Supplementary Information for

Capsule-like DNA Hydrogels with Patterns Formed by Lateral Phase Separation of DNA Nanostructures

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Supplementary Information Text

Supplementary Note 1: Generation of lipid vesicles whose inner leaflet was covered with the phase-separated DNA hydrogels

The lipid-surrounded W/O droplets were generated using DOPC and DOTAP. The lipids were dissolved in a mixture of chloroform/methanol at a 2:1 volume ratio. Each lipid solution was added to round-bottom glass tubes. The organic solvent was evaporated under a nitrogen gas flow and vacuumed for over 2 h to completely remove organic solvents, followed by the addition of liquid paraffin to the glass tube to obtain a 2 mM lipid concentration. The liquid paraffin containing the lipid was sonicated for over 1 h at 50 °C, and then, equal volumes of the lipid solutions were mixed to obtain a 2 mM lipid solution (1 mM each). Using this lipid mixture, droplets containing Y- and ortho-Y-motifs (5 μM each) with or without 0.1 μM X-linker were generated using the same method as for the Span 80/oleylamine mixture. Note that 142 mM (10% w/v) of iodixanol was additionally mixed in aqueous solution for the droplet generation to create a density difference between the inside and outside of the lipid vesicles. For an outer leaflet of lipid vesicles, a mixture of DOPC and cholesterol (9:1 molar ratio, 5 mM in total) dissolved in liquid paraffin was used. The outer lipid solution was prepared by the same method used for preparing the DOPC/DOTAP mixture. The outer lipid mixture was poured on the outer aqueous solution (20 mM Tris-HCl (pH 8.0), 350 mM NaCl, and 142 mM sucrose) in a 1.5 ml tube and incubated for over 15 min. The W/O droplet after annealing was gently poured on the outer lipid mixture and subsequently centrifuged at 8,000 g at 4 °C for 15 min. After centrifuging, the remaining liquid paraffin was removed using a micropipette. The precipitated lipid vesicles were dispersed and collected using a micropipette.

To visualize the generated lipid vesicles, a 1.5-mm-thick silicone rubber sheet with a punch hole (5 mm in diameter) was placed on a BSA-coated glass (24 mm by 36 mm, with a thickness of 0.17 mm). The lipid vesicle solution (29 μL) was poured in the hole, and a coverslip (22 mm by 22 mm, with a thickness of 0.17 mm) was placed on the silicone rubber to avoid the evaporation of the solution during observation.

To confirm the bilayer formation and removal of lipids after the extraction, 0.1 mol% Lissamine Rhodamine B 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (Thermo Fisher Scientific, Pittsburgh, PA, USA) was mixed in the outer lipid solution and visualized before and after Triton-X100 addition.
Supplementary Note 2: Enzymatic degradation of the extracted DNA hydrogels

Exonuclease I (for degradation of single-stranded DNA) and III (for degradation of double-stranded DNA) were added to the solution containing the extracted gel capsules.

The lipid vesicle solution containing the gel capsules (15 µL) was poured in the hole. After 10 min, 1 µL of 10% (v/v) Triton-X 100 in the buffer was dropped into the vesicle solution in the hole, and subsequently, 2 µL of NEbuffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM MgCl₂, and 1 mM DTT, pH 7.0) (New England Biolab) was dropped into the hole. Before exonuclease addition, 20 µL of liquid paraffin was placed on the solution in the hole to avoid evaporation of the solution during observation. Finally, 2 µL of exonuclease I (20 U) and III (100 U) solution was dropped into the hole and visualization was performed using a confocal microscope without any temperature control.
Supplementary Note 3: Method used to analyze the patch size in the phase-separated hydrogels

The patch size in the phase-separated hydrogels became smaller with the increase in the amount of X-linkers added (Figure 4b in the main text). To quantitatively evaluate it, we performed Fourier transform to the fluorescence intensity profiles of the imY-motif region on the interface. The intensity line profiles were obtained using the image analysis software ImageJ. We arbitrary drew the lines to the images that visualized the droplet interfaces. The line length was shorter than the droplet because the intensity gap at the oil/water boundary influenced the result. The obtained intensity was standardized so that the average intensity value becomes 0 and dispersion becomes 1. The standardized data were analyzed using the Fast Fourier Transformation (FFT) with Hanning window function using the Origin software (OriginLab Corp. v 9.60). Twenty droplets were analyzed in each condition. Maximum values in each analysis were used to obtain the mean value, shown in Figure S6.

Supplementary Note 4: Sigmoidal curve fitting of Mix pattern appearance frequency

We used the following equation for sigmoidal curve fitting:

\[ \text{Mix pattern } [\%] = 100 \times \frac{[X - \text{linker}]^n}{(K_d + [X - \text{linker}]^n)} \]

where \( K_d \) is the apparent dissociation constant and \( n \) is the Hill coefficient. \(^1\) \( K_d \) and \( n \) values were determined using a latest square method. The fitting results are shown in Figure S7.
### Table S1: Oligonucleotide sequences

| Name | Sequences (5'–3') |
|------|-------------------|
| Y-1  | GCTCGAGCAGTGAGGACGGAAGTTTGCCTAGCATCAGCAC | CAGTGAGGACGGAAGTTTGCCTAGCATCAGCAC |
| Y-2  | GCTCGAGCCAACCACGCTGCTACCTACTCCGTCCTACCTGTGACAGC | GCTCGAGCCAACCACGCTGCTACCTACTCCGTCCTACCTGTGACAGC |
| Y-3  | GCTCGAGCGGTACGTCTACTACCTCTTGGAATGCAGGCTGTGTG | GGTACGTCTACTACCTCTTGGAATGCAGGCTGTGTG |
| orthY-1 | CTCGCCAGAAGAGAAGATCTCTACGCTGAGAAGCATC | AAGAGAAGATCTCTACGCTGAGAAGCATC |
| orthY-2 | CTCGCCAGATCGCTGTACCTTTGTTCTCATCAGTGAAGAGCATC | ATCGCTGTACCTTTGTTCTCATCAGTGAAGAGCATC |
| orthY-3 | CTCGCCAGATCGCTGTACCTTTGTTCTCATCAGTGAAGAGCATC | ATCGCTGTACCTTTGTTCTCATCAGTGAAGAGCATC |
| X-1  | CTCGCCAGATCGCTGTACCTTTGTTCTCATCAGTGAAGAGCATC | ATCGCTGTACCTTTGTTCTCATCAGTGAAGAGCATC |
| X-2  | GCTCGAGCGGTACGTCTACTACCTCTTGGAATGCAGGCTGTGTG | GGTACGTCTACTACCTCTTGGAATGCAGGCTGTGTG |
| X-3  | CTCGCCAGAAGAGAAGATCTCTACGCTGAGAAGCATC | AAGAGAAGATCTCTACGCTGAGAAGCATC |
| X-4  | CTCGCCAGAAGAGAAGATCTCTACGCTGAGAAGCATC | AAGAGAAGATCTCTACGCTGAGAAGCATC |
| Y-2_0_FAM | [6-FAM]-CAGTGAGGACGGAAGTTTGCCTAGCATCAGCAC | [6-FAM]-CAGTGAGGACGGAAGTTTGCCTAGCATCAGCAC |
| orthY-2_0_Alexa405 | [Alexa405]-CAGTGAGGACGGAAGTTTGCCTAGCATCAGCAC | [Alexa405]-CAGTGAGGACGGAAGTTTGCCTAGCATCAGCAC |
| X-2_0_Cy3 | [Cy3]-GCTCGAGCGGTACGTCTACTACCTCTTGGAATGCAGGCTGTGTG | [Cy3]-GCTCGAGCGGTACGTCTACTACCTCTTGGAATGCAGGCTGTGTG |

### Table S2: Melting temperature ($T_m$) of the sequences for the Y- and orthY-motifs. These parameters were obtained using DINAMelt² (Two State Melting Hybridization mode) with 350 mM Na+ and 2.5 µM DNA (for the nanostructures) and 7.5 µM DNA (for the sticky ends)

| Sequence pairs | $T_m$ [°C] |
|----------------|------------|
| CAGTGAGGACGGAAGT vs ACTTCCGTCCTACTG | 62.2 |
| GCTCGAGCATCAGCAC vs GGTGCGATGCTACGAC | 64.8 |
| CAACCACGCTTGTGCCCCTCGGGAGGAGCAGTGG | 65.9 |
| AAAGGAACCTCTCAGCCG vs CGCGGAGGAGTTCTTTT | 63.5 |
| GACAAAAGCGACACGTG vs ACGTGCTGCTTTTTC | 64.5 |
| GCCTCTCTGCTGCATCTC vs GATGCCGACACAGAGGC | 64.8 |
| GCTCGAGC vs GCTCGAGC | 43.7 |
| CTCGCCAG vs CTCGCCAG | 43.2 |
Figure S1: Size distribution of generated droplets. The generated droplet radius was 4.7±1.3 µm (mean ± standard deviation). The number of measured droplets was 203.
Figure S2: Effects of the Span 80/oleylamine ratio in the adsorption of DNA nanostructures onto the droplet interface. Note that the Y- and orthY-motif concentrations were 5 µM each. Representative confocal microscopy images using Span 80/oleylam at the ratios of 3:1, 1:1, and 1:3 (a) magnified images (b) (from left to right). At the Span 80/oleylamine ratios of 3:1 and 1:1, DNA hydrogel particles were formed inside the droplets, indicating that DNA nanostructures were not fully adsorbed onto the interface. Scale bars: 50 µm in (a) and 20 µm in (b). (c) Fluorescence intensity profiles on the yellow lines shown in (b).
Figure S3: Confocal microscopy images of the droplets containing Y- and orthY-motifs without sticky ends, each at a concentration of 2.5 µM. No patterns were observed, suggesting that the formed patterns were due to the orthogonality of sticky end sequences in the motifs. Scale bar: 20 µm.
Figure S4: Confocal microscopy images of the droplets containing only \textit{\textsuperscript{only}}-Y-, Y- or X-linkers, each at a concentration of 5 µM. Scale bars: 20 µm.
Figure S5: Size evaluation of the orthoY-motif region on the droplet interface analyzed using Fourier transformation. The data show the mean ± standard error.
Figure S6: Fitting of appearance frequency of the Mix pattern to a Hill-type sigmoidal curve. (a) Y/orthY-motif = 2.5/2.5 μM/μM. (b) Y/orthY-motif = 1.5/3.5. (c) Y/orthY-motif = 3.5/1.5. Yellow dots and black solid lines represent experimental results and fitting curves, respectively. Red dashed lines show the X-linker concentrations resulting in a 50% Mix pattern, which were 0.43 μM in (a), 0.29 μM in (b), and 0.20 μM in (c).
**Figure S7**: X-linker distribution at $Y_{\text{orth}}$/$Y_{\text{-}}$/X-linker= 2.5/2.5/0.25 µM. (a) Microscopy images of the droplet surfaces. Scale bar: 20 µm. (b) Normalized intensity profiled at the lines shown in the right images in (a). (i), (ii), and (iii) correspond to the white lines in the image. The blue, green, and magenta colors represent the $Y_{\text{-}}$, $Y_{\text{orth}}$, and the X-linkers, respectively.
Figure S8: Microscopy images of the droplet surfaces under imbalanced Y/\textsuperscript{ortho}Y-motif concentration with different X-linker concentration. (a) 3.5/1.5 μM of Y/\textsuperscript{ortho}Y-motifs with 0.1, 0.25, 0.5, and 1 μM X-linkers. (b) 1.5/3.5 μM of Y/\textsuperscript{ortho}Y-motifs with 0.1, 0.25, 0.5, and 1 μM X-linkers. Scale bars: 20 μm.
Figure S9: Microscopy images of phase-separated hydrogels formed on droplet surfaces that were generated using an equimolar mixture of DOPC and DOTAP. (a) 5/5/0 µM of Y-motif/orthY-motif/X-linker. (b) 5/5/0.1 µM of Y-motif/orthY-motif/X-linker. Phase-separated DNA hydrogels were observed on the surface, similar to those formed on the W/O droplets composed of Span80/oleylamine. Scale bar: 20 µm.
Figure S10: Microscopic images of lipid vesicles with the phase-separated DNA hydrogels and the extracted hydrogel capsule. (a) Cross-section images of the lipid vesicle in which phase-separated hydrogels were formed on the inner leaflet. Rhodamine-labeled lipids in the outer leaflet were observed. (b) Cross-section images of the extracted hydrogel capsule. A rhodamine signal was not detected on the gel surface. Scale bar: 20 µm. (c) and (d) Intensity profiles of rhodamine signal at the line shown in the right images in (a) and (b), respectively.
Figure S11: Microscopic images of extracted DNA hydrogels prepared under 0 µM X-linker conditions. Only broken filaments or particles of the DNA hydrogels were observed. Scale bar: 30 µm.
Figure S12: Sequential microscopic images of the extracted hydrogel capsules before and after the addition of exonuclease solution. Scale bar 30 µm