Manipulation of Cell-Sized Phospholipid-Coated Microdroplets and Their Use as Biochemical Microreactors

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Cell-sized water droplets coated by a phospholipid layer mimicking the inner surface of living cells were manipulated by laser tweezers and used as biochemical microreactors. The cell-sized phospholipid-coated microdroplets (CPMDs) consisted of a water droplet in mineral oil with a diameter of 1−100 μm and coated by 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine. We monitored the time development of biochemical reactions in a single CPMD obtained after the controlled fusion of two CPMDs containing a substrate and an enzyme, respectively. We present results on two enzymatic reactions: calcein production in the presence of esterase and green fluorescence protein expression.

Introduction

In nature, most biochemical reactions are realized in micrometric closed units, such as the cellular structure, in which the typical size of the core (1−100 μm) and the nature of the membrane are both of crucial importance. The cellular membrane consists of a bilayer composed mainly of phospholipids with various functional proteins embedded in the membrane bilayer.[1,2] Owing to their biological significance, phospholipid membranes have been extensively studied up to the present.[3−5] Mainly two types of artificial cell-sized microstructures have been studied: (i) vesicles and (ii) water microdroplets. Vesicles, closed membranes separating two water media, are called liposomes when the membrane is composed of lipids. Within the range of 1−100 nm in outer diameter, liposomes are mainly employed for transfection applications[6] and are not suitable candidates for cell-mimicking properties. In contrast, cell-sized liposomes (10−100 μm) have been actively studied as cell models due to their similarities with natural cell structures in terms of size and membrane composition.[7−12] However, manipulation of cell-sized liposomes is usually hampered by the difficulty in fusing desired vesicles or encapsulating biological macromolecules and their high sensitivity to osmotic or mechanical stresses.[13−16] On the other hand, microdroplets of water in oil have also been studied with an eye toward their use as cell models or microreactors.[17−21] Water-in-oil microdroplets can be formed through simple mixing of oil, water, and surfactant (e.g., Span 80, Tween 20) or by using microfluidics technology to obtain nearly monodisperse simple or multiple microemulsions.[22−25] Water-in-oil microdroplets have many advantages: they are easily manipulated or fused, they offer a good resistance to osmotic or physical stresses, it is possible to conduct experiments under physiological ionic conditions, and molecules of interest (e.g., biological macromolecules) can be easily encapsulated without denaturation. Various biochemical reactions have been successively conducted in water-in-oil microdroplets, such as green fluorescence protein (GFP) expression[17] or polymerase chain reaction.[18] However, since, in this case, the composition of the water/oil interface (mainly non-phospholipid surfactants such as Tween 20, Span 80, or Triton X-100) is very different from that of a cell membrane, the use of water-in-oil microdroplets for studies on artificial cells is somewhat limited. Therefore, in order to combine the advantages of cell-sized liposomes (the presence of a phospholipid membrane) and water-in-oil microdroplets (facile manipulation), we recently developed cell-sized phospholipid-coated microdroplets (CPMDs) as a new type of cell-sized microstructure.[26] A CPMD consists of a water-in-oil droplet with a phospholipid layer present at the oil/water interface. Such CPMDs may be suitable models of living cells, since phospholipid molecules are arranged on the surface with their hydrophilic moieties oriented toward the inner aqueous phase, as in the cytoplasmic membrane. As we have shown recently, CPMDs can also be used as a

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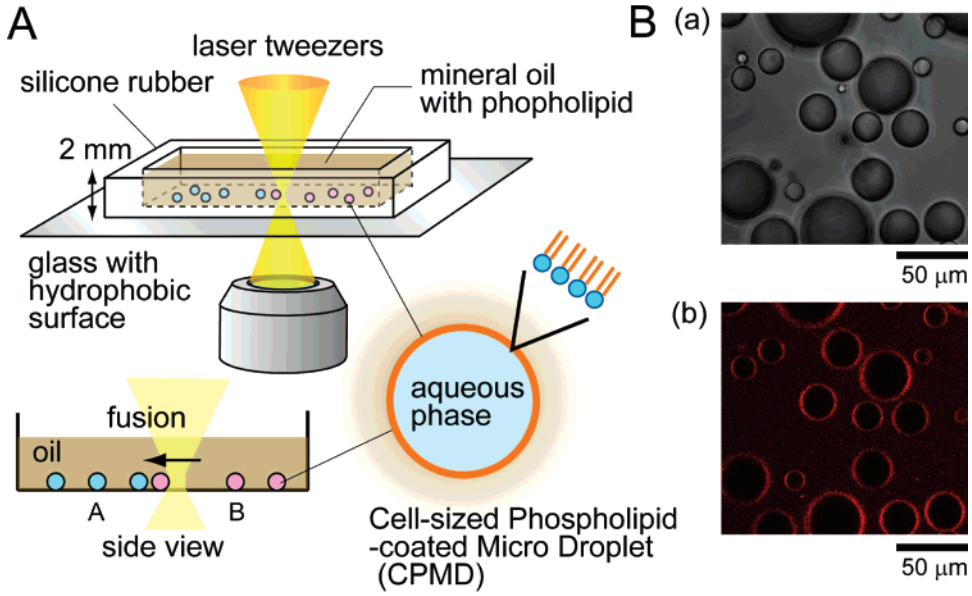

Figure 1. (A) Experimental setup. CPMDs dispersed in oil are contained in a chamber made of silicon rubber on a hydrophobic microscopy glass slide. CPMDs are observed by microscopy and manipulated by laser tweezers through the glass slide. Side view: a CPMD with an inner aqueous compartment of composition B can be fused to a CPMD with an inner compartment of composition A by using the repulsive force from the focus point of laser tweezers. (B) Typical microscopy images of CPMD observed by transmission (a) and fluorescence (b) microscopy. For this experiment, we used 0.5 mM DOPE phospholipid containing 0.1% Texas Red DHPE fluorescent phospholipid.

precursor to produce liposomes.[27] In this letter, we describe the manipulation of CPMDs by using laser tweezers and the time-monitoring of biochemical reactions in a single CPMD obtained after the controlled fusion of two CPMDs containing a substrate and an enzyme, respectively. Two examples of biochemical reactions are presented: calcein production in the presence of esterase and GFP expression.

Experimental Section

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), calcein acetoxymethyl ester (calcein-AM), and Fura 2 were purchased from Sigma. Mineral oil was obtained from Nacalai Tesque. The fluorescent phospholipid, Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Texas Red DHPE) was obtained from Molecular Probes. For the experiment on cell-free gene expression, a PROTEINscript-PRO Kit, which included *E. coli* S30 extract, T7 RNA polymerase, Master Mix, and amino acids excluding L-methionine, was purchased from Ambion. L-Methionine was purchased from Nacalai Tesque and Plasmid pQBI T7 DNA (5115 bp) encoding a T7 promoter and an rsGFP gene was obtained from Nippon Gene as template DNA. We used nuclease-free water from Otsuka Pharmaceutical Factory for the gene expression experiment.

Microscopy and Laser Tweezers. We used a Nikon TE300 microscope and a Hamamatsu Photonics EBCCD camera and performed the measurement at ambient temperature (around 20 °C). For the observation of GFP, we used an Olympus IX-70 microscope and an Andor iXon DV887 EMCCD camera equipped with a temperature controller (TOKAI HIT microscope incubation system). For laser tweezers, a Nd:yttrium-aluminum garnet (YAG) laser (SL902T, Spectron) with a TEM_{00} beam at a wavelength of 1064 nm was used.

CPMD Preparation. CPMDs were prepared as follows. First, DOPE was dissolved in mineral oil by ultrasonication for 90 min at 50 °C and used within 24 h. NMR analyses demonstrated that the phospholipid molecules were not damaged during the ultrasonication treatment. The concentration of DOPE was 1 mM for all experiments presented here except for the microscopy image of Figure 1 (0.5 mM). Then, 2 μL of the aqueous solution of interest (water, water + enzyme, etc.) was added to 200 μL of mineral oil containing DOPE in a test tube and mixed by pipetting up and down for ~2 min, which resulted in the formation of CPMDs dispersed in the oil phase.

Observation and Manipulation of CPMDs. Right after preparation, the oil phase containing CPMDs was transferred to an observation chamber, as illustrated in Figure 1A. This chamber (10 × 10 × 2 mm) was carved in silicon rubber and glued on a hydrophobic microscopy glass slide (Matsunami). The use of an hydrophobic surface was essential to prevent CPMDs from sticking and spreading on the surface. Such a chamber allowed for real-time microscopy observation of CPMDs and their manipulation by laser tweezers. The repulsive force generated from the focus point of the laser tweezers was used to displace a desired CPMD to a desired place. In particular, we used this methodology to induce the fusion between a CPMD of inner composition A (e.g., a substrate) with another CPMD of inner composition B (e.g., an enzyme).

Results and Discussion

Figure 1B shows typical transmission and fluorescence microscopy images of CPMDs formed in the presence of DOPE containing 0.1% Texas Red DHPE fluorescent phospholipid. First, it can be noticed that, for all the microdroplets observed, the fluorescence intensity is significantly larger at the microdroplet surface than in the bulk medium, which suggests that most of the fluorescent phospholipids are present at the surface of the CPMDs. Since the Texas Red DHPE concentration was 1000 times lower than DOPE, it is expected that DOPE molecules surround the CPMD surface and eventually form a layer with hydrophilic moieties oriented toward the inner aqueous compartment. Moreover, the diameter of the thus obtained CPMDs were typically within the range of 5−100 μm, which corresponds well with typical eukaryotic cell dimensions. As indicated in Figure 1B, CPMDs obtained by our simple mixing procedure are polydisperse. When control of the size is necessary, it would be more appropriate to use more elaborate procedures to control interface formation, such as microdroplet generation by microfluidics technology.[22] In the present study, we used DOPE as a phospholipid because it is known to be a major component

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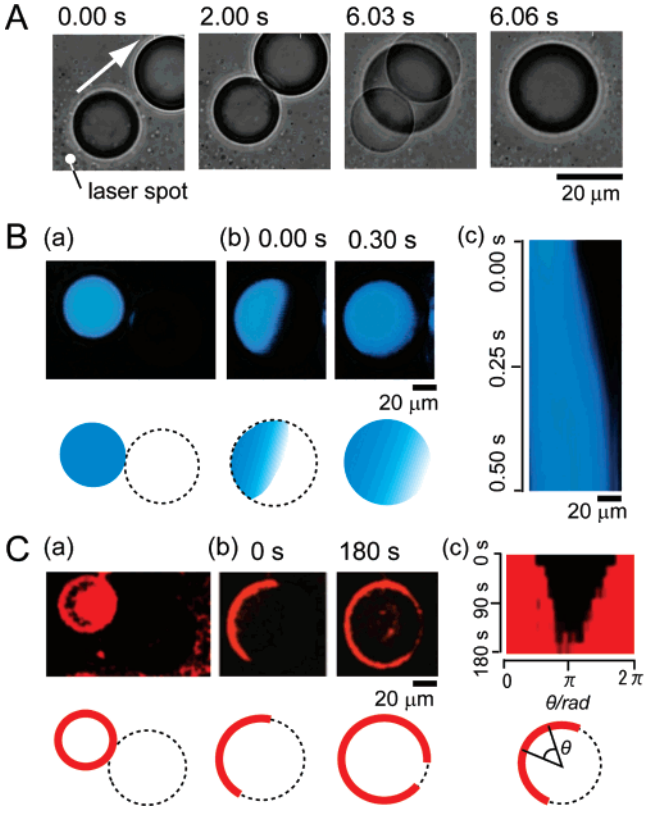

Figure 2. (A) Phase contrast images of the fusion of two CPMDs by the use of laser tweezers. The white dot indicates the position of the laser focus point, and the white arrow indicates the direction of the pushing force at $t = 0$ s. The forced contact is initiated at $t =$ 1.8 s. At $t = 2$ s, the two CPMDs are still in contact; at $t =$ 6.03 s, the two CPMDs coalesce, and the images before and after coalescence overlap, which indicates that fusion is completed within the video frame rate (30 ms); at $t = 6.06$ s, the resulting CPMD is formed after fusion. (B) (a,b) Fluorescence microscopy images (top) and schematic illustrations (bottom) of the fusion of two CPMDs containing 50 μM Fura 2 fluorescent dye and pure water, respectively: (a) before fusion, the CPMD containing Fura 2 (left) was placed in contact with the CPMD containing water (right); (b) after fusion, Fura 2 diffuses at $t = 0$ (fusion) and $t = 0.3$ s in the resulting CPMD. (c) Spatio-temporal image of the centerline of the resulting CPMD, where $t = 0$ corresponds to the fusion of the two initial CPMDs. (C) Fluorescent microscopy images (top) and schematic illustrations (bottom) of the fusion of the two CPMDs with and without Texas Red DHPE fluorescent phospholipid (1% of DOPE), respectively: (a) before fusion; (b) diffusion of Texas Red DHPE on the resulting CPMD surface at $t = 0$ (fusion) and $t = 180$ s. (c) Spatio-temporal image of the circular arc of the CPMD. For all experiments, the initial DOPE concentration in the oil phase was 1 mM.

of the inner cytoplasmic membrane.[28] However, it has been reported that DOPE usually forms an inverted hexagonal structure and does not form stable vesicles in aqueous solutions under usual physiological solution conditions.[29] To the best of our knowledge, no previous studies have successfully used DOPE to form a model membrane system with an inner water compartment and a typical size within the range of 1−100 μm. Thus, our report describes a new useful methodology for preparing a cell-sized model membrane system containing DOPE.

Figure 2A illustrates the typical procedure used to fuse two CPMDs, where the droplet in the lower left-hand corner is pushed toward the droplet in the upper right-hand corner by using laser tweezers (frame $t = 0$ s). In this experiment, forced contact was initiated at $t = 1.80$ s, and the two droplets stayed in contact for 4.23 s (frame $t = 2$ s) prior to fusion. The fusion was observed at $t = 6.03$ s, and the entire fusion process was completed in less than 30 ms, that is, within the video frame-rate (frame $t = 6.03$ s). The volume of the new fused CPMD (frame $t = 6.06$ s) was essentially the same as the sum of those of the two original CPMDs (frame $t = 0$ s). In order to qualitatively characterize the mixing of the inner compartments after fusion, we used the procedure described in Figure 2A to fuse microdroplets containing a fluorescent dye and pure water, respectively. For all droplet sizes studied within the range of 1−100 μm, we observed that the homogenization of the fluorescent dye concentration in the resulting CPMD required less than 1 s after fusion. Figure 2B shows a typical fluorescence microscopy observation before (a) and after (b) the fusion of two CPMDs containing 50 μM Fura 2 fluorescent dye and water, respectively, together with a spatio-temporal representation of the centerline of the resulting CPMD. It indicates that, in this case, the homogenization of Fura 2 concentration in the resulting CPMD was reached after approximately 0.3 s. Furthermore, we observed the diffusion of the lipids within the surface before and after the fusion. For this purpose, by using the technique described in Figure 2A, we fused CPMDs with CPMDs containing Texas Red DHPE fluorescent phospholipid. Figure 2C shows typical fluorescence microscopy images before (a) and after (b) the fusion of two CPMDs where the CPMD of the left was labeled by Texas Red DHPE (1% of 1 mM DOPE). Right after fusion ($t = 0$ s), one observes that the fluorescence emission coming from Texas Red DHPE is first concentrated on the left hemisphere of the resulting CPMD. Then, the front of the fluorescence can be visualized as it diffuses on the CPMD surface. The fluorescence intensity is homogeneously distributed on the whole CPMD surface after approximately 3 min. These observations show that phospholipid molecules are constantly reorganizing on the CPMD surface, as is the case in the membrane of living cells. It also indicates that the CPMD obtained after fusion is covered by a phospholipid layer essentially similar to that of CPMDs before fusion. It should also be mentioned that the characteristic time of phospholipid diffusion in the CPMD membrane is on the order of a few minutes, which is a significantly larger value compared to the characteristic time for a two-dimensional Brownian diffusion in the membrane[30] (∼10 s for the fused CPMD) and experimental results reported on cell-sized liposomes (∼7 s).[15] This increase in diffusion time might be explained by the viscosity of the oil phase used for this study,[31] which was measured to be approximately 13 times larger than that of water. Therefore, all these observations show that the coalescence of two desired CPMDs can be easily obtained through mutual contact by the use of laser tweezers. In contrast, it has been reported that it is rather difficult to produce the fusion of two cell-sized liposomes, such as giant vesicles. To fuse giant vesicles, chemicals, such as surfactants or multivalent ions, are usually added to the vesicle solution to destabilize the bilayer.[32,33] The other known method for vesicle fusion is the application of a rather strong electric field pulse.[16] However, with these methodologies, it is practically difficult to fuse a desired pair of vesicles. In contrast, we showed here that the fusion of two desired CPMDs with specific compositions of the inner compartments can be easily achieved. Moreover, we would like to address the benefits of a CPMD as

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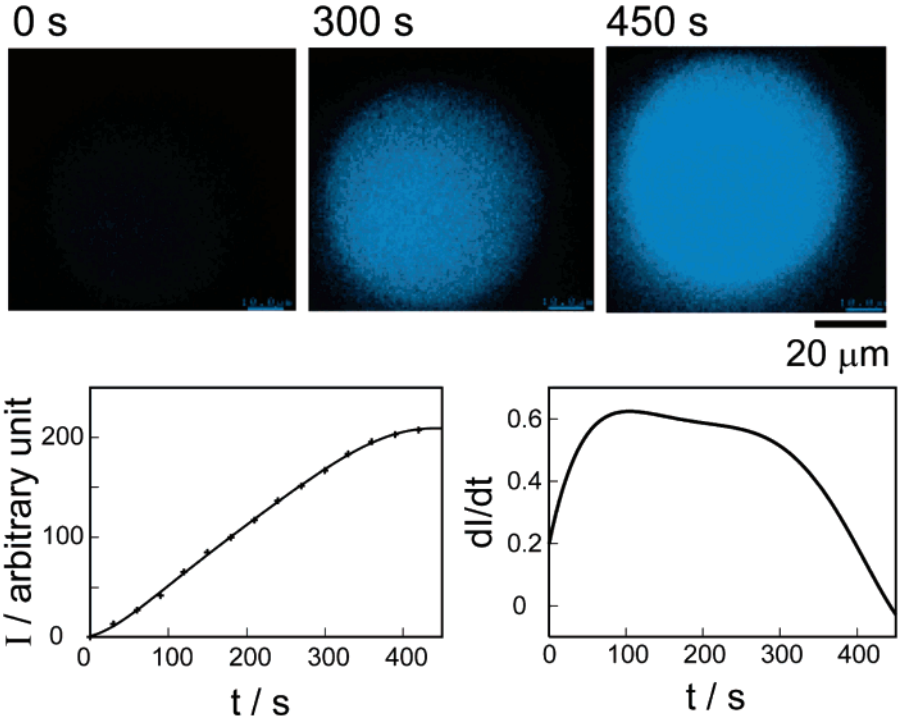

Figure 3. Production of fluorescent calcein in a single CPMD obtained from the fusion of two CPMDs containing 50 μM calcein-AM and 2.5 μg/mL esterase, respectively. Top: fluorescence microscopy images of the resulting CPMD as a function of time. Bottom: fluorescence intensity I (left) and the time derivative dI/dt as a function of time t. $t = 0$ corresponds to the fusion of the two initial CPMDs.

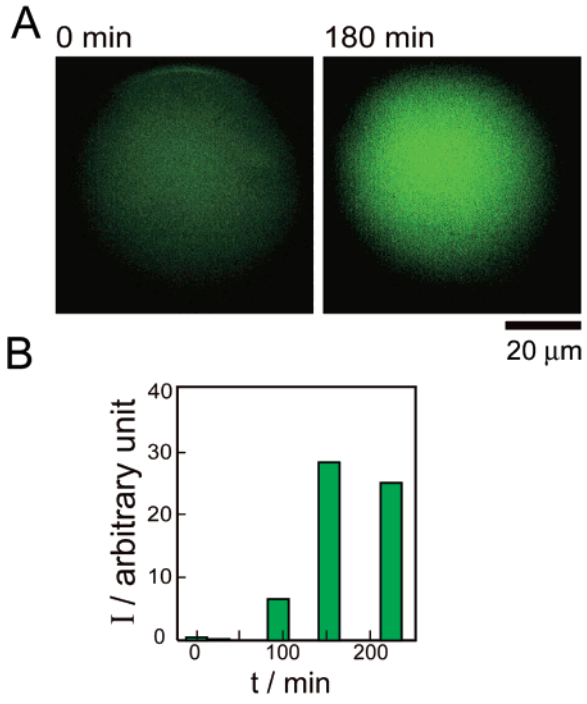

Figure 4. Production of GFP protein in a single CPMD obtained from the fusion of two CPMDs containing DNA (0.25 μg/μL pQBI T7 plasmid DNA) and expression medium (*E. coli* S 30 extract, Master Mix, 100 μM each amino acid, and 2.5 U/μL T7 RNA polymerase) at 37 °C. $t = 0$ corresponds to the fusion of the two initial CPMDs. (A) Fluorescence microscopy images of the resulting CPMD at $t = 0$ and $t = 180$ min. (B) Fluorescence intensity I as a function of time.

a small biochemical reactor. However, one usual problem with the use of microreactors is the mixing of reactants, which cannot be achieved quickly by conventional methods due to the confinement (no convective mixing). For instance, the characteristic diffusive time τ for molecules in water with a diffusion coefficient $D \sim 10^{-10}$ m^2·s^{-1} typically ranges from ~1 to ~100 s when the characteristic size varies between 10 and 100 μm. Our experiments showed that the mixing was much faster and obtained in a time significantly shorter than 1 s for comparable sizes (e.g., Figure 2B). This rapid mixing within the fused droplet is an important advantage and might be explained by the presence of recirculating flows generated in the fused CPMD, in agreement with previously reported observations on microdroplets.[24]

To examine further the applicability of a CPMD as a microscopic biochemical reactor, we performed model biochemical reactions induced by the fusion of two CPMDs containing a substrate and an enzyme, respectively. First, we studied the reaction between the enzyme esterase and calcein-AM as a substrate, in which the release of the acetoxymethyl (AM) group from calcein-AM can be followed by the fluorescence emission of the product calcein. Although a full kinetic analysis would require taking into account the effects of a small leakage of dye or photobleaching,[12] we will consider here only the fluorescence intensity as a function of time, which can give a semiquantitatively good description of the time course of the enzymatic reaction. Figure 3 shows fluorescence microscopy images, the fluorescence intensity I, and the derivative dI/dt as a function of time of a single CPMD obtained from the fusion of two CPMDs containing 50 μM calcein-AM and 2.5 μg/mL esterase, respectively. First, it demonstrates that the production of calcein is localized inside the resulting CPMD. Moreover, it shows that the fluorescence intensity associated with calcein concentration increases with time. Furthermore, the derivative of the intensity dI/dt associated with reaction rate clearly presents three parts. The initial increase in dI/dt may be attributed to the formation of the substrate and enzyme complex, which can be observed since the characteristic mixing time is less than 1 s after CPMD fusion, as discussed before. Then, it is followed by a region where the reaction rate is almost constant, which may indicate that the concentration of the enzyme−substrate complex is almost constant. Finally, the decrease in reaction rate may be

attributable to a deficiency of the reaction substrate. All these kinetics features are typical of enzymatic reactions. Therefore, it is clear that a CPMD can be used as a micro-biochemical reactor, with no apparent inhibitory effect on the enzyme activity. As a second example of biochemical reaction, we followed the production of GFP in a single CPMD obtained from the fusion of two CPMDs containing DNA (0.25 μg/μL pQBI T7 plasmid DNA) and expression medium (*E. coli* S 30 extract, Master Mix, 100 μM each amino acid, and 2.5 U/μL T7 RNA polymerase[34]), respectively. The temperature was controlled at 37 °C. Fluorescence images of Figure 4 show that GFP is actually produced inside the resulting CPMD. The fluorescence intensity associated with GFP production increases progressively and reaches a maximum after approximately 120 min. The further decrease in fluorescence intensity may be attributed to spontaneous degradation.

Conclusion

In this letter, we showed that CPMDs can be easily manipulated and used as useful microreactors for biochemical reactions. CPMDs combine the advantages of cell-sized liposomes (the presence of a membrane) and microdroplets (facile manipulation). In particular, CPMDs can be fused by the use of laser tweezers to induce a biochemical reaction of interest, and CPMDs can also serve as simple models of living cells. The new experimental methodology reported here may contribute to a deeper understanding of the mechanism of biochemical reaction networks in the microscopic environment of living cells.

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