Supporting Information

Macromolecularly crowded protocells from reversibly shrinking monodisperse liposomes

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1. Materials

To prepare monodisperse liposomes attached with an oil droplets from templates of W/O/W double emulsions, an aqueous solution with 8 wt.% polyethylene glycol (PEG, $M_w = 6,000 \text{ g mol}^{-1}$, VWR) and 2 wt.% polyvinyl alcohol (PVA, $M_w = 13,000-23,000 \text{ g mol}^{-1}$, 87-88% hydrolysed, Sigma-Aldrich), a mixture of chloroform and hexane (30:70, v/v) containing 5 mg mL$^{-1}$ L-α-phosphatidylcholine (egg PC) or E. coli lipids (Avanti Polar Lipids) as well as an aqueous solution with 2 wt.% PEG, 8 wt.% PVA and 0.0-0.2 wt.% Pluronic® F-68 (ThermoFisher Scientific) were respectively utilized as inner water phase (W0), middle oil phase (O) and outer water phase (W1). To visualize as-formed liposomes, water-soluble dyes including fluorescein isothiocyanate-dextran (FITC-Dextran, $M_w = 40,000 \text{ g mol}^{-1}$, Sigma-Aldrich) or Alexa Fluor 647 (A647, Invitrogen) were added in the inner water phases; a labelled lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (18:1 NBD-PE, 810145, Avanti Polar Lipids, Inc.) was added into the oil phase.

2. Microfluidics

2.1 Microfluidic devices. The microfluidic devices used here are assembled from round and square glass capillaries reported by the Weitz group. Briefly, two cylindrical capillaries of outer diameter 960 µm, inner diameter 400 µm were precisely
tapered to achieve orifice sizes of about 40-60 and 80-120 µm in diameter by using a capillary puller (PN-31, Narishige) and a microforge (MF-830, Narishige). The microcapillary with smaller tip modified by trimethylsilyl chloride (Sigma-Aldrich) into hydrophobic was used for flowing inner phase, while the capillary with larger diameter was treated by 2-[methoxy(polyethyleneoxy)propyl] trimethoxy silane (Gelest, Inc.) to render its surface hydrophilic, and used as the collection tube. Both of two cylindrical capillaries were inserted into a square capillary of inner diameter 1.00 mm from its two opposite ends. The gaps between the square capillary and round capillary are used as two channels for flowing middle and outer phase as shown in Figure S1a. Lastly, dispensing needles used as inlets of fluids were connected at the junctions between capillaries or their ends by using a transparent 5 minute® Epoxy (Devcon).

2.2 Manipulation. To generate the double emulsions, all fluids were pumped into the capillary microfluidic devices by using syringe pumps (PHD 2000 series, Harvard Apparatus) at desired flow rates. Typical flow rates of the inner, middle and outer phases are 300-500, 500-1000, and 5,000-10,000 µL h⁻¹, respectively. The formation process of emulsion drops was monitored by using an inverted optical microscope (IX71, Olympus) equipped with a high-speed camera (Miroex4, Phantom, Vision Research). The freshly prepared emulsion templates were collected in a semi-enclosed silicone isolation chamber (diameter 9 mm, height 0.12mm, SecureSeal™) covered with a glass coverslide for further characterization. The dewetting process and resultant samples were observed by either an inverted optical
microscope equipped with a high-speed camera or a confocal laser scanning microscope (CLSM) (SP8x, Leica).

2.3 Reversible swelling and shrinking partially dewetted liposomes: Partially dewetted liposomes were prepared using a combination of fluids as follows: 1) inner phase: aqueous solution with 1.7 wt.% PEG and 170 mM sucrose; 2) middle phase: 4 mg mL\(^{-1}\) egg PC dissolved in a mixture of chloroform and hexane (3:7 v/v); 3) outer phase: an aqueous solution with 10 wt.% PVA and 0.05 wt.% F-68. For visualizing the shrinkage and swelling process, the freshly prepared liposomes were then pipetted into a µ-Slide VI 0.4 (Ibidi) with a channel volume of 30 µL. To shrink the liposomes, we added about 7 µL hypertonic solution (an aqueous solution of 750 mM sucrose and 0.05 wt.% F-68) to the reservoir to increase the outer osmotic pressure. The process was recorded using an inverted optical microscope (IX71, Olympus) equipped with a high-speed camera (Miriox4, Phantom, Vision Research). Please note that the addition of buffer solution into outer phase needs to be done gently, otherwise it may cause instability of the samples. Long reach pipette tips (Eppendorf Microloader\textsuperscript{TM} 20 uL) is advised for adding the solution. Subsequently, we swelled the shrunk liposomes by adding about 7 µL hypotonic solution (an aqueous solution of 0.05 wt.% F-68) was added to the reservoir. Images were again recorded every 15 seconds.

3. Criteria of partial dewetting of W/O/W double emulsion droplets

The dewetting of double emulsions is determined by the spreading coefficient that is defined as \( S_i = y_{jk} - (y_{ij} + y_{ik}) \), where \( y_{ij} \) is the interfacial tension between fluids \( i \)
To illustrate, we take W1/O/W2 double emulsions for example: when \( S_o < 0 \), i.e., \( \gamma_{lw1w2} - (\gamma_{ow1} + \gamma_{ow2}) < 0 \) (1), the dewetting will occur spontaneously to minimize the total interfacial energies (Figure S1). However, if the structures maintain the snowman-like configuration, it also requires a balance of interfacial tensions, i.e., \( \gamma_{lw1w2} + \gamma_{ow1} > \gamma_{ow2} \) (\( S_w < 0 \)) (2) and \( \gamma_{lw1w2} + \gamma_{ow2} > \gamma_{ow1} \) (\( S_w < 0 \)) (3). Therefore, the combination of equation (1), (2) and (3) is the criterion for partial dewetting of W1/O/W2 emulsion drops. This criterion agrees well with the adhesion energy \( \Delta F \) derived from the Young-Dupre equation of the droplet system, \( \Delta F = \gamma_{ow2} + \gamma_{lw1w2} - \gamma_{ow1} \) (4). When \( S_w < 0 \), \( \Delta F \) is positive, meaning that there is some adhesion energy between the two drops.

4. Impact of the surfactant and oil on the properties of protocells

In our method, the surfactant Pluronic F-68 may adhere to the outer bilayer leaflet, and no surfactant exists inside the liposome or adheres to the inner bilayer leaflet. The reason is as follows: as reported previously, when the surface pressure of lipid monolayer is low, the surfactant Pluronic F-68 adsorbs to the oil-water interface and physically occupies a portion of the available area, but when the surface pressure is increased to a threshold, F-68 cannot penetrate into the membrane any more. Pluronic F-68 has 27 PO units (hydrophobic part, see Figure R1) that cannot achieve a membrane spanning configuration, because a spanning configuration requires at least 40 PO units. We only use the F-68 in outer water phase, therefore, the F-68 only can adhere to the outer bilayer leaflet, and no surfactant exists inside the
liposome or adheres to the inner bilayer leaflet. Therefore, semi-permeability of the membrane should be similar to the pure membrane. Besides, it has been reported that the use of F-68 in liposomes also can improve the stability of liposomes\(^6\).

The oil phase we used is a mix of hexane and chloroform. Previous research (Ref. 1b) has found that the residual oil in bilayer membranes prepared from this system is very little, nearly undetectable, because the dewetting process happens spontaneously and the presence of small molecules amongst longer lipid tails is energetically unfavourable as it forms defects in the ordered structure of lipids and can disrupt the hydrophobic interactions. However, the present of chloroform do have some drawbacks, for example, it may hinder some bioreactions or cause degeneration of proteins. We have found chloroform and hexane have a slightly negative effect on protein expression when statured in water (a roughly 25% drop in protein yields) (Ref. 1b in main text).

In general, different surfactants or/and different oil phases may result in protocells with different properties. For example, if ionic surfactant is used in W2 phase, the osmotic pressure inside the liposome may differ from that prepared by using same concentration of nonionic surfactant. As the reviewer mentioned, the first rule of choosing surfactant and oil is that interfacial energies of the multiple phases must meet the criteria of partial dewetting.
5. Calculation of the diameter of liposomes after osmotic shock

As Figure S8 shows, the initial diameter and salt (or polymer) concentration are respectively $D_0$ and $C_0$; the final diameter and salt (or polymer) concentration are respectively $D_i$ and $C_i$ (after osmosis). Because salt (or polymer) cannot transfer across the bilayer membrane, the total amount of salt (or polymer) molecules keeps the same inside the inner water compartment. Therefore, we can get

$$C_0 \cdot \frac{4}{3} \pi \left(\frac{D_0}{2}\right)^3 = C_i \cdot \frac{4}{3} \pi \left(\frac{D_i}{2}\right)^3$$

$$\Rightarrow D_i = D_0 \cdot \sqrt[3]{\frac{C_0}{C_i}}$$

(5)

As a consequence, the final dimension of liposomes can be easily predicted and tuned via changing the applied osmotic pressure.

6. Isolation of nucleoids from *E. coli*

We used an osmotic shock-based procedure to isolate the nucleoids from *E. coli* BL21 Ros2 as reported previously. Briefly, there are three main steps: (1) plasmolysis (shrinking of the cell cytoplasm due to a loss of water); (2) breaking of the peptidoglycan layer; (3) Applying hypotonic shock to break the inner cell membrane to release the nucleoids. We used lysozyme (1 mg/mL) in step (2) to degrade the peptidoglycan in the bacterial cell membrane. Once the nucleoids were properly isolated, a continuous sucrose gradient was employed to purify them. To visualize the nucleoids, SYTOX orange (0.5 μM) was used to dye the nucleoid DNA.

7. *In vitro* gene expression in crowded and non-crowded protocells
Escherichia coli cell lysate and feeding buffer were prepared and stored according to a recent publication of our group. To investigate the effect of the crowded interior on gene expression, we encapsulated the IVTT mixtures in liposomes made from eggPC. The IVTT mix consisted of one-third Escherichia coli cell lysate (100 μL) and two-thirds feeding buffer of the following composition: 50 mM HEPES, 100 mM potassium glutamate, 6 mM magnesium glutamate, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/ml tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 0.5 mM of each amino acid, 1 mM spermidine and 30 mM 3-PGA. To this mixture we also added 7.5 nM of plasmid DNA coding for monomeric red fluorescent protein (mRFP) (pRSETa mRFP Spi-32R) and 2% (v/v) T7 RNA polymerase (6 µL) to observe the expression. To enhance stability of liposomes, 3% PVA was also added to the inner water phase. When the double emulsion templates were prepared, we collected them into two containers (one with 3 M sucrose solution, the other without), to prepare shrunk protocells and normal protocells. Then the two samples were monitored by an Olympus IX81 confocal microscope to observe the expression of mRFP inside the protocells. Images showing fluorescence intensity of mRFP inside the liposomes were analyzed by a free software ImageJ associated with TrackMate.

8. Dynamic molecular crowding and Phase separation in protocells

Escherichia coli cell lysate was prepared and stored according to a recent publication of our group. The density of the cell lysate is about 60 g·L⁻¹. To encapsulate E. coli cell extracts into liposomes, we use the as-prepared cell lysate as inner water
phase to prepare double emulsion droplets, meanwhile we also added 300 mM sucrose solution into the outer phase to balance the osmotic pressure. As the liposomes were formed, we then add 1 M and 2M sucrose into the collection phase in order with an interval about 1 min to observe the shrinking process. To investigate dynamic molecular crowding inside liposomes, FRAP experiments were carried out before and after shrinking on an Olympus IX81 confocal microscope, equipped with an Andor iXon3 camera, Andor 400-series solid-state lasers, a Yokogawa CSU-X1 spinning disk, and an Andor FRAPPA photobleach module. Recovery of the fluorescence intensity was recorded every 30 ms or 2s.

To carry out the coacervation of cell lysate inside liposomes, the IVTT mixtures mainly consisting of one-third Escherichia coli cell lysate (100 µL) and two-thirds feeding buffer (200 µL), were loaded into the liposomes. To this mixture we also added 20 mM magnesium glutamate, 200 mM potassium glutamate and 5 nM of linear DNA coding for enhanced green fluorescent protein (eGFP) to observe the expression. When the samples were prepared, we gradually added 0.5 M, 1 M, 2M and 4M sucrose solution into the collection container to shrink the liposomes, thus triggering the phase separation of the interior in high salt conditions. The whole process was recorded by an inverted optical microscope (IX71, Olympus) equipped with a high-speed camera (Miroex4, Phantom, Vision Research). Finally, the expression of eGFP inside the protocells was checked by an Olympus IX81 confocal microscope.
Part II. Supplementary Figures S1-S10

Figure S1. (a) Schematic illustration showing partial dewetting process of W/O/W double emulsion droplet and the analysis of interfacial energies.

Figure S2. Optical images of liposomes containing two, three and four liposomal compartments (transparent ones).
Figure S3. (a) Snapshot of the microfluidic preparation of W/O/W double emulsion templates. (b-d) Confocal images of as-formed monodisperse W1/O/W2 double emulsion droplets (b), liposomes in 5 min (c) and in 7h (d). The membrane surface area changes slightly due to the evaporation of the solvents in the emulsion shells as the sample were incubated for 7 h.
Figure S4. Optical images of liposome samples captured in 4 days after preparation.

Figure S5. The shrinking kinetics of the liposomes. $D$, $S$ and $V$ respectively represent diameter, surface area and volume of the shrinking liposomes; $D0$, $S0$ and $V0$ respectively represent the initial diameter, surface area and volume of the liposomes before shrinking.
Figure S6. Stability of liposomes without attached oil drop (a) and the ones with attached oil drop (b) in response to osmotic shock (1 M NaCl). Liposomes without attached oil drop burst immediately, while the ones with attached oil drop shrink to balance the osmotic difference. Because we used 8% PEG and 2% PVA as inner phase, the interior of liposomes without attached oil drop will first form phase separation, then popped as osmotic shock was applied.
Figure S7. Reversible shrinking and swelling of oil-attached liposomes in response to the change of osmotic pressure. The inner phase and outer phase for preparing the liposomes were respectively 1.7 wt.% PEG + 170 mM sucrose as well as 10 wt.% PVA + 0.05 wt.% F-68.
Figure S8. Schematics of size change of liposomes due to the change of osmotic pressure.

Figure S9. (a) Confocal images of recovery of fluorescence of the A647 dyed inner water droplet after photobleaching. (b) the relevant fluorescence intensity-distance profiles along the red line in a1.
Figure S10. Optical and confocal images showing phase separation of cell lysate inside liposomes.
Figure S11. Confocal image of small-sized liposomes formed via shrinking method. The conventional methods to such small structures, such as extrusion through porous membranes, electroformation, hydration or swelling, always lead to polydisperse structures and show inefficient encapsulation. Current droplet microfluidic techniques are also difficult to make monodisperse vesicles in that small sizes, but enable to prepare droplets in 10-20 μm easily which can be employed as templates to form sub-micron vesicles via shrinking during dewetting process.
Part III. Supplementary Movies S1-S5

**Movie S1.** Shrinking process of the protocells via hypertonic shock.

**Movie S2.** Increase of the concentration of fluorescent molecules in protocells during shrinking.

**Movie S3.** Recovery of fluorescence of the bilayers after photobleaching.

**Movie S4.** *In vitro* transcription and translation in crowded and non-crowded protocells.

**Movie S5.** Complex coacervation of cell lysates inside protocell.
Part IV. Supplementary References

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