Supporting Information

Microfluidic Assembly of Monodisperse Vesosomes as Artificial Cell Models

Nan-Nan Deng, Maaruthy Yelleswarapu, Lifei Zheng and Wilhelm T. S. Huck*

Institute for Molecules and Materials, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

*Corresponding Author: Wilhelm T.S. Huck (w.huck@science.ru.nl)

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Part I. Supplementary Experimental Section

1. Materials

To prepare monodisperse single liposomes from templates of W/O/W double emulsions, an aqueous solution with 8 wt.% polyethylene glycol (PEG, M_W= 6,000 g mol^{-1}, VWR) and 2 wt.% polyvinyl alcohol (PVA, M_W= 13,000-23,000 g mol^{-1}, 87-88% hydrolysed, Sigma-Aldrich), a mixture of chloroform and hexane (36:64, v/v) containing 5 mg mL^{-1} L-α-phosphatidylcholine (egg PC, Avanti Polar Lipids) as well as an aqueous solution with 2 wt.% PEG, 8 wt.% PVA and 0.5 wt.% Pluronic® F-68 (ThermoFisher Scientific) were respectively utilized as inner water phase (W0), middle oil phase (O) and outer water phase (W1). To prepare monodisperse vesosomes, the as-formed liposomes dispersed in phase W1 were injected into the microfluidic devices as inner phase. The middle oil phase was the same as that in preparation of single liposomes. The outer water phase was 10 wt.% PVA with 1.0 wt.% F-68. To visualize as-formed vesosomes, water-soluble dyes including fluorescein isothiocyanate-dextran (FITC-Dextran, M_W= 40,000 g mol^{-1}, Sigma-Aldrich), rhodamine B isothiocyanate-Dextran (RITC-dextran, M_W= 70,000 g mol^{-1}, Sigma-Aldrich) and Alexa Fluor 647 (A647, Invitrogen) were added in the relevant phases.

2. Microfluidics

2.1 Microfluidic devices. The microfluidic devices used here are assembled from

round and square glass capillaries reported by the Weitz group.[1,2] 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).

To prepare vesosomes with distinct liposomes from the templates of double emulsion droplets, the devices were designed with two-inlets for pumping two kinds of liposome. In short, two cylindrical microcapillaries of outer diameter 170 μm, inner diameter 100 μm were inserted into a bigger cylindrical microcapillary of outer diameter 960 μm, inner diameter 400 μm, which were used as co-inlets in device assembly. The rest fabrication is the same as described hereinabove.

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 150-500, 300-800, and 2,000-8,000 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). In the first dewetting process, the as-formed double emulsion droplets were collected in a PETF tube (inner diameter 0.81 mm) which was subsequently placed vertically for 10 min to remove residual oil droplets formed during the dewetting of emulsion templates, because oil droplets (density of 30:70 chloroform and hexane, less than 0.905 g mL-1) floats into the top of the tube. Later, the pure liposome samples were injected into the microcapillary device to prepare second level of double emulsion templates, producing liposome-in-liposome vesosomes. The freshly prepared emulsion templates were collected in a semi-enclosed silicone isolation chamber (diameter 9 mm, height 0.12mm, SecureSeal^{TM}) covered with a glass coverslide for further characterization. The resultant labeled vesosomes were observed by a confocal laser scanning microscope (CLSM) (SP8x, Leica).

3. IVTx in nucleus liposomes and IVTT

For the *in vitro* transcription (IVTx) reactions, we used a reaction buffer without any cell lysate for cell-free transcription reactions as previously reported.[3] The reaction buffer consisted of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 8.0), 3 mM guanosine triphosphate, 1 mM each of adenosine triphosphate, cytidine triphosphate and uridine triphosphate, 0.66 mM spermidine, 0.5

mM cyclic adenosine monophosphate, 0.22 mM nicotinamide adenine dinucleotide, 0.17 mM coenzyme A, 20 mM 3-phosphoglyceric acid, 0.045 mM folinic acid, 0.13 mg ml^{-1} transfer ribonucleic acid and 1 mM of each amino acid. This reaction buffer was premixed and stored in aliquots at -80 ^{o}C after flash-freezing. To visualize and real-time detect the RNA generation, DNA template coding for Spinach2 aptamer with tRNA scaffold (15 nM) was used. To this reaction buffer we also supplemented 100 U of RNase inhibitor (human placental, NEB), 60 uM of 5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) (Tocris), 70 mM potassium glutamate, 15 mM magnesium glutamate, 1.5 U of pyrophosphatase, inorganic (yeast, Sigma), 300 U of T7 polymerase. To perform the IVTx in liposomes, we used the mixed reaction buffer with 2 wt.% PEG as the inner water phase to prepare liposomes. PEG used here is to increase the stability of resultant liposomes. The collected samples were incubated at 28 ^{o}C and monitored for 3 hours by a confocal laser scanning microscope (CLSM) (SP8x, Leica) equipped with a box temperature control system (Life Imaging Services, Switzerland). The detection interval was 5 min. Images showing fluorescence intensity of Spinach2-DFHBI inside the liposomes were analyzed by a software ImageJ.

For IVTT, the reaction mixtures mainly consisted of one-third *Escherichia coli* cell lysate (100 µL) and two-thirds feeding buffer (200 µL), which were prepared and stored according to a recent publication of our group.[3] To this mixture we also added 12.5 mM magnesium glutamate, 70 mM potassium glutamate and 4 nM of pRSET5d-mRFP1 plasmid coding for monomeric red fluorescent protein (mRFP) to

observe the mRFP expression.

Before encapsulating single liposomes containing IVTx reaction mixtures into larger liposomes that contain IVTT mixtures to build the architecture of eukaryotic cell models, we first test the feasibility to form liposomes by using IVTx reaction mixtures and IVTT reaction mixtures as inner and outer water phases respectively as well as by using IVTT reaction mixtures and 10 wt.% PVA with 1.0 wt.% F68 as inner and outer phases respectively. To trigger the dewetting of double emulsion droplets and increase the stability of as-formed liposomes, 0.5 wt.% F68, 4.0 wt.% PEG and 1.0 wt.% PVA were added into the IVTT reaction mixtures. The results show that both systems can form monodisperse liposomes (Figures S16 and S17). To mimic the architecture of eukaryotic cells, the single liposomes containing IVTx mixtures formed in IVTT mixtures were reinjected into the microfluidic device to prepare vesosomes.

4. Insertion of nanopores into nucleus liposomes of vesosomes

To incorporate the membrane protein into the nucleus liposomes, melittin (2 μM, Sigma-Aldrich) together with calcein (10 μM, pH 7.35-7.45, M_W= 623 g mol^{-1}) were loaded in the inner phase and Rhodamine B isothiocyanate-dextran (10 μM, RTIC-Dextran, M_W= 70,000 g mol^{-1}) was loaded in the outer water phase to prepare liposomes. Then, the as-prepared liposomes dispersed in RTIC-Dextran-labeled solution were used as inner phase to prepare vesosomes. Commercial lyophilized powder of melittin was first solubilized in milli-Q water, and then added into W0 phase to desired concentrations. As-formed vesosomes were collected in a semi-enclosed

silicone isolation chamber, and then recorded automatically by an inverted microscope (IX81, Olympus) equipped with a sensitive EMCCD camera (iXon3; Andor) illuminating from a mercury lamp. The interval time of detection was 0.5 min and the mercury lamp exposure time was 500 ms. Images were processed and analyzed by ImageJ.

Part II. Supplementary Figures S1-S17

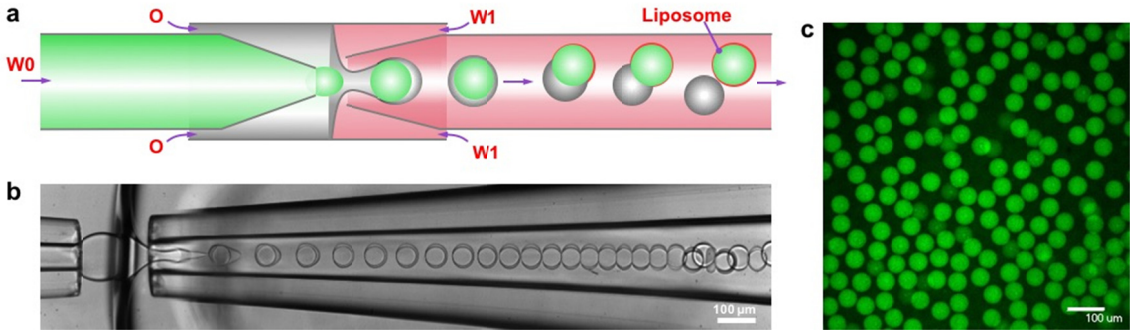

Figure S1. (a) Schematic illustration and (b) snapshot of the microfluidic preparation of double emulsion templates to liposomes. (c) Confocal image of single monodisperse liposomes formed from dewetting of double emulsion templates. Scale bars, 100 μm

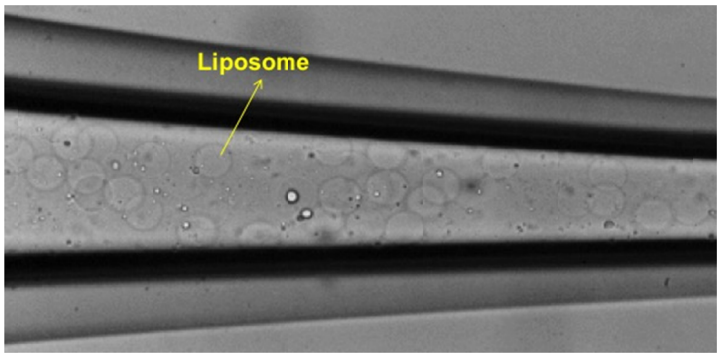

Figure S2. Optical image shows the reinjection of liposomes into the microfluidic device.

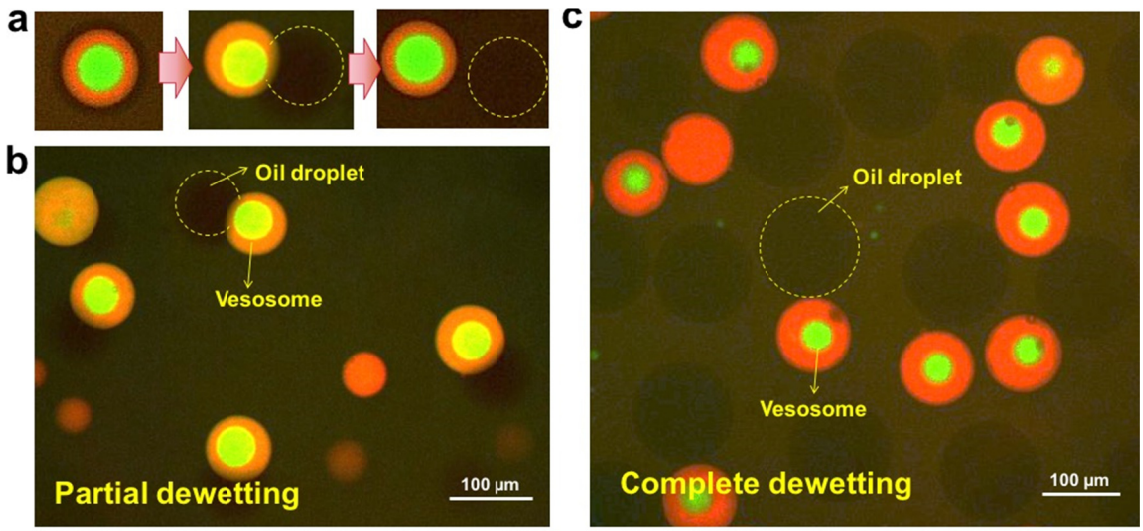

Figure S3. Confocal images showing the dewetting process of double emulsion droplets to form vesosomes (a) and partial dewetting state (b) and complete dewetting state (c). The brightness and the contrast of the images are modified in propose to show the oil droplets. Scale bars, 100µm

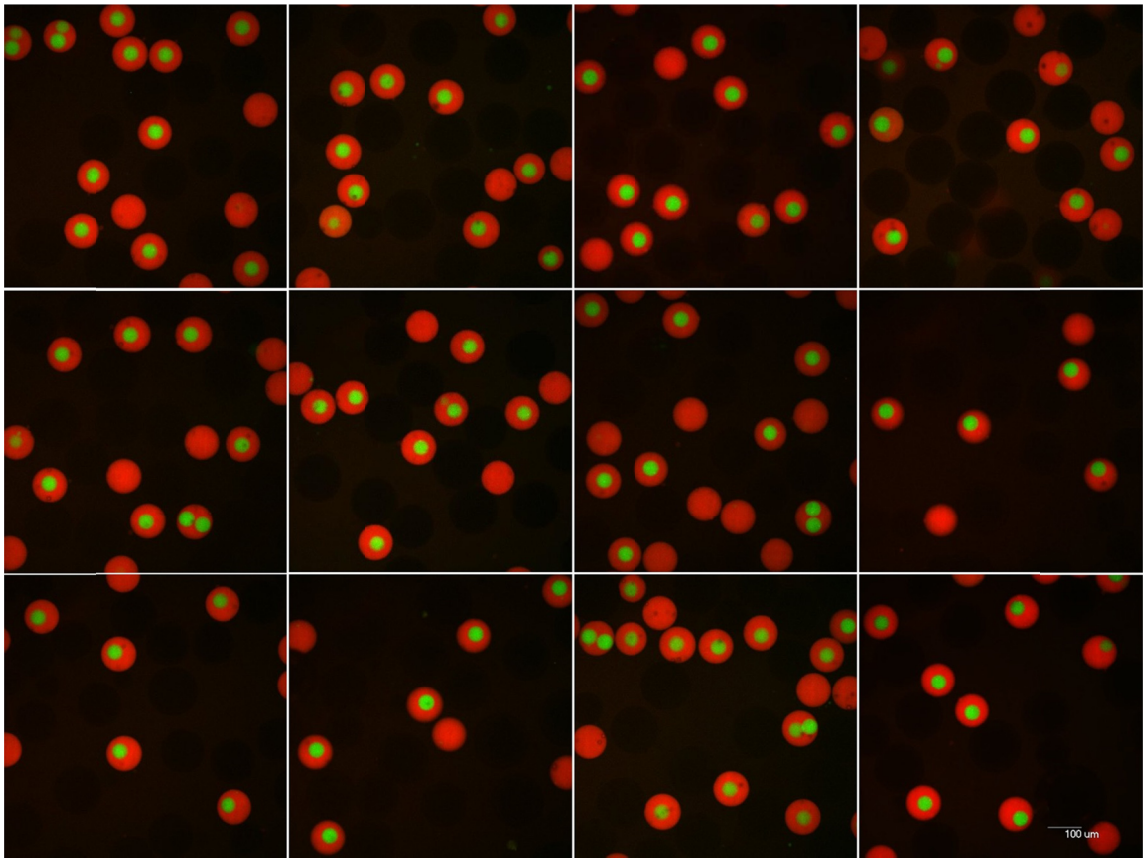

Figure S4. Confocal images of the as-prepared vesosomes with single liposome. Scale bar, 100μm

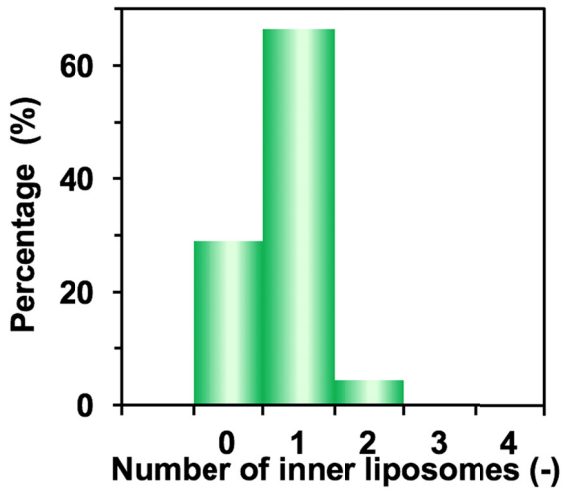

Figure S5. Distribution of the number of inner liposomes in vesosomes as partially shown in Figure S4. N= 262.

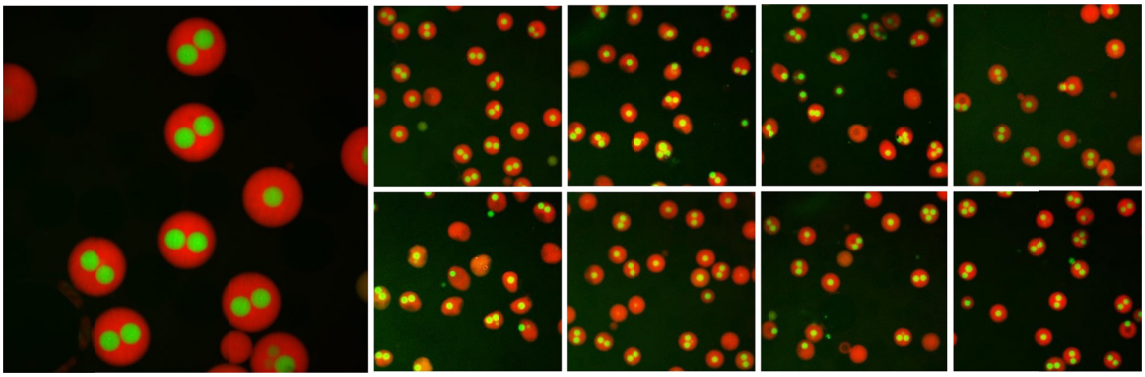

Figure S6. Confocal images of the as-prepared vesosomes mainly with one or two inner liposomes. The black dots in the images are the residual oil droplets.

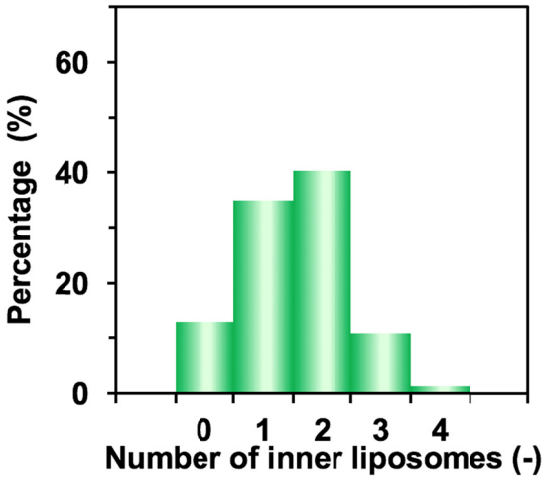

Figure S7. Distribution of the number of inner liposomes in vesosomes as partially shown in Figure S6. N= 251.

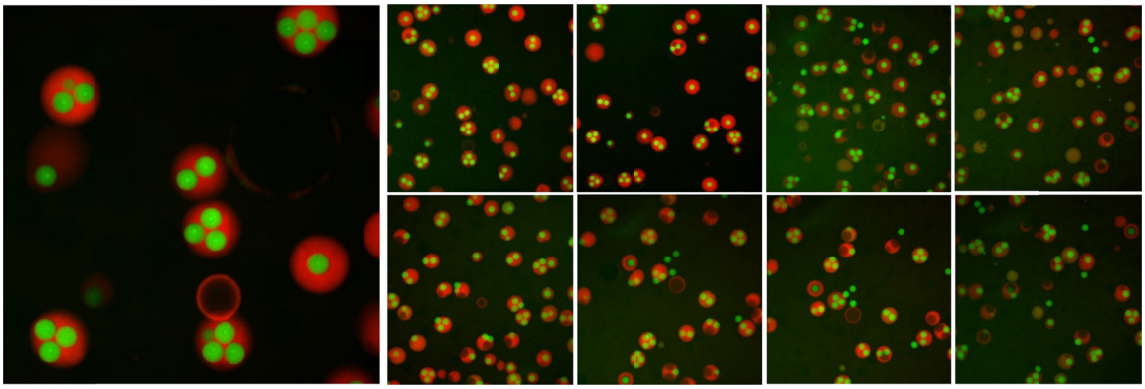

Figure S8. Confocal images of the as-prepared vesosomes that mainly contain three, one or two inner liposomes. The black dots in the images are the residual oil droplets.

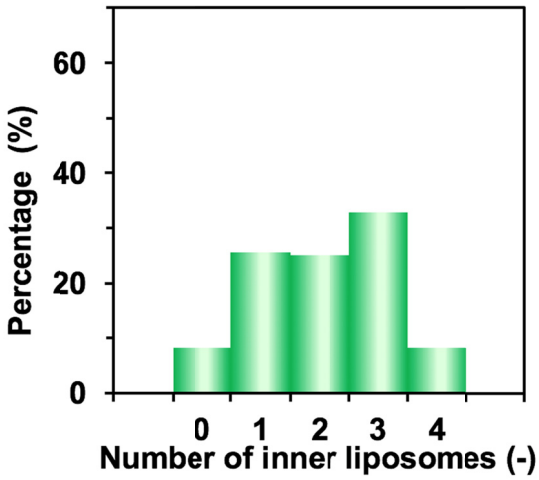

Figure S9. Distribution of the number of inner liposomes in vesosomes as partially shown in Figure S8. N= 195.

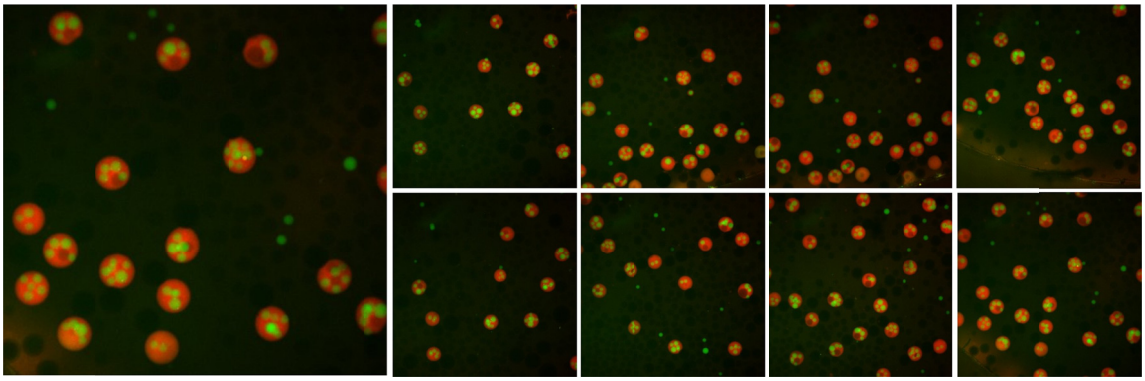

Figure S10. Confocal images of the as-prepared vesosomes mainly containing three, four or five inner liposomes. The black dots in the images are the residual oil droplets.

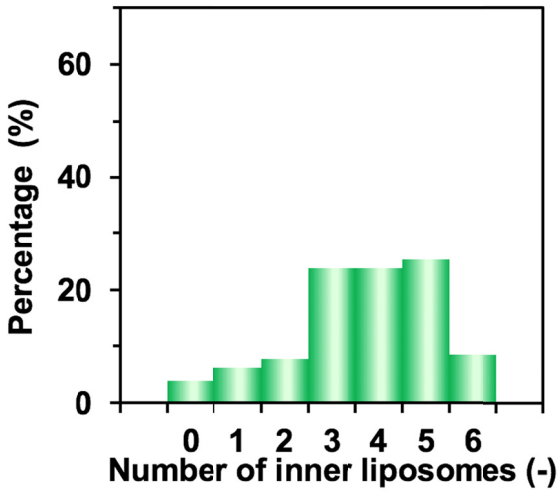

Figure S11. Distribution of the number of inner liposomes in vesosomes as partially shown in Figure S10. N= 129.

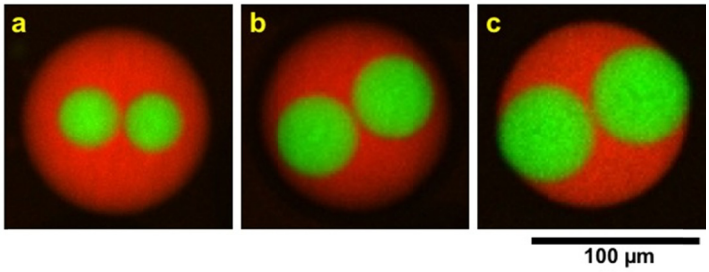

Figure S12. Confocal images of the examples of as-prepared dual-core vesosomes with different shell thickness. Scale bar, 100μm.

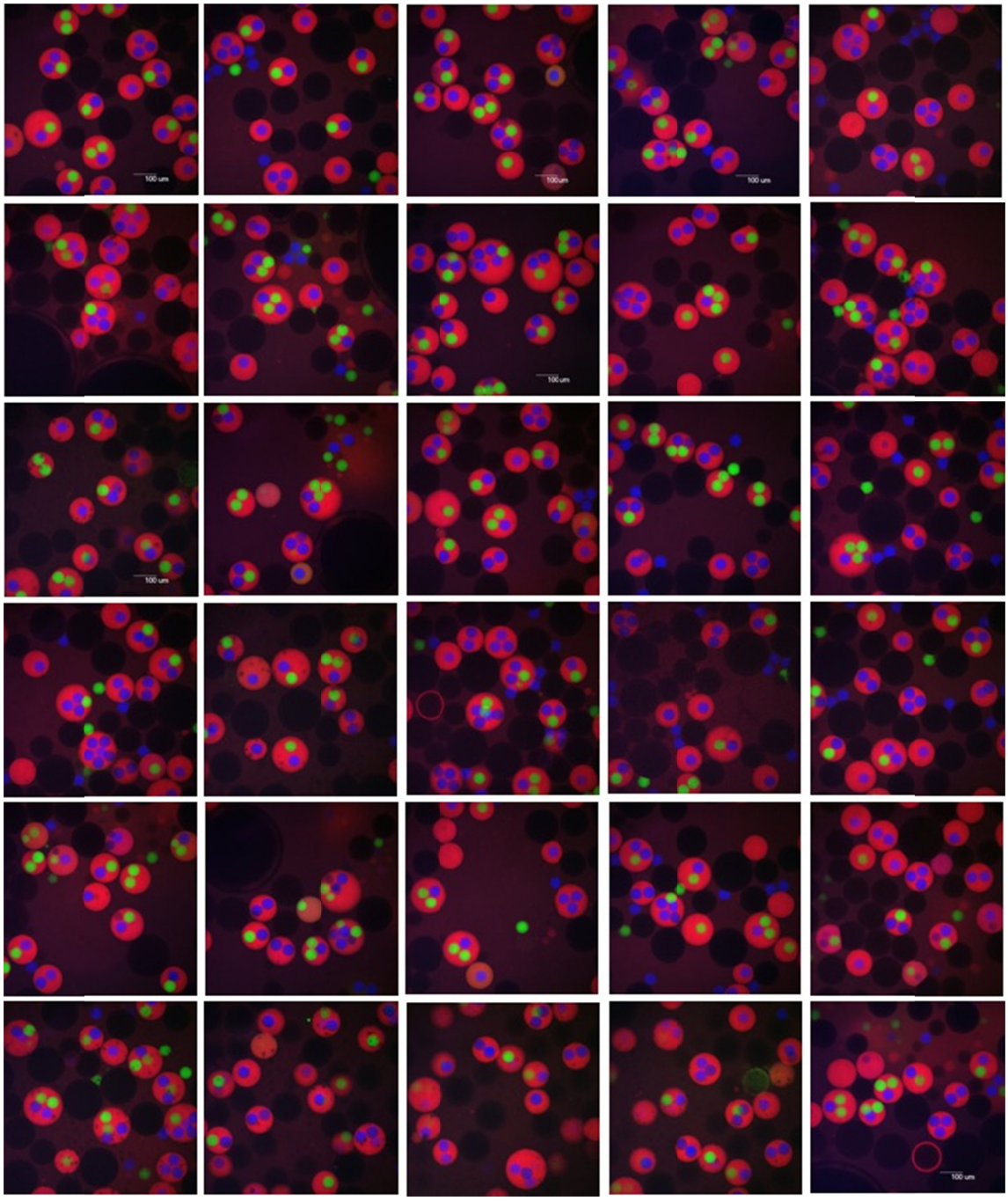

Figure S13. Confocal images of the as-prepared vesosomes with diverse inner structures. The black dots in the images are the residual oil droplets. Scale bar, 100μm.

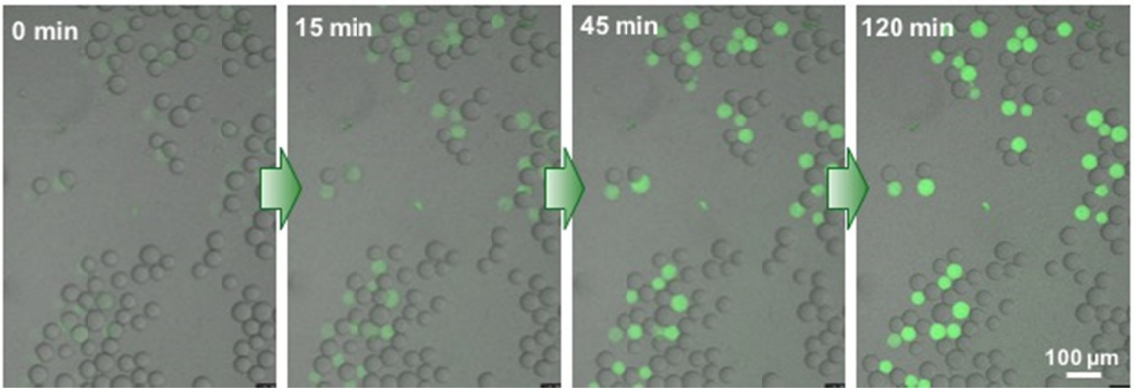

Figure S14. Sequence confocal images show IVTx in liposomes in large quantities. Scale bar, 100μm

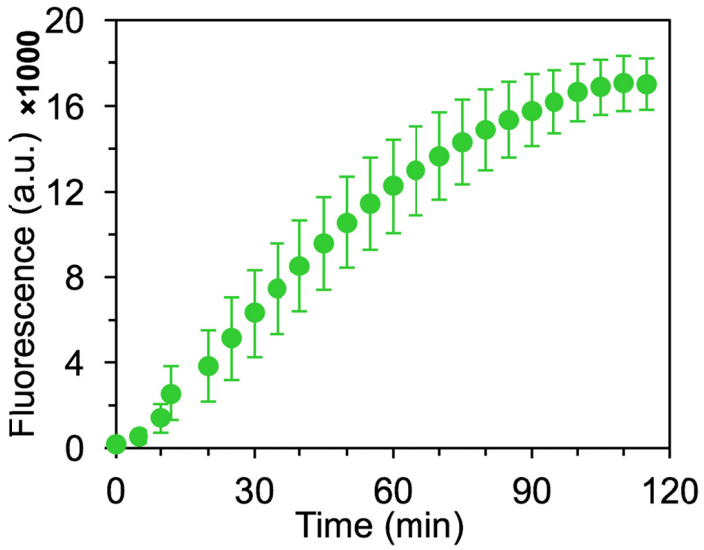

Figure S15. The expression kinetics of the IVTx in bulk recorded by a plate reader.

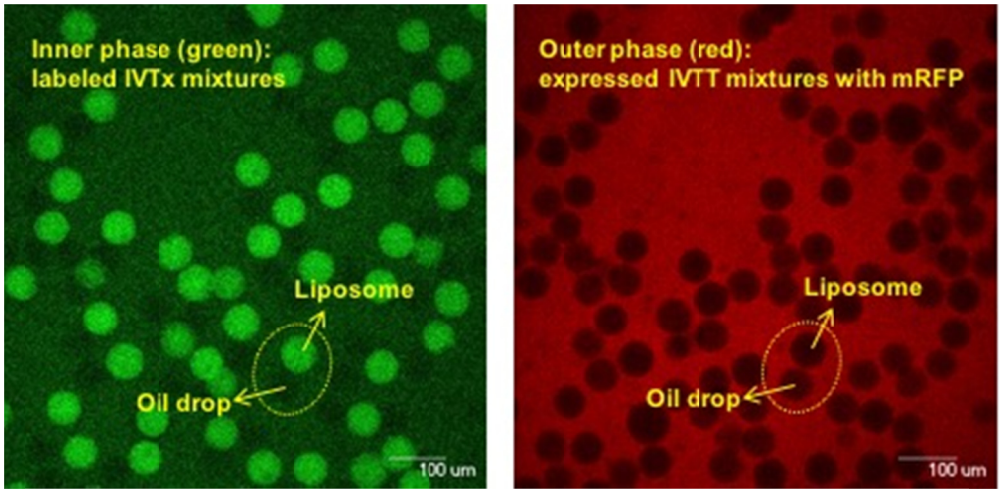

Figure S16. Confocal images show the liposomes prepared by using IVTx reaction mixtures and IVTT reaction mixtures as inner and outer water phases respectively.

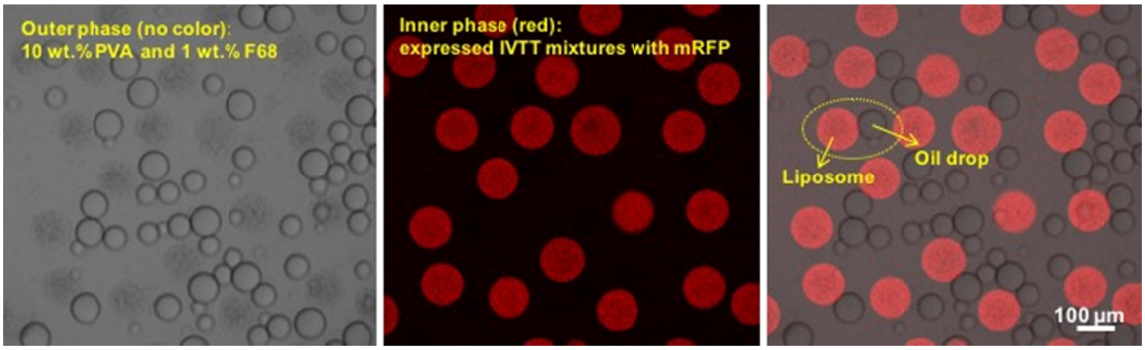

Figure S17. Confocal images of liposomes by using IVTT reaction mixtures (mRFP) and 10 wt.% PVA with 1.0 wt.% F68 as inner and outer phases respectively.

Part III. Supplementary Movies S1-S5

Movie S1. Reinjection of liposomes into the microfluidic device.

Movie S2. Microfluidic preparation of W/O/W emulsion droplets loaded with single liposomes.

Movie S3. Microfluidic preparation of W/O/W emulsion droplets loaded with two liposomes.

Movie S4. *In vitro* transcription and real-time detection of RNA in liposomes.

Movie S5. Insertion of nanopores into the bilayers of nucleus liposomes to transport fluorescent molecules.

Part IV. Supplementary References

(1) Utada, A. S.; Lorenceau, E.; Link, D. R.; Kaplan, P. D.; Stone, H. A.; Weitz, D. A. *Science* **2005**, *308*, 537-541.
(2) Chu, L.-Y.; Utada, A. S.; Shah, R. K.; Kim, J.-W.; Weitz, D. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 8970-8974.
(3) Hansen, M. M. K.; Ventosa Rosquelles, M.; Yelleswarapu, M.; Maas, R. J. M.; van Vugt-Jonker, A. J.; Heus, H. A.; Huck, W. T. S. *ACS Synthetic Biology* **2016**, DOI: 10.1021/acssynbio.6b00010.