the liposomes. DNA quickly binds to the internal surface of the ruptured liposome, and the process repeats until the intact liposomes completely disappear. On the other hand, at high DNA concentrations, multilayered structures of the type of cationic lipoplexes in a DNA–PC liposome–Mg^{2+} system can be obtained (Fig. 8a). Treatment with DNAse I discontinued an interaction between liposomes and disrupted most of the DNA threads on their surface (Fig. 8b).

In conclusion, we have shown DNA condensation on the liposome surface and the formation of contacts between small liposomes ($\leq$50 nm), which can grow to fusion, for TC with Mg^{2+} cations. Three different cations—Mg^{2+}, Ba^{2+}, and Tb^{3+}—led to a considerable difference in interactions between DNA and PC liposomes. Our study verified the direct involvement of DNA in TC formation.

Data on fine structure of contact between liposomes obtained with high-resolution cryo-TEM support an earlier proposed model of TC and nuclear pore formation (Kuvichkin 1983, 2002).

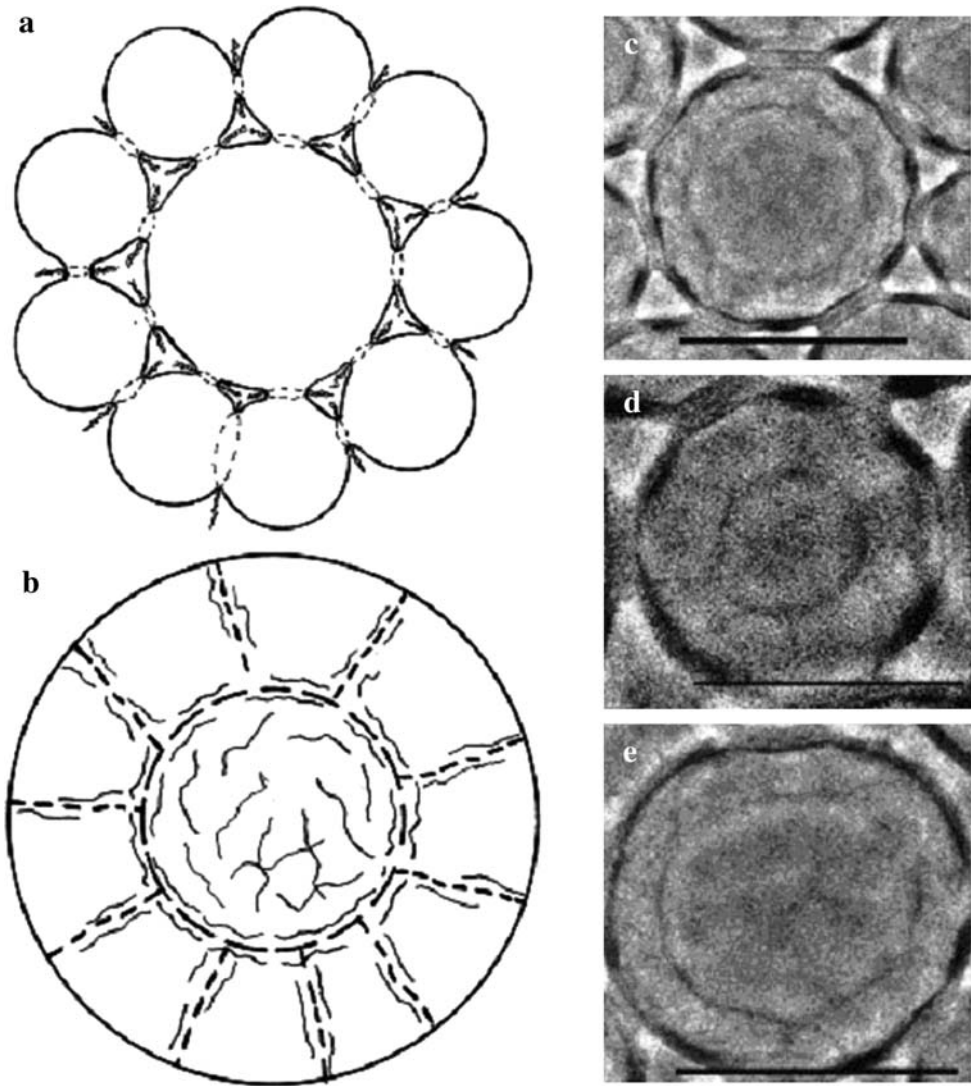

Fig. 7 Scheme illustrating the mechanism of small PC liposome fusion around the large liposome induced by dsDNA located between two liposomes. **a** DNA condensed on PC liposomes (gray) and binding two liposomes (black). **b** Tight contacts between these liposomes after fusion induced by DNA (*dashed line,* ssDNA). **c–e** Cryo-TEM data on PC liposome DNA-induced fusion

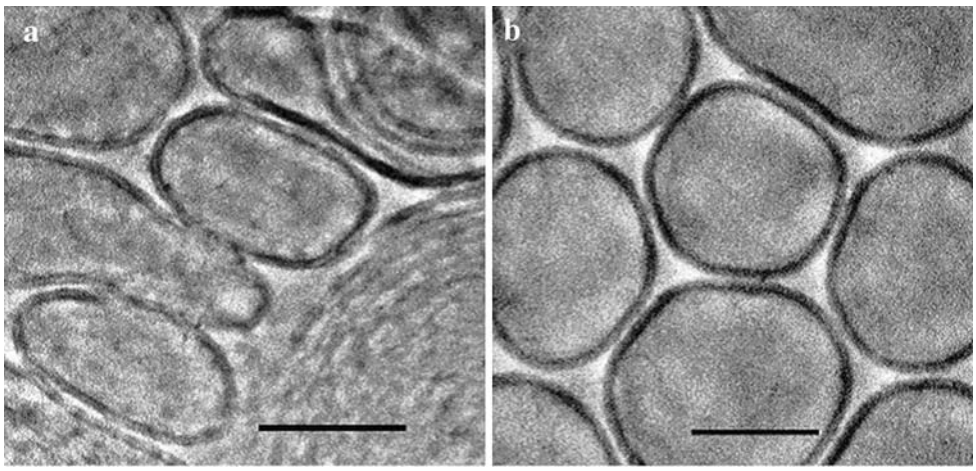

Fig. 8 Formation of multilamellar structures in TC. **a** Multilayer structures as in DNA–cationic liposome complex. **b** After DNAse I treatment of (**a**). Concentrations: DNA, 20 μg/ml; egg PC, 1 mg/ml; $MgCl_2$, 5 mM; initial liposome size, 100 nm

DNA unwinding in the nuclear pore area results in advantages at the initiation of transcription. Genes adjacent to a nuclear pore have higher expression than genes distant from DNA contacts with the nuclear membrane. The evidence for this, as proposed by Kuvichkin (1983), can be found in recent publications that have uncovered the dynamic associations between nuclear pore complexes and actively transcribed genes (Taddei 2007; Brown and Silver 2007).

TC can be formed in a cell anywhere where conditions are suitable—as in DNA located between a double lipid bilayer and divalent metal cations. By this process, annulate lamellae, some type of contacts between cells, cellular membranes, and organelles can be generated. The TC system thus provides possibilities for in vitro studies and for research into the role of TC in the cell.

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