Direct Visualization of DNA Duplex Formation on the Surface of a Giant Liposome

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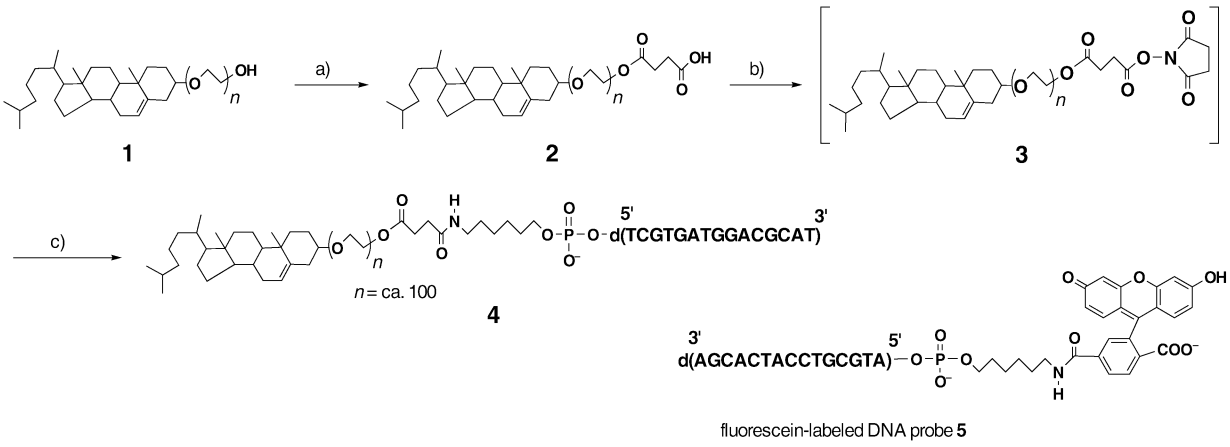
KEYWORDS:

DNA · liposomes · optical microscope · origin of life

The origin of life is one of the most fundamental questions for the intellectual human being. Although various types of life models have been advocated so far,[1–5] there is no doubt that our direct ancestor must certainly be a cellular life form. The primordial cellular life form (protocell) requires both genomic entities and a component that separates it from the outer world.[6–9] Nowadays, a polyribonucleotide is considered to be a candidate of the first genomic molecule because RNA both contains information and has catalytic activities (ribozyme), which include catalysis of RNA-dependent RNA polymerization.[10, 11] However, a bilayer membrane constructed of self-assembling lipids is regarded as a component of primitive life. We recently developed a self-replicating liposome system as a model of the protocell.[12] However, in order to realize an advanced protocell model, coupling of the liposome system with a self-replicating information-carrying substance is essential. If the two major anionic biomaterials—polynucleotide and lipid—that form the protocell are able to communicate with each other in spite of the electrostatic repulsion between them, they could cooperate and coevolve effectively.

As a novel approach to couple these two inhabitants of discrete worlds at the molecular level, we designed a DNA molecule connected to a lipophilic cholesterol through a poly(ethylene glycol) (PEG) spacer (compound **4**, Scheme 1). This cholesterol-tagged DNA (**4**) is expected to be fixed inside the membrane of a liposome by means of the anchoring ability of the cholesterol part of the molecule. Since such a liposome bears genetic information, it can be regarded as an advanced protocell model. DNA anchoring onto the membrane is similar to the partial adhesion of genomic DNA to the membrane of a prokaryotic cell.[13] Such a design might be a molecular fossil of the hypothetical lipid–RNA (or DNA) conjugate. Moreover, if cholesterol-tagged DNA **4** is not released into the aqueous phase but hybridizes with its complementary DNA or RNA, it will become a bridging molecule between the RNA and the lipid worlds. DNA is very similar to RNA because polydeoxyribonucleotides also possess catalytic activity (deoxyribozyme) as well as genomic information.[14] Since polydeoxyribonucleotides lack 2′-hydroxy groups, they are chemically and biologically more stable than polyribonucleotides. Accordingly, polydeoxyribonucleotides have several merits for synthesis, handling, and storage.

Although a lipid–DNA hybrid with a relatively short spacer inbetween the two components has been reported, such a



Scheme 1. *a) Succinic anhydride, Et_3N, N-dimethylaminopyridine, CH_2Cl_2, 98%; b) water-soluble carbodiimide, N-hydroxysuccinimide, CH_2Cl_2; c) 5′-aminohexyl oligodeoxyribonucleotide, $NaHCO_3$, dimethylformamide/H_2O (1:1, v/v), 38% (based on the 5′-modified DNA).*

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hybrid can be used mainly for detection of a DNA fragment of a target microbe.[15] A hybrid of this kind is also effective for investigation of a protein that binds a specific DNA sequence made by embedding a cholesterol-tagged DNA 31-mer in a monolayer of a phospholipid on a gold substrate.[16] In these examples, however, DNA was introduced after the membranes were formed. Accordingly, these methods cannot produce liposomes that can carry DNA directly to the inner water pool.

Our question herein is whether a giant liposome containing such a cholesterol – DNA hybrid can be constructed in spite of the charge repulsion between the anionic surface of a liposome and a polyanionic DNA fragment. For this purpose we designed a cholesterol – DNA hybrid with a spacer made of PEG. The PEG unit was expected to be a sufficiently long spacer to separate the two types of anionic materials—DNA and phospholipid—enough to lessen the electrostatic repulsion between them when the cholesterol-tagged DNA **4** is anchored into the membrane of an anionic liposome.

Cholesterol-tagged DNA **4** was synthesized from cholesterol – PEG (**1**).[17] The starting material **1** was treated with succinic anhydride in the presence of a catalytic amount of *N*-dimethylaminopyridine. Subsequently, the carboxyl group of compound **2** was converted into active ester **3** by treatment with a combination of *N*-hydroxysuccinimide and water-soluble carbodiimide. The resulting urea derivative and excess carbodiimide were extracted into the aqueous phase. The DNA section, modified with an aminohexyl group at the 5′-terminal site, was synthesized separately by the standard phosphoramidite procedure.[18, 19] The final amide condensation was performed in an aqueous solution under mild alkaline conditions[20] and yielded the desired cholesterol-tagged DNA 15-mer **4**, the sequence of which is shown in Scheme 1.[21]

To demonstrate the presence of the cholesterol-tagged DNA 15-mer on a membrane, we employed a giant liposome produced by the electroformation method for direct visualization with an optical microscope.[22] First, 10 μL phospholipid solution in chloroform (10 mM; DOPC/POPG/DSPE-PEG$_{5000}$ = 20:20:1; for definitions, see ref. [23]) was mixed with 2.5 μL cholesterol-tagged DNA **4** in methanol (1 mM). We changed the composition of phospholipids systematically and found that those liposomes including POPG and DSPE-PEG$_{5000}$ produced better results in microinjection experiments.[24] A pair of parallel platinum electrodes (0.4 mm in diameter with a separation of 1 mm) was fixed in a plastic vessel, which was coated with a mixture of the phospholipids and **4** (Figure 1), and the organic solvent was completely removed under reduced pressure. An alternating electric field (2 V, 10 Hz) was applied and the film of the electrodes was slowly soaked with sodium phosphate buffer (50 mM, pH 7.0). After an hour, many giant liposomes with a diameter larger than 1 μm were observed on the electrodes under the optical microscope.

The growth of the liposome was halted by turning off the alternating electric field to keep the giant liposome on the electrodes. Fluorescein-labeled (λ_{em} = 515 nm) DNA 15-mer **5**, whose sequence is complementary to that of cholesterol-tagged DNA 15-mer **4** was added to the vessel filled with the buffer solution (8 μL 25 μM solution in water) to act as a fluorescence probe. After spontaneous diffusion of the fluorescein-labeled DNA probe **5**, the stained buffer solution was continuously washed with a fresh buffer solution through charging and discharging ducts connected with the vessel (see Figure 1 b).

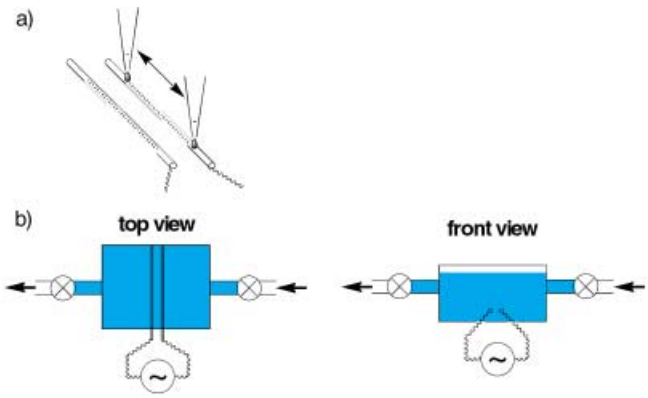

Figure 1. a) Lipid solution was spread onto the parallel Pt electrodes; b) electroformation system with two ducts attached for replacing buffer solution. The length, width, and height of the vessel were 20, 28, and 14 mm, respectively. The arrows represent the current of buffer solution when both valves are open.

Figure 2 a shows phase-contrast and fluorescence microscopic images of the giant liposome containing the cholesterol – tag-

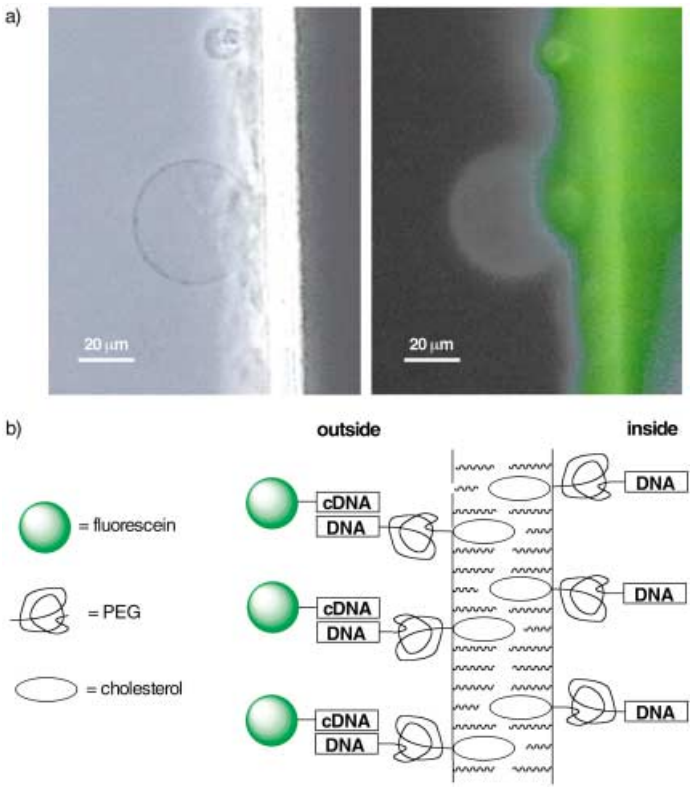

Figure 2. a) Phase-contrast (left) and fluorescence (right) microscopic images of the same giant liposome growing on the platinum electrode. The right-hand halves of the images are the electrodes. In the image on the right, a band of fluorescence near the giant liposome represents the fluorescein-labeled DNA probe that hybridized with cholesterol-tagged DNA in the nonswelling lipid spread over the electrode; b) diagram of the hybridization event occuring on the surface of the giant liposome shown above.

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ged DNA 15-mer colored with the fluorescein-labeled DNA probe with the complementary sequence. In the phase-contrast image, a spherical giant vesicle of 40-μm diameter containing the cholesterol-tagged DNA was observed. The fluorescence microscopic image showed clearly that the surface of the giant liposome was fluorescent. This result indicates that cholesterol-tagged DNA **4** anchored in a membrane hybridizes the complementary DNA probe **5**. It is notable that the cholesterol-tagged DNA was held on the membrane even after a more anionic double-stranded DNA duplex was formed. A schematic diagram of the hybridization event on the lipid bilayer is shown in Figure 2b. Since the fluorescence probe was added to the solution outside the giant liposomes, the hybridization event occurred only on the outer membrane of the lipid bilayer. However, this result provides strong support for the capability of the DNA probe to interact with the cholesterol-tagged DNA **4** when it is located on the inner layer of the liposome.

It is known that a high salt concentration intrinsically disturbs formation of giant liposomes by the electroformation method.[25] Although giant liposomes swelled easily in a low salt concentration such as 10 mM, the DNA duplex on the membrane became considerably unstable, presumably as a result of the electrostatic repulsion between the strands at a low salt concentration (data not shown). For stable hybridization of DNA, especially in the case of the short (15 bps) duplex, high salt concentration (for example, the concentration of physiological saline is around 150 mM) is required. However, we managed to find that the salt concentration of 50 mM satisfied such contradictory requirements. The success in the formation of giant liposomes under such a relatively high salt condition (50 mM) may be attributed to the presence of DSPE-PEG_{5000}.

Since a two-base mismatched DNA probe (5′-fluorescein-ATGCCTCCATGACGA, underline: mismatch base) never stained any membranes of the giant liposomes under the same conditions as described above, the precise recognition of the DNA strand is indispensable for the fixation of the fully-matched DNA probe with cholesterol–PEG–DNA conjugate **4**.

It may be concluded that the long poly(ethylene glycol) spacer played a crucial role in DNA duplex formation on the polyanionic membrane by suppressing the electrostatic repulsion between the membrane and the duplex. Since the average degree of polymerization of PEG used in this study was approximately one hundred (MW: 4400), the diameter of the intact PEG_{4300} molecule was estimated to be 66 Å.[26] The electrostatic repulsion between the polyanionic membrane on which **4** is anchored and the polyanionic DNA head of **4** would stretch the ends of the cholesterol–PEG–DNA conjugate apart. In contrast, the conformation of PEG is likely to be such that the molecule is folded according to the gauche effect around the O–C–C–O bonds,[27] which brings the DNA head close to the membrane. Under such a circumstance, the PEG part of the cholesterol–PEG–DNA conjugate behaves as if it were a molecular spring on the membrane.

Another purpose of using PEG as the spacer of the conjugate **4** is to suppress the interaction between the membrane and the proteins that will be encapsulated in a giant liposome in our prospective studies.[28] Unless a PEG molecule of a sufficient length links covalently to the liposome, the encapsulated enzyme will be gradually inactivated through interaction with the membrane of the liposome. Another merit of the cholesterol–PEG–DNA conjugate is that, since the DNA is anchored on the membrane of a liposome, the information of the DNA sequence must be delivered from mother to daughter liposomes upon division of the self-replicating mother liposome, regardless of the type of division (for example separation, birthing, and so on).[29] Such a self-replicating liposome with genomic information on the inner membrane will become a promising model of the protocell.

This work was supported by grants-in-aid from the Center of Excellence (Study of Life Science as Complex Systems) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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[21] Compound **4** was characterized by MALDI-TOF MS spectrometry (Voyager DE, Applied Biosystems Inc.) with 3-hydroxypicolinic acid, citric acid diammonium salt, and trifluoroacetic acid (50:2.5:1, w/w/w) in CH_3CN-H_2O (1:1, v/v) as a matrix solution; $[M+H]^+$: calcd.: 9657 (the degree of polymerization of the PEG unit is estimated to be 100); found: 9600 ± 800 (a cluster of multiplets due to diversity of the molecular weight of the PEG unit).

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[23] The phospholipids used in this study were abbreviated to DOPC (dioleoyl-phosphatidylcholine), POPG (palmitoyl-oleoyl-phosphatidylgly-

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cerol), and DSPE-PEG$_{5000}$ (*N*-PEGylated distearoyl-phosphatidylethanolamine).
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Received: March 10, 2003 [Z 593]

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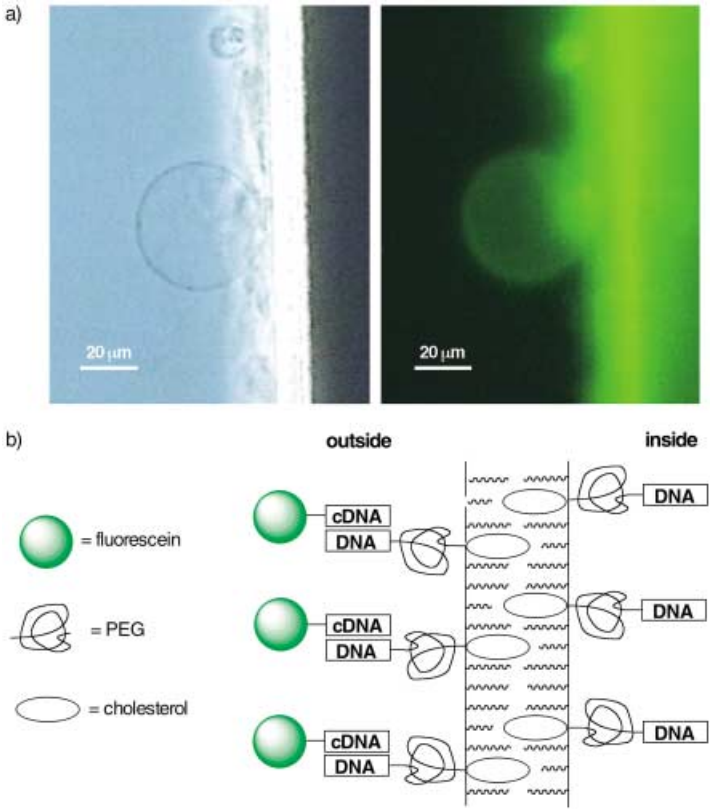
CORRIGENDUM

In the communication by **T. Sugawara et al.** in Issue 8 (*ChemBioChem*, **2003**, *4, 778 – 781*) Figure 2 was incorrectly reproduced. The correct version of Figure 2a should appear as shown below.



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