VIP *Very Important Paper*

Self-Emergent Protocells Generated in an Aqueous Solution with Binary Macromolecules through Liquid-Liquid Phase Separation

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Recently, liquid–liquid phase separation (LLPS) has attracted considerable attention among researchers in the life sciences as a plausible mechanism for the generation of microstructures inside cells. LLPS occurs through multiple nonspecific interactions and does not always require a lock-and-key interaction with a binary macromolecular solution. The remarkable features of LLPS include the non-uniform localization and concentration of solutes, resulting in the ability to isolate certain chemical systems and thereby parallelize multiple chemical reactions within the limited space of a living cell. We report that, by using the macromolecules, poly(ethylene glycol) (PEG) and dextran, that exhibit LLPS in an aqueous solution, cell-sized liposomes are spontaneously formed therein in the presence of phospholipids. In this system, LLPS is generated through the depletion effect of macromolecules. The results showed that cell-like microdroplets entrapping DNA wrapped by a phospholipid layer emerge in a self-organized manner.

All living organisms have complicated but self-organized intracellular structures with various specific functions to maintain their lives. Our understanding of gene production has increased over the past several decades; however, we still have only a primitive understanding of how gene production is self-organized in a systematic manner. In other words, the generation of cellular structures cannot be explained simply in terms of the accumulation of gene products. To gain insight into the fundamental mechanism of the self-organization of cells, studies on the construction of cell-like systems through real-world modeling, or through synthetic biology, are being developed.

Liquid–liquid phase separation (LLPS) is considered to play an important role in the generation of microstructures inside cells.[1] LLPS occurs through multiple nonspecific interactions in a global manner, in contrast to biochemical lock-and-key interactions such as between enzymes and their substrates.[2] LLPS can induce the non-uniform localization and concentration of solutes, resulting in sophisticated, specific chemical reactions.[3] While providing the space for certain reactions to take place selectively, LLPS could also be involved in the generation of intracellular structures[4] that differ with respect to the exchange of substances as well as morphology and size, and could have different roles for organelles covered with lipid membranes.

As characteristically stable compartments in which the solute concentration is different from that of the surroundings that emerge in solution even without lipid membranes, LLPS has been considered to play an essential role in the origin of membraneless organelles such as nucleoli, P-bodies and some stress granules.[5] On the other hand, there are indication that LLPS is to be associated with the development of diseases, especially cancers or diseases associated with degeneration of the nervous system such as Alzheimer's or Parkinson's disease,[6] which are caused by abnormal gene expression or the denaturation of proteins. Through the use of LLPS, it might be possible to manipulate localized or concentrated target substances or realize advanced layered chemical systems through the parallelization of multiple chemical reaction pathways.

It is well known in polymer physics that a mixed solution of different polymers tends to undergo phase separation because of the minimum contribution of mixing entropy, in contrast to a solution containing different chemicals of low molecular weight. Thus, aqueous two-phase systems (ATPSs) are usually prepared from mixed polymer aqueous solutions.[7] It is also well known that a solution consisting of poly(ethylene glycol) (PEG) and dextran (DEX) produces LLPS.[8] Here, we found that phospholipids added

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to such a solution spontaneously assemble at the interface between the PEG-rich (PEG phase) and DEX-rich phases (DEX phase), and demonstrated that the assembled lipid structures remained even after one phase (DEX phase in this case) vanished upon dilution.

In actual cells, there are substantial biopolymers like proteins and nucleic acids that have basically electric charges and can undergo micro phase separation apparently through attractive interaction due to their electrostatic and specific properties; generally, intracellular phase segregation, or LLPS, is usually interpreted in terms of associative interaction such as polyion condensation, complex coacervation, and polyelectrolyte complexation. In contrast, mixed solution with plural number of soluble polymers undergoes phase separation through depletion interaction[9] accompanied by the entropically unfavorable interaction between different polymers. In this study, we employed a mixed solution with PEG and DEX to generate micro phase segregation caused by the depletion interaction. Here, it is noted that cytoplasmic solution, or intracellular solution, contains of 0.3 to 0.4 g/mL macromolecules, including skeletal proteins, enzymes, RNA, DNA, etc.[9b,c] This suggests possible important effect of depletion interactions on cellular structure and function.[9d,e]

Our previous study revealed that when double-stranded (ds) DNA and actin filament, both of which are biological components essential for living cells, are added to LLPS in a two-component system of PEG and DEX at the same time, they selectively accumulate together in the DEX phase.[10] This finding could lead to a useful method for encapsulating localized or concentrated target substances into membrane vesicles.

It has been reported that lipid vesicles can be formed by the simple hydration of lecithin powder.[11] So, as our first attempt, as a convenient method for adding lipids to an LLPS system, the lipid-rich portion of a solution in which lecithin powder was hydrated was mixed into a PEG/DEX-binary solution. Figure 1a shows the generation of a large number of lipid vesicles together with aggregates. Their shape is mostly spherical and their size varies from sub-micrometer to tens of micrometers. Because the fluorescence intensities of vesicles and aggregates that were stained with rhodamine B are not uniform, they are presumed to be composed of various different structures, including vesicles formed from several lipid bilayers, many densely stacked membranes, and droplets that do not have any layered structure.

After the suspension is mixed into the PEG/DEX-binary solution, the fluorescence signal showing the distribution of lipids is mainly observed between the PEG and DEX phases (Figure 1b). Under the conditions used in this study, spherical DEX-rich regions (DEX phases) are dispersed in the PEG phase, which produces LLPS (Figure 1b-i, left). Phospholipids are localized to the spherical surface of each DEX phase (Figure 1b-i, right), thus indicating that the mixing of lipids does not affect phase separation or the shape of each phase. The profile of the fluorescence intensity obtained from the cross-sectional image of a certain DEX phase shows that such phospholipids are concentrated in a very narrow place, just like the boundary between two phases (Figure 1b-ii). As a result, micrometer-sized structures made of phospholipid that encapsulate a DEX phase are constructed. Figure 1b-ii shows the accumulation occurred on the boundary, and it is noted that what

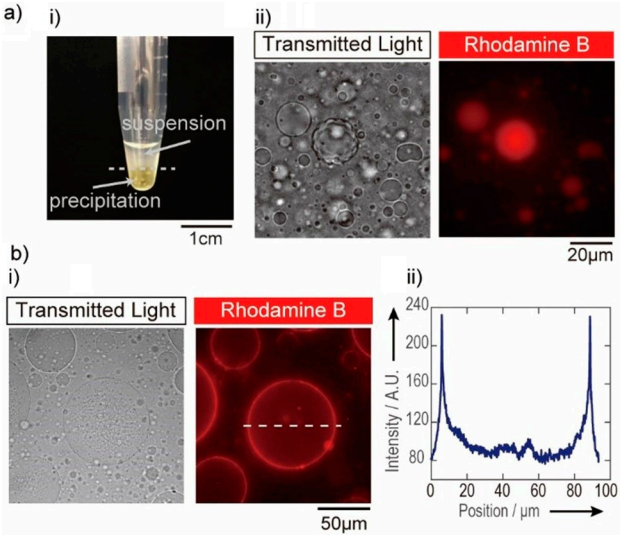

Figure 1. Accumulation of phospholipids at the interface of a droplet. a) Lipid suspension prepared by the simple hydration method. i) The hydrated solution. Powdered lecithin from soybean was immersed in water. ii) Microscopic images of the part of the suspension rich in lipids. b) The PEG/DEX-binary solution mixed with the lipid suspension. i) Microscopic images. ii) The spatial profile of the fluorescent intensity corresponds to the broken line.

type of structure the assembled lipids assumed remained to be solved.

These vessels, which are the DEX phases surrounded by lipids, are stable compared to DEX phases that usually form in LLPS, that is, in the absence of lipid. When two DEX phases are in contact, fusion occurs to form a larger spherical DEX phase on a time-scale of min to hour. However, in the case of DEX phases encapsulated within a lipid layer, individual spheres are stable for more than several hours, highly reducing the frequency of deformation or fusion (Figure S1).

It is also observed that dot-like small clumps are scattered inside DEX phases wrapped by a lipid layer (Figure 1b-i). These may be vesicles or aggregates made of lipids that have remained without accumulating at the boundary between the two phases. On the other hand, almost no such fluorescent signal of phospholipids is observed in the PEG phase.

To investigate the applicability of the assembling of lipid in LLPS found in this study, the same experiments were performed using other lipids (synthesized phosphatidylcholine (PC) or phospholipids other than PC) and different preparation method for obtaining lipid suspension. Even when the species of lipid and/or the method was changed, DEX phases were wrapped by lipid layers (Figure S2). The state of LLPS and the morphology of each phase, except for fusion between the same phases, were not affected by the addition of lipid suspension regardless of which lipid was used. However, the efficiency of wrapping the DEX phase by lipid layers depended on the amount of membrane vesicles in the lipid suspension added. Since a relation between the lipid composition and the degree of vesicle formation has been reported with well controllable protocols,[12] the selection of the lipid composition used will be important.

To further monitor the mechanism of lipid accumulation in this system, we tried to directly mix the components of phospholipid with microdroplets prepared in advance on the stage of the microscope. First, a mixture of DEX and PEG was injected into an observation chamber to generate DEX-rich microdroplets in the PEG-rich phase. Next, a phospholipid suspension was gently injected into the chamber via its opening and attached to the solution previously placed. To prevent dilution of the PEG phase, we used a lipid suspension hydrated with 5% PEG solution. Figure 2a shows membranous structures at the boundary around the DEX droplets. In addition, around and inside these droplets, small lipid vesicles or clumps were floating. In the region far from the attaching point to the phospholipid suspension in the LLPS solution, neither such membranous structures nor vesicles or clumps were found (Figure 2b). Thus, lipids may not yet have reached that area. The results observed are consistent with the notion that lipids are supplied by the diffusion of these lipid vesicles or clumps.

When lecithin was added to pure water and then applied to an LLPS solution, the polymers were diluted and thus phase separation was not maintained. This caused competition between the formation of lipid-surrounded DEX droplets by lipid supply and disappearance of the DEX phases by dilution. Next, we examined the durability of the lipid accumulation surrounding the DEX phases. If this is a semipermeable barrier like a lipid bilayer membrane, it might be affected by the perfusion of aqueous solutions with different osmolality values. Therefore, as above, the responses of the lipid-surrounded DEX phases were observed under changes in the osmotic pressure. In a chamber for microscopy, an LLPS solution to which lecithin was added to form a lipid-surrounded DEX phases was attached to an aqueous solution containing a high concentration of salt (5% NaCl), and observed in real time (Figure 3). In the present condition, salts do not induce aggregation of vesicles to form large clumps in the absence of a PEG/DEX ATPS (Figure S3a). After the two solutions are attached, due to diffusion into the LLPS solution, the salt concentration will be increased and the polymers (in this case mainly PEG) will be diluted.

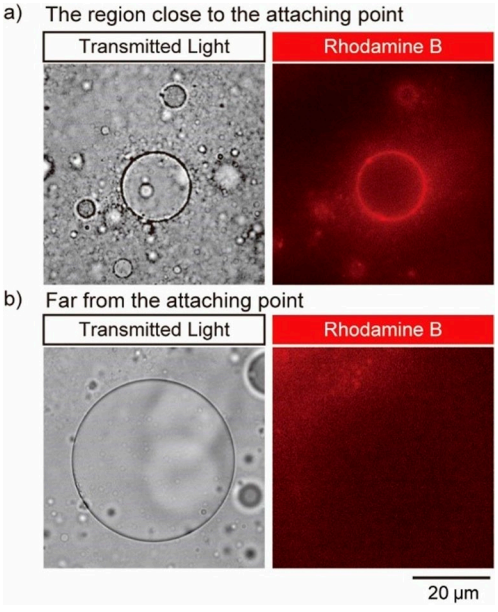

Figure 2. Accumulation of lipid vesicles at the interface of a DEX-rich droplet by mixing a lipid suspension in PEG/DEX-binary solution. Transmitted light and fluorescent images of the region a) close to and b) far from the attaching point. In the region far from the attaching point, a lipid membranous structure is scarcely formed.

In the region close to the attaching point to the salt-containing solution in the LLPS solution, lipids that had been distributed throughout the boundary around a droplet, which is the DEX phase, gathered at one location as if it was shrinking, and simultaneously the droplet itself disappeared. As a result, depending on the degree of lipid-gathering, a lipid-surrounded DEX phase finally turned into a collapsed clump (Figure 3a-i), a remnant of a ruptured vesicle (Figure 3a-ii), or a shrunken vesicle (Figure 3a-iii). In the area far from the point of attachment, the lipid-surrounded DEX phases tended to maintain their spherical shapes (Figure 3b). The lipids surrounding them continued to be distributed throughout the boundary. However, the dot-like lipid clumps that were inside them came to the boundary, so that their interiors became clear (Figure 3b-i, right), or moved due to the convection generated (Figure 3b-ii). It is noted that a binodal line of the phase diagram can be shifted downwards in the presence of salt, in the present case, and so dilution was attributed to the disappearance of droplets; instead, the order of addition of the salt is more significant in determining the localization of lipid molecules. When the PEG/DEX solution, lipid suspension and NaCl stock solution are mixed initially, lipid aggregation inside droplets was observed more dominantly than lipid layer formation at the interface between the two phases (Figure S3b), and the difference in the preference was not changed in the presence of DNA entrapped inside droplets (Figure S3c-i and c-ii).

As salt diffusion will cause a gradient of salt concentration in the LLPS solution, at the region nearer to where the salt solution was introduced, the changes in osmotic pressure will be rapid and the solution outside of the lipid-surrounded DEX phases will be more hypertonic. When the region outside of a lipid membrane vesicle quickly becomes very hypertonic, probably because a large amount of water has moved out of the vesicle, the membrane can rupture, resulting in leakage of the contents, rather than simple shrinkage of the lipid membrane vesicle.[13] Therefore, the change to a collapsed clump of lipids or remnant of a ruptured vesicle may be due to breakage of the membrane caused by a large difference in osmotic pressure (Figures 3a-i and 3a-ii).

In the region of the LLPS solution further from where the salt solution was applied, the changes in osmotic pressure will be slow and the effect will be smaller. The lipid-surrounded DEX phases that maintained their spherical shape would have been able to maintain the integrity and morphology of the membrane due to the slow outflow of water molecules (Figures 3b). The redistribution from the interior to the surface (Figure 3b–i) or the flowing movement of the dot-like lipid clumps, as shown by particle image velocimetry (PIV; Figure 3b-ii), might be due to the flow generated with the slow outflow of water. As well as its response to changes in osmotic pressure, clarifying the behavior of the lipid layer wrapping the DEX phases should be important to understand the properties of lipids or vesicles in polymers.[14]

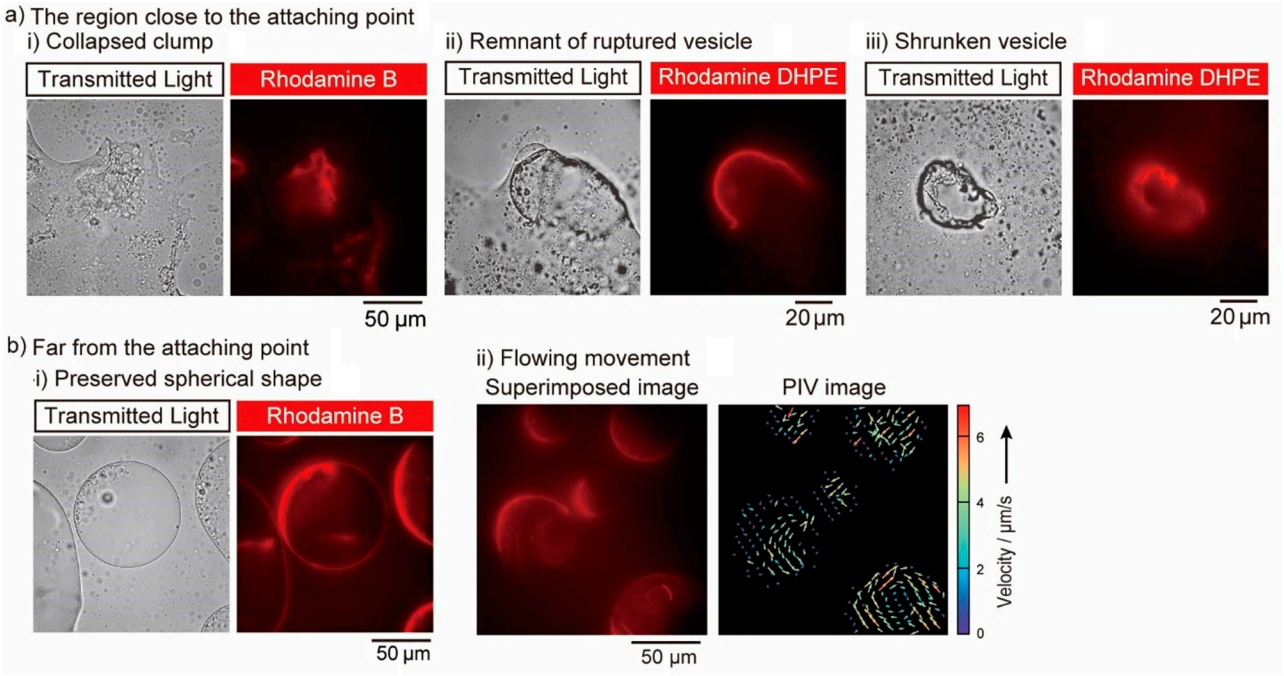

Figure 3. Morphological changes in lipid membranous structure due to the injection of a NaCl solution. a) The various morphological changes depending on the degree of lipid gathering in the region close to the point of attachment. DEX-rich droplets tend to disappear and become homogeneous with the PEG-rich outer solution under the addition of a NaCl solution, i) collapsed clump, ii) remnant of a ruptured vesicle, iii) shrunken vesicle. b) Far from the point of attachment. i) Due to the effect of NaCl, the interior of the droplet became clear. ii) Convection emerged inside the droplet. Left: Superimposed image of convection (duration: 8 s). Right: Particle image velocimetry image of convection.

This spontaneous accumulation of lipid at the interface between the two phases enables us to construct lipid-surrounded droplets encapsulating DNA. Our previous studies revealed that long dsDNAs accumulate in the DEX phase.[10,15] When λ DNA is mixed with a PEG/DEX-binary solution together with a lipid suspension, λ DNA accumulates inside the droplets of DEX phase and lipids accumulate at the interface of the droplets, as shown in Figure 4.

Here, lipid was added to a PEG/DEX-binary solution using a hydrated suspension of multilamellar lipid film. Similar results are observed regardless of the method used to prepare the lipid suspension: simple hydration of lipid powder or hydration of lipid dry films.

As described, we first observed that when phospholipids are mixed with an LLPS produced in such a PEG/DEX binary solution, DEX microdroplets are wrapped with lipids and become more stable. As previously reported,[10] long semiflexible DNA and actin molecules are concomitantly partitioned into droplets and further assume subcellular domains. In the present work, where the DNA and phospholipid are mixed within the simple system, they are spontaneously sorted to an appropriate location due to their own characteristics. This trend was also observed by Keating and collaborators, who were the pioneering group in the study of

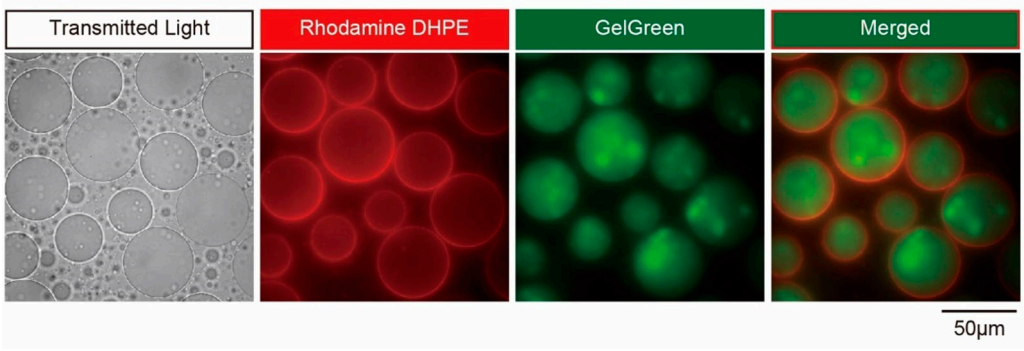

Figure 4. Spontaneous encapsulation of DNAs into micro compartments wrapped by lipid layers. A hydrated suspension of multilamellar lipid film was mixed, and lipids were labeled with a fluorescent dye (Rhodamine-DHPE).

microsystems based on the ATPS technology; in their work, small liposomes are closely arranged over a microdroplet containing nucleic acids.[4] In the one-batch system investigated here, which is quite simple compared to well-designed microsystems,[16] interestingly, the trend is so robust that the droplets uniformly assume a similar appearance (Figure 4).

In addition, if phospholipid molecules are provided abundantly to the system, lipid aggregates could gather completely over the surface area of the microdroplet. Next, very interestingly, the membranous aggregates appeared to burst in response to the osmotic shock applied just to the stage of the microscope, implying that the structure can function as a barrier to small electrolytes, though such solutes could usually be permitted to cross over the interface of the droplets. The oil and water interface generated through LLPS of water and oil with higher surface energy can make phospholipid molecules accumulate into monolayered structures, which can in turn be converted into bilayers by binding. Even with a low surface energy, the aqueous/aqueous interface can collect lipid aggregates like vesicles and probably serve as a template that makes the phospholipids localized thereon assume a membranous morphology.

If we consider, for instance, actual intracellular systems where biomacromolecules widely exhibit dynamic assembly, it is well known that nuclear envelopes must repeatedly disassemble and reassemble for the division of eukaryotic cells. At the end of mitosis, when the chromosomes are wrapped up after separation along with other nucleic components, fusion of vesicles derived from the endoplasmic reticulum (ER) or reshaping of ER membrane takes place, resulting in regeneration of the nuclear envelope, that is, formation of the daughter cell's nucleus.[17] Autophagy, which is also essential for eukaryotic cells, includes a process in which the cytoplasmic compartment that appears to be generated by phase separation is covered and isolated by a lipid membrane.[18] The origin of this lipid membrane is thought to be the isolation membranes derived from ER.[19]

The microscopic observation in the present work implies the intriguing possibility that when condensing biopolymers give rise to micro phase-separation, phospholipid fragments would in turn be sorted onto the newly generated surfaces, resulting in the emergence of membranous structures that can attenuate the diffusion of small polar solutes including salts and energy-related molecules like ATPs. The compartmentalization by membranes may affect the behavior of entrapped macromolecules, as observed in Figure 4. Aqueous droplets in LLPS solution can be stabilized by being covered with micro particles, that is, formation of Pickering emulsion,[20] and cell-sized systems could develop autonomously into protocell models by the further combination of biosynthesis reactions of aggregates of lipids and proteins, as an extension of the present study.

We also found that the membranes can follow the geometry of the interfaces of microdroplets. Therefore, if the shape of the interface can be controlled, the morphology of the lipid membranes can be manipulated. We previously showed that polymerized actin filaments or their assembled bundles were localized in droplets, resulting in changes in the morphology of the droplets.[10] Actin has several binding factors including various molecular motors (myosins) with cargo adaptors,[21] so their entrapment would be advantageous when mimicking cell motility. Thus, it would be worthwhile to develop a study on LLPS microdroplets stabilized by lipid layers, through the spontaneous inclusion of desired substances, and the strategy may be biologically important together with the utilization of lipid membrane vesicles, which is to principally function in microcompartmentalization in the living cell system.[22]

Experimental Section

Aqueous/aqueous microdroplets: We adopted an aqueous two-phase system of polyethylene glycol (PEG) and dextran (DEX). At a composition of PEG/DEX = 5 wt %:5 wt %, 10 to 100 μm droplets, the interiors of which contain the DEX phase, emerge in the PEG phase. The molecular weight (M_w) of DEX was 180 000 to 210 000 Da and that of PEG was 7 300 to 9 300 Da. They were purchased from Wako Pure Chemical Industries (Osaka, Japan). These polymers were dissolved in nuclease-free water (Milli-Q, 18.2 MΩ·cm) at a stock concentration of 20 wt %, and were mixed using vortex mixer immediately before observation so that the droplets became the above size.

Phospholipids: In this study, two methods were used to prepare the suspension of lipids for mixture in the PEG/DEX-binary system. First, lipid vesicles were formed by the simple hydration of lecithin powder from soybean (Nacalai Tesque, Kyoto, Japan). A suspension containing lipid vesicles and aggregates could be obtained by hydrating lecithin powder. To visualize the lipids, lecithin was hydrated by a solution of rhodamine B (Wako Pure Chemical Industries, Osaka, Japan). The excitation (ex) and emission (em) wavelengths were 554 and 576 nm, respectively. The lecithin powder was firstly hydrated to be 50 mg/mL by the hydration solution containing 50 μM rhodamine B. Then, the solution was diluted 10 times by nuclease-free water (Milli-Q, 18.2 MΩ·cm) to obtain a 5 mg/mL stock solution. The vesicular structures or aggregates of lipids were able to be observed with the fluorescence of rhodamine B (Figures S1 and S3a). Second, a suspension was produced by the hydration of lipid dry film. Lipid molecules were dissolved in an organic solvent, trichloromethane (chloroform; Wako Pure Chemical Industries, Osaka, Japan), and a volatilized multilamellar lipid film was obtained. Using this method, we performed experiments using not only lecithin obtained from nature, but also purer synthetic dioleoyl-phosphatidylcholine (DOPC; 1,2-dioleoyl-*sn*-glycero-3-phosphocholine) or dioleoyl-phosphatidylethanolamine (DOPE; 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; Tokyo Chemical Industry, Tokyo, Japan). For observation by a fluorescent microscope, *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanol amine, triethylammonium salt (Rhodamine-DHPE; Molecular Probes) was used. $\lambda_{ex} = 560$ nm and $\lambda_{em} = 580$ nm. The dry film of phospholipid was hydrated by the nuclease-free water for 200 μM as stock solution. Rhodamine-DHPE was contained 2 % of the total amount of lipids (Lecithin, DOPC or DOPE).

DNA: λ DNA of dsDNA, with a M_W of 31.5 MDa (48.5 kbp), was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). It was dissolved in nuclease-free water at a stock concentration of 770 μM (nucleotide concentration). For fluorescence observation, λ DNA was labelled with GelGreen as a fluorescent dye (Biotium Inc., Fremont, CA, USA), with $\lambda_{ex} = 500$ nm and $\lambda_{em} = 530$ nm. It was dissolved in nuclease-free water to give a 0.5 mM stock solution. The antioxidant 2-mercaptoethanol (2ME) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The buffer solution of tris(hydroxymethyl)aminomethane-HCl (1 M Tris-HCl, pH 7.5) was

purchased from Nippon Gene, and diluted with nuclease-free water as a 200 mM stock solution.

Microscopy: Images were obtained with a fluorescent microscope. The microscope was an Axio Observer. A1 (Carl Zeiss, Germany) equipped with a 40X objective lens. Images were obtained with a CCD digital camera (C11440, Hamamatsu Photonics, Hamamatsu, Japan). The obtained images were analysed by using ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2018). The compositions of the solutions for the samples for microscopic observation in this work are listed in Tables S1–S10 in the Supporting Information.

Author Contributions

H.S.,[a] F.F.,[a] T.H.,[b] M.H.,[c] K.Takiguchi,[d] K.Tsumoto,[e] and K.Y.[a,f] conceived this project. H.S. and F.F. conducted experiments. H.S., K. Takiguchi, and K. Tsumoto wrote and summarized the manuscript with feedback from the coauthors. K.Y. provided supervision.

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Conflict of Interest

The authors declare no conflict of interest.

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