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Directed aggregation and fusion of lipid vesicles induced by DNA-surfactants

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ABSTRACT.

We investigated DNA-directed aggregation of vesicles using DNA-surfactants. Following tethering of single-stranded DNA oligonucleotides to vesicles using DNA-surfactant, the tethered vesicles were assembled with other vesicles bearing complementary strands. The vesicle aggregation was strongly affected by the salt concentration and by temperature according to the characteristics of DNA hybridization. Restriction enzyme, which can hydrolyze the double-stranded DNA used in the present study, dissociated the vesicle aggregates. Exploration using fluorescently-labeled vesicles suggested that the DNA-directed vesicle aggregation took place in a sequence-specific manner through DNA-duplex formation. Interestingly, the DNA-directed aggregation using short DNA-surfactant induced the fusion of vesicles to produce giant vesicles, resulting in an enzymatic reaction in the giant vesicle.

Introduction

Conventional synthetic strategies have provided a number of attractive molecular devices [1], but the sizes of the devices produced using these strategies are limited to a few hundred nanometers at most. Molecular self-assembly is an alternative approach for constructing systems that are larger (submicrometers to micrometers) and more complex [2-4]. In particular, lipid vesicles, which are spherical bilayer shells encapsulating an aqueous interior, have been widely studied as a biomimetic example of molecular self-assemblies [5]. Such lipid vesicles can range in size from 50 nm to ~1 mm, and contain various compounds, including metal ions, macromolecules and biomolecules. Furthermore, modification of the lipid bilayer can confer diverse functions onto lipid vesicles. Higher order or secondary levels of self-assembly of lipid vesicles have been achieved through vesicle aggregation using electrostatic and ligand-receptor interactions [6-8]. These higher-ordered assemblies of lipid vesicles were also studied as a simple model for biological tissues [8].

Over the last decade, oligonucleotides have attracted much attention as intelligent materials due to their highly specific and designable binding properties toward their complementary sequences. Many researchers have employed oligonucleotides as building blocks to construct nanostructured systems with well-designed geometric shapes and sizes [9-13]. Besides, oligonucleotides have been used as molecular glues for cross-linking, immobilizing and aggregating proteins, nanoparticles, colloids and cells [14-19]. Oligonucleotides were also introduced to the vesicles and utilized for the immobilization of vesicles on an electrode or a plate [20-28] Patolsky et al utilized the DNA-tethered vesicles for amplification of hybridization signal [20,21]. Yoshina-Ishii and Boxer first reported the sequence-specific immobilization of lipid vesicles [22-24]. These studies suggested the utility of DNA-tethered vesicles. Most of them mainly focused on surface modification and nanofabricated structures, rather than on the construction of macroscopic structured systems.

Wu et al. reported that an extremely high concentration (10 mg/ml) of long linear DNA induced non-selective aggregation of a small portion of zwitterionic vesicles, where the aggregation was supposed to be induced not by the DNA hybridization but simply by the electrostatic interaction [29]. Graneli et al. first reported the interaction among DNA-tethered vesicles on a DNA-modified gold substrate and

succeeded in the formation of three-dimensional vesicle matrices based on DNA hybridization [28]. There are, however, considerable uncertainties on the DNA-directed aggregation of vesicles in solution. For example, are the DNA-tethered vesicles responsive to external-stimuli such as temperature, ionic strength etc? Here, we investigate DNA-directed aggregation of vesicles in solution using DNA-surfactants and clarify if the DNA-tethered vesicles possesses the DNA-derived characteristics to be responsive to external stimuli (Scheme 1). Furthermore, we successfully accomplished DNA-directed fusion of vesicles based on this assembly procedure, leading to the production of a microreactor involving an enzyme-catalyzed reaction in the giant vesicle produced.

Experimental

Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was kindly provided by Nippon Fine Chemical Co (Osaka, Japan). Synthesized DNA oligonucleotides were purchased from Genenet (Fukuoka, Japan). A restriction enzyme *Hha*I was purchased from Takara Bio Inc. (Ohtsu, Japan). Fluo3 was purchased from Dojindo Laboratories (Kumamoto, Japan). FITC-labeled casein (EnCheck Protease Assay Kit) was purchased from Invitrogen Co. (Carlsbad, CA). Fluo3 was purchased from Dojindo Laboratories (Kumamoto, Japan). 5(6)-Carboxy-X-rhodamine and α-chymotrypsin were purchased from Sigma (St. Luis, MO). Other chemicals were of analytical grade.

Synthesis of DNA-surfactants

5'-Aminated DNA oligonucleotides (0.5 mM) in phosphate buffer (50 mM, 10 μl) were mixed with dimethylsulfoxide solution (54 μl) containing oleic acid N-hydroxysuccinimide ester (1 mM). Following incubation of the mixtures at 40 ºC for 24 h, the synthesized 5'-oleoyl DNA oligonucleotides (DNA-surfactants) were purified by HPLC (Jasco HPLC 2000 system) equipped with an Inertsil ODS-3 column (4 × 250 mm, GL Science) initially eluted with 10% acetonitrile in 0.1 M triethylamine acetate buffer for 5 min, then with a linear gradient of 10 to 90% acetonitrile over 30 min and isocratic 90 %

acetonitrile for 10 min at a flow rate of 1.0 ml/min. The synthesis of each DNA-surfactant [30] was confirmed by MALDI-TOF-MS analysis.

DNA-tethered vesicle preparation

Large unilamellar vesicles were prepared from thin film of POPC at room temperature using the extrusion technique and a polycarbonate membrane with a pore size of 200 nm according to the literature [31]. The POPC concentration was 3.1 mM, and the buffer was 100 mM Tris-HCl (pH 8.0). The vesicle solution (0.125 ml) was added to the DNA-surfactant solution (0.675 μM, 0.1 ml) to allow the formation of the DNA-tethered vesicles. The vesicles with complementary oligonucleotides were prepared in a similar way. The nucleobase sequences of the DNA-surfactants used in the present study are listed in Table 1.

Vesicles containing fluorescent probes (fluo3-Ca^{2+} complex or 5(6)-carboxy-X-rhodamine) were also prepared by the extrusion technique using 100 mM Tris-HCl solutions (pH 8.0) containing each compound (5 μM for fluorophores and 10 μM for $CaCl_2$). Non-entrapped compounds were separated from the vesicles by gel permeation chromatography using a Sepharose 4B CL column (length: 25 cm; diameter: 1.4 cm; GE Healthcare Bio-Sciences KK) [33]. Vesicles containing α-chymotrypsin or FITC-labeled casein were prepared from 50 mM HEPES buffer (pH 8.0) containing 15 mg/ml α-chymotrypsin or 10μg/ml FITC-labeled casein. These vesicles were also subjected to the gel permeation chromatography. Next, the DNA-surfactants were inserted to the vesicles as described above. The vesicle aggregation formed was observed using an inverted microscope (IX70; Olympus) equipped with a mercury lamp and fluorescence filters.

TEM observation

A vesicle assembly solution (10 μl) was placed on a carbon-coated grid at room temperature, and the solution was dried in vacuo for 12 h. The dried sample was stained with uranyl acetate solution (10 mM, 10 μl) and allowed to dry in vacuo for 5 h. TEM images of the vesicle aggregation were taken using a JEOL 200 CX TEM at 120 kV.

Dissociation of vesicle aggregation by a restriction enzyme

The solution of the aggregated vesicles with DNA-surfactants **1** and **2** (0.45 ml) containing POPC (3.1 mM) and DNA-surfactants (0.15 μM each) was added by a restriction enzyme *Hha*I (20 units, 2 μl) at room temperature. The dissociation of the vesicle aggregates was periodically observed on a glass plate at room temperature.

Effect of salt concentration on the vesicle aggregation

The DNA-tethered vesicle solution was prepared using a Tris-HCl buffer (10 mM) containing NaCl using DNA-surfactants **1** and **2**. NaCl concentration was varied from 0 to 10 mM. The aggregation was monitored by measuring the optical density of the solution at 550 nm using a UV-vis spectrophotometer.

Fusion of DNA-tethered vesicles

Equivalent volumes of DNA-tethered vesicles containing α-chymotrypsin (the interior concentration was 15 mg/ml) and complement-tethered vesicles containing FITC-labeled casein (the interior concentration was 10 μg/ml) were mixed at 25 ºC. The FITC-labeled casein shows green fluorescence when the casein is hydrolyzed by the enzyme. Mixing the vesicles with the different DNA-surfactants allowed immediate aggregation of the vesicles.

Results and discussion

DNA-surfactants were synthesized by conjugation of oleic acid and amine-terminated oligo DNA. The aggregation of DNA-tethered vesicles was investigated using 24-mer DNA-surfactants **1** and **2 (**Table **1**). The DNA-tethered vesicle solution (Fig. 1A) was mixed with a solution of DNA-tethered vesicles with the complementary sequence. Immediately, insoluble aggregates were observed in the mixed vesicle solution (Fig. 1B), indicating the aggregation of the DNA-tethered vesicles. Vesicles with noncomplementary oligonucleotides did not cause any changes in the vesicle solution (data not shown). Microscopic observation confirmed the presence of vesicle aggregates measuring dozens of micrometers (Fig. 1C). TEM observation reveals that these aggregates in the micron range were formed via the assembly of nano-scale vesicles (Fig. 1D). Observed vesicles were in size from 100 nm to 200 nm, identical to the sizes of the original vesicles. Under the present experimental conditions, a vesicle

possessed 90 molecules of the DNA-surfactant. Decreasing the number of the DNA-surfactant per vesicle reduced the vesicle aggregation (Fig. 2). More than 40 molecules of DNA-surfactants per vesicle allowed us to recognize the vesicle aggregation by naked eyes.

In order to obtain evidence that DNA hybridization on the vesicle surfaces caused aggregation of the vesicles, three different experiments were carried out. The first experiments investigated temperature control for dissociation of the vesicle aggregation. The calculated dissociation temperature (Tm) of the duplex oligonucleotides was 58 ºC in the presence of NaCl (100 mM). Rapid heating the vesicle aggregation solution to 70 ºC resulted in the disappearance of the micro-scale vesicle aggregation and produced the original small vesicles within 1 min. The turbidity measurements also confirmed the dissociation of the vesicle aggregation when heating gradually (Fig. 3). Cooling the resultant vesicle solution reproduced the vesicle aggregates. Tm of the vesicle aggregation was around at 60 ºC, which was comparable to the calculated Tm of the DNA-surfactants **1** and **2**. These results demonstrate that the aggregation of the DNA-tethered vesicles was thermally reversible and that the processes of the aggregation and dissociation were very fast.

The second experiments investigated sequence-specific cleavage of the DNA-surfactants catalyzed by a restriction enzyme. The nucleobase sequences of the DNA-surfactants **1** & **2** had a cleavage site for *Hha*I (nucleobases underlined in Table 1) [34]. Therefore, if the vesicle aggregation was formed by DNA duplexes on the vesicle surfaces, the aggregation would be dissociated by *Hha*I-catalyzed cleavage of these duplex oligonucleotides. Microscopic investigation revealed the dissociation of the aggregates of DNA-tethered vesicles after addition of *Hha*I (Fig. 4). The aggregates gradually became smaller over several hours. After 3 h, the largest assembly observed was one-third the original size. However, the digestion with longer time did not achieve complete dissociation of aggregated vesicles. This could be attributed following three reasons. One is that steric hinderance due to the vesicle surface prevented the restriction enzyme from accessing DNA duplex. Second is that even though the duplex was digested by the restriction enzyme, the resultant DNA fragments could rehybridize with non-digested DNA-surfactants. In fact, *Hha*I digests DNA duplex to produce a protruding end, which tends to induce the strand-exchange reaction to produce another DNA duplex [35,36]. Third is that the

digestion was carried out in a thin space between slide and cover glasses without mixing, which would inhibit the diffusion of the enzyme. The thermal reversibility and restriction-enzyme sensitivity of the vesicle aggregation revealed that the aggregation of the DNA-tethered vesicles was induced by duplex formation of the DNA-surfactants on the vesicle surface.

Third, the effect of salt concentration on the vesicle aggregation was studied, since DNA hybridization strongly depends on a salt concentration. The DNA-tethered vesicle solution was prepared in diluted Tris-HCl solution containing NaCl at varied concentrations (Fig. 5). The turbidity of the DNA-tethered vesicle increased with the increase of a NaCl concentration, while there was no change in the turbidity of the intact vesicle solution (without DNA-surfactants). These results demonstrate that hybridization of DNA-surfactants induced the vesicle aggregation and that the aggregation was responsive to the external stimuli according to the characteristics of DNA. These results revealed that the DNA-tethered vesicles retain the stimuli-responsive properties of DNA duplex to produce macroscopic aggregates.

One of the advantages of using DNA-surfactants as molecular glues is the diversity of possible nucleobase sequences in the DNA-surfactants. The biotin-avidin interaction also allows the aggregation of vesicles, but this strategy offers only a few types of interactions and is incapable allowing multi-type assemblies of vesicles. To verify the diversity of the vesicle aggregation using the DNA-surfactants, we employed two different complementary sets of 8-mer oligonucleotides as the DNA-surfactants. Four types of vesicles with different sequences were prepared. The vesicles with DNA-surfactant **3**, which were designed to assemble with vesicles with DNA-surfactant **4**, were dyed with fluo3 in the presence of Ca^{2+} to show green fluorescence (Fig. 6A). The vesicles with DNA-surfactant **5**, which were designed to assemble with vesicles with DNA-surfactant **6**, were dyed with 5,6-carboxy-X-rhodamine to show red fluorescence (Fig. 6B). We mixed the four types of vesicles at the same time and observed the resultant vesicle aggregation using a fluorescence microscope (Figs. 6C and 6D). The vesicle aggregation was color-coded in green or red, suggesting that the vesicles with DNA-surfactant **3** were assembled selectively with vesicles with DNA-surfactant **4** and that vesicles with DNA-surfactant **5** were assembled selectively with vesicles with DNA-surfactant **6**. However, Fig. 6D shows the

aggregation of green-coded clusters and red-coded clusters. This was probably due to the following two reasons. One is that the microscale aggregates tend to spontaneously assemble themselves due to their low mobility in solution. Another is the possible migration of DNA surfactants between vesicles because DNA surfactants are soluble in aqueous solution. Although we tried to measure the concentration of DNA-surfactant, which was solubilized into a bulk phase in the presence of vesicles, the DNA-surfactant was not detected in the bulk phase probably due to its too low concentration.

Finally, we investigated DNA-directed fusion of vesicles using DNA-surfactants with 12-mer sequences (DNA-surfactants **7** and **8**). DNA-tethered vesicles containing α-chymotrypsin and complement-tethered vesicles containing FITC-labeled casein were mixed. The FITC-labeled casein shows green fluorescence when the casein is hydrolyzed by the enzyme. Mixing the vesicles with the different DNA-surfactants allowed immediate aggregation of the vesicles. At 24 hours after mixing, a giant vesicle more than 10 μm was observed (Fig. 7A), which was not present just after the formation of the aggregation. The giant vesicle exhibited green fluorescence (Fig. 7B), suggesting fusion between a vesicle containing α-chymotrypsin and one containing FITC-labeled casein. The vesicle fusion initiated α-chymotrypsin-catalyzed hydrolysis of the FITC-labeled casein, resulting in green fluorescence [37]. This fusion experiment was reproduced more than triplicate. In the absence of DNA-surfactants, we neither observed vesicle aggregation nor a giant vesicle.

Interestingly, DNA-surfactants with 24-mer sequences (DNA-surfactants **1** and **2**) did not allow the fusion of the vesicles although the vesicle aggregation was observed. The use of DNA-surfactants **3** and **4** (8-mer) resulted in the vesicle fusion to produce a giant vesicle. These results indicate that the distance between vesicles in the vesicle aggregation plays a key role for the vesicle fusion. There remains considerable uncertainty on the vesicle fusion induced by the DNA hybridization. The detail investigation on the vesicle fusion is currently studied.

The present study reveals that the DNA-tethered vesicles possess the stimuli-responsive characteristics of DNA duplex to produce macroscopic aggregates. We report here not only the DNA-directed aggregation but also the fusion of lipid vesicles via sequence-specific hybridization of DNA-

surfactants on the vesicle surfaces. Our present strategy highlights the potential of DNA for construction of macroscopic systems, and validates the use of DNA-surfactants in DNA/RNA-based nanotechnology.

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FIGURE CAPTIONS

Scheme 1 Schematic illustration of the aggregation of DNA-tethered vesicles responsive to external stimuli.

Figure 1 Aggregation of DNA-tethered lipid vesicles. (A, B) Photographs of a solution of vesicles with DNA-surfactant **1** before (A) and after (B) mixing with vesicles with DNA-surfactant **2**. The solutions were centrifuged at 2500 × g for 2 min. (C, D) Bright field microscope and TEM images of aggregation of DNA-tethered vesicles. Kinked redline represents the cleavage site catalyzed by restriction enzyme *Hha* I.

Figure 2 Effect of the number of DNA-surfactant per vesicle on the vesicle aggregation. The solutions were centrifuged at 2500 × g for 2 min.

Figure 3 Effect of temperature on the aggregation of DNA-tethered vesicle. The vesicle solution was heated (gray line) and then cooled (black line). The ramp rate was 0.5 ºC/min.

Figure 4 Dissociation of a vesicle aggregation catalyzed by the restriction enzyme *Hha*I at (**A**) 0 h, (**B**) 1 h, (**C**) 2 h and (**D**) 3 h after the addition of *Hha*I. Scale bars: 20 μm.

Figure 5 Effect of NaCl concentration on the vesicle aggregation. Closed circles are the results of DNA-tethered vesicles and open circles are those of intact vesicles. An aqueous solution was 10 mM Tris HCl (pH 8).

Figure 6 Fluorescence and phase-contrast microscope images of sequence-specific vesicle aggregation. (**A**) Aggregation of vesicles with DNA-surfactants **3** and **4** containing fluo3 (5 μM)and $CaCl_2$ (10 μM). (**B**) Aggregation of DNA-tethered vesicles with DNA-surfactants **5** and **6** containing 5,6-carboxy-X-rhodamine (5 μM). (**C, D**) Phase-contrast (C) and fluorescence microscope (D) images of aggregation of vesicles with DNA-surfactants **3, 4, 5** and **6**, in which the vesicles with DNA-surfactant **3** contain

fluo3 and calcium ions, and the vesicles with DNA-surfactant **5** contain 5,6-carboxy-X-rhodamine. Scale bars: 10 μm.

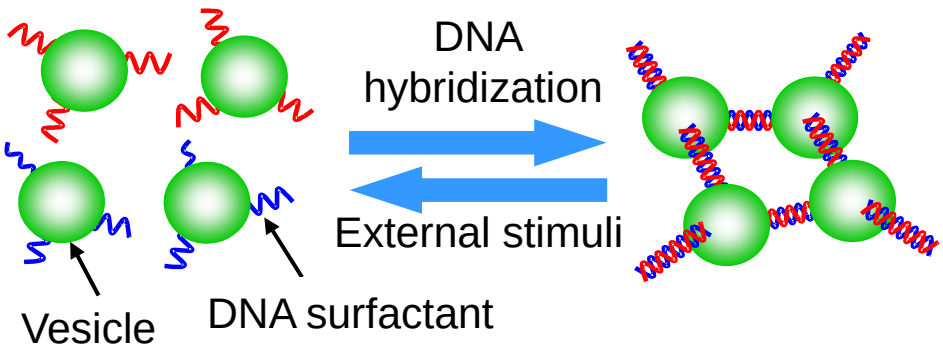
Figure 7 Vesicle fusion induced by the aggregation of DNA-tethered vesicles. Vesicles with DNA-surfactant **7** containing α-chymotrypsin and vesicles with DNA-surfactant **8** containing FITC-labeled casein were mixed, followed by 24 h incubation. **A**) Bright field microscopic image, **B**) fluorescent microscopic image. Scale bars: 10 μm.

Table 1. Sequences and Tm values of the DNA-surfactants

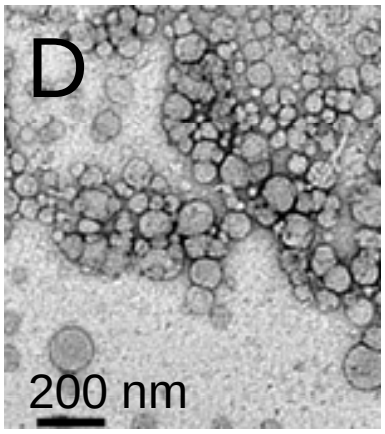
Table 1. Sequences and Tm values of the DNA-surfactants

	Sequence	Tm*
DNA-surfactant **1**	5'-TTTTTTGCACGCGCCACAAAGAAA-3'	58 ºC
DNA-surfactant **2**	3'-AAAAAACGTGCGCGGTGTTTCTTT-5'	
DNA-surfactant **3**	5'-ACAGTCTA 3'	22 ºC
DNA-surfactant **4**	5'-TGTCAGAT-3'	
DNA-surfactant **5**	5'-CTCGCAAA-3'	24 ºC
DNA-surfactant **6**	5'-TTTGCGAG-3'	
DNA-surfactant **7**	5'-GCACGCGCCGCA-3'	44 ºC
DNA-surfactant **8**	5'-TGCGGCGCGTGC-3'	

*The Tm values of the oligonucleotides in NaCl (0.1 M) were calculated using the Wallace method [32].

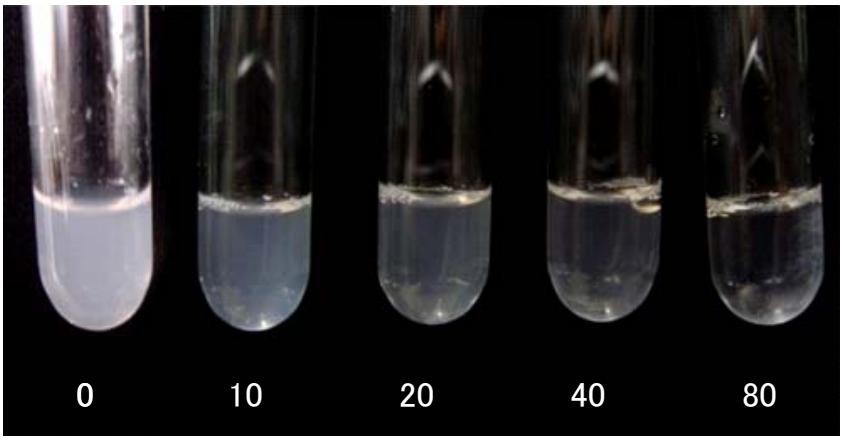

DNA
hybridization
External stimuli
Vesicle
DNA surfactant

Scheme 1



DNA surfactant 1: Oleoyl-5'-TTTTTTGCACGCGCCACAAAGAAA-3'
DNA surfactant 2: 3'-AAAAAACGTGCGCGGTGTTTCTTT-5'-Oleoyl

Fig. 1



DNA-surfactant / vesicle

Fig. 2

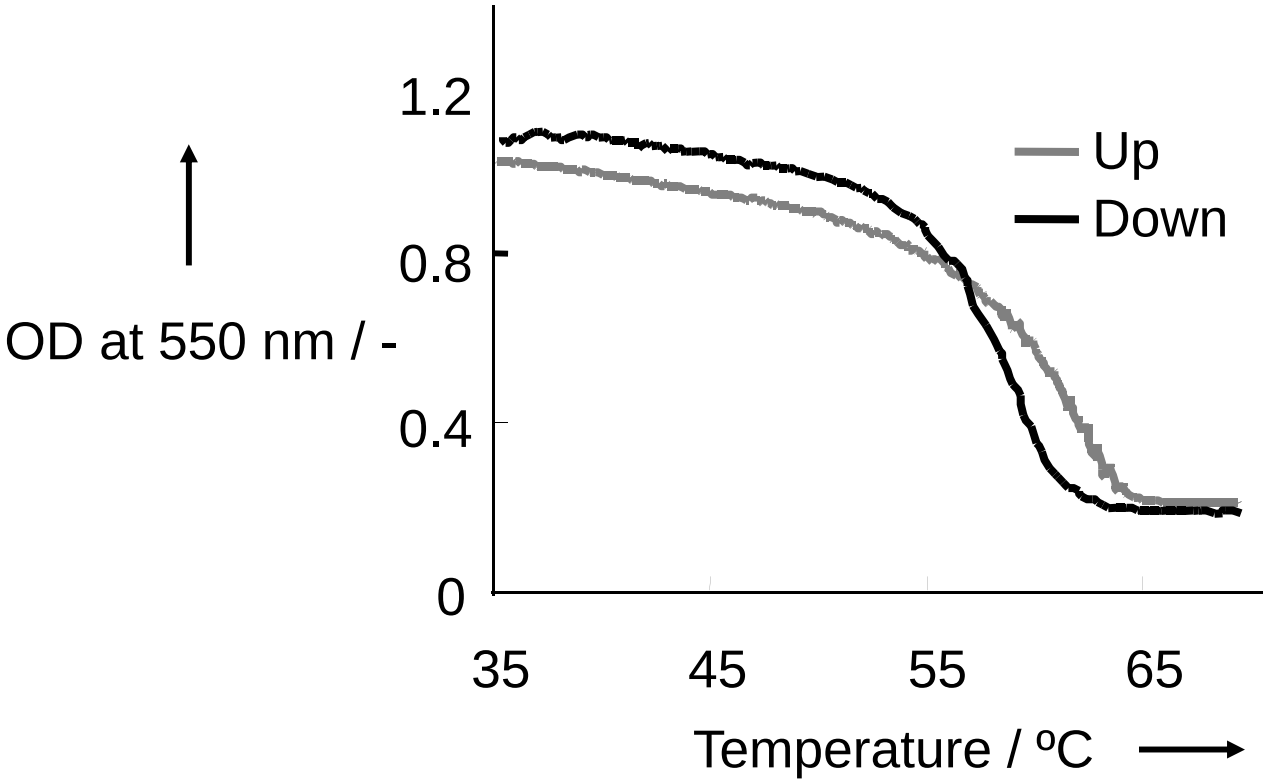


Fig. 3

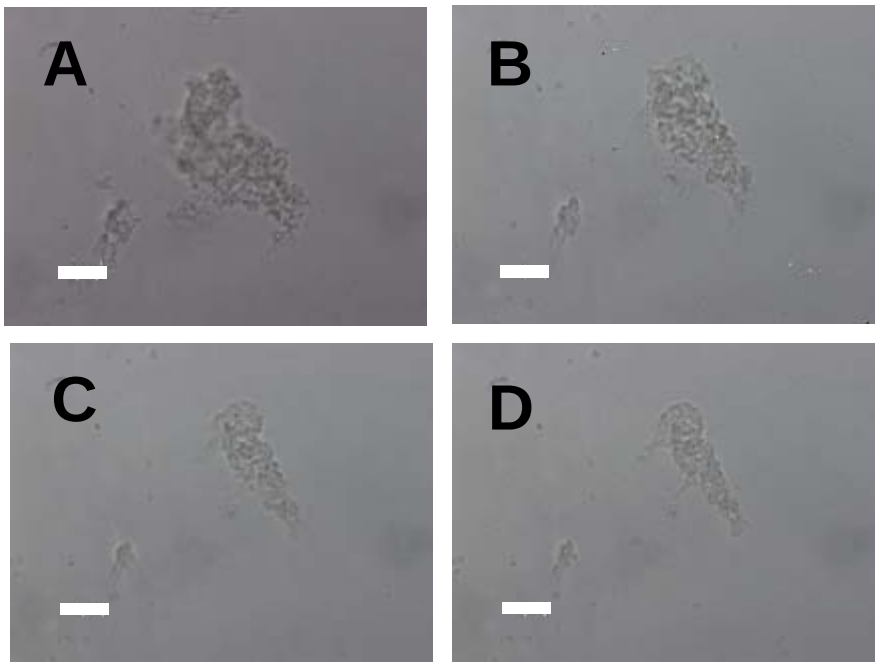


Fig. 4

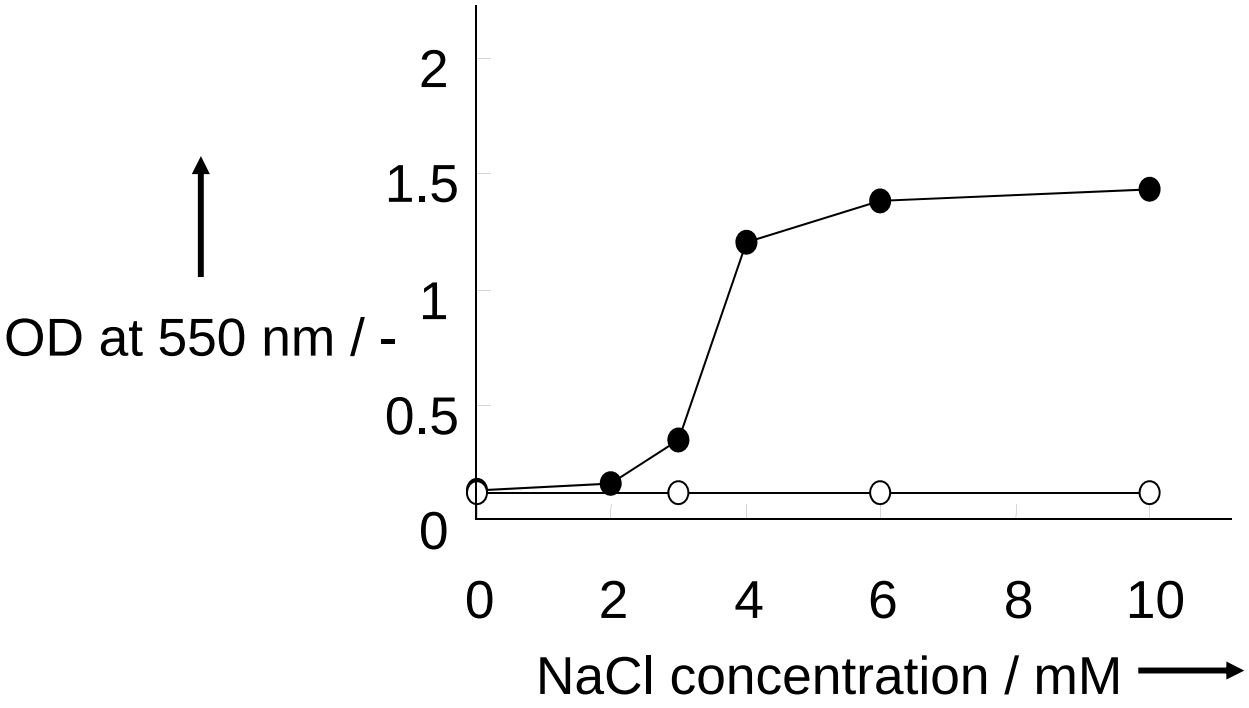


Fig. 5

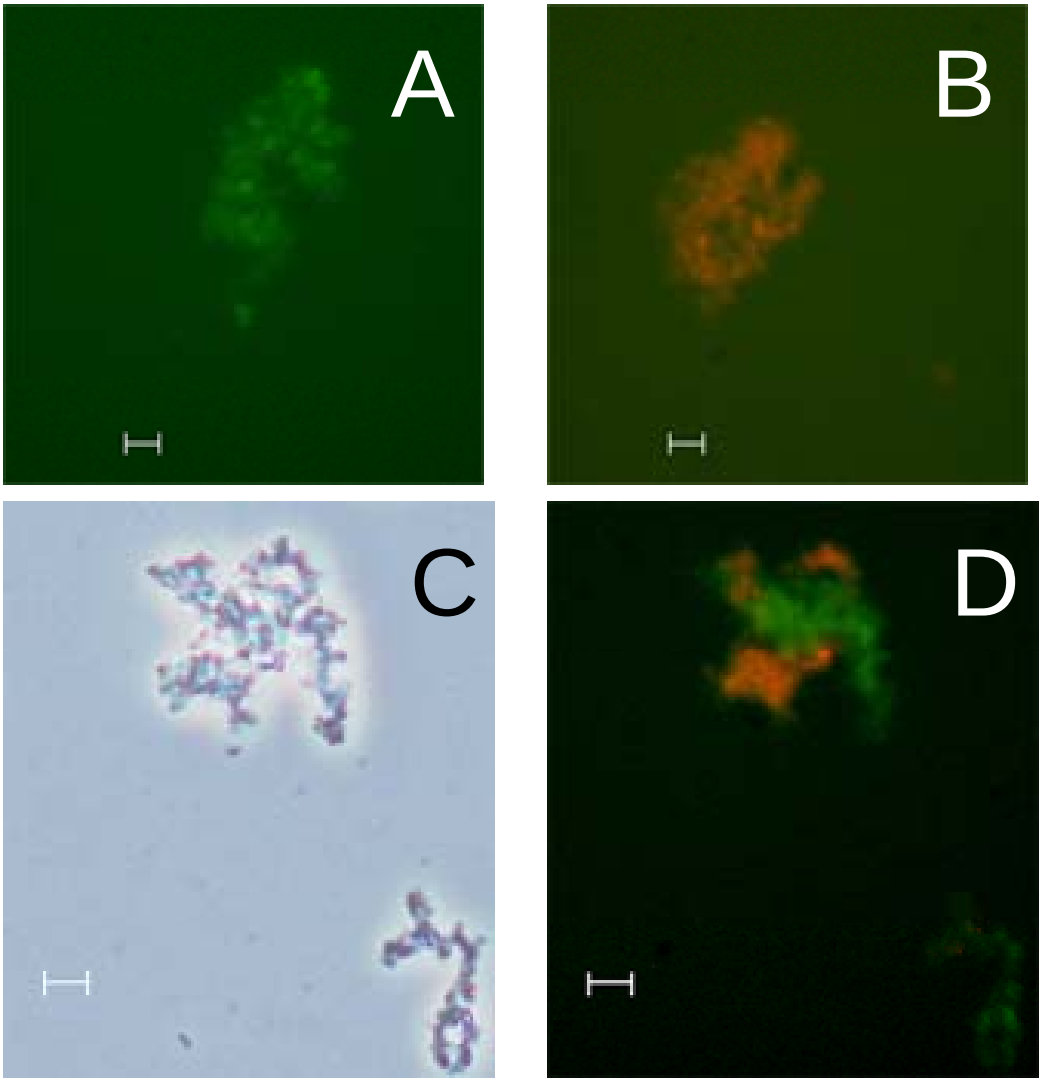


Fig. 6

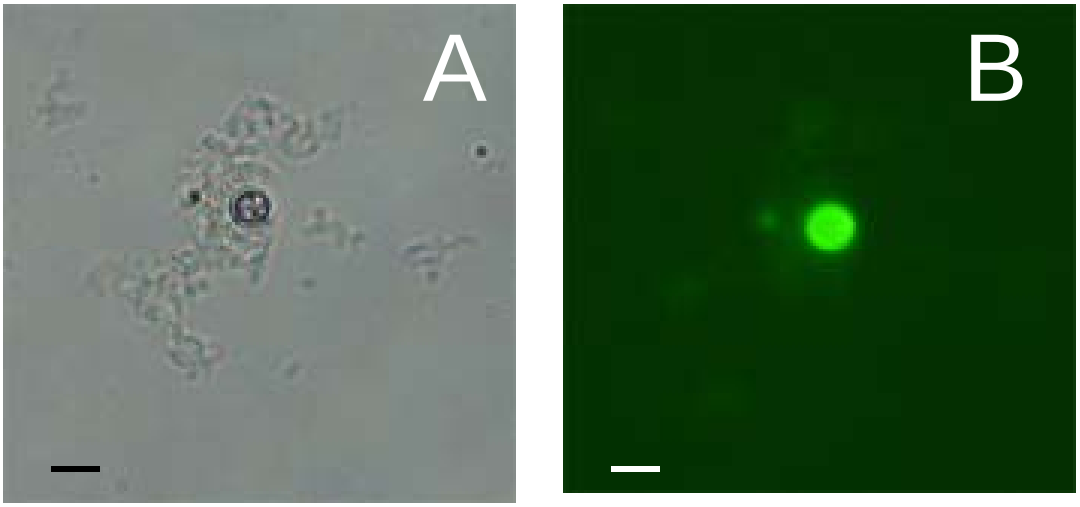


Fig. 7

Supplementary Information

Re: Aggregation and fusion of lipid vesicles induced by DNA-surfactants

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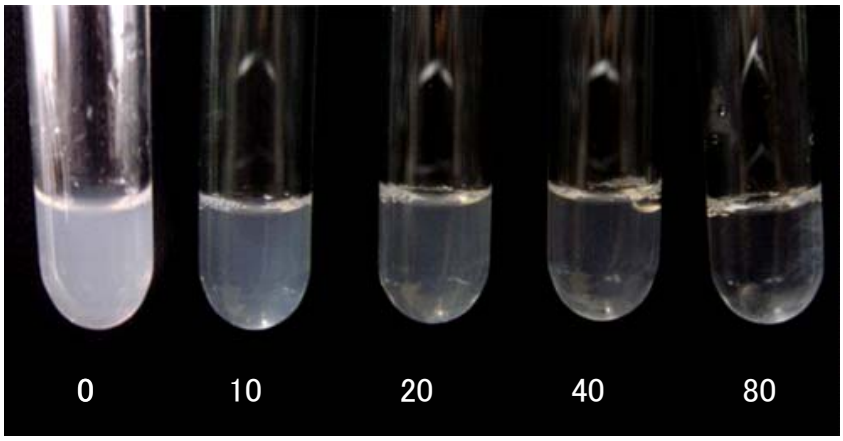

Fig. S1 Effect of the number of DNA-surfactant per vesicle on the vesicle aggregation. The solutions were centrifuged at 2500 × g for 2 min.