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

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Supporting Information

Supplementary figures

**Figure S1.** Morphology and stability of the dextran (DEX) phase wrapped by a lipid layer. The images were obtained 1 hour after preparation. Lecithin and the DEX phase were labelled by the mixing of rhodamine B and FITC-Dextran (final 5% (w/w) against non-labelled DEX; Sigma-Aldrich, St. Louis, MO, USA, average Mw 250,000 Da).

In contrast, if lipid was not added, the DEX phases fused to each other without delay after attachment, so that cell-size DEX phases did not appear to remain more stable compared to the case with Figure S1.

**Figure S2.** Spontaneous accumulation of mixed suspensions from a variety of lipids. Lipid used was lecithin in (a), or DOPC (top) or DOPE (bottom) in (b). Left column: fluorescent microscopic image of each lipid suspension, middle and right: transmitted light and fluorescent microscopic images of the PEG/DEX-binary solution mixed with each lipid suspension, respectively.
In the main text, when lipid suspensions were obtained by the simple hydration of lipid powder, they were labeled by mixing with rhodamine B for fluorescent microscopy. Rhodamine B was selected here because it is water soluble and can be mixed into an aqueous solution without worrying about aggregation that would interfere with observation. If there are lipid membranes or lipid clumps in the aqueous solution, the dye stains them preferentially. On the other hand, when synthetic lipid like DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) or DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) was used, instead, lipid vesicles were obtained by the hydration of a dry film of lipid, and labeled by mixing with rhodamine-DHPE (N-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanol amine, triethylammonium salt) for fluorescent microscopy. Two fluorescent reagents were used simply because of the difference in the amount of lipid required for each method (the simple hydration of the lipid powder requires a large amount of lipid). This difference did not affect the results. In Figure S2, however, the hydration of a dry lipid film was applied to preparation of each sample from both lecithin and synthetic lipids (DOPC or DOPE).

Lecithin obtained from nature contains many components derived mainly from biological membranes in addition to phospholipid (phosphatidylcholine, PC). Therefore, to investigate the use of purer synthetic PC or the use of a phospholipid other than PC, similar experiments were performed using DOPC or DOPE instead of lecithin. Lecithin and DOPC give similar results ((a) and the top of (b) in Figure S2). In the case of DOPE (the bottom of (b) in Figure S2), larger aggregates are formed, and conversely, fewer vesicles are formed than with lecithin or DOPC. These results are consistent with the finding that DOPE is difficult to hydrate and thus vesicles are not easily formed when only phosphatidylethanolamine (PE) is used, as reported in many previous studies on the preparation of lipid membrane vesicles (ref. 12 in the main text).

After the suspension obtained by the hydration of either DOPC or DOPE is mixed with the PEG/DEX-binary solution, the fluorescence signal showing the distribution of lipids is mainly observed between the PEG and DEX phases ((b) in Figure S2), as with the use of lecithin. In particular, when DOPC is used, DEX phases are more clearly enclosed with lipid (the top of (b) in Figure S2). The fluorescence indicating the distribution of lipids is much more intense at the boundary of the two phases than in the case of lecithin, indicating a greater accumulation of DOPC. Moreover, there is almost no dot-like aggregate, unlike when lecithin is used. This may be because the samples made from DOPC contain much less contaminants compared to the case of lecithin, i.e., a greater amount of DOPC is contained in the same amount of sample. Again, even if two lipid-surrounded DEX phases meet, they do not fuse together easily and remain in contact.

When DOPE is used, the enclosure of the DEX phase with lipids is nonuniform, and dot-like aggregates are also observed (the bottom of (b) in Figure S2). We surmise that this reflects the difference in the efficiency of the preparation of membrane vesicles between DOPE and other phospholipids described above. In the case of DOPE, lipid bilayer membranes are less likely to be prepared, and insoluble lipid aggregates, rather than membrane vesicles, are obtained. The former may cause insufficient enclosure of DEX phases with lipid, and the latter may cause the appearance of dot-like aggregates inside the DEX phases. It is noted that the degree of aggregates observed or non-uniformity of the fluorescence intensity depended on not the species of the dye used but that of the phospholipid, indicating that they are derived from the structure made by lipids.

Notably, the state of LLPS and the morphology of each phase, except for fusion between the same phases, are not affected by the addition of phospholipid regardless of the phospholipid used.
Figure S3. The effects of salt on the distribution of lipid vesicles. Only 2.5 mg/ml of lipid vesicle suspension (a), lipid vesicles in PEG/DEX solution (b), and lipid vesicles and 19.25 μM λ DNA in PEG/DEX solution (c). All samples were mixed without (i) or with 1\% (w/v) NaCl (0.17 M) (ii) before microscopic observation. Lipid suspensions of lecithin were obtained by simple hydration method. Lipid and DNA were fluorescently labelled by rhodamine B (red) and GelGreen (green), respectively.

To investigate the effect of salt on the distribution of lipid vesicles, we mixed NaCl and sample suspensions before microscopic observation. In the case of hydrated lipid vesicles alone, the presence of NaCl (1\%) exhibited little influence (Figure S3 (a-i)); thus, lipid aggregates could be observed similarly compared with the result in the absence of NaCl (Figure S3 (a-ii)). In the case of lipid vesicle was present in PEG/DEX solution, lipid vesicles were localized on the PEG/DEX interphase without NaCl (Figure S3 (b-i, c-i)), same as Figure 1 (b) in the main text. However, in the presence of 1\% NaCl, lipid aggregates were localized within DEX droplets (Figure S3 (b-ii)). No effects of salt on the distribution of DNA were observed (Figure S3 (c-ii)). On the other hand, in the main text, we injected NaCl solution into the observation chamber which contained lipid vesicles in PEG/DEX solution (Figure 3). The lipid layers formed at PEG/DEX interphase collapsed to be clumps or ruptured vesicles. The difference in the distribution pattern of lipid vesicles between in the main text and here might be due to whether the osmotic pressure change in the observation chambers was gradual or instantaneous.
Experimental solution details

All the samples tested in the present work are formulated as follows except for the experiment for Figure 1 (a). In preparation, the indicated stock solutions were added into a single microtube in the order from the top to the bottom of each table.

In the experiment for the Figure 1 (a), as described in its caption, some amount (~10 mg) of lipid powder was simply put together with enough volume of water for hydration at room temperature, and the resulting suspension was then subjected to fluorescent microscopic observation to demonstrate formation of lipid vesicles.

| Table S1. Experimental solution for Figures 1 (b), 3 (a-i, b)\(^a\), S3 (b-i). |
|-----------------|----------------|----------------|----------------|
| **Materials**   | **Stock solution** | **Volume added (µL)** | **Final solute concentration** |
| DEX             | 20 %            | 10              | 5 %            |
| PEG             | 20 %            | 10              | 5 %            |
| Lipid (Lecithin) | 5 mg/mL         | 8               | 1 mg/mL        |
| (Rhodamine B)\(^b\) | 5 µM          |                 | 1 µM          |
| Nuclease-free water | -           | 12              | -              |
| (Total)        | 40 µL           |                 | -              |

\(^a\) In the experiment for Figure 3 (a-i, b), 10 µL of this solution was injected into the observation chamber at first, and then 2.5 µL of 5% NaCl solution was added.

\(^b\) The solution containing Rhodamine B was used for suspending the lipid.

| Table S2. Experimental solution for Figure 2.\(^a\) |
|-----------------|----------------|----------------|----------------|
| **Materials**   | **Stock solution** | **Volume added (µL)** | **Final solute concentration** |
| DEX             | 20 %            | 10              | 5 %            |
| PEG             | 20 %            | 10              | 5 %            |
| Nuclease-free water | -           | 20              | -              |
| (Total)        | 40 µL           |                 | -              |

\(^a\) In the experiment for Figure 2, 10 µL of this solution was injected into the observation chamber at first, and then 2.5 µL of 5 mg/mL lecithin suspension was added. This suspension was obtained by the simple hydration method with 5% PEG solution.

| Table S3. Experimental solution for Figures 3 (a-ii, a-iii)\(^a\), S2. |
|-----------------|----------------|----------------|----------------|
| **Materials**   | **Stock solution** | **Volume added (µL)** | **Final solute concentration** |
| DEX             | 20 %            | 10              | 5 %            |
| PEG             | 20 %            | 10              | 5 %            |
| Lipid (Lecithin, DOPC, DOPE) | 200 µM | 100 µM |
| (Rhodamine-DHPE)\(^c\) | Label Ratio 2 % | Label Ratio 2 % |
| (Total)        | 40 µL           |                 | -              |

\(^a\) In the experiment for Figure 3 (a-ii, a-iii), 10 µL of this solution (using lecithin) was injected into the observation chamber at first, and then 2.5 µL of 5% NaCl solution was added.

\(^b\) Lecithin (soybean lecithin) was considered to be 758.06 in molecular weight.

\(^c\) The lipid dye was dissolved in the lipid solution, which was in turn subjected to the evaporation to form the dry films.
Table S4. Experimental solution for Figure 4.

| Materials       | Stock solution | Volume added (µL) | Final solute concentration |
|-----------------|----------------|-------------------|----------------------------|
| DEX             | 20 %           | 10                | 5 %                        |
| PEG             | 20 %           | 10                | 5 %                        |
| GelGreen        | 0.5 mM         | 0.4               | 5 µM                       |
| Tris-HCl        | 200 mM         | 1.6               | 8 mM                       |
| 2ME             | -              | 1.6               | 4 %                        |
| λ DNA           | 770 µM<sup>a</sup> | 1              | 19.25 µM                   |
| Lipid (Lecithin)<sup>b</sup> | 200 µM       | 15                | 75 µM                      |
| (Rhodamine-DHPE)<sup>c</sup> | Label Ratio 2 % |                | Label Ratio 2 %            |
| Nuclease-free water | -            | 0.4               | -                          |

(Total) 40 µL

<sup>a</sup> Nucleotide concentration.
<sup>b</sup> Lecithin (soybean lecithin) was considered to be 758.06 in molecular weight.
<sup>c</sup> The lipid dye was dissolved in the lipid solution, which was in turn subjected to the evaporation to form the dry films.

Table S5. Experimental solution for Figure S1.

| Materials       | Stock solution | Volume added (µL) | Final solute concentration |
|-----------------|----------------|-------------------|----------------------------|
| DEX             | 20 %           | 9                 | 4.5 %                      |
| FITC-DEX        | 20 %           | 1                 | 0.5% (Label Ratio 10 %)    |
| PEG             | 20 %           | 10                | 5 %                        |
| Nuclease-free water | -            | 20                | -                          |

(Total) 40 µL

Table S6. Experimental solution for Figure S3 (a-i).

| Materials       | Stock solution | Volume added (µL) | Final solute concentration |
|-----------------|----------------|-------------------|----------------------------|
| Lipid (Lecithin)| 5 mg/mL        | 20                | 2.5 mg/mL                  |
| (Rhodamine B)<sup>a</sup> | 5 µM        | 20                | 2.5 µM                     |
| Nuclease-free water | -            | 20                | -                          |

(Total) 40 µL

<sup>a</sup> The solution containing Rhodamine B was used for suspending the lipid.
Table S7. Experimental solution for Figure S3 (a-ii).

| Materials          | Stock solution | Volume added (µL) | Final solute concentration |
|--------------------|----------------|-------------------|----------------------------|
| Lipid (Lecithin)   | 5 mg/mL        | 20                | 2.5 mg/mL                  |
| (Rhodamine B)      | 5 µM           |                   | 2.5 µM                     |
| NaCl               | 5 %            | 8                 | 1 %                        |
| Nuclease-free water| -              | 12                | -                          |
| (Total)            |                | 40 µL             | -                          |

* The solution containing Rhodamine B was used for suspending the lipid.

Table S8. Experimental solution for Figure S3 (b-ii).

| Materials          | Stock solution | Volume added (µL) | Final solute concentration |
|--------------------|----------------|-------------------|----------------------------|
| DEX                | 20 %           | 10                | 5 %                        |
| PEG                | 20 %           | 10                | 5 %                        |
| Lipid (Lecithin)   | 5 mg/mL        | 8                 | 1 mg/mL                    |
| (Rhodamine B)      | 5 µM           |                   | 1 µM                       |
| NaCl               | 5 %            | 8                 | 1 %                        |
| Nuclease-free water| -              | 4                 | -                          |
| (Total)            |                | 40 µL             | -                          |

* The solution containing Rhodamine B was used for suspending the lipid.

Table S9. Experimental solution for Figure S3 (c-i).

| Materials          | Stock solution | Volume added (µL) | Final solute concentration |
|--------------------|----------------|-------------------|----------------------------|
| DEX                | 20 %           | 25                | 5 %                        |
| PEG                | 20 %           | 25                | 5 %                        |
| GelGreen           | 0.5 mM         | 1                 | 5 µM                       |
| Tris-HCl           | 200 mM         | 4                 | 8 mM                       |
| 2ME                | -              | 4                 | 4 %                        |
| λ DNA              | 770 µM         | 2.5               | 19.25 µM                   |
| Lipid (Lecithin)   | 5 mg/mL        | 20                | 1 mg/mL                    |
| (Rhodamine B)      | 5 µM           |                   | 1 µM                       |
| Nuclease-free water| -              | 18.5              | -                          |
| (Total)            |                | 100 µL            | -                          |

* Nucleotide concentration.

*b The solution containing Rhodamine B was used for suspending the lipid.
Table S10. Experimental solution for Figure S3 (c-ii).

| Materials          | Stock solution | Volume added (µL) | Final solute concentration |
|--------------------|----------------|-------------------|----------------------------|
| DEX                | 20 %           | 25                | 5 %                        |
| PEG                | 20 %           | 25                | 5 %                        |
| GelGreen           | 0.5 mM         | 1                 | 5 µM                       |
| Tris-HCl           | 200 mM         | 4                 | 8 mM                       |
| 2ME                | -              | 4                 | 4 %                        |
| λ DNA              | 770 µM<sup>a</sup> | 2.5             | 19.25 µM                   |
| Lipid (Lecithin)   | 5 mg/mL        | 20                | 1 mg/mL                    |
| (Rhodamine B)<sup>b</sup> | 5 µM |                 | 1 µM                       |
| NaCl               | 10 %           | 10                | 1 %                        |
| Nuclease-free water| -              | 8.5               | -                          |
| (Total)            |                | 100 µL            | -                          |

<sup>a</sup> Nucleotide concentration.

<sup>b</sup> The solution containing Rhodamine B was used for suspending the lipid.