Facile and Programmable Capillary-Induced Assembly of Prototissues via Hanging Drop Arrays

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ABSTRACT: An important goal for bottom-up synthetic biology is to construct tissue-like structures from artificial cells. The key is the ability to control the assembly of the individual artificial cells. Unlike most methods resorting to external fields or sophisticated devices, inspired by the hanging drop method used for culturing spheroids of biological cells, we employ a capillary-driven approach to assemble giant unilamellar vesicles (GUVs)-based protocells into colonized prototissue arrays by means of a coverslip with patterned wettability. By spatially confining and controllably merging a mixed population of lipid-coated double-emulsion droplets that hang on a water/oil interface, an array of synthetic tissue-like constructs can be obtained. Each prototissue module in the array comprises multiple tightly packed droplet compartments where interfacial lipid bilayers are self-assembled at the interfaces both between two neighboring droplets and between the droplet and the external aqueous environment. The number, shape, and composition of the interconnected droplet compartments can be precisely controlled. Each prototissue module functions as a processor, in which fast signal transports of molecules via cell−cell and cell-environment communications have been demonstrated by molecular diffusions and cascade enzyme reactions, exhibiting the ability to be used as biochemical sensing and microreactor arrays. Our work provides a simple yet scalable and programmable method to form arrays of prototissues for synthetic biology, tissue engineering, and high-throughput assays.

KEYWORDS: giant unilamellar vesicles (GUV), droplet interface bilayer (DIB), prototissues, wetting, capillary, cell aggregation, cell-environment communication

Living tissues are functional multicellular structures with spatially organization of specialized cells, which communicate with each other and exhibit higher-order collective behaviors within interactive environments. 1,2 To bridge the gap between nonliving matter and living organisms, bottom-up approach has been adopted to create artificial tissue-like constructs by assembling multiple protocells. 3−9 The synthetic biology approach deepens understanding of intercellular mechanism in tissues, and also breeds bioinspired materials and microengineered devices. 10−13

Protocells based on vesicles, 8,14−20 proteinosomes, 11,21−23 capsules, 24 and water-in-oil droplets, 2,5,25−28 assemble to form prototissues with spatial architectures, such as spherical clusters and planar aggregates. Most efforts have been centered to assemble vesicles. 24−15,29−35 Vesicles with a cell-mimicking lipid bilayer membrane can aggregate and hemifuse, through which the cell−cell communications can be regulated. For example, by means of a microfluidic device, multicore lipid-stabilized water-in-oil-in-water (W/O/W) emulsion droplet can be generated and then dewetting of its aqueous and oil phases gives rise to assembled lipid vesicles. 36 However, high-throughput production of prototissue arrays with programmable architectures remains unresolved. Recently, breakthrough reports have shown that a large scale of prototissue arrays can be spatiotemporally constructed through the use of external fields, such as acoustics 17 and magnetic fields. 15,16 For
example, magnetic and acoustic approaches allow for the generation of arrays of spatially programmed architectures of GUVs. In magnetic manipulation, the dissolution of a high concentration of toxic MnC in aqueous medium for its paramagnetic property is required, which can be undesirable for applications that involve bioactive molecules. In acoustic trapping, GUVs can be spatially grouped together by leveraging standing waves, but this necessitates the establishment of a density gradient of sucrose and glucose across the vesicle membrane, which effectively generates an acoustic contrast between the vesicles and the aqueous surroundings, enabling the trapping process. Therefore, integrating vesicles into structured assemblies with tight cell–cell conjunctions in a controlled yet mild manner that facilitates subsequent cell–cell and cell-environment communications still remains a considerable challenge.

In addition to vesicle-based prototissues, prototissues can be constructed by covalently cross-linking two different types of proteinosome-based protocells into spherical clusters. They exhibit astonishing reversible contractions mediated by enzymes, which have important implications for mechanochemical transduction. The controllable fabrication of such prototissues is later demonstrated by using a microfluidic platform, where the number and type of linked protocells can be tuned. Alternatively, lipid-laden water-in-oil droplets can be versatilely three-dimensionally printed into networked tissue-like materials, where droplets are connected via adhesive droplet interface bilayers (DIBs). These water-in-oil droplet-based protocells demonstrate cell–cell communications mediated by membrane protein, light, and electrical signals. However, since DIBs are operated in bulk oil phase, communication between cell and bulk aqueous environment is hindered in such prototissues.

In this work, inspired by the hanging drop method employed for culturing cell spheroids, we describe a facile, scalable, and programmable capillary-driven assembly of GUV-based protocells into colonized prototissue arrays. Each prototissue in arrays has controllable compositions and 3D architectures in
which cell−cell and cell-environment communications are gated by lipid bilayers. We use a microfluidic platform to generate water-in-oil-in-water (W/O/W) double emulsion droplets, where lipids are self-assembled at water/oil interfaces. The oleic shell of each double emulsion wets an oleophilic coverslip and merges into a bulk oil phase, resulting in the encapsulation of multiple water droplets. Subsequently, these droplets are packed together by an attractive capillary force and are interconnected by interfacial lipid bilayers. Thus, signals can be transmitted among these droplets. In contrast to traditional DIBs where signals are screened by surrounding oil phase, the DIBs formed in our method are able to exchange substance with an external aqueous environment, realizing both cell−cell and cell-environment communications. We demonstrate that both the number and the type of protocells within each prototissue as well as the spatial organizations of prototissue arrays can be precisely controlled. Cell−cell and cell−environment communications have been demonstrated via cascade enzyme reactions within resultant prototissues.

Although we use microfluidics for uniform vesicle colonies as prototissues that are otherwise unattainable, the capillary-induced assembly approach can also be used for polydisperse vesicles as building blocks. Because of the modularity of such arrays, they can be used for biochemical sensing and microreactor arrays. Our work provides a simple yet efficient method to form arrays of prototissues for synthetic biology, tissue engineering, and high-throughput assays. We envision such a method can be also engineered for producing hybrid cell/protocell spheroids as biomaterials and in vitro models.

RESULTS AND DISCUSSION

To produce monodisperse giant unilamellar vesicles (GUVs) for their manipulations, we use double-emulsion template method by microfluidics. Monodisperse water-in-oil-in-water (W/O/W) double emulsions are generated by a classic flow-focusing glass capillary device (Figure 1A and Movie S1). Both the shell thickness and the size of the double emulsion can be controlled by adjusting flow rates. Since all solvents in the shell are removed subsequently, the shell thickness does not affect the final size of lipid vesicles, and the size of the resultant lipid vesicle depends on that of the core droplet. In capillary microfluidics, the inner aqueous phase is 0.3 wt % Pluronic F-68 aqueous solution and the outer aqueous phase is 10 wt % poly(vinyl alcohol) (PVA) solution with 0.3 wt % Pluronic F-68 to match the osmolality difference between the inner and outer phases, thus inhibiting water diffusion across the middle solvent phase. The middle solvent phase is a mixture of chloroform and hexane (with a volume ratio of 36:64) with 5 mg mL⁻¹ phospholipid, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), which absorbs at the inner/middle and middle/outer water/oil interfaces.

The resultant double emulsion droplets are collected in a self-made polydimethylsiloxane (PDMS) cuboid container filled with deionized (DI) water. RBITC-dextran (red fluorescence) is used to show the spatial positioning of the core water droplets of double emulsions. Scale bars = 100 μm.
Since chloroform is more volatile than hexane, the middle phase is gradually chloroform-depleted and becomes a poor solvent for DOPC. As DOPC precipitates at the confined oil/water and water/oil interfaces, they stick to each other and self-assemble into a lipid bilayer membrane; meanwhile, the excessive hexane is expelled. Thus, as the good solvent chloroform starts to evaporate, a typical partial wetting morphology (dumbbell-like shape) is obtained. To visualize lipid vesicles, rhodamine B isothiocyanate-dextran (RBITC-dextran, red) is added in the inner aqueous phase to distinguish lipid vesicles from oil phase that is labeled by Coumarin 6 (C6, green) (Figure 1B). As green hexane droplets are lighter than red lipid vesicles, the green droplets float in the aqueous bulk phase (Figure 1B(ii)). Within 15 min, upon hexane evaporation, a complete dewetting pattern between red and green droplets can be formed. As hexane droplets are surrounded by the bulk aqueous solution, their evaporation is hindered such that these droplets are still visible in Figure 1B(ii) (indicated by green fluorescence).

Surprisingly, we observed an interesting phenomenon of collected W/O/W double emulsion droplets in a container sealed with an oleophilic coverslip at the top. After the droplets are collected, the coverslip is quickly put at the top of the open container to ensure that the dewetting process of the double emulsion is not completed. Then, the core–shell droplet floats to the top due to buoyance and its oil shell spreads onto the...
oleophilic coverslip, forming an oil raft encapsulating several water droplets, as indicated by the yellow dashed line in Figure 1C(i). Simultaneously, the core water droplets spontaneously self-assemble and pack together tightly (Figure 1C(i), Figure S1 and Movie S2). These closely packed droplets are suspended at the interface between the oil raft and the aqueous bulk solution (Figure 1C(ii)). The process of wetting of oil shell and subsequent self-assembly of several core water droplets is typically completed within seconds, and it may take a few minutes to assemble dozens of droplets. To determine how the surface hydrophobicity of the coverslip will affect the wetting and self-assembly process, we test several commercial coverslips (see Figures S2 and S3, and Table S1 in the Supporting Information). Our findings indicate that as long as the coverslip exhibits an oil contact angle of $<90^\circ$, the wetting and self-assembly of droplets are identical. During the dynamic self-assembly of lipid-laden double emulsions, lipid bilayer structures emerge at two distinct interfaces: one between the tightly packed water droplets and another between the water droplets and their aqueous surroundings. Both of them are important for communication and substance exchange among assembled lipid-laden droplets.

To illustrate the mechanism of spontaneous assembly, we monitored the entire process from droplet collection to aggregation (Figures 2A and 2B). After the double emulsions are generated and collected in the container, these core—shell droplets float on top of the container, because of their smaller density, compared to the bulk DI water (Figure 2B(i)). As we place an oleophilic glass coverslip on top of the container, the oleic shell of droplets instantly wets and spreads onto the glass, deforming the core—shell droplets and forming a spreading oleic raft (Figure 2B(ii)). The neighboring oleic rafts contact with each other and coalesce into a bulk oil phase, encapsulating more than one lipid-coated core droplet (Figure 2B(iii)). These aqueous droplets rest and hang on the interface between the oleic raft and bulk DI water. Inside the oleic raft, the encapsulated lipid-coating droplets are subjected to long-range capillary attractions, rendering them densely packed (Figure 2B(iv)).88–62 The key step is the wetting and spreading of oleic shells onto the coverslip, while the encapsulated lipid-coated core droplets are hung by the oil—water interface. As the oleic rafts spread onto the coverslip and contact with each other, the surface tension leads to their coalescence into a bulk oil phase (yellow region in Figure 2C(i)). The hanging lipid-coating droplets (blue in Figure 2C(ii)) will deform the oil/water interface, resulting in a radius of curvature $R_2$ that is much smaller than the radius of curvature $R_1$ in the peripheral region (Figure 2C(ii)). According to Young—Laplace equation, the pressures at different regions have the relationship:

$$P_0 - P_1 = \frac{2\gamma}{R_1}$$

$$P_0 - P_2 = \frac{2\gamma}{R_2}$$

where $\gamma$ is the interfacial tension, and $P$ with subscripts “0”, “1”, and “2” denote the pressure at different regions as shown in Figure 2C(ii). As $R_1 \gg R_2$, it yields $P_1 \gg P_2$. Therefore, the lateral pressure difference pushes the droplets to be tightly packed. Therefore, the wetting behavior between the oleic shells and the coverslip can be manipulated to form stable vesicle aggregations.

To verify whether the vesicle aggregation is triggered by the wetting between the core—shell droplets and the coverslip, we made the coverslip hydrophilic. Indeed, we coat the oleophilic coverslip with a PVA layer, and the aggregation of vesicles disappears (Figure S4). Therefore, if we treat a coverslip with variable wettability patterns, then the size and geometry of vesicle aggregations can be manipulated.

To control the size and geometry of the vesicle aggregations, we selectively modify the wettability of the glass coverslip in certain regions. We used PVA coatings to render the oleophilic coverslip hydrophilic. The oil contact angle of PVA coated coverslip is 126.17°, much higher than that of the uncoated one (49.96°; see Figure S5 in the Supporting Information). Then, we selectively coated the coverslip with PVA solution by a stamp imprinting method (Figure 3A). We microfabricated stamps with variable geometries by a CO$_2$ laser engraver. By dipping engraved stamps into PVA solutions, and then transferring the stamps onto the glass coverslip, upon drying, a hydrophilic coverslip with oleophilic domains of engraved patterns is obtained. Such a coverslip is placed on top of the container, and the vesicle aggregation appears only in the oleophilic domains, as indicated by the assembled droplets in the oleophilic domains and core—shell droplets in the hydrophilic domains, respectively (Figure 3A).

By changing the area of oleophilic domains, we can precisely control the number of aggregated vesicles can be precisely controlled. For instance, as we increase the area of oleophilic domains of the stamp, the number of aggregated vesicles grows from two to nine (Figure 3B). The number of assembled vesicles varies linearly with the area of the oleophilic domain, as shown in Figure 3C. The slope is $\sim5.71$ mm$^{-2}$, which is of the same magnitude as $1/S$, where $S$ is the cross-sectional area of a typical double emulsion. We explain that the area of the oleophilic domain determines the volume of the oil it can hold and, consequently, controls the number of double emulsions and aggregated vesicles. This process represents a simple way to regulate the assembled GUVs. Moreover, by changing the geometry of oleophilic domains from circular to rectangular, the aspect ratio of the vesicle aggregation colony can also be altered significantly. For instance, by designing oleophilic domains with dimensions of 0.25 mm $\times$ 0.8 mm, 10 vesicles are aggregated in two rows (Figure 3D). Multiple colonies of aggregated vesicles can be achieved by using stamps with a sudoku grid (Figure 3E). We also demonstrate that different populations of GUVs can be grouped into a single colony, where two species of GUVs containing RBITC-dextran and fluorescein isothiocyanate-dextran (FITC-dextran) are denoted by red and green fluorescences, respectively (Figure 3F). As shown in Figure 3F, some larger vesicles are surrounded by smaller ones. The presence of larger vesicles distorts the oil—water interface and gives rise to capillary attraction to smaller vesicles, forming the surrounding satellite structure of vesicle colonies. Size and composition of vesicles can be easily tuned by the microfluidic double-emulsion template method. In the preparation of double emulsions as shown in Figure 1A, we normally fix the flow rates of inner and middle phases and adjust the flow rate of outer phase such that size of the vesicle can be tunable: the larger the flow rate of outer phase, the smaller the diameter of vesicle. Furthermore, a user-defined water-soluble composition was fed into the inner phase. Macromolecules will be confined in formed vesicles due
to a barrier of the lipid bilayer. Consequently, these macromolecule-laden vesicles can be used as microreactors to achieve specific functions. Therefore, controlling the wetting behavior between droplet templates and a coverslip is a simple, scalable, and versatile method to precisely control the size and geometry of vesicle aggregations as microreactor arrays.

Cell–cell and cell–environment signaling and communication are vital for vesicle-based prototissues. To demonstrate cell–cell communication, we first investigated whether a lipid bilayer structure is formed between assembled GUVs (Figure 4). We use melittin to create nanopores within lipid bilayer membranes. These nanopores with characteristic size permit passage of smaller molecules while effectively restrict larger ones. We generate two species of GUVs, one containing calcein (Mw = 623 g/mol, green fluorescence) and another containing RBITC-dextran (Mw = 70,000 g/mol, red fluorescence) and melittin (2 μM). By assembling these two different populations of GUVs into a single colony by a coverslip, we observe that green fluorescent calcein diffuses to the surrounding GUV through the lipid bilayer, while RBITC-dextran remains in the original GUVs (see Figure 4A(ii), as well as Movie S3). In the absence of melittin, neither calcein nor RBITC-dextran molecules can transport between different species of GUVs (Figure 4B). These results demonstrate controlled cell–cell communication via melittin-induced nanopores within lipid bilayer structures formed between assembled GUVs.

Subsequently, for a proof of concept, we exploit a simple and well-established signal transduction pathway, the enzyme cascade reaction (Figure 5A), to demonstrate the successful processing of cell-environment and cell–cell communications of our prototissues. We prepare two populations of vesicles, which are “signal transmission module” and “signal processing module”, respectively. The red “Vesicle A” responsible for signal transmission is labeled by RBITC-dextran. Melittin, a toroidal pore-forming cationic peptide, is inserted in the lipid membrane of “Vesicle A”, which ensures the uptake of glucose from the external aqueous environment. Because of the lipid bilayer formed between two modules, environmental input can be transmitted from “Vesicle A” to nonfluorescence-labeled

Figure 4. Formation of a typical bilayer structure at the interface between two assembled GUVs. (A) Schematic (i), fluorescent microscopic images (ii), and a plot of fluorescence intensity against time illustrate the controlled signal transmission phenomenon via assembled GUVs with membrane protein (melittin) reconstitution. Melittin creates nanopores within lipid bilayer structures formed between two closely packed vesicles. Through these melittin-induced nanopores, the smaller green-fluorescent calcein molecules are able to pass through the nanopores, while the larger red-fluorescent RBITC-dextran molecules cannot. (B) Schematic (i), fluorescent microscopic images (ii), and a plot of fluorescence intensity against time illustrate no signal transmission phenomenon via assembled GUV without nanopores as a control group, where neither calcein nor RBITC-dextran can pass the lipid bilayer membrane formed between assembled GUVs. Scale bars are 100 μm and error bar denotes standard deviation.
“Vesicle B”. “Vesicle A” encapsulates glucose oxidase (GOx) and “Vesicle B” contains horseradish peroxidase (HRP). Meanwhile, during the preparation of “Vesicle A” and “Vesicle B” in the microfluidic device, o-phenylenediamine (o-PD) is added in the middle solvent phase as the report molecule. As such, glucose in the container is an input to trigger the cascade reaction. “Vesicle A” takes up glucose from an external aqueous environment and then oxidizes it to glucuronic acid by GOx, generating hydrogen peroxide (H_{2}O_{2}) as byproducts. Subsequently, via intervesicular transfer, H_{2}O_{2} diffuses into “Vesicle B”, initiating the HRP-catalyzed oxidization of nonfluorescent o-PD into 2,3-diaminophenazine (DAP), generating green fluorescence. Therefore, for vesicle colonies containing both “Vesicle A” and “Vesicle B” in the presence of melittin exhibits green fluorescence. Scale bars = 100 μm.

**CONCLUSIONS**

In summary, we constructed a prototissue array for processing chemical signals in a modular manner. Each prototissue based on GUV (giant unilamellar vesicle) aggregation in the array is formed within an oleophilic domain of a substrate. GUVs are prepared by a soft template method which uses a water-in-oil-in-water (W/O/W) double emulsion produced in a flow-focusing glass capillary microfluidic device. The double emulsions with lipid-coated aqueous core and oleic shell aggregation entities are entrapped in oleic rafts containing nonfluorescent o-PD. As a negative control, no fluorescence output is observed for the vesicle colony with “Vesicle A” and “Vesicle B” but without melittin (Figure 5B(ii)) and for the vesicle colony with only “Vesicle A” or “Vesicle B” (Figure 5B(iii) and 5B(iv)), while green fluorescence is observed for the vesicle colony with both “Vesicle A” and “Vesicle B” in the presence of melittin (Figure 5B(i) and Figure S6). Therefore, the vesicles aggregated by the capillary attraction exhibit elementary functions as basic prototissue models.
phases are collected in an open DI-water-filled container, which is covered by an oleophilic glass coverslip after collection. It is observed that the oleic shell of each floating double emulsion wets the oleophilic coverslip and merges into a bulk oil phase that contains multiple vesicles. These vesicles hang on the water/oil interface since their density is intermediate between those of the DI water and the bulk oil phase. Two neighboring vesicles deform the water–oil interface between them, inducing an attractive capillary force that can assemble them. As a result, these hanging vesicles are tightly packed to form a prototissue. Because of the formation of lipid bilayers among packed vesicles and between vesicles and bulk DI water, messages can be transmitted from the external environment (bulk DI water) to the internal prototissue. By varying the mixed population of double emulsions and the area of oleophilic domain of the glass coverslip, the number and composition of vesicle assembly can be well controlled. For instance, vesicles from two different populations with predetermined numbers can be assembled into a prototissue in a modular manner. By manipulating wettability domains on a substrate, a hanging array of nine multicompartmental processors that operate in an aqueous environment is demonstrated. Through incorporating melittin, a toroidal pore-forming cationic peptide, in the lipid bilayer, the intake of small-molecular signals from bulk aqueous environment can be transported and processed via vesicle compartments, which can finally output fluorescent signals. For instance, by combining different components of modular vesicles, we built two-compartmental processors that received and processed chemical signals and produced fluorescent outputs. Although we just demonstrate a single signal pathway, an instance, by combining different components of modular vesicles, we built two-compartmental processors that received and processed chemical signals and produced fluorescent outputs. However we demonstrate a single signal pathway, multiple signal pathways can also be activated and processed in such multicompartmental processor arrays in high throughput.

Our work provides a robust and versatile method to obtain modular prototissue arrays. It is inspired by the hanging method to assemble biological cells and does not rely on any external fields. The bottom-up prototissue array constructed in this study demonstrates the rapid transportation of molecular signals not only across multiple lipid bilayers among droplet compartments but also across lipid bilayers between the compartment and bulk external environment. The size-selective pores in lipid bilayers ensure the fast diffusion of small molecules while quiescenting large enzymes within compartments, triggering an organized cascade chemical reactions that can occur sequentially and orderly. These demonstrations help us validate effective communications within assembled GUVs and between GUVs and external environment, as a robust mimic of cell–cell and cell–environment interactions. Prototissue arrays can be efficiently produced with a high throughput by the proposed method and can be transported from one aqueous environment to another. These advantages may unleash its potential as a robust platform for a large variety of applications such as sensing and microreactor arrays. For example, the encapsulation of suitable signal reporters in different prototissues in the array would allow parallel processing in high throughput sensing and diagnostics. The multicompartment processor arrays might be useful for enhancing drug efficacy by integrating multiple biomarkers to produce outputs. Such a prototissue array can even be engineered for producing hybrid cell/protocol spheroids as biomaterials and in vitro models.

**METHODS**

**Fabrication of Microfluidic Device.** The device was assembled by two round and one square glass capillaries (World Precision Instrument, Inc.). The injection and the collection round capillaries were placed inside a square capillary. The inner and outer diameters of the round glass capillaries are 300 and 400 μm, respectively, and the inner diameter of square glass capillary is 400 μm. After being tapered by a micropipet puller (Sutter Instrument, Inc.), the injection and collection round capillaries were polished to the diameters of ~20–50 μm and 100–150 μm, respectively.

**Production of Double Emulsion.** To prepare stable W/O/W double emulsions, the inner water solution contains 0.3 wt % pluronic F-68 (Thermo Fisher Scientific) and was labeled by FITC-dextran (Mw = 150 000 g/mol, Sigma–Aldrich) or RBITC-dextran (Mw = 70 000 g/mol, Sigma–Aldrich) to better visualize vesicles observed under inverted fluorescence microscope (Eclipse Ti2, Nikon) and confocal microscope (AX/AX R, Nikon). To produce GUVs in microfluidics, we adopted the chemicals used in ref 36. The middle oil phase was a mixture of chloroform and hexane (Sigma–Aldrich) with a volume ratio 36:64, and contained 5.0 mg/mL 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids). The mixing ratio of chloroform and hexane can affect interfacial tensions among different phases and so does the formation of GUVs.36 DOPC is the one of the commonly chosen lipids to form vesicles.36 The outer water phase contained 10.0 wt % poly(vinyl alcohol) (PVA, Mw = 13 000–23 000 g/mol, 87%–88% hydrolyzed, Sigma–Aldrich) and 0.3 wt % pluronic F-68. PVA is used to improve emulsion preparation and stability.36 The inner, middle, and outer liquid phases were fed by the syringe pumps (Longer Pump, Inc.) into the microfluidic channel by plastic syringes connected by microtubings (Scientific Commod- ities, Inc.). Typical flow rates of the inner, middle, and outer liquid phases were 5, 10, and 30–180 μL/min, respectively. We increased the flow rate of the outer phase to decrease the diameter of droplets. A high-speed camera (Phantom V10.1, Vision Research, Inc.) was used to observe the generation of a double emulsion.

**Collection of Aggregated Vesicles.** After double emulsions were stably generated, they were then collected in a cubic polydimethylsiloxane (PDMS)-based container with a length of side of 12 mm. To realize aggregation of vesicles, a coverslip (20 mm × 20 mm, CITOTEST Inc., China with Product No. 80340-1630) was placed over the top of the container before the oil and water phases of the emulsion became completely dewetted. Then, the aggregation of the vesicle could be observed. Oleophilic coverslip can be used to assemble GUVs (Table S1 in the Supporting Information). The pristine coverslips provided by CITOTEST Inc., China, with no additional treatment, were used for the demonstration, unless otherwise specified.

**Patterning of Vesicle Arrays.** Wettability of the coverslip was important in triggering the aggregation of vesicles. In experiments, the coverslip without any treatment could aggregate vesicles. But the patterns were random. To design specific aggregation patterns, surface wettability treatments were required. Here, we used the solution containing 5.0 wt % poly(vinyl alcohol) (PVA, Mw = 13 000–23 000 g/mol, 87%–88% hydrolyzed, Sigma–Aldrich) to render the coverslip less oleophilic. In this way, the area coated with the PVA solution could not aggregate vesicles. Thus, specific aggregation patterns could be constructed by designing treated and untreated areas. We microfabricate stamps with variable geometries by a CO2 laser engraver. The stamp was dipped in PVA ink. Then we impressed the stamp on the coverslip and dried the coverslip. This coverslip was placed on top of the container after the container collected the vesicles. The aggregation of vesicles could be observed in the untreated area of the coverslip.

**Proof of Bilayer Structure Formed at Fused Site of Assembled GUVs.** Using a glass capillary microfluidic device, we fabricated two species of GUVs: one containing calcine (green fluorescence, Mw = 623 g/mol, Sigma–Aldrich) and the other containing red dye RBITC-dextran (red fluorescence, Mw = 70 000 g/mol, Sigma–Aldrich) and melittin (2 μM, Sigma–Aldrich). The
components were fed into the inner phases of the microfluidic devices. After formation of double emulsions, these two species of GUVs were assembled by collecting them in a container covered by an oleophilic coverslip (20 mm × 20 mm, CITOTEST Inc., China with Product No. 80340-1630). Melittin can form nanopores at the lipid bilayers of two neighboring GUVs. The smaller green fluorescent calcein molecules were able to pass through the nanopores, while the larger red-fluorescent RBITC-dextran molecules could not. The fluorescent images showing the process of molecular diffusion were collected by the software NIS-Elements AR 5.41 and the corresponding fluorescence intensities were analyzed by the software ImageJ 1.48 V. When we recorded fluorescent images by Nikon ECLIPSE Ti2, in the preference panel of the software NIS-Elements AR 5.41, the analog gains of green and red fluorescence were 20.9 and 38.4X, respectively, and the exposure time was 300 ms. In addition, the light intensity was set to be 100% in the controller of the mercury lamp (by choosing the setting “1” in Nikon C-HGFIE HG Controller).

Enzyme Cascade Reaction. To explore enhancement of communication between aggregated vesicles, we prepared two classes of vesicles denoted by “Vesicle A” and “Vesicle B”, which were used to demonstrate the enzyme cascade reaction. The “Vesicle A” contained 2 μM melittin (Sigma—Aldrich), 0.05 U/mL glucose oxidase (GOx, ≥10000 units/g, Sigma—Aldrich), and 10 μM RBITC-dextran (MW = 70 000 g/mol, Sigma—Aldrich), while the “Vesicle B” contained 0.05 U/mL horseradish peroxidase (HRP, ≥250 units/mg, Sigma—Aldrich). To validate the production of the cascade reaction, 0.5 mM o-phenylenediamine (o-PD, Sigma—Aldrich) was added into the oil phase (mixture of chloroform and hexane) to convert non-fluorescent o-PD into green-fluorescent 2,3-diaminophenazine (2,3-DAP). In the experiment, the “Vesicle A” and “Vesicle B” were collected in the same PDMS container, and a coverslip (20 mm × 20 mm, CITOTEST Inc., China with Product No. 80340-1630) was placed over the top of the container before the oil and water phases of the emulsion became completely dewetted. Note that o-PD is insoluble in hexane but is soluble in chloroform. During the experiment, chloroform barely evaporates from the oil phase owing to the presence of the covered coverslip such that the oil phase can still dissolve o-PD in subsequent observations. For a while after collection, we could observe aggregation of vesicles. Randomly, there were three cases: (1) aggregation of “Vesicle A” only; (2) aggregation of “Vesicle B” only; and (3) aggregation of “Vesicle A” and “Vesicle B”. Only in the last case there was green fluorescence. The fluorescent images showing the reaction outcomes were collected by the software NIS-Elements AR 5.41 and the corresponding fluorescence intensities were analyzed by the software ImageJ 1.48 V.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsnano.3c03516.

Fluorescent images of assembled GUVs; corresponding contact angles of different commercial coverslips; statistic data of GUVs assembly on different several commercial coverslips; nonassembled GUVs by collecting them in a container covered with a PVA-coated coverslip; measurement of contact angle under different scenarios; confocal microscopic images of each channel of GUVs aggregation colonies consisting of both “Vesicle A” and “Vesicle B” with the addition of melittin; comparisons of coverslips bought from different brands (PDF)

Movie S1: Generation of water-in-oil-in-water double emulsions by flow focusing capillary microfluidics (AVI)

Movie S2: Process of vesicle assembly in a PDMS container covered by an oleophilic coverslip (MP4)

Movie S3: Diffusion of calcein through nanopores formed at droplet interface bilayers (MP4)

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C. Qi, Z. Liu, and T. T. Kong conceived, designed, and supervised the project. X. D. Ma, J. F. Zhong and J. Y. Fang performed experiments. C. Qi, Z. Liu, and T. T. Kong wrote the manuscript. All authors analyzed the data and commented on the paper.

Notes
The authors declare no competing financial interest.

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