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Investigation of Ternary Complexes: DNA–Phosphatidylcholine Liposomes–Mg^{2+} by Freeze-Fracture Method and Their Role in the Formation of Some Cell Structures

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Abstract Long-term investigations of ternary complexes: DNA–zwitterionic liposomes–divalent metal cations have revealed many details of their structure; but some questions need additional study. The conditions under which fusion or aggregation of liposomes occurs during such complex formation remain obscure. The DNA structure in the ternary complex is still unclear. In this work, using a freeze-fracture method, author demonstrate the thin structure of a complex (early attempts to observe this structure employing other electron microscopic methods, in particular cryo-TEM, have not met with success). After treatment of ternary complexes with nuclease S1, which is able to digest single-stranded DNA, local DNA unwinding in such complexes was confirmed. Author describe how the curvature of liposomes as the main factor may determine the interaction between liposomes and DNA, especially aggregation or fusion of liposomes during ternary complex formation. Therefore, interaction between lipids of membrane vesicles in cell and chromatin DNA can be the first stage of a nuclear envelope and pore complex assembly.

Keywords DNA · Zwitterionic liposome · Divalent metal cation · Nuclease S1 · Freeze-fracture electron microscopy

Introduction

The study of ternary complexes (TCs), DNA–phosphatidylcholine (PC) liposomes–Mg^{2+}, with cryo-TEM (Kuvichkin et al. 2009) has shown the coexistence of a set of structures in TCs from DNA-induced liposome aggregation and their fusion to multilayered structures observed in cationic lipoplexes. Though the method of high-resolution cryo-TEM has many advantages (e.g., high object magnification and high image sharpness, preservation of native structure of TCs due to quick freezing), it exhibits a number of disadvantages. Among them is a flat image of the object, which is similar to the X-ray picture of 3D objects. 3D structures (liposomes) of size 50–100 nm in a layer of 500 nm at the projection on a plane form 2D images are difficult to identify. The existing methods of 3D reconstruction of the object, cryo-electron tomography, do not yield the resolution required. My interest is in comparing the findings obtained using the method of cryo-TEM and those resulting from the freeze-fracture technique. The freeze-fracture method provides a relief image of split surfaces without penetrating the depth of the object. In addition, author used the enzyme nuclease S1, which has action on single-stranded DNA, with the aim of elucidating whether it influences TC structure. The use of these complementary methods has provided the possibility of clearing up the TC structure, which was impossible when they were employed separately.

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Materials and Methods

Chemicals

Calf thymus DNA, egg yolk 3-sn-phosphatidylcholine (PC), $MgCl_2$ and NaCl, 2-(4)2-Hydroxyethyl-1-piperazinylethene-sulfonic acid (HEPES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The DNA solution and liposomes were prepared in 0.01 M HEPES buffer, pH 7.2. DNA segments with length 400 bp were prepared by ultrasonic treatment of

DNA solution for 20 min in ultrasonic bath, as in Uhrikova et al. (2005). Nuclease S1 from *Aspergillus oryzae* (Sigma–Aldrich) was dissolved in HEPES buffer ("Serva"), 0.01 M NaCl (pro-analysis), pH 7.2.

Liposome Preparation

A liposome suspension was prepared by extrusion, as described in Frisken et al. (2000). Liposome suspensions were extruded after a freeze–thaw 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.

Samples of TC for freeze-fracture EM were prepared traditionally (Fikhte et al. 1973).

Xenopus laevis Egg Extract Isolation

The egg extract of *Xenopus laevis* was prepared according to Hola et al. (1994) in our laboratory. *X. laevis* eggs were centrifuged on a Centrikon T-2070 (Kontron Instruments, Munich, Germany), rotor TST555 of 24,000–30,000 rpm (75,000–120,000×*g*), for 40 min. The clean extract fraction and the membrane fraction were collected separately, and the extract was centrifuged repeatedly for complete purification from membrane vesicles. The results of fusion of liposomes in *Xenopus* egg extracts were observed using light and electron microscopes (by negative contrast and freeze-etching methods).

Results and Discussion

We previously demonstrated the use of the cryo-TEM method for DNA condensation on the liposome surface (Kuvichkin et al. 2009). However, for the analysis of DNA location in TCs, the DNA image against lipids was not sharp and contrasting. Thus, we tried to use the freeze-fracture method as the cryo-TEM method preserves the native TC structure due to high-speed freezing. Unlike the cryo-TEM method, the freeze-fracture technique provides a relief image of the split.

The typical image of TCs using the freeze-fracture method is shown in Fig. 1b. Figure 1a shows the control liposomes and liposomes in the presence of Mg^{2+} ions or at DNA addition without Mg^{2+}, which are the same. However, the addition of Mg^{2+} ions (5 mM) to the system of DNA liposomes resulted in the formation of aggregates and multiple extended structures, which were visible under the light microscope. The fine structure of such aggregates was revealed only by the method of freeze-etching (Fig. 1b–d). The formation of two main structure types is shown (Fig. 1c, d). The first type of TC (Fig. 1c) is the tubular liposome, belted with rims in several places. The second type of liposome has a rim along the equator and two fibrils going out from the rim in opposite directions (Fig. 1b, d). Graphic interpretations of the TCs shown in Fig. 1c and d are depicted in Fig. 1c_1 and d_1, respectively. Dotted lines are single-stranded DNA sites that appeared during TC formation.

Nuclease S1 Effect on TC

Since the main question arising during the study of TCs concerns DNA unwinding and the presence of single-stranded DNA in TCs, we studied how the enzyme digesting single-stranded DNA–nuclease S1 influences TC structure. The results are presented in Fig. 2.

It is the absence of spherical liposomes which predominate in Fig. 1a–c that attracted my attention first. Almost all have the shape of a disk with two or more strands going outward, and author suppose this to be a double-stranded DNA (Fig. 2a, b) because S1 endonuclease has no effect on double-stranded DNA. This DNA is in the complex with lipids since its width is larger than that of nude DNA. Honeycomb (cavernous, spongy) structures (Fig. 2c) also show less relief; in some places they fuse with the lipid bilayer of the larger size (Fig. 2d). Several liposomes belted with a rim of single-stranded DNA (Fig. 2a, b) are fused; this is not the case in the absence of nuclease S1, when they can form aggregates only (Fig. 1d). As a result, all liposomes are completely fused with the formation of wide lipid bilayers, which are very often multilayered (Fig. 2a, d).

Observation of Single-Stranded DNA During TC Formation in *X. laevis* Egg Extract

When salmon sperm DNA is added to *X. laevis* egg extract, a nuclear envelope with pores is known to assemble from membrane vesicles of the extract. To elucidate a role of lipids in this process, in our experiment we added small PC liposomes to *Xenopus* egg extract. To accelerate TC formation, 10 mM Mg^{2+} was also injected into this extract. As a result, pieces of nuclear envelope with pores of various diameters were observed (Fig. 3b).

But the most remarkable fact is the appearance of pore-like structures on the liposome surface (Fig. 3a). The size of these pores nearly corresponds to the diameter of nuclear pores in cells. Their shape, that of a nuclear pore complex, is far from ideal, although the freeze-fracture method demonstrates their considerable similarity to pores observed in the cell nucleus (Fig. 3b). In my opinion, what I observed in liposomes is a result of the binding of some proteins–nucleoporins on the liposome surface to single-

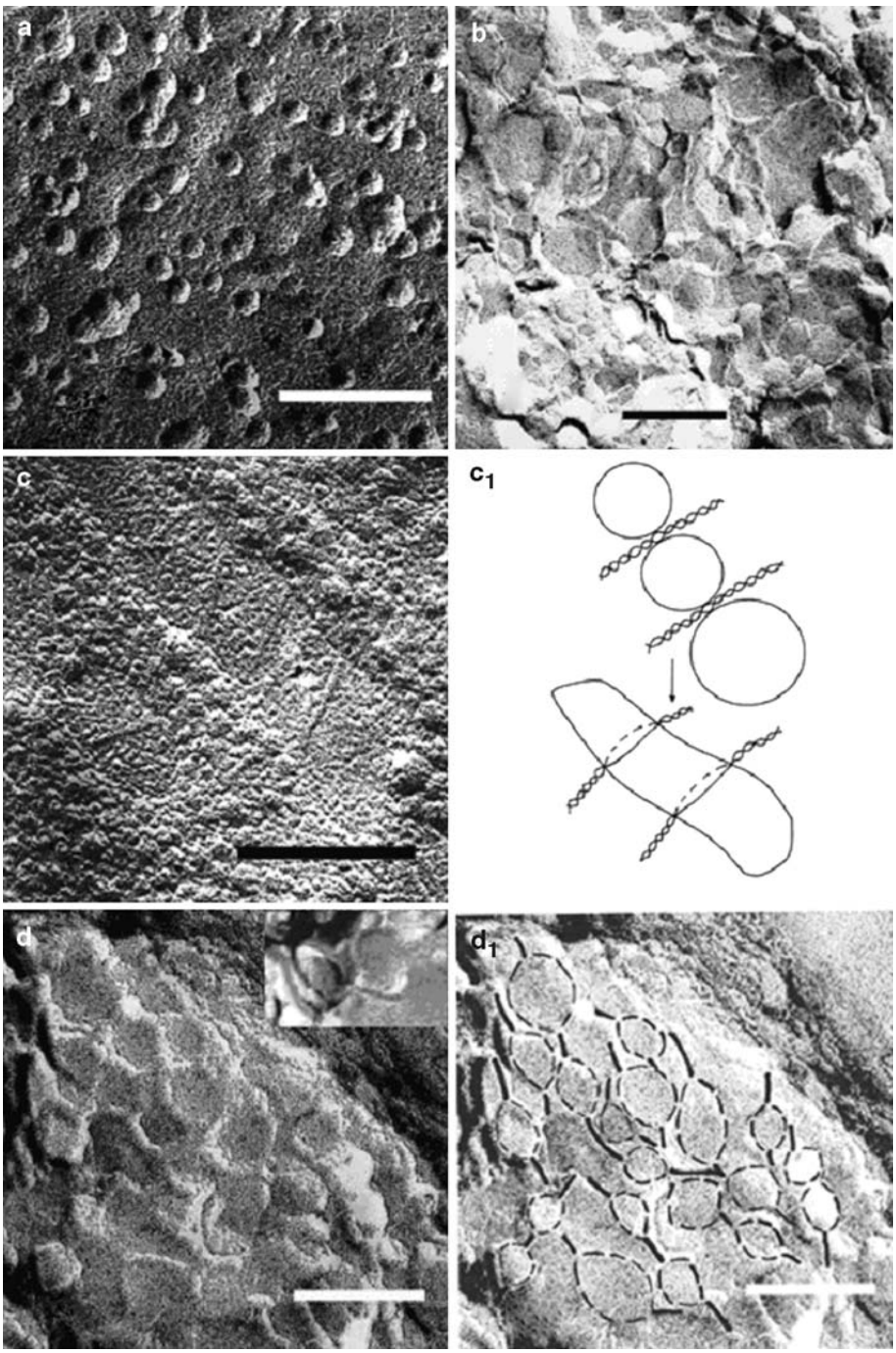

Fig. 1 Freeze-fracture electron microscopic images of a TC (DNA–PC liposomes–Mg^{2+}). Concentration of PC liposomes 1.0 mg/ml, DNA 20 μg/ml, $MgCl_2$ 5 mM; bar = 150 nm

stranded DNA, which appeared in the course of fusion of a small liposome and a large liposome. The mechanism of the appearance of single-stranded DNA on a large liposome surface has been described in previously (Kuvichkin et al. 2009). A single-stranded DNA is not dyed with uranyl acetate, but it becomes visible after binding to *Xenopus* egg extract proteins, which stained well with uranyl acetate.

A Model of DNA-Induced Liposome Fusion

Here, author explain these findings with the use of the TC model described in Kuvichkin (2002) and graphically depicted in Fig. 4a–e. It is known that between zwitterionic liposomes there exists a water layer 2–3 nm thick. The first stage of TC formation is the approach of two liposomes to the width of DNA (2 nm). When DNA is located between

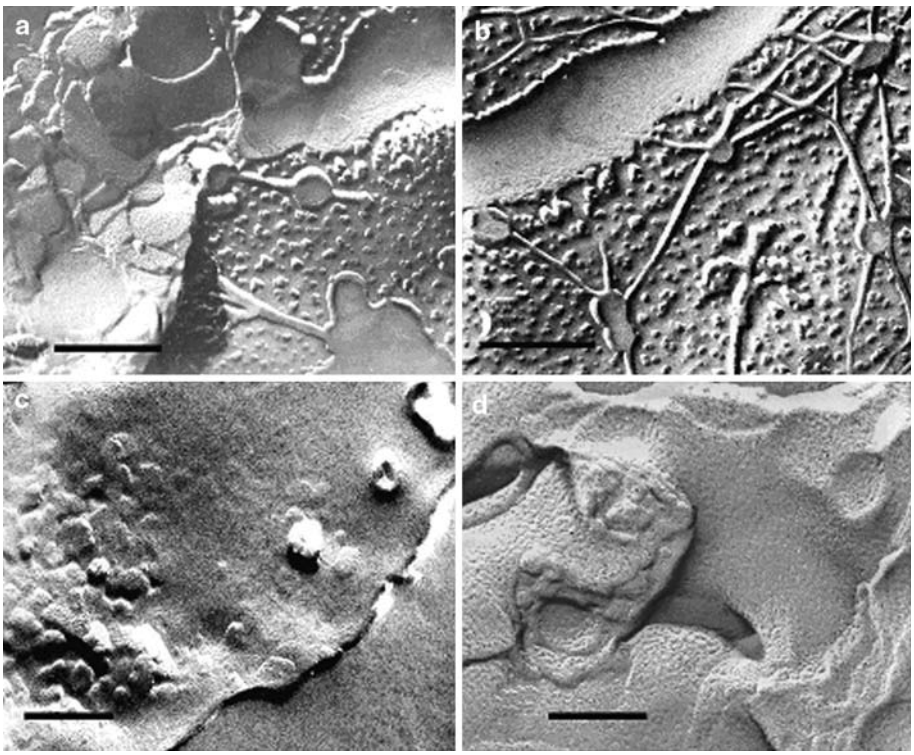

Fig. 2 Effect of nuclease S1 on a TC (freeze-fracture method). Concentration of DNA 20 μg/ml, PC liposomes 0.5 mg/ml, $MgCl_2$ 5 mM, nuclease S1 1 μg/ml; bar = 100 nm (details in text)

liposomes in the presence of cations of divalent metals, it makes a bridge between the liposomes along which lipids transfer that supports the phenomenon of lipid mixing, seen in a previous experiment (Kuvichkin 2009). In the case of smaller liposomes, their fusion occurs in parallel with DNA unwinding around the fusion area. The energy of interaction between two DNA phosphates located on opposite sides of a double helix via a Mg^{2+} bridge is 13–14 kcal/mol (Fedorov et al. 1999), which is comparable to the energy of DNA helix stabilization due to hydrogen bonds between bases plus stacking of about 6–15 kcal/mol (Breslauer et al. 1986). Stacking interactions between the aromatic planar bases are estimated to be about 4–15 kcal/mol per dinucleotide. The energy of hydrogen bounds is about 3–7 kcal/mol (Sinden et al. 1998). Hydrogen bonding and base stacking contribute to the stability of the DNA double helix. Cation Mg^{2+} in concentration of 5–10 mM had no effect on DNA stability in the presence of 10^{-2} M NaCl in our buffer solution (Korolev et al. 1998). DNA partially unwinds and new DNA phosphates interact with liposome phosphates cooperatively. Since DNA has a double helical structure, the interaction spread along the helix results in a liposome bilayer approach. This may occur due to liposome shape deformation (protruding) rather than their actually moving toward each other (Fig. 4b).

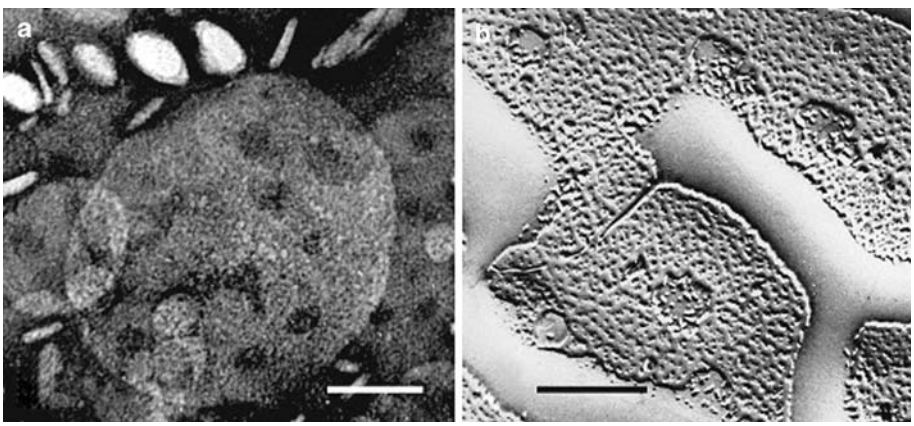

Fig. 3 Pore-like structure on the PC liposomes added to *Xenopus* egg extract. **a** Negative contrast staining. **b** Pores in lipid bilayers (freeze-fracture method). Concentration of DNA 20 μg/ml, PC liposomes 0.5 mg/ml, $MgCl_2$ 5 mM; bar = 50 nm

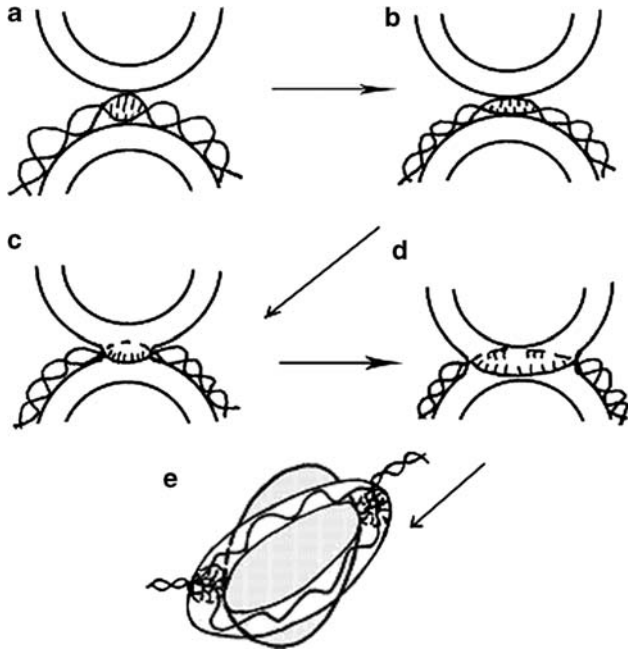

Fig. 4 The scheme of TC formation. **a** First contact of DNA with two PC liposomes. **b** The approach of liposomes with DNA unwinding. **c**, **d** Liposome hemifusion. **e** Liposome full fusion

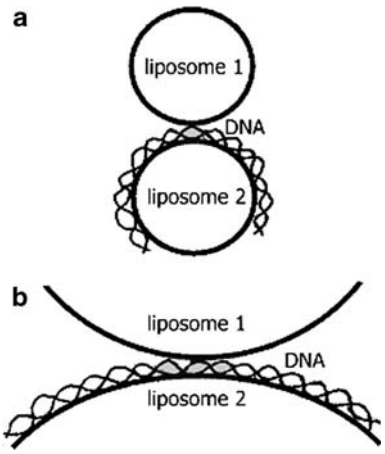

Fig. 5 Effect of curvature on PC liposome fusion (**a**) or aggregation (**b**). *Gray* color fill indicates DNA turns interacting simultaneously with two liposomes

Direct liposome contact (Fig. 4c) starts the program of liposome fusion following a well-known pathway, resulting in their hemifusion and further DNA unwinding (Fig. 4d). The next stage is full fusion of liposomes and localization of single-stranded DNA around the fusion zone on the equator of a newly forming large liposome (Fig. 4e). More detailed information can be obtained by PC simulation of the process of DNA interaction with liposomes, which will be done in further studies.

This model is used to explain the formation of structures shown in Fig. 1. In my view, these structures are formed as a result of DNA-induced fusion of small liposomes (diameter $\leq$ 50 nm). Single-stranded DNA, which belts the liposome along its diameter, most likely possesses a supercoiled structure and forms a specific complex with lipid and Me^{2+}. This leads to the fact that the width of the rim is much more than that of single-stranded DNA. That is why it is clearly seen on splits (Fig. 1). The rim is tight to liposome, but it slides down when the freeze-fracture method is used and is observed separately (Fig. 1e). Honeycomb (spongy) structures are very probably the result of detachment of the rims from liposomes (Fig. 1d). Simultaneously, both structures are easily visible on an insert in the right top corner of Fig. 1d.

As an alternative explanation, it can be the result of full fusion of small liposomes with a large liposome when on the surface of the large liposome only the rim containing single-stranded DNA remains. This has been discussed previously (Kuvichkin et al. 2009).

Significance of Liposome Curvature in Interaction of PC Liposomes with DNA and Divalent Metal Cations

How can the results that DNA induces both liposome aggregation and fusion, using our cryo-TEM method, be interpreted? In my opinion key role in the realization of one or another method of DNA interaction with liposomes belongs to the liposome curvature. Liposomes of small diameter ($\leq$50 nm, large curvature) interact with each other and DNA binds with one of them so that only one turn of the DNA helix is involved in the interaction between two liposomes (Fig. 5a). In this case, this turn and neighboring ones may unwind successively, interacting with lipids in the area of liposome fusion.

When the diameter of a liposome is three or four times larger, a minimum of three turns of the DNA helix bind simultaneously to two liposomes due to the smaller curvature. This prevents DNA unwinding, and in this case, only local change of the secondary structure (within one turn) is possible (Fig. 5b). Lipid exchange between the liposomes via the DNA bridge resulting in lipid mixing is supposed. No complete fusion or hemifusion of liposomes occurs.

Thus, this report shows that the curvature of lipid bilayers determines their ability to fuse or aggregate.

Conclusion

The nuclear envelope in a cell can be assembled from membrane vesicles 70 nm in diameter, which can fuse by

DNA-induced fusion using zwitterionic lipids of vesicles. The structure shown in Fig. 2e may be the initial stage in nuclear pore complex assembly. Their fusion with other types of membrane vesicles can form a nuclear envelope with pores.

This is the simplest analysis of the role of direct DNA–lipid–Me^{2+} interactions in nuclear envelope and pore complex assembly. Our model may serve as a basis for the construction of nuclear pore complexes with the participation of many known proteins–nucleoporins.

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