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A sustainable self-reproducing liposome consisting of a synthetic phospholipid

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ABSTRACT

A novel phosphoric membrane lipid (phospholipid) bearing an oleyl group as one of the hydrophobic chains formed a liposome with a thin lamella in water. Since the anionic membrane of pre-existing liposomes acted as a catalytic surface in phosphate buffer, membrane lipids could be generated from their precursor in an auto-catalytic manner without the inclusion of catalytic amphiphiles in the liposome. The morphological changes of this anionic liposome were monitored both by flow cytometry and optical microscopy, and it was found that the liposomes deformed into a budding shape, followed by division, after the addition of a membrane precursor. Hence, this anionic monocomponent liposome could be regarded as a sustainable self-reproducing system. This liposome was also able to provide a reaction cavity for enzymatic reactions, such as DNA amplification by a polymerase chain reaction.

1. Introduction

A protocell is characterized by a sustainable self-reproducing molecular system comprising fundamental organic molecules, macromolecules, and metal ions that may have existed in the prebiotic era (Walde, 2010). The construction of a model protocell is an appropriate approach for revealing the intrinsic properties of cellular life. A model protocell consists of a *compartment* that separates the inner volume from the outer environment, a *catalyst* that catalyzes metabolic reactions, and *information* that transmits characteristic properties of the cell to its descendants (Szostak et al., 2001). For the compartment, a liposome composed of phospholipids is often used if the size of the compartment is larger than 1 μm because liposomes are structurally similar to the membranes of living cell (Luisi, 2006; Sugawara, 2009; Walde, 2010; Kurihiara et al., 2011; Sugawara et al., 2013; Kurihara et al., 2015; Tu et al., 2016; Bhattacharya et al., 2017).

A self-reproducing catalyst is a prerequisite for the sustainability of a giant liposome-based model protocell; otherwise, the amount of catalysts decreases with each division step. A self-reproducing liposome composed of phospholipids, the membrane of which served as a catalytic surface for the formation of phospholipids from its precursor, has been reported (Takahashi et al., 2010). However, the liposome was formed with cholesterol as a helper lipid in an aqueous medium containing alcohol, which was not appropriate for enzymatic reactions. Devaraj et al. has extensively studied artificial phospholipids, including a synthetic self-reproducing metal ion-based catalyst, and emphasized the importance of an autocatalyst (Bissette and Fletcher, 2013) that drives lipid synthesis, leading to the continual formation of artificial phospholipids (Hardy et al., 2015). Scott et al. prepared a model protocell in which phospholipids were produced by membrane proteins, but no division of the liposome was observed (Scott et al., 2016).

Here, we describe a novel anionic liposome composed of a synthetic phospholipid (**V$^-$*ole***) carrying an oleyl chain as one of its hydrophobic chains. Without using helper lipids, inorganic catalysts, or membrane proteins, our monocomponent liposome composed of **V$^-$*ole*** was able to repeatedly self-reproduce because the membrane served as a catalytic surface for the production of phospholipids from their precursors (Fig. 1). Moreover, it was shown that this self-reproducing liposome could serve as an excellent vessel in which enzymatic reactions could proceed.

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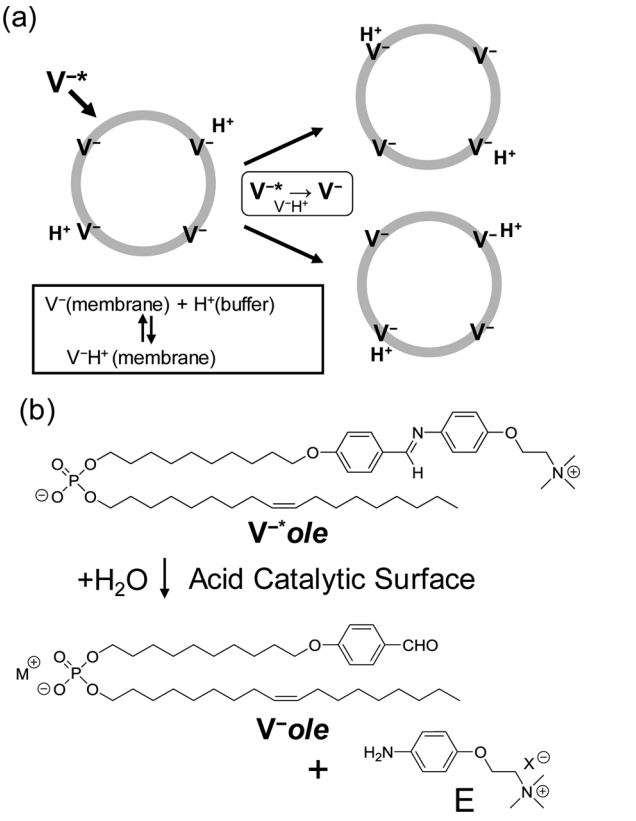


Fig. 1. Scheme of the self-reproducing liposomes: (a) Schematic illustration of the self-reproduction of phospholipid liposomes, the membrane of which works as a catalytic surface. (b) Hydrolysis of the phospholipid precursor (**V⁻*ole**) into phospholipid **V⁻ole** and electrolyte **E** in the presence of a catalytic surface.

2. Material and methods

2.1. General

All chemicals were purchased from a commercially source and used without further purification. Nuclear magnetic resonance (NMR) spectra were measured by a Fourier transform NMR spectrometer, ECS-400S (JEOL, Japan). Ultraviolet-visible (UV–vis.) spectra were measured by a UV–vis. spectrometer, UV-1850 (SHIMADZU, Japan). Electrospray ionization mass spectra (ESI-MS) were measured by an electrospray ionization mass spectrometer, JMS-T100LC (JEOL, Japan). Intensities of forward scatter (FS) and fluorescence (FL) were measured by a flow cytometer (FCM), SH 800 (SONY, Japan).

2.2. Synthesis

2.2.1. Synthesis of membrane phospholipid (V⁻ole)

Phospholipid, potassium (*Z*)-octadec-9-en-1-yl 10-(4-(((4-(2-(trimethylammonio)ethoxy)phenyl)imino)methyl)phenoxy)decyl phosphate (**V⁻ole**) was synthetic by using an amidite method (Beaucage and Caruthers, 1981), modifying the reported procedures (Takahashi et al., 2010) (Scheme 1).

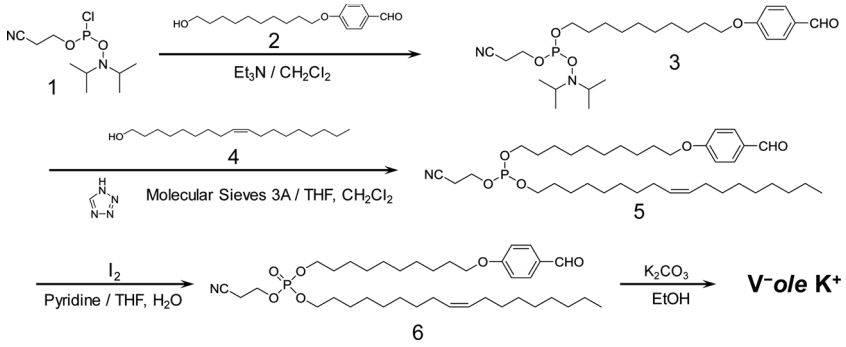
2.2.1.1. 2-Cyanoethyl 10-(4-formylphenoxy)decyl (Z)-octadec-9-en-1-yl phosphate (6). Under a nitrogen atmosphere in a glove-box, 4-((10-hydroxydecyl)oxy)benzaldehyde (**2**) (300 mg, 1.1 mmol) was placed in a vessel, and 5 mL of a dichloromethane solution of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (**1**) (260 mg, 1 mmol) and trimethylamine (100 mg, 1 mmol) was added through a dropping funnel. A mixture was stirred for 15 min at room temperature. After the solvent was evaporated, oleyl alcohol (**4**) (560 mg, 2 mmol) was added to the vessel, together with a small amount (ca. 100 mg) of molecular sieves 3 A A mixture was evacuated by a rotary pump for 1 h. Then, 10 mL of a THF and acetonitrile (1:1 v/v) solution of tetrazole (280 mg, 4 mmol) was added to the vessel and a mixture was stirred for 1.5 h to give a hetero-phosphite diester, (*Z*)-2-cyanoethyl-10-(4-formylphenoxy)decyl octadec-9-en-1-yl phosphite (**5**). Cautious oxidation of the phosphite to phosphate was conducted by adding a 10 mL of a mixed solution of THF-H_2O (1:1 v/v) of iodine (349 mg, 1.2 mmol), containing several drops of pyridine to the above solution during 1.5 h in a drop by drop manner. The addition was stopped before the color of the solution changed brown. After solvent was removed by evacuation, 10 mL of water was added, and the solution was extracted by chloroform three times, and the organic layer was dried over sodium sulfate. After drying reagent was filtered off, the filtrate was evaporated to give 2-cyanoethyl 10-(4-formylphenoxy)decyl (*Z*)-octadec-9-en-1-yl phosphate (**6**) as a crude product. The crude product was purified by gel-permeation chromatography in a recycle mode, using chloroform as an eluent. The final product **6** was obtained as an oil (300 mg, 47%). ^{1}H NMR(400 MHz; $CDCl_3$): δ(ppm) = 0.9 (3H, t, Me), 1.6-1.2 (34H, m, alkyl CH_2), 1.7 (m, 4H, $POCH_2CH_2$) 1.8 (2H, m, $PhCOCH_2CH_2$), 2.0 (4H, br m, CH_2CH = $CHCH_2$), 2.8 (3H, t, $CNCH_2$), 4.1 (6H, m, $COCH_2$, $POCH_2$), 4.2 (2H, m, $CNCH_2CH_2$), 5.4 (2H, m, *cis*-C*H*=C*H*), 7.0 (2H, d, Ph(3,5)), 7.8 (2H, d, Ph(2,6)) and 9.8 (1H, s, CHO).

2.2.1.2. (Z)-10-(4-formylphenoxy)decyl octadec-9-en-1-yl phosphate (V⁻ole). Cyanoethyl-protected phospholipid (**6**) (53 mg, 0.1 mmol) was dissolved in 1 mL of ethanol, and potassium carbonate (14.2 mg, 0.1 mmol) was added, and the mixture was stirred for 2 h, and the same amount of potassium carbonate was added again 1 h after the first addition. After the solvent was removed by evacuation, water was added to the reaction mixture and the de-protected product was extracted by chloroform three times. The organic layer was dried over sodium sulfate, and the drying reagent was filtered off to afford a pure product, (*Z*)-10-(4-formylphenoxy)decyl octadec-9-en-1-yl phosphate (**V⁻ole**) as an oil. (47 mg, 93%). ^{1}H NMR spectrum was shown in Figure S1. ^{1}H NMR(400 MHz; $CDCl_3$): δ(ppm) = 0.9 (3H, t, Me), 1.5-1.0 (34H, m, alkyl CH_2), 1.6 (m, 4H, $POCH_2CH_2$), 1.8 (2H, m, $PhOCH_2CH_2$), 2.0 (4H, br m, CH_2CH = $CHCH_2$), 2.8 (3H, t, $CNCH_2$), 3.8(4H, m, $POCH_2$) 4.0 (2H, t, $COCH_2$), 5.3 (2H, m, *cis*-C*H*=C*H*), 7.0 (2H, d, Ph(3,5)), 7.8 (2H, d, Ph(2,6)) and 9.9 (1H, s, CHO), ESI-MS: *m/z* = 631.4048 (M^+ + H + Na. $C_{35}H_{61}O_6PNa$ requires 631.4103).

2.2.2. Synthesis of phosphate lipid precursor (V⁻*ole)

Membrane precursor, (*Z*)-octadec-9-en-1-yl (10-(4-((*E*)-((4-(2-(trimethylammonio)ethoxy)phenyl)imino)methyl)phenoxy)decyl) phosphate (**V⁻*ole**) was synthetic by a dehydro-condensation between membrane lipid and electrolyte 2-(4-aminophenoxy)-*N,N,N*-trimethylethan-1-aminium bromide (**E**) (Takakura and Sugawara, 2004; Suzuki et al., 2009) in an organic solvent, followed by the removal of the cyanoethyl group. However, the yield of **V⁻*ole** was low in this scheme, and crude **V⁻*ole** was difficult to purify because of its poor solubility in organic solvents. Hence, we used the tertiary amine compound **D**, instead of **E** which has a tertiary ammonium salt (Scheme 2).

2.2.2.1. N-(4-(2-bromoethoxy)phenyl)acetamide (9). In a 300 mL flask was placed 100 mL of anhydrous ethanol, and Na (1.5 g, 65 mmol) was added to form sodium ethoxide. Then 4-hydoroxyacetoanilide (9.9 g, 65 mmol), 1,2-dibromoethane were added, and the mixture was refluxed for 15 h. After the reaction mixture was filtered, ethyl acetate and 1 M aqueous NaOH were added to the filtrate, and the product was extracted by ethyl acetate. After the organic layer was removed by evaporation, addition of hexane precipitated the product *N*-(4-(2-bromoethoxy)phenyl)acetamide (**9**) as a solid (3.9 g, 24%). ^{1}H NMR(400 MHz; DMSO-d_6): δ(ppm) = 2.0 (3H, s, COMe), 3.7 (2H, t, $BrCH_2$), 4.2 (2H, m, OCH_2), 6.8 (2H, d, Ph(3,5)) and 7.4 (2H, d, Ph (2,6)), 9.8 (1H, s, NH).



Scheme 1. Synthesis of membrane phospholipid (**V⁻*ole***).

2.2.2.2. N-(4-(2-(dimethylamino)ethoxy)phenyl)acetamide (10). In a high-pressure tolerant sealed vessel was placed **9** (2.01 g, 7.8 mmol), followed by the addition of 10 mL of aqueous dimethyl amine (50%). The mixture in a pressure-resistant container was heated in an oil bath at 80 °C for 80 h. After filtration of the reaction mixture, ethanol as added to the filtrate, and water in the filtrate was removed by an azeotropic mixture. Addition of acetone to the concentrated filtrate precipitated solids. Filtration of the mixture gave *N*-(4-(2-(dimethylamino)ethoxy)phenyl)acetamide (**10**) as a white solid. ^{1}H NMR(400 MHz; DMSO-d_6): δ(ppm) = 2.0 (3H, s, COMe), 2.8 (6H, s, NMe_2), 3.4 (2H, t, NCH_2), 4.2 (2H, m, OCH_2), 6.9 (2H, d, Ph(3,5)) 7.5 (2H, d, Ph(2,6)) and 9.8 (1H, s, NH).

2.2.2.3. 4-(2-(dimethylamino)ethoxy)aniline (D) dihydrochloride salt. In a 100 mL flask was placed the product **10** (0.91 g 4.2 mmol) and 30 mL of 3 M HCl was added. Under nitrogen atmosphere, the mixture was refluxed for 18 h. After addition of ethanol to the reaction mixture, solvents were removed by evaporation to afford the product (**D**) as a solid of dihydrochloride salt (0.71 g, 93%). ^{1}H NMR(400 MHz; DMSO-d_6): δ(ppm) = 2.1 (6H, s, NMe_2), 2.5 (2H, t, NCH_2), 3.8 (2H, t, OCH_2), 4.5 (2H, m, NH_2), 6.4 (2H, d, Ph(3,5)) and 6.6 (2H, d, Ph(2,6)).

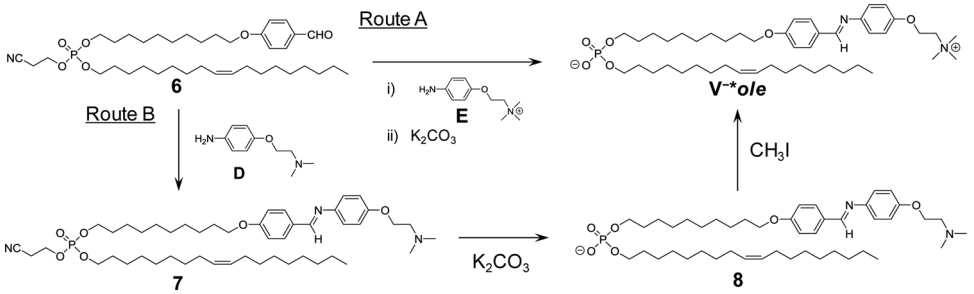
2.2.2.4. 2-cyanoethyl 10-(4-(((4-(2-(dimethylamino)ethoxy)phenyl) imino)methyl)phenoxy)decyl (Z)-octadec-9-en-1-yl phosphate (7). Dihydrochloride salt of **D** was neutralized by washing in an aqueous solution of potassium carbonate. A precursor of the membrane lipid (**6**) (150 mg, 0.23 mmol) and **D** (64 mg, 30 mmol) were dissolved in 20 mL of benzene and (20 mL), and a drops of acetic acid was added. The mixture was refluxed for 24 h. The solvent was removed by evaporation. The crude product was purified by gel-permeation chromatography (GPC) in a recycle mode, using chloroform as an eluent. The solvent was removed by evaporation to afford yellow viscous solid (**7**). ^{1}H NMR(400 MHz; DMSO-d_6): δ(ppm) = 0.9 (3H, t, Me), 1.2–1.6 (34H, br, alkyl CH_2), 1.7 (4H, m, $POCH_2CH_2$), 1.8 (2H, m, $PhOCH_2CH_2$), 2.0 (4H, br m, CH_2CH = $CHCH_2$), 2.3 (6H, s, NMe_2), 2.7 (2H, t, $Me_3N^+CH_2$), 2.8 (3H, t, $CNCH_2$), 4.1 (8H, m, $PhOCH_2$, $POCH_2$), 4.5 (2H, t, CH_2CH_2CN), 5.3 (2H, m, *cis*-CH_2=CH_2), 7.0 (4H, d, N = CH*Ph*(3,5), CH = N*Ph*(2,6)), 7.2 (2H, d, CH = N*Ph*(3,5)), 7.9 (2H, d, N = CH*Ph*(2,6)) and 8.5 (1H, s, N = CH)).

2.2.2.5. Potassium 10-(4-(((4-(2-(dimethylamino)ethoxy)phenyl)imino) methyl)phenoxy)decyl (Z)-octadec-9-en-1-yl phosphate (8). A condensed compound with **D** (**7**) (53 mg, 0.1 mmol) was dissolved in 1 mL of ethanol. Potassium carbonate (14.2 mg, 0.1 mmol) was added, and the same amount of potassium carbonate was added again 1 h after the first addition and stirred for 2 h at room temperature. Reaction solution was filtered. The filtrate was evacuated to give a yellow viscous solid of Potassium 10-(4-(((4-(2-(dimethylamino)ethoxy)phenyl)imino)methyl) phenoxy)decyl (*Z*)-octadec-9-en-1-yl phosphate (**8**) (47 mg, 93%).^{1}H NMR(400 MHz; DMSO-d_6): δ(ppm) = 0.9 (3H, t, Me), 1.2–1.6 (34H, br, alkyl CH_2), 1.7 (4H, m, $POCH_2CH_2$), 1.8 (2H, m, $PhOCH_2CH_2$), 2.0 (4H, br m, CH_2CH = $CHCH_2$), 2.3 (6H, s, NMe_2), 2.7 (2H, t, $Me_3N^+CH_2$), 4.1 (6H, m, $COCH_2$, $POCH_2$), 4.5 (2H, t, $PhOCH_2$), 5.3 (2H, m, *cis*-CH_2=CH_2), 7.0 (4H, d, N = CH*Ph*(3,5), CH = N*Ph*(2,6)), 7.2 (2H, d, CH = N*Ph*(3,5)), 7.9 (2H, d, N = CH*Ph*(2,6)) and 8.5 (1H, s, N = CH)).

*2.2.2.6. V⁻*ole*. Amine derivative (**8**) (38 mg, 0.05 mmol) was dissolved in 30 ml of chloroform, and methyl iodide (25 mg, 0.18 mmol) was added, and mixture was stirred for 2 h. The precipitated solid was filtered, and the desired compound (**V⁻*ole**) was obtained as a pale-yellow power (30 mg, 95%). ^{1}H NMR spectrum was shown in Figure S2. ^{1}H NMR(400 MHz; DMSO-d_6): δ(ppm) = 0.9 (3H, t, Me), 1.1–1.6 (34H, br m, alkyl CH_2), 1.8 (2H, m, $PhOCH_2CH_2$), 2.0 (4H, br m, CH_2CH = $CHCH_2$), 3.3 (9H, s, N^+Me_3), 3.8 (6H, m, $COCH_2$, $POCH_2$), 4.1 (t, 2H, $Me_3N^+CH_2$), 4.5 (2H, t, $PhOCH_2$), 5.3 (2H, m, *cis*-CH_2=CH_2), 7.0 (4H, d, N = CH*Ph*(3,5), CH = NPh(2,6)), 7.2 (2H, d, CH = N*Ph*(3,5)), 7.8 (2H, d, N = CH*Ph*(2,6)) and 8.5 (1H, s, N = CH)), MS(ESI) *m/z* = 785.5521 (M^+ + H. $C_{46}H_{78}N_2O_6P$ requires 785.5598), UV–vis (Phosphate Buffer): λ_{max} (ε) = 284.5 nm (5300), 327 nm sh (4230).

2.3. Preparation of giant liposome composed of V⁻ole in phosphate buffer

A chloroform solution (100 μL, 10 mM) of synthetic phosphate membrane lipid (**V⁻*ole***) was poured to a vial, and the solvent was



Scheme 2. Synthesis of phosphate lipid precursor (**V⁻*ole**).

removed by rotating the vial and a thin film of the lipid was form on the vial wall. The formed thin film was dried in vacuo for 2 h. A phosphate buffered solution of 500 μL (67 mM, pH = 5.7) was added to the vial and mixed them for 10 s by sonification and incubated for 24 h in a constant temperature bath at 25 °C. Microscopy observation was conducted by a phase contrast microscope IX70 (Olympus, Japan) or an inverted microscope Eclipse Ti (Nikon, Tokyo, Japan) equipped with a confocal laser scanner unit CSU-W1 (Yokogawa electric corp., Tokyo, Japan) (excitation 532 nm / emission 560–585 nm) and a sCMOS camera unit Zyla 4.2 plus (Andor Technology Ltd., Belfast, United Kingdom).

2.4. Phase-contrast microscopy observation of morphological changes of anionic liposome induced by the addition of V⁻*ole

Anionic liposomes were prepared from 2 mM **V⁻*ole*** in a 67 mM phosphate buffer (NaH_2PO_4, Na_2HPO_4) of pH = 5.7, and morphological changes of anionic liposomes were observed by phase contrast microscope IX70 (OLYMPUS, Japan) in a micro-chamber placed on a heated stage at 40 °C after the addition of 3 mM of **V⁻*ole***.

2.5. UV spectroscopic monitoring of the hydrolysis of V⁻*ole in a phosphate buffer dispersion

A solution of precursor of membrane lipid **V⁻*ole*** (0.1 mM) was prepared by dissolving **V⁻*ole*** of the weighed amount in a phosphate buffer (67 mM, pH 5.7, 6.7, 7.7 or 8.7) in the absence of membrane lipid **V⁻*ole***. The rate of the decrease of **V⁻*ole*** concentration was measured by tracing the decrease of a UV absorption band at 327 nm which was assignable to the benzylideneaniline unit of **V⁻*ole.*** The UV absorption of **V⁻*ole*** was measured in a wave-length range of 250–400 nm with a 5 min's interval by UV spectrometer. The similar measurements were conducted in the presence of membrane lipid **V⁻*ole*** by varying its concentration, such as 100, 25, and 6 μM, respectively.

2.6. Flow cytometry measurement of population analysis of anionic liposomes induced by the addition of V⁻*ole

A dispersion of anionic liposomes for flow cytometry measurement was prepared by a thin-film method using **V⁻*ole*** (1.0 mM) and containing 0.1 mol% of 2-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-HPC) (Thermo Fisher Scientific K.K., Japan) soaked with phosphate buffer (67 mM, pH = 5.7). After addition a **V⁻*ole*** solution (1 mM) of the same volume, the change of the fluorescence intensities of a vesicular dispersion was measured using FCM (excitation wave length of 488 nm) with a count number of particles of 10,000 per each measurement, and 10 min interval for 60 min.

2.7. Enzymatic DNA amplification in self-reproductive liposome

A stock lipid solution was prepared by dissolving of **V⁻*ole*** (10 mM) in chloroform. The 100 μL of the lipid solution was added to a vial and evaporated for 2 h to remove the organic solvent. Liquid paraffin of 1 mL (d = 0.86–0.89 g/cm^3) was added to the resulted lipid thin film. The liquid paraffin solution of lipid (1 mM) was incubated at 80 °C for 2 h. A PCR solution, which was encapsulated by a liposome as an inner aqueous phase, was composed of deionized water (220 μL), sucrose aq. (1 M, 75 μL), PCR buffer (10 × KOD-plus- buffer, 50 μL) (Takara Bio, Japan), $MgSO_4$ aq. (25 mM, 20 μL), a dNTPs mixture solution (2 mM each, 40 μL), a forward primer (5′-GACAGCATCGCCAGTCACTA-3′, 100 μM, 15 μL), a reverse primer (5′-TTTGCGCATTCACAGTTCTC-3′, 100 μM, 15 μL), 1164-bp DNA template (200–1363 excised from pBR322 plasmid, 10 nM, 5 μL), DNA polymerase (1.0 U/μL, KOD-Plus-, Mg^{2+} free, 10 μL) (Takara Bio, Japan) and *N*',*N*'-dimethyl-*N*-[4-[(*E*)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-*N*-propylpropane-1,3-diamine (SYBR Green I) aq. (Takara Bio, Japan) (×100, 50 μL). A 300 μL of PCR solution was emulsified into 1 mL of the liquid paraffin solution by using a vortex for 30 s. Another 1 mL of solution composed of deionized water (690 μL), glucose aq. (1 M, 150 μL), PCR buffer (10 × KOD -plus- buffer, 100 μL), $MgSO_4$ aq. (25 mM, 40 μL) and DNase I (5000 U/μL, 20 μL) (Merck, Germany), which was supposed to become an outer aqueous phase of liposomes, was poured into a 1.5 mL microtube, and layered a 300 μL of the water-in-oil emulsion containing the PCR solution onto it. The tube was subjected to incubation for 10 min at 25 °C and centrifugation at 18,800*g* for 30 min at 25 °C. The bottom of the tube was pierced with a pushpin to replace the precipitated liposomes to another 1.5 ml microtube. Obtained liposome dispersion was diluted by ten times with the outer aqueous solution. The liposome dispersion was subjected to thermal cycles, 94 °C for 2 min and [94 °C for 15 s and 68 °C for 90 s] × 20 cycles, to amplify template DNA in the liposome. Then, the liposome dispersion was placed into a frame-chamber with a size of 9 mm × 9 mm with a volume of 25 μL on a glass plate with cover glass, and was observed by using an inverted microscope (Eclipse Ti, Nikon, Japan) equipped with a confocal laser scanner unit (CSU-W1, Yokogawa electric Corp., Japan) and a sCMOS camera unit (Zyla 4.2 plus, Andor Technology Ltd., United Kingdom) with 1024 × 1024 active pixels (excitation 488 nm / emission 500–550 nm) to detect the fluorescence emission from SYBR Green I intercalated to amplified dsDNA.

3. Results and discussion

3.1. Synthesis of phospholipids and preparation of synthetic liposomes

A novel phospholipid (**V⁻*ole***) was designed according to our previous report (Takakura and Sugawara, 2004; Takahashi et al., 2010). **V⁻*ole*** possesses a phosphate group as a hydrophilic head, an oleyl group as one of the hydrophobic chains and a 4-formylphenyloxydecyl group as the other (Fig. 1, center). Replacement of a saturated chain with an unsaturated one increases the membrane fluidity and decreases the temperature of sol-gel phase transition (T_m). Reduction of T_m facilitates the construction of liposomes at r.t. because unsaturated phospholipids geometrically disrupt an integrity of saturated phospholipids in the membrane and augment the membrane fluidity (Leekumjorn et al., 2009; Mansilla et al., 2004). To improve the synthetic yield of **V⁻*ole***, we substantially revised the process for amidite synthesis (Beaucage and Caruthers, 1981; Takahashi et al., 2010).

Monocomponent liposomes composed of synthetic **V⁻*ole*** were formed in deionized water by a thin-film hydration method (Walde et al., 2010). The microscopy image in Fig. 2 shows that the lamellarity of liposomes was low and that the liposomes contained an inner water phase in which enzymatic reactions couldproceed. The formation of liposomes was confirmed at higher pH value than 5 (Figure S3).

The membrane precursor **V⁻*ole*** was synthesized by dehydro-condensation between the membrane lipid **V⁻*ole*** and electrolyte **E** in an organic solvent (route A in Scheme 2) (Takakura and Sugawara, 2004; Takahashi, et al., 2010). However, the yield of **V⁻*ole*** was low in this route, and crude **V⁻*ole*** was difficult to purify because of its poor solubility in organic solvents. Hence, we revised the route to produce **V⁻*ole*** (route B in Scheme 2).

3.2. Kinetic measurements of the hydrolysis of the precursor of membrane lipid V⁻*ole in the presence of pre-existing V⁻ole

It was found that the rate of hydrolysis of **V⁻*ole*** into **V⁻*ole*** and ***E*** (Fig. 1) heavily depended on the concentration of pre-existing **V⁻*ole,*** as was shown in a previous report (Takahashi et al., 2010). The hydrolyses of **V⁻*ole*** (1.0×10^{-1} mM) in the presence of pre-existing concentrations of **V⁻*ole*** ranging from 6.0×10^{-3} - 1.0×10^{-1} mM in a phosphate buffer (pH = 5.7) at 25 °C were monitored using the UV

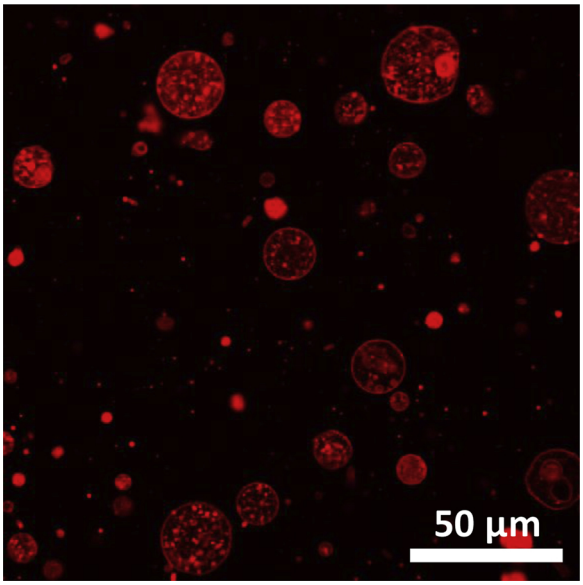


Fig. 2. Confocal laser scanning fluorescence microscope image of large liposomes composed of the synthetic phospholipid **V⁻*ole*** in deionized water. The membranes were stained with Texas Red-DHPE (0.1 mol%).

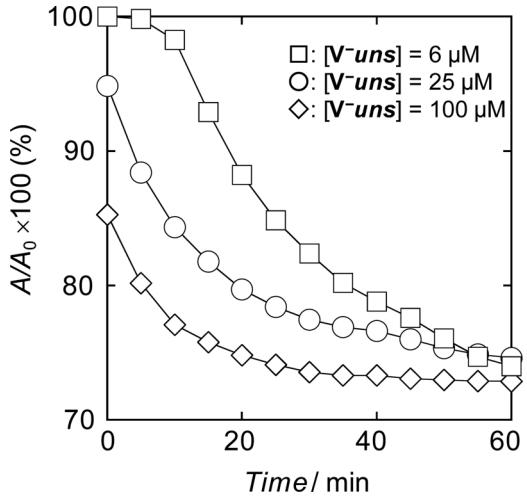


Fig. 3. Dependence of the conversion rate of **V⁻*ole*** into **V⁻*ole*** on the concentration of pre-existing **V⁻*ole*** (6, 25 and 100 μM) as measured by UV spectroscopy. The rates were measured by monitoring the decrease in the benzylideneaniline absorption peak of **V⁻*ole*** at 327 nm. The vertical axis represents A/A_0, where A_0 denotes the initial absorbance of **V⁻*ole*** at 6 μM **V⁻*ole***, and A denotes the absorbance of **V⁻*ole*** at other concentrations of pre-existing **V⁻*ole*** (25 and 100 μM).

absorption band at 327 nm, which was assignable to the benzylideneaniline unit of **V⁻*ole*** (Figure S4) (Takakura and Sugawara, 2004). The rate of hydrolysis of **V⁻*ole*** was initially slow for the low **V⁻*ole*** concentrations in the buffer, but the rate increased rapidly after ca. 10 min, resulting in a sigmoidal decay curve (plot of [**V⁻*ole***] = 0 μM, Fig. 3). This result was interpreted as the observed autocatalytic formation of phospholipids being promoted by the membranes of the anionic liposomes, which served as a catalytic surface. The generated **V⁻*ole*** phospholipids expanded the surface membrane and increased the catalytic activity to accelerate the hydrolysis of the precursors of the **V⁻*ole*** phospholipids.

To identify the origin of the autocatalytic effect of the anionic liposomes, we examined the dependence of the rate of hydrolysis of the imine bond of **V⁻*ole*** (which is an acid-catalyzed reaction) on the pH

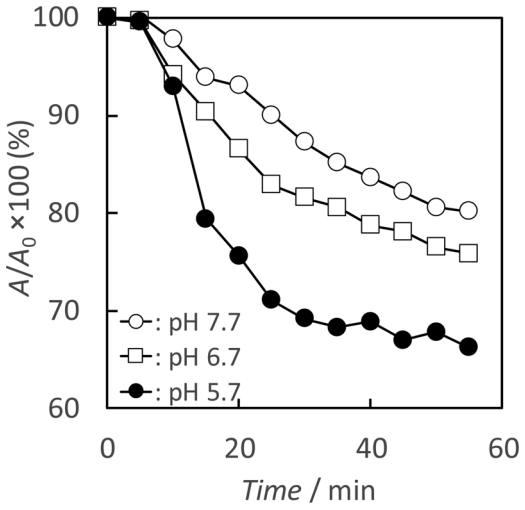


Fig. 4. Dependence of the conversion rate of **V⁻*ole*** on the pH, where pH = 7.7, 6.7, and 5.7, as monitored by UV spectroscopy for the decrease in absorption of the benzylideneaniline group of **V⁻*ole*** (500 μM) in the absence of membrane lipid **V⁻*ole***.

of the phosphate buffer. As the pH of the dispersion decreased from 7.7 to 5.7, the rate of hydrolysis increased, as shown in Fig. 4, possibly because an increase in the local proton concentration at the surface of the liposome membranes prompted an exchange of the sodium ions paired with the phospholipids with the protons in the phosphate buffer (Figure S5). The enhanced nonlinear behavior of the hydrolytic decay of **V⁻*ole*** at pH = 5.7 may be explained by activation of the autocatalytic effect at lower pH values, and the decrease in the decay rate at later stages presumably comes from interference by the increased amount of aniline-type amphiphilic **E,** which is a competitor for accepting the catalytic protons.

3.3. Morphological changes of the anionic liposomes as monitored by flow cytometry and optical microscopy

The morphological changes of the synthetic liposomes were monitored by flow cytometry and microscopy after the addition of **V⁻*ole***. The anionic liposomes for this measurement were prepared by a thin-film hydration method (Walde et al., 2010) in a phosphate buffer (67 mM, pH = 5.7) using 1.0 mM of the membrane lipid **V⁻*ole***, and the liposome membranes were stained with 0.1 mol % BODIPY-HPC. The temporal changes in the fluorescence intensty of the membrane-stained liposomes were traced after the addition of 1.0 mM **V⁻*ole*** (Fig. 5) by flow cytometry, which is able to measure 1×10^4 specimens per minute in a rapid flow. The fluorescence intensities of each liposome were measured at 5, 30, and 60 min after the addition of **V⁻*ole***. Whereas no changes in the samples were observed without **V⁻*ole*** (Figure S6), the addition of **V⁻*ole*** (1.0 mM; 5–10 min) led to a new population that was different from the original population. In a two-dimensional plot of the flow cytometry data with respect to two parameters (size of the liposome vs fluorescence intensity of each liposome), the fluorescence intensity decreased by an order of magnitude after several divisions because the amount of dye in each liposome decreased by half for each division. If the division volume was not equivalent, i.e., 9:1, 8:2, 7:3, and 6:4, the amount of the fluorescent dye in the divided liposomes became continuous. In such cases, a two-dimensional plot of the flow cytometry data with respect to the two parameters shows a broad band (Toyota et al., 2008). In this experiment, the fluorescence intensity had decreased by an order of magnitude 5 min after the addition of **V⁻*ole***, suggesting that nearly equivolume division occurred several times during this time period. Thereafter, the decrease in the fluorescence intensity slowed considerably, suggesting that the frequency of

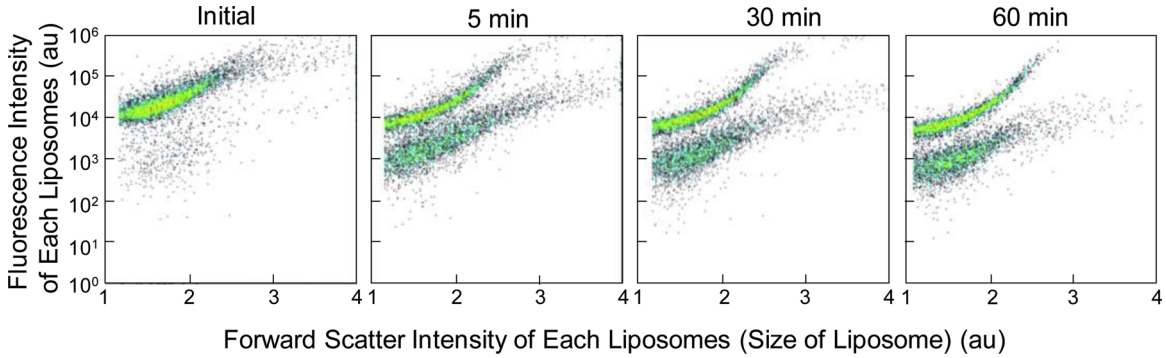


Fig. 5. 2D population analysis of the fluorescence intensity of BODIPY vs the size of the anionic liposomes before and 5, 30 and 60 min after addition of the precursor of the $\mathbf{V}^{-}$**ole* membrane lipid.

liposomal division decreased because of a decrease in the concentration of $\mathbf{V}^{-}$**ole*. The high rate of liposomal division, in particular, in the early stages, may be ascribed to the considerable disturbance, e.g., the flow of water caused by the addition of $\mathbf{V}^{-}$**ole* and the gentle stirring during mixing of the added $\mathbf{V}^{-}$**ole*. It was conformed that no division occurs in the flow cytometry measurement 334 if no $\mathbf{V}^{-}$**ole* was added (Figure S6).

Using phase-contrast microscopy, we also examined the morphological changes of the anionic liposomes. Division was observed 15 min after the addition of 3 mM $\mathbf{V}^{-}$**ole* to a microchamber placed on a heated stage set to 40 °C, and the deformed liposomes had almost completely divided after 20 min (Fig. 6). The division dynamics were much slower than those in the low cytometry measurements, even though the concentration of $\mathbf{V}^{-}$**ole* was higher and the temperature was slightly elevated, likely because the liposomes in the microchamber were not disturbed by the microscopy observations. An advantage of the current experiment on the morphological changes of liposomes is the absence of additional components. It is advantageous that a monocomponent anionic liposome composed of a synthetic phospholipid was free from phase separation or inhomogeneity of the membrane composition.

3.4. Enzymatic reaction in an anionic monocomponent liposome

Enzymatic reactions in liposomes have drawn a considerable amount of interest, and a number of excellent experiments have been reported (Walde et al., 1994; Oberholzer et al., 1995; Nomura et al., 2003; Ishikawa et al., 2004; Noireaux and Libchaber, 2004; Sunami et al., 2006; Kurihiara et al., 2011; Scott et al., 2016). It is worth testing whether this liposome can be used as a vessel for enzymatic reactions. A buffered solution containing PCR reagents, template DNA, dNTPs, two kinds of primers, DNA polymerase, Mg^{2+}, and SYBR Green I for probing dsDNA was encapsulated in the liposomes through a centrifugal precipitation method that is widely used for the preparation of liposome-encapsulated macromolecules (Pautot et al., 2003). A buffered solution of DNase was used as the outer aqueous phase to decompose the template DNA and primers outside the liposomes. A dispersion of the liposomes was subjected to thermal cycling to amplify the DNA. The resulted dispersion was observed by a confocal laser scanning fluorescence microscope and showed green fluorescent emission from inside of the liposomes, indicating the amplification of dsDNA by the polymerase (Fig. 7a) (Shohda et al., 2011). A line profile of the fluorescence emission from an intercalated complex of SYBR Green I with dsDNA (Dragan et al., 2012) in the liposome showed a concave shape (Fig. 7b), indicating that DNA was dissolved in the aqueous phase of the liposomes, not localized in the liposomal membranes. In addition, fluorescence was detected from almost all liposomes that were 5 μm in diameter or larger. On the other hand, almost no fluorescence was observed from liposomes that were smaller than 5 μm in diameter (Figs. 7c and S7), which derived from the failure to encapsulate all the necessary components in the small liposomes. These data were direct proof of successful and high-efficiency DNA amplification in these anionic liposomes.

4. Conclusion

Replacement of a dodecyl group in the previously reported phospholipid, dodecyl-10-(4-formanylphenoxydecanyl)phosphate, with an oleyl group enabled the formation of anionic liposomes in water without the addition of cholesterol or alcohol. Monocomponent liposomes made of $\mathbf{V}^{-}$*ole* exhibited self-reproducing dynamics (e.g., a budding deformation followed by division) induced by the addition of the precursor of the $\mathbf{V}^{-}$**ole* membrane lipid to the aqueous buffer. Because the pre-existing liposomal membrane acts as a catalytic reaction field, $\mathbf{V}^{-}$*ole* phospholipids can be generated autocatalytically without the need for catalytic amphiphiles. Since the synthetic liposomes exhibited thin lamellarity and contained an inner aqueous solution, an enzymatic reaction, i.e., DNA replication, was achieved within these liposomes. These anionic liposomes could be utilized as the compartment of a protocell that expresses a specific function with/without a transcription and translation system. The plausibility of

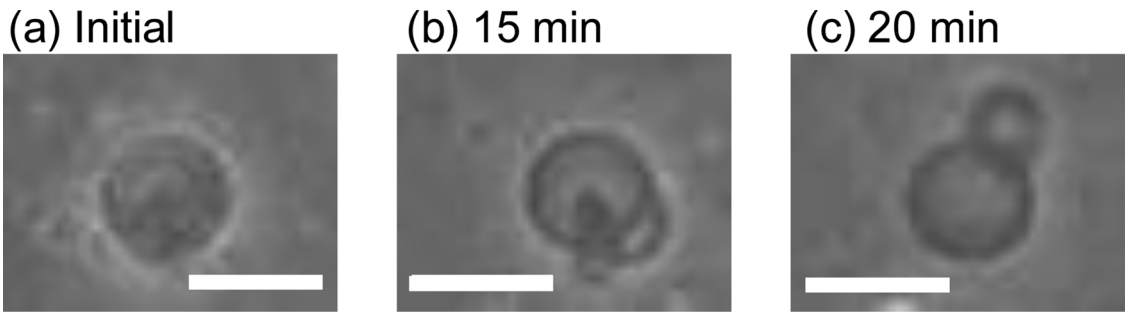


Fig. 6. Self-reproduction of synthetic liposomes. Phase-contrast microscopy images of the self-reproduction dynamics of an anionic liposome (1 mM) at 40 °C immediately (a), at 15 min (b) and at 20 min (c) after the addition of 3 mM $\mathbf{V}^{-}$**ole*. Scale bars represent 20 μm.

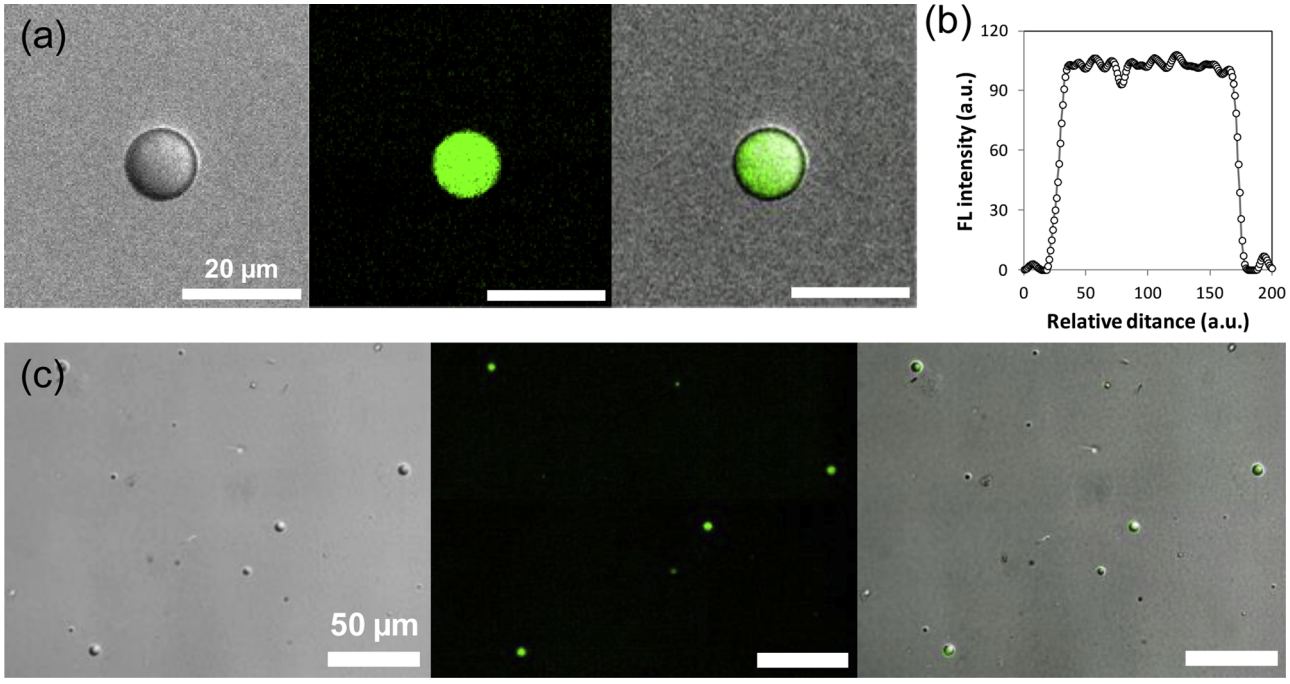


Fig. 7. Differential interference contrast (DIC) and confocal fluorescence microscopy images of an anionic liposome after PCR treatment. (a) DIC (left), fluorescence (middle) and merged (right) microscopy images of a liposome subjected to PCR treatment. (b) A fluorescence line profile of a liposome subjected to PCR treatment. (c) DIC (left), fluorescence (middle) and merged (right) microscopy wide images of a liposome dispersion subjected to PCR treatment.

combining the liposomal division in a manner of autocatalytic self-reproduction with the DNA amplification in the liposome could be a future direction in protocell research. Such cooperative interaction between the sustainable self-reproduction of liposomes and the replication of DNA may involve a primitive transfer system of information carried on DNA to the protocell.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chemphyslip.2019.04.007.

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