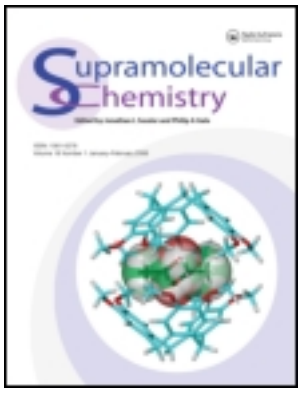

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[a] Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara, Japan

[b] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda, Tokyo, Japan

[c] Research Laboratories, NTT DOCOMO, Inc., Yokosuka, Kanagawa, Japan

[d] Department of Information and Computer Science, University of California, Irvine, CA, USA

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Specific delivery of transport vesicles mediated by complementary recognition of DNA signals with membrane-bound oligonucleotide lipids

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[a]*Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara, Japan;* [b]*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda, Tokyo, Japan;* [c]*Research Laboratories, NTT DOCOMO, Inc., Yokosuka, Kanagawa, Japan;* [d]*Department of Information and Computer Science, University of California, Irvine, CA, USA*

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Molecular communication is a novel communication paradigm inspired by information processing in biological systems using molecules as information medium. In this study, we achieved the selective propagation of transport vesicles which is an essential technique to establish a molecular communication system using artificial cells. The hybridisation of DNA was employed to conduct a signal-induced delivery of transport vesicles from a sender to a receiver vesicle formed with phospholipids. Oligonucleotide lipids each with a different single-stranded DNA, which acts as recognition tags, were embedded in the sender, receiver and transport vesicles. The addition of a DNA signal with a complementary sequence to connect two oligonucleotide lipids induced the assembly of two types of large unilamellar vesicles as well as recombination of the vesicles. In addition, the selective delivery of transport vesicles between sender and receiver giant vesicles is discussed, based on the results of fluorescence microscopic observations.

Keywords: lipid vesicle; DNA hybridisation; oligonucleotide lipid; molecular communication

1. Introduction

In biological systems, a variety of biochemical reactions are performed to handle energy conversion, material transport and information processing, which are essential to the preservation of life (*1*). Target-specific delivery of biologically important molecules is a fundamental vital process. Membrane trafficking including vesicle transport is a standard way of selectively transporting bioactive molecules, including membrane proteins and subcellular organelles in the cell (*2*). In the process of membrane trafficking, molecular recognition by certain proteins on the membrane surfaces enables the association of transport vesicles with the appropriate membrane organelle (*3*). Recently, we have proposed a concept of molecular communication, which is a new communication paradigm inspired by information processing in biological systems using molecules as information medium (*4*). In contrast to the conventional communication technologies using electromagnetic waves, molecular communication operates by transmitting information slowly and locally. Moreover, the molecular communication technology has the significant advantage of being capable of handling a wide variety of biochemical information and resulting biological phenomena directly, which are unable to be treated by a conventional communication technology approach.

The molecular communication system is essentially constituted with several elemental processes, including encoding of molecular information at a sender, sending of the molecules entrapped in molecular capsules from the sender, propagation of the molecular capsules to a target receiver and receiving and decoding of the molecular information at the receiver. Currently, we have developed various elemental techniques for molecular communication in artificial cellular systems, such as budding and fission of molecular capsules (*5*), propagation of molecular capsules controlled by ionic (*6–8*) or photonic (*7, 8*) stimuli and decoding of molecular information by artificial receptor-mediated enzymatic reactions (*8, 9*). In order to extend such a supramolecular system to a multicast molecular communication, it is necessary to develop a propagation method to cover the variety of combinations between a sender and a receiver. DNA hybridisation offers a potent approach to introduce diversity into the molecular communication system, due to the enormous variety and high specificity of DNA base sequences.

Recently DNA-tethered vesicles have been utilised for the manipulation of vesicles on a micro- or nanometre scale. For example, there have been several reports on the tethering of vesicles onto a substrate (*10*), environment-sensitive control of vesicular aggregation (*11*), controlled

*Corresponding author. Email: jkikuchi@ms.naist.jp

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fusion of membranes (*12*) and the detection of a hybridisation signal (*13*). Most of these reports focused on the highly ordered organisation of multi-vesicular assemblies rather than on the transportation of vesicles, which can carry various functional molecules.

In this article, we present diffusion-based selective transportation of molecular capsules between artificial cells with high specificity by employing DNA hybridisation on the membrane surface as shown in Figure 1. The present system provides the temporally controlled, stimuli-triggered and target-selective delivery of molecular capsules or transport vesicles, formed with phospholipid molecules. Sender, receiver and transport vesicles were functionalised with different oligonucleotide lipids, each having a single-stranded DNA (ssDNA) at the hydrophilic head moiety. The present transportation system consists of three elemental stages: loading of molecular capsules to a sender, release of loaded capsules and docking of the capsules to a receiver. Release or docking of the transport vesicles was triggered by the addition of a signal DNA, which is an ssDNA with a complementary sequence against two oligonucleotide lipids, under the conditions of changing temperature or salt concentration. This technique can be applied to the propagation of not only large unilamellar vesicles (LUVs) but also cell-sized giant vesicles. Initially, the essential characteristics of the signal ssDNA-induced association of vesicles were characterised by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The recombination of assembled vesicles was subsequently achieved through the strand exchange of DNA by employing a second signal ssDNA. Finally, the signal ssDNA-induced release and docking of transport vesicles was examined using giant vesicles to discuss the possibility of selective delivery of molecular capsules from a sender vesicle to a receiver vesicle, thereby representing a molecular communication system.

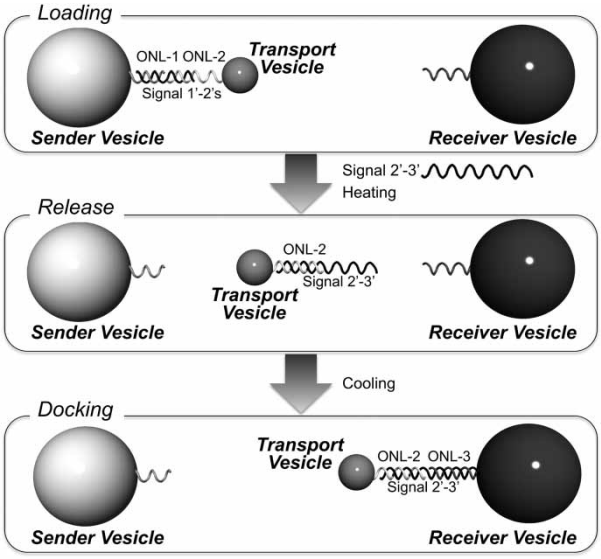

Figure 1. Schematic illustration of the signal DNA-directed transportation of vesicles between a sender and a receiver. The DNA-directed vesicle delivery system consists of loading, release and docking of a transport vesicle as elemental processes. Each process of vesicle delivery was triggered by the addition of a signal DNA and temperature change.

2. Experimental

2.1 Materials

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). *N*-(Lissamine rhodamine B sulphonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine ammonium salt (Rh-PE) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE) were purchased from Molecular Probes (Eugene, OR, USA). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals used in this study were of guaranteed grade and used without further purification. Custom-made oligonucleotides and oligonucleotide lipids, DNA derivatives of 16-(5-cholesten-3β-oxycarboxamine)-2-hydroxy-4,7,10,13-tetraoxohexadecyl phosphate, were purchased from Nippon EGT Co., Ltd. (Toyama, Japan) and Eurogentec SA (Seraing, Belgium), respectively.

2.2 Preparation of oligonucleotide lipid-modified vesicles

LUVs were prepared by the hydration of a lipid film. An appropriate amount of DMPC was dissolved in chloroform. The solvent was evaporated under nitrogen gas flow and the residual trace solvent was completely removed *in vacuo* to obtain a thin lipid film on the wall of a round-bottomed flask. The lipid film was hydrated with an appropriate amount of aqueous HEPES buffer (10 mM) containing NaCl (100 mM) and sucrose (12 mM) at pH 7.4 and 40°C using vortex mixer, followed by five freeze-and-thaw cycles at − 196 and 50°C. The aqueous dispersion of multi-walled liposomes was extruded 21 times with an Avestin LiposoFast mini-extruder using a 100-nm polycarbonate membrane filter above the phase transition temperature of DMPC (*14*). Giant vesicles were prepared by the natural swelling method as follows. A solution of appropriate amounts of phospholipid and fluorescent dye-labelled lipids in chloroform was dried under vacuum for 3 h to obtain the lipid thin film. The film was gently hydrated with aqueous HEPES buffer (10 mM) containing sucrose (12 mM), which assists the quantitative formation of giant vesicles (*15*), at pH 7.4 and 40°C for 12 h. After the formation of giant vesicles, sodium chloride was added to the suspension of giant vesicles and the concentration was set to 100 mM. Oligonucleotide lipids were incorporated

into the membrane of LUVs as well as giant vesicles by the addition of their stock solutions in acetonitrile–water (1:1 v/v) and subsequent incubation at room temperature for 30 min.

2.3 Determination of the denaturation temperature of double-stranded DNA

The denaturation experiments were carried out using an UV–vis spectrophotometer (U-3310, Hitachi, Tokyo, Japan) equipped with a peltier-type temperature controller. The sample solution was pre-equilibrated in a cuvette at 10°C for 10 min, and then heated from 10 to 70°C at a rate of 1.0°C min^{-1}. The melting temperature was determined as the first derivative of the temperature dependence of the absorbance at 260 nm.

2.4 DLS measurements

Hydrodynamic diameters of vesicles and their assembly were measured by a DLS spectrometer equipped with a He–Ne laser at 633 nm (DLS-6000, Otsuka Electronics, Osaka, Japan). Time course of the light scattering from the sample was analysed using the Cumulant method at an angle of 90° from the incident light.

2.5 Cryogenic TEM (Cryo-TEM)

The specimen for Cryo-TEM was prepared by rapid freezing of a vesicular dispersion. A copper microgrid with 200 meshes was pre-treated with a glow-discharger (HDT-400, JEOL, Tokyo, Japan) to obtain a hydrophilic surface. An aliquot (3 μl) of a vesicular dispersion was placed onto the grid and immediately plunged into liquid propane using a specimen preparation apparatus (EM CPC, Leica, Vienna, Austria). The temperature of the specimen was maintained by a cryotransfer holder (Model 626.DH, Gatan, Inc., Pleasanton, CA, USA) at a temperature lower than −140°C during the TEM observation. Microscopic observation was carried out using a transmission electron microscope (JEM-3100FEF, JEOL) at an acceleration voltage of 300 kV with the zero-loss imaging mode. The lipid concentration was set to 500 μM for the Cryo-TEM observations.

2.6 Fluorescence microscopy

Optical microscopic observations were carried out using an epifluorescence microscope (IX71, Olympus, Tokyo, Japan), and the obtained images were recorded using a colour CCD camera (DP70, Olympus). Both phase-contrast and fluorescence modes were used for the observations. All of the images were acquired at the 5-min time point following the addition of signal DNA.

3. Results and discussion

3.1 Design of oligonucleotide lipid and signal DNA

The molecular structure of the oligonucleotide lipid (ONL-*X*) as a recognition tag used in this study is shown in Chart 1. In order to effectively anchor the DNA chain on the surface of a phospholipid vesicle, a hydrophobic cholesteryl group was connected to the 5′- or 3′-end of the ssDNA via a hydrophilic spacer. ssDNA sequences of the oligonucleotide lipids and of the signal ssDNA molecules are listed in Table 1. The base sequences in ONL-1, ONL-2 and ONL-3 were chosen to prevent the formation of G-quadruplexes (*16, 17*), hairpin structures (*16, 18*) and self-dimerisation. In addition, unexpected hybridisation with non-target DNA was prevented. The denaturation temperatures (T_m) of completely matched double-stranded DNA were calculated as 56–62°C for all sequences using the Wallace–Itakura formula (*19*). We also designed two unmodified ssDNAs (signals 1′-2′s and 2′-3′) as molecular signals to control vesicle transport. Each signal ssDNA has fully or partly complementary sequences against two different oligonucleotide lipids to ensure their selective crosslinking. For the release of transport vesicles from a sender vesicle, signal 1′-2′s was designed to be readily substituted with signal 2′-3′ upon hybridisation with ONL-2 because of the relatively low stability of the shorter duplex. Thus, the reassembly of transport vesicles from a sender to a receiver vesicle can be achieved by the exchange of the hybridised signal DNA.

The denaturation temperature (T_m) of hybridised DNA was experimentally determined by measuring the temperature dependence of the absorbance at 260 nm. Table 2 summarises the T_m values for the possible duplexes of oligonucleotide lipids and signals. The T_m values for fully complementary combinations were found to be ∼60°C, which were consistent with the calculated values (entries 1, 3 and 4 in Table 2). Signal 1′-2′s, which has a shorter complementary sequence against ONL-2, displayed a much lower T_m (entry 2 in Table 2) than signal 2′-3′ upon hybridisation with ONL-2. Thus, it is possible that signal 1′-2′s hybridised with ONL-2 can be easily substituted with signal 2′-3′ in a temperature range higher than the T_m values. In addition, the coexistence of vesicles did not affect the denaturation temperature in any combinations, suggesting that duplex formation on the surface of lipid membranes is analogous to that observed in an aqueous solution.

3.2 Sequence-selective assembly of lipid vesicles

Signal ssDNA-induced assembly of vesicles was evaluated from an average hydrodynamic diameter (D_{hy}) of vesicular assembly using DLS. The D_{hy} value was measured upon the addition of a signal ssDNA to the mixture of two different types of vesicles modified with oligonucleotide

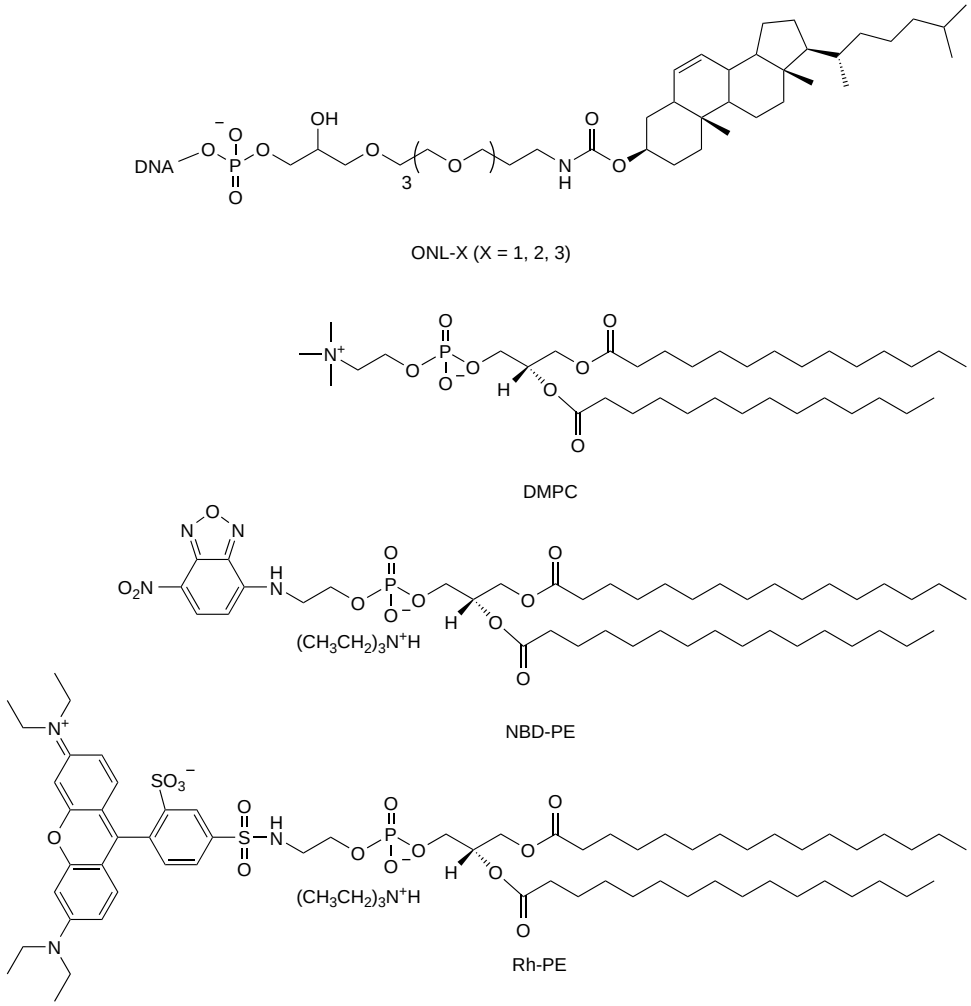

Chart 1. Chemical structures of lipids used in this study.

lipids (vesicles A and B). Since the modification of oligonucleotide lipids to LUV did not affect the D_{hy} value, oligonucleotide lipids alone do not induce the aggregation of LUV. The result of DLS measurements for various vesicular systems is listed in Table 3. When the sequence of signal ssDNA was matched with that of oligonucleotide lipids modified on both vesicles A and B, we observed a significant increase in the D_{hy} value (entries 3 and 6, Table 3). In contrast, such an increase of D_{hy} was not induced by the addition of non-complementary signal ssDNA (entry 2, Table 3) or a signal ssDNA with a complementary sequence against the single oligonucleotide lipid (entries 1, 4 and 5, Table 3). The results clearly indicate that sequence-selective assembly of vesicles was achieved by the crosslinking of two oligonucleotide lipids tethered to vesicles by a signal ssDNA. In addition, we examined the effect of temperature and salt concentration on the assembly of vesicles. The D_{hy} value did not increase upon the addition of complementary signal DNA (entry 7) when the salt concentration decreased to 50 mM. This is consistent with the requirement that hybridisation of DNA strands generally requires a high salt condition to diminish the electrostatic repulsion of phosphate groups in the chain (*20*). Furthermore, the assembly of vesicles induced by DNA duplex formation was strongly inhibited at a temperature above the T_m (entry 8). Thus, changing the medium conditions such as salt concentration or temperature is an effective way to control the assembly of vesicles mediated by DNA hybridisation.

Table 1. ssDNA sequences of the oligonucleotide lipids and signal DNA.

DNA	Sequence ($5'-3'$)[a]
ONL-1	Chol-GAG CGT GGG TAG AGA GAG G
ONL-2	GAG TAT TCA ACA TTT CCG TG-Chol
ONL-3	Chol-GGA GAG AGA TGG GTG CGA G
Signal $1'$-$2'$s	GTT GAA TAC TCC CTC TCT CTA CCC ACG CTC
Signal $2'$-$3'$	CAC GGA AAT GTT GAA TAC TCC TCG CAC CCA TCT CTC TCC

[a] Chol represents the cholesterol functionalised anchor part of the oligonucleotide lipid.

Table 2. Denaturation temperatures (T_m) of the DNA duplexes.[a]

Entry	DNA duplex	T_m (°C)		
		Obs. with vesicles	Obs. without vesicles	Calc.[b]
1	ONL-1 and signal 1′-2′s	61	61	62
2	ONL-2 and signal 1′-2′s	29	29	30
3	ONL-2 and signal 2′-3′	54	55	56
4	ONL-3 and signal 2′-3′	60	61	62

[a] [DMPC], 0.1 mM; [DNA], 1.0 μM; [Sucrose], 12 mM; in HEPES buffer (10 mM, pH 7.4).
[b] Calculated denaturation temperatures were obtained using the Wallace–Itakura equation.

Table 3. Effect of DNA sequence, salt concentration and temperature on the assembly of the oligonucleotide lipid-modified vesicles.[a]

Entry	Vesicle A	Vesicle B	Signal	NaCl (mM)	Temperature (°C)	D_{hy}[b] (nm)
1	ONL-1	ONL-1	1′-2′s	100	20	140
2	ONL-1	ONL-1	2′-3′	100	20	140
3	ONL-1	ONL-2	1′-2′s	100	20	1500
4	ONL-1	ONL-2	2′-3′	100	20	140
5	ONL-2	ONL-3	1′-2′s	100	20	140
6	ONL-2	ONL-3	2′-3′	100	20	1200
7	ONL-1	ONL-2	1′-2′s	50	20	140
8	ONL-1	ONL-2	1′-2′s	100	60	130

[a] [DMPC], 0.1 mM; [DNA], 1.0 μM; [Sucrose], 12 mM; in HEPES buffer (10 mM, pH 7.4).
[b] The D_{hy} value was measured 30 min after the addition of signal DNA.

3.3 Signal DNA-induced recombination of vesicular assembly

We next evaluated the recombination behaviour of vesicular assembly induced by the exchange of hybridised signal ssDNA. Three different vesicles incorporating ONL-1, -2 and -3 were prepared individually, and signal ssDNA was sequentially added to the mixture of vesicles concomitant with a change in temperature. The time course of the D_{hy} value for the vesicular assembly upon the addition of signal ssDNA, as evaluated by DLS, is shown in Figure 2. Before the addition of signal ssDNA, the equimolar mixture of vesicles modified with ONL-1 and vesicles modified with ONL-2 displayed a D_{hy} value of 140 nm, which corresponds to the size of individual intact vesicles. Upon addition of signal 1′-2′s to the mixture, the D_{hy} value gradually increased and reached 1500 nm in 30 min, indicating the formation of multi-vesicular assembly. Judging from the results in Table 3, the vesicular assembly was formed by the signal 1′-2′s-mediated crosslinking of ONL-1 and ONL-2 embedded in the vesicular membrane. Subsequently, signal 2′-3′, which has higher affinity towards ONL-2 than signal 1′-2′s, was added to the vesicular mixture and the temperature was elevated to 60°C. The D_{hy} value rapidly decreased to 140 nm in 10 min, reflecting that the assembled vesicles dissociated into isolated individual vesicles. Complete recovery of the initial D_{hy} value indicates that the addition of signal 1′-2′s has led to the association of vesicles without remarkable morphological perturbation of vesicles such as membrane fusion. When signal 2′-3′ was added at room temperature without heating, a significant decrease in D_{hy} was not observed (data not shown). The results show that the exchange of the DNA chain requires the denaturation of the present preformed DNA duplex. After the complete disassembly, the mixture was cooled again to room temperature. Upon addition of ONL-3-modified vesicles to the vesicular dispersion, the D_{hy} value gradually increased with time, reaching 1700 nm in 30 min (closed circles in Figure 2). In contrast, the time-dependent

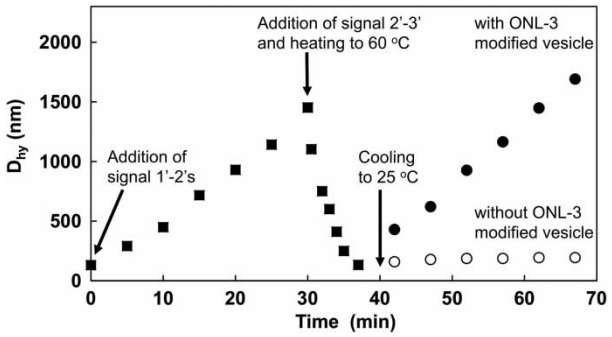

Figure 2. Time-course of the hydrodynamic diameter (D_{hy}) of vesicle assembly upon the sequential addition of DNA signals. Signals 1′-2′s and 2′-3′ were added at 0 and 30 min, respectively. The vesicle dispersion was heated to 60°C at 30 min and cooled to 25°C at 40 min. Closed circles and opened circles correspond to the presence and absence of ONL-3-modified vesicles, respectively. [DMPC], 0.1 mM; [signal DNA], 1.0 μM; [NaCl], 100 mM; [Sucrose], 12 mM; in HEPES buffer (10 mM, pH 7.4).

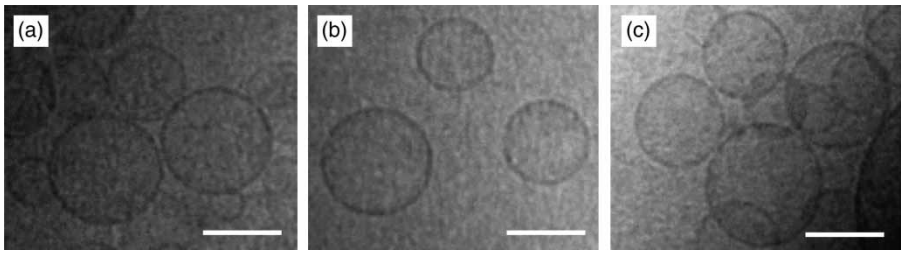

Figure 3. Cryo-TEM images for the recombination of vesicle assembly. The initial assembly of ONL-1-modified and ONL-2-modified vesicles in the presence of signal 1′-2′s (a), followed by disassembly induced by signal 2′-3′ (b), and the reassembly of ONL-2-modified vesicles with ONL-3-modified vesicles (c). Images (a)–(c) correspond to snapshots in the time course of the DLS measurement shown in Figure 2 at 30, 40 and 70 min, respectively. [DMPC], 0.1 mM; [DNA], 1.0 μM; [NaCl], 100 mM; [Sucrose], 12 mM; in HEPES buffer (10 mM, pH 7.4) and bar = 100 nm.

change of the D_{hy} value was not observed in the absence of ONL-3-modified vesicles (opened circles in Figure 2). The observed difference in the D_{hy} increase indicates that the initially hybridised signal 1′-2′ was completely substituted with signal 2′-3′ at 40 min. In addition, we confirmed that not only the temperature change but also variation in the salt concentration is able to control signal DNA-mediated recombination of vesicular assembly (data not shown). Accordingly, the binding partner of ONL-2-modified vesicles was successfully switched from the ONL-1-modified vesicle to the ONL-3-modified vesicle through the substitution of hybridised signal DNA on the vesicular surface.

We also carried out Cyro-TEM observations for the direct visualisation of the microscopic structure of vesicular assembly as triggered by the sequential addition of signal DNA. Each Cryo-TEM images shown in Figure 3 corresponds to a snapshot of an elemental step on the recombination of vesicular assembly. The addition of signal 1′-2′s induced the assembly of vesicles with ONL-1 and vesicles with ONL-2 while maintaining the spherical shape of the vesicles (Figure 3(a)). The estimated gap length between the lipid membranes was about 10 nm, corresponding to the theoretical extended length of the B-type double-stranded DNA with 30 base pairs (*21*). The assembled vesicles were dissociated into individual vesicles upon the addition of signal 2′-3′ and the increase in temperature (Figure 3(b)). Vesicles were assembled again by the addition of ONL-3-modified vesicles at room temperature in the presence of signal 2′-3′ (Figure 3(c)). Thus, a series of DLS measurements and Cryo-TEM observations clarified that the recombination of oligonucleotide lipid-modified vesicles was induced by the sequential addition of signal ssDNA with concomitant exchange of the hybridisation pair without membrane fusion.

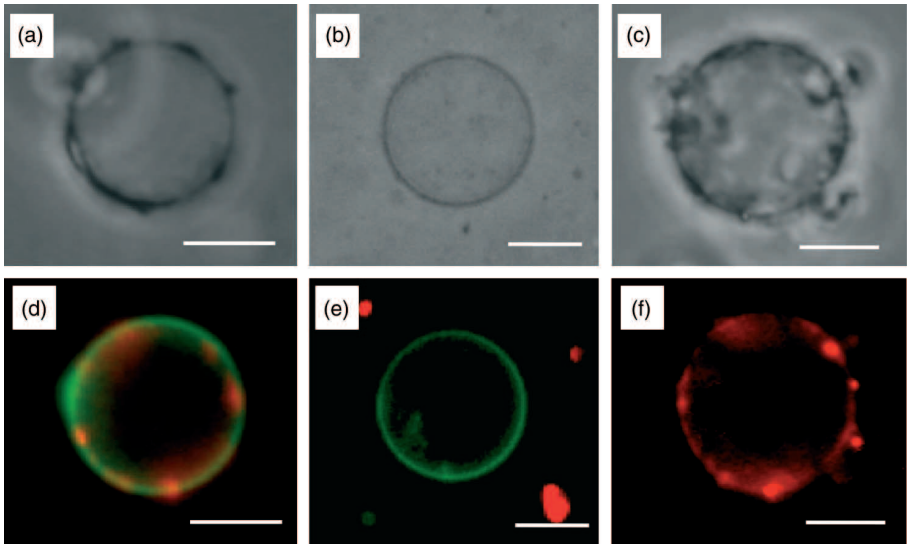

Figure 4. Phase-contrast (a)–(c) and fluorescence (d)–(f) microscopic images for the elemental steps of vesicle delivery. Loading to a sender (a, d), release (b, e) and docking to a receiver (c, f) of the transport vesicles. A giant sender vesicle was stained with green fluorescent dye (NBD-PE) and both transport vesicles and a giant receiver vesicle were stained with red fluorescent dye (Rh-PE) and bar = 5 μm.

3.4 Specific delivery of transport vesicles from a giant sender vesicle to a giant receiver vesicle

Signal DNA-induced recombination of vesicle assembly using LUVs, as mentioned above, was extended to the delivery of large unilamellar transport vesicles between a giant sender vesicle and a giant receiver vesicle. We prepared sender and receiver vesicles by modifying giant phospholipid vesicles with oligonucleotide lipids, ONL-1 and ONL-3, respectively. ONL-2 was only introduced to the LUVs to form transport vesicles. For direct observation by fluorescence microscopy, the sender was stained with green fluorescent dye (NBD-PE), and red-coloured fluorescent dye (Rh-PE) was incorporated in the receiver and transport vesicles. The phase-contrast and fluorescence microscopic images corresponding to the elemental steps of vesicle delivery are shown in Figure 4. First of all, transport vesicles were loaded onto the surface of the sender by adding signal 1′-2′s (Figure 4(a),(d)). In both phase-contrast and fluorescent images, we confirmed the attachment of small vesicles onto the surface of a giant vesicle. Since the red-coloured fluorescent lipid was localised without migration into the membrane of a sender, the transport vesicles were attached to the surface of the sender vesicle without lipid mixing. The release of the transport vesicles from the sender vesicle was induced by the addition of signal 2′-3′ and the elevation of temperature to 60°C. Transport vesicles showing red fluorescence were completely detached from the surface of the sender vesicle and the surface of the sender vesicle became smooth (Figure 4(b),(e)). Upon addition of a receiver vesicle modified with ONL-3 at room temperature, the transport vesicles were delivered to the surface of the receiver vesicle (Figure 4(c),(f)). Therefore, it was confirmed that the elemental steps for the signal ssDNA-induced delivery of transport LUVs between a sender and a receiver, both formed with giant vesicles, were successfully achieved by the rearrangement of DNA duplexes on the membrane surface.

4. Conclusion

In this article, we demonstrated signal ssDNA-triggered delivery of molecular capsules from a sender to a receiver vesicle. Oligonucleotide lipids were used for the incorporation of ssDNA into the lipid membrane. These ssDNA molecules acted as recognition tags. The addition of signal DNAs, which have complementary sequences against two different oligonucleotide lipids, induced the selective assembly of vesicles. In addition, we revealed that the exchange of hybridised signal DNA is an effective way to switch the recognition and assembly target. Fluorescence microscopic observations using giant vesicles illustrated the possible selective delivery of transport vesicles from a sender vesicle to a receiver vesicle. Consequently, the strategy presented in this study has great potential to be a key technique for the target-selective propagation of molecular information. We believe that the supramolecular chemistry-based approaches should provide inimitable features to construct bio-inspired molecular communication systems in the near future.

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