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Spontaneous and Driven Growth of Multicellular Lipid Compartments to Millimeter Size from Porous Polymer Structures**

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This report describes a method to obtain multicellular shaped compartments made by lipids growing from a sponge-like porous structure. Each compartment is several tens of micrometers in diameter and separated by membranes comprised of phospholipid and amphipathic molecules. The multi-compartment structure spontaneously grew to a millimeter scale, driven by an ionic concentration difference between the interior and exterior environments of the sponge. These compartments can also easily incorporate hydrophilic species as a well as smaller materials such as liposomes. Additionally, we showed that mechanical squeezing of the sponge was also effective in producing multicellular bodies. These simple methods to obtain large-scale multicellular compartment of lipid membrane will help future designs and trials of chemical communications on artificial cells.

Introduction

With the rapid development in our understanding of chemistry and biochemistry, there have been many reports on artificial (synthetic) cells designed based on the self-assembly of biomolecules to mimic the structure of living cells.[1,2] Many of the studies use giant liposomes (spherical closed lipid membranes) as independent units for encapsulation of functional contents.[3] The essential molecular systems of living cells, such as gene expression,[4,5] evolution,[6] photosynthesis,[7,8] replication,[9,10] have been studied to emulate their function in a bottom-up manner. There have also been reported liposome-based structures containing artificially designed molecular systems which aims to introduce information processing circuits not used by natural cells.[11,12] Just as organisms in nature have evolved from unicellular to multicellular, research on multi-compartmentalization of artificial cells has received much attention.[13,14] There have been reports on the adhesion of post-formed liposomes,[15,16] multicompartment emulsions containing drugs by centrifugal sedimentation,[17] 3D-printing injection molding of water-in-oil emulsion droplets,[18] and re-exposure of the structure to water to form lipid bilayers.[19] These methods typically require sophisticated machinery such as specialized microfluidic channels, modified 3D printers, etc. If macroscopic multicellular bodies could be prepared under simpler physiological conditions, it would accelerate the study of molecular communication between compartments, complex information processing, and demonstrations used in macroscopic field. Inspired by the formation of soap bubbles from macroscopic sponges, we describe here a simple method for spontaneously generating multicellular lipid-based compartments with millimeter scale dimensions from sponge-like porous gels.

Results and Discussion

For forming multi-cellular lipid compartments, lipid solutions containing phospholipid (DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and the commercial food-grade surfactant polyglycerol-polyricinorate (PGPR) were used as the basis to form the amphiphilic membrane structure. Porous silicone-based marshmallow-like gel (MG) sponges were prepared according to previous reports.[20,21] Figure 1a outlines the procedure used to generate multicellular lipid-based compartments, firstly MG sponges were soaked with the lipid solution (DOPC and PGPR dissolved in chloroform) and subsequently allowed to dry. An aqueous inner buffer was then added to the lipid coated MG-sponge without oversaturating, once fully absorbed, the inner

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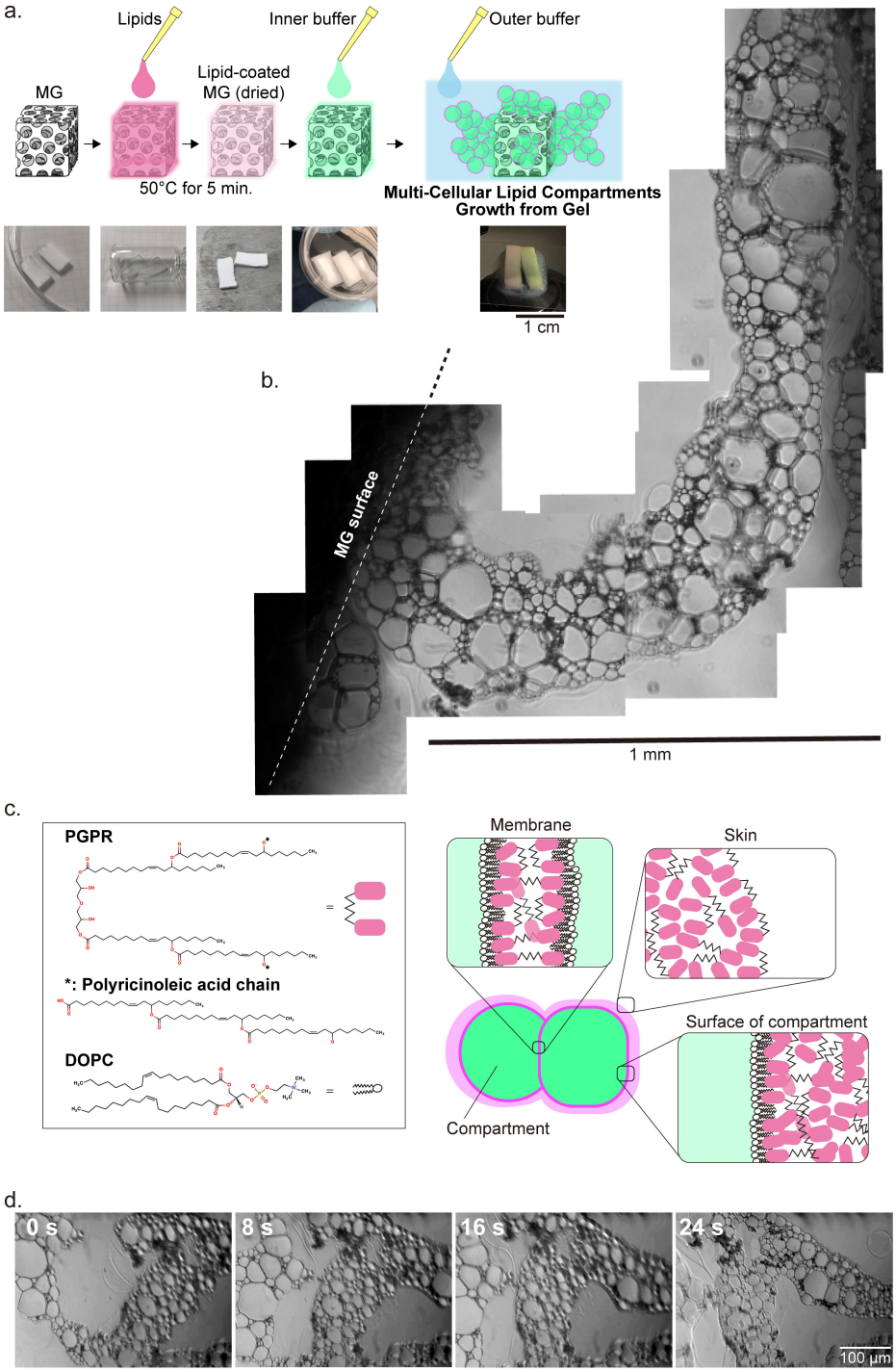

Figure 1. Growth of multicellular lipid compartments (MCLCs) swelling from marshmallow-like gels (MGs). a) Scheme of MCLC preparation. b) Phase-contrast microscopy image of MCLCs growing from MG surface (composed image). c) Schematic of MCLC structure. d) Time-lapse image showing the formation of MCLC flowing outward from the MG.

buffer loaded MG-sponge was then placed in a dish containing the aqueous outer buffer solution. Within a few minutes, the spontaneous growth of multi-cellular lipid compartments (MCLC) was observed, with sizes exceeding 1 mm (Figure 1b, Movie S1). The compartment sizes were not uniform, but the majority had diameters larger than 100 µm, interestingly this was larger than the microporous structure of the MG-sponge (typical pore size up to 10 µm, Figure S1). Figure 1c shows the molecular structures DOPC and PGPR. The growth of MCLC continued for about 50 minutes gradually expanding out from the sponge (Figure 1d, Movie S2).

To understand the molecular structure of the compartments, MCLC was formed with addition of DiO (hydrophobic fluorescent dye, 3,3′-dioctadecyloxacarbocyanine perchlorate) in the lipid solution, and calcein (hydrophilic fluorescent dye, bis[*N, N* − bis(carboxymethyl)aminomethyl]fluorescein) in the inner buffer (Figure 2a, b). As a result, the hydrophobic lipid-based membranes and the hydrophilic aqueous internal compartments could be easily distinguished by fluorescence microscopy. The structures were regarded as clusters of water-in-oil-in-water emulsions that are in contact with each other. The compartments are separated by a lipid-based membrane formed from PGPR and DOPC, resulting in structures which appear similar to the hemifused intermediate structures formed as liposomes coalesce together.[22] Unlike typical unilamellar lipid bilayers, with addition of α-hemolysin no connecting pores were formed in the MCLC from which we infer that the Phospholipid-PGPR hybrid membrane is thicker pure phospholipid membranes (Figure S2). By mixing hydrophilic materials such as small liposomes (1 μm in diameter) in the inner buffer before absorbing into the MG-sponge, the resulting multicellular compartments included the liposomes inside (Figure 2c, Movie S3).

We examined the conditions for obtaining MCLC growth. The key features in formation were found to be the difference in ion concentration (specifically the difference NaCl concentration) between the inner and outer buffers, and the ratio between DOPC and PGPR in the lipid solution (and therefore adsorbed on the MG-Sponge), the optimal conditions for MCLC formation were obtained by the mixing ratio of the PGPR with DOPC for the lipid solution and the NaCl concentration difference between the internal and external aqueous buffers (Figure 3a). Large bulging MCLCs were observed in the appropriate lipid composition and NaCl concentration range (50–500 mM) (circle). When the NaCl concentration difference or lipid composition ratio was out of the range, insufficient swelling (triangle) or oily/collapsed aggregates without swelling were observed (cross).

In the case of DOPC without PGPR or PGPR without DOPC, the MCLC formation was not observed indicating that both compounds are necessary for the membrane formation. The necessity for NaCl difference between inside and outside of the MG-sponge indicated a concentration gradient was necessary as the driving force to form and to growth the MCLC (Figure 3b).

Separately from spontaneous formations, MCLCs were also obtained by mechanical squeezing of the lipid-soaked sponge, similar as when creating macroscopic soap bubbles. We found that the MG-Sponge described above does not withstand the squeezing operation, so the following experiment was conducted using a natural rubber sponge (NR-Sponge) (Figure S3). We developed a computer controlled mechanical device to squeeze the NR-sponge with a motor and syringe pumps (Figure 4a). The NR-sponge soaked with lipid solution and dried was attached to the device. Then, inner buffer was dropped onto the lipid loaded NR-sponges, and the sponge absorbed the buffer. The buffer-soaked NR-sponge, while at one end was attached to a fixed point, the opposing end was attached to a motor, when the motor was activated the sponge twisted

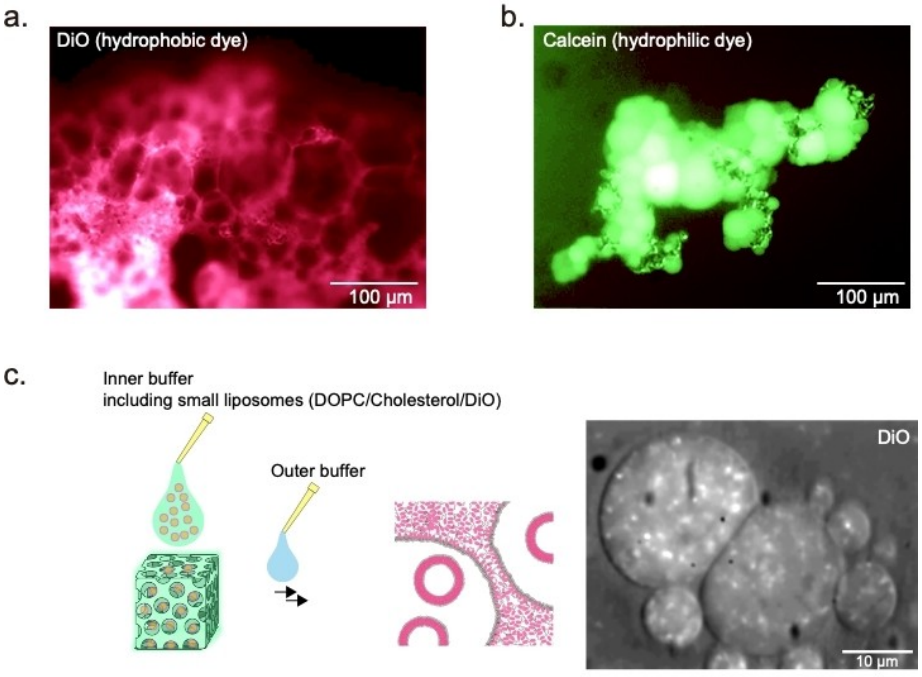

Figure 2. Characterization of the MCLCs by imaging by using fluorescence microscopy. a) The MCLC membrane image was obtained by staining with hydrophobic dye DiO (0.5 μM) that was pre-mixed with lipids. b) Using the hydrophilic dye calcein (100 μM, premixed in the inner buffer) showed the stained compartments inside of the MCLC. d) Internal buffer containing preformed liposomes (about 1 μm in diameter, stained with DiO (0.5 μM)), MCLCs incorporating liposomes were observed. The internalized liposomes showed Brownian motion but did not escape from the compartment (Movie S3).

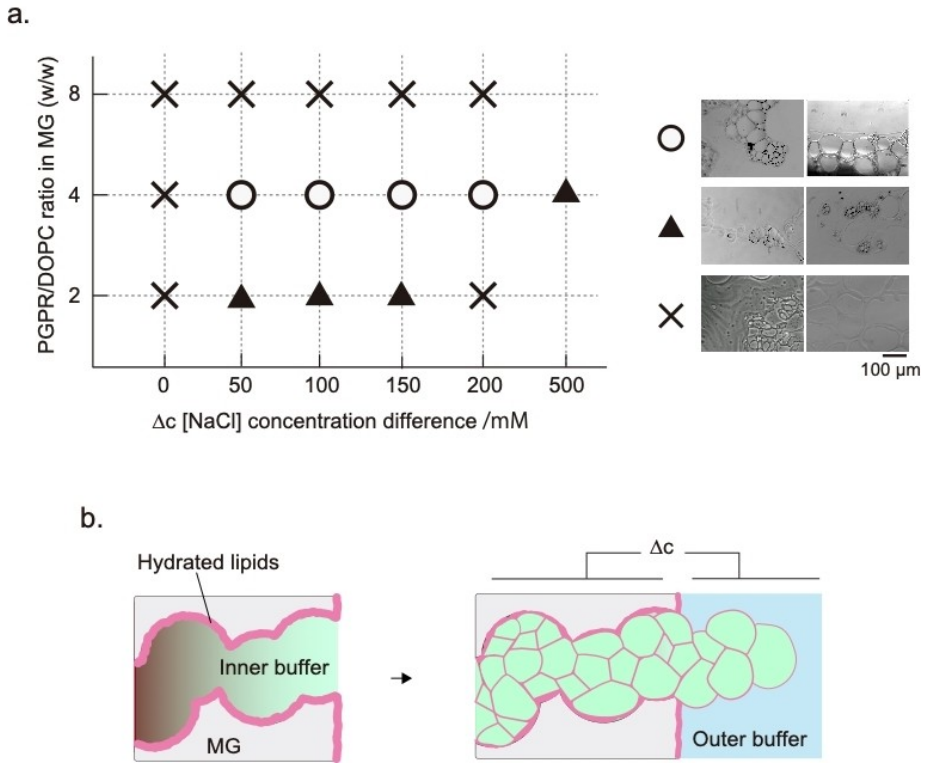

Figure 3. Preparation of MCLCs by the buffer condition difference. a) Phase diagram of spontaneous MCLC growth from a porous gel. The horizontal axis represents the net concentration difference of NaCl between the buffer inside the gel and the buffer outside. The vertical axis represents the mixing ratio of viscous surfactant (PGPR 20% (w/w)) and phospholipid (DOPC 5% (w/w)) in $CHCl_3$ solution. Circles: well-formed MCLCs, triangles: partially formed MCLCs, crosses: no compartments were observed. b) Imaginary diagram of the MCLC formation. Swelling of hydrated lipids from MG with driving force of concentration difference of solute, then the compartment would be closed balancing of the swelling force and the membrane surface tension.

causing a squeezing effect, simultaneously outer buffer was dropped from a syringe pump onto the NR-sponge surface. This resulted in a secreted solution which fell into a buffer solution placed below the system (Movie S4). The secreted MCLC solution was examined by fluorescence microscopy, the structures were found to be typically larger than 1 mm^2, Figure 4b. Figure 4c showed size comparisons between the MCLCs produced through spontaneous formation by immersion in outer buffer (Figure 4c, i) or automated squeezing by twisting (Figure 4c, iii, iv) were assessed through light microscopy and population distributions. For the NR-sponge, MCLCs produced by pouring outer buffer over the surface without squeezing operations were examined to investigate the clipping effect of the sponge (Figure 4c ii, Movie S5) The size of MCLCs produced by squeezing the NR-sponge by twisting for one or three turns was also examined. It was found that the largest MCLC sizes were produced by either the pouring method or squeezing for one turn while squeezing by three rotations produced comparable sizes to the immersion in outer buffer method. These results may suggest that the optimum external force parameter is between 0 (no twisting, pouring method) and the force applied after one twisting rotation.

We have previously reported the formation of polydisperse multi-lamellar liposomes by squeezing lipid loaded MG-sponges in water.[20] In this study, MCLCs with multiple connected compartments were formed by the addition of PGPR. The size of the individual compartments are larger than 50 micrometers which exceeds typical sizes expected of individual lipid only liposomes, indicating that PGPR plays a role in both multicellularity and large compartment sizes observed. Based on the experimental results using the two different methods, NaCl concentration difference, and squeezing pressure, the mechanism for spontaneous MCLC formation can be considered as follows. First, the lipid solution containing phospholipid and PGPR forms a dry lipid film on the hydrophobic surface of the porous MG-sponge. When the sponge is immersed in inner buffer, the lipid molecules are hydrated from the surface. However, due to the interfacial tension of the air-liquid interface at the sponge surface, the lipid molecules do not flow outward (Figure 3b, left). When the sponge encounters the outer buffer, the chemical potential difference causes an exchange of solutes between the outside and the inside of the sponge. When the inner buffer's salt concentration was higher than the outer buffer, the external solution flowed into the sponge displacing the hydrated lipid molecules in the sponge and thereby causing the internal solution to flow out. In the case of PGPR without DOPC, only the amorphous oily liquid flows out (Figure 3a, cross). When the phospholipid DOPC is added, which constitutes a planar membrane, lipid molecules flow onto the oily surface and reorient simultaneously, forming a closed compart-

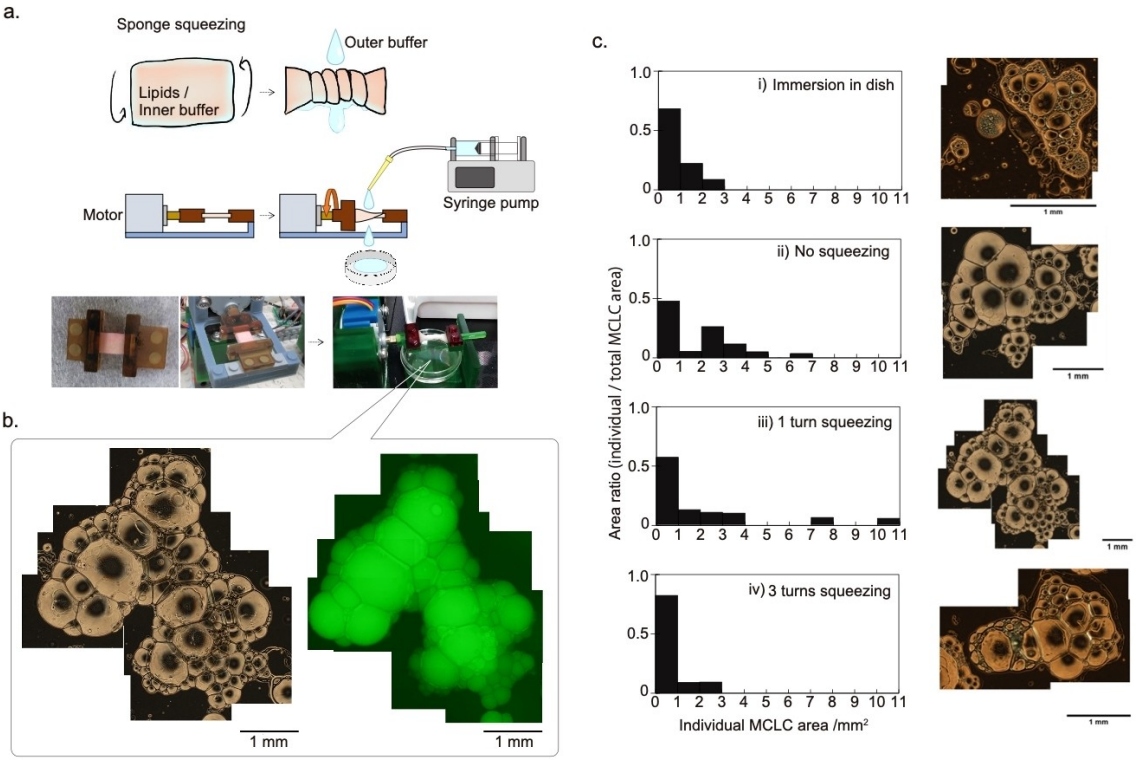

Figure 4. Preparation of MCLC by mechanical squeezing. a) Schematic representation of mechanical squeezing of a natural rubber (NR) sponge soaked in lipid solution. The NR sponge is fixed to a computer controlled automatic squeezer system (home-made, Figure S4) equipped with a motor and syringe pump. b) microscopic images of the obtained MCLC. Left: transmitted light image, right: fluorescence microscopy image of MCLCs encapsulating the hydrophilic dye calcein solved in the inner buffer. c) Histogram of area distribution of MCLC's obtained under different conditions of mechanical squeezing. i): simple immersion in a dish. A lipid soaked NR sponge with inner buffer was placed in the dish and the outer buffer was dropped. ii): The sponge was attached to the squeezer and the outer buffer was dropped to the sponge directly without squeezing. iii): One-turn squeeze was performed with dropping the outer buffer. iv: Results of 3-turn squeezing. The values are obtained from the trials with N = 3, 4, 6, and 3, respectively. The images on the far right are typical micrographs for each condition.

ment (water-in-oil-in-water) (Figure 3b right). Weinberger et al. reported that giant liposomes formed from hydrophilic polymer substrates and noted it is important that water uptake from the substrate-side.[23] In our experiment, the substrate of porous sponges can provide many pathways for water uptake. While there is a chemical potential difference between the inside of the sponge and the outside, the formation of compartments and efflux to the outside continues. In the case of squeezing NR-Sponges, inner buffer and lipids inside the sponge flow out at once. Funakoshi et al. reported a method of forming giant liposomes by spraying microjet pulses on lipid membranes, just like when making soap bubbles.[24] Our present system can be considered as a large-scale and spontaneous generation of such micron compartments by using sponges. The formation of a compartment that is larger than the void of the sponge means that expansion has occurred. The driving force for the expansion was the concentration difference and squeezing operation. When the concentration difference of the buffer is used as the driving force, all the inner buffer and lipid contained in the MG are not fully released, and the exchange of solutes reaches equilibrium due to the tension of the lipidic membrane, which probably causes the growth of MCLC to stop in the middle. On the other hand, in the squeezing operation, all inner buffer and lipid structures are released into the outer buffer in a short time. In the squeezing process, the lipid orientation of the membrane would be greatly disrupted, and water influx would occur, creating a huge compartment by swelling to the limit that balances the interfacial tension.

These methods provide millimeter sized multicellular lipid compartments system with simple procedures. The presence of PGPR in the lipidic membrane likely increases the thickness from the typical bilayer observed in liposomes. The addition of α-hemolysin, a common test for bilayer confirmation, failed to produce pores in the lipid-PGPR structure, supporting the notion of a thicker hybrid membrane wall. In future studies it may be studied to change the lipid system and remove PGPR to produce an artificial multicellular model in which membrane proteins and pore receptors may be integrated. Recently, research of artificial cells has been focusing on molecular communication between the compartments.[16,18,19,25–28] In the near future, various artificial, natural or hybrid chemical systems might be installed for functioning as simple analogues to living systems. Using this model as a vessel for artificial cells, research on the transfer of molecular information between artificial cells, memory, learning, and intercommunication with the outside macroscopic world would surely be advanced.

Conclusion

In this study, we reported the spontaneous formation of multi-compartment lipid structures from porous polymer sponges. The driving force is the difference in salt concentration between the inside and outside of the sponge, and we have shown that structurally similar, but even larger millimeter scale structures could be obtained by squeezing the macroscopic sponge. The method of compartmentalizing supramolecular assemblies under simple external conditions is expected to contribute to the study of molecular systems engineering for the integrated operation of molecular devices and the design of information transfer and higher-order functions through the interaction of artificial cell groups and their mass production.

Experimental Section

Materials

Acetic acid was purchased from Wako Pure Chemical Industries, Ltd., Japan, and urea was obtained from Hayashi Pure Chemical Industry, Ltd., Japan. Pure lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc., USA. Polyglycerol-polyricinorate (PGPR); Poem-100, was purchased from RIKEN Vitamin, Japan. Methyltrimethoxysilane (MTMS), dimethyldimethoxysilane (DMDMS), and *n*-hexadecylammonium chloride (CTAC) were obtained from Tokyo Chemical Industry, Ltd., Japan. α-Hemolysin, 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), bis[*N*, *N* − bis(carboxymethyl)aminomethyl]fluorescein (calcein), NaCl, $MgCl_2$, were obtained from Merck Sigma-Aldrich, Inc., Germany. Phosphate buffered saline (PBS) was obtained from OXOID, Ltd., UK. All reagents were used as received.

Preparation of marshmallow-like silicone gels (MG)

Samples were prepared based on a previous report.[20] Specifically,*x* mL of 5 mM aqueous acetic acid (typically, $x = 150$), $0.33 \cdot x$ g of urea, and 10 g of CTAC were mixed in a sealed PFA bottle. Thirty mL of MTMS and 20 mL of DMDMS were added under stirring at room temperature for 20 min. The molar ratios of acetic acid:urea and MTMS:DMDMS:CTAC were $1.0:3.3\times10^{3}$ and $3.0:2.0:4.3\times10^{-1}$, respectively. The obtained sol was heated at 80 °C for 6 h to complete gelation and aging. The resultant wet gels were removed from the bottle and washed with water and ethanol by soaking to remove residual CTAC and other chemicals. The samples were finally dried in an oven at 80 °C and (MT10, Giorik, Italy).

Characterization of MG

The microstructure of monolithic MGs was observed by scanning electron microscopy (SEM; TM3030Plus, Hitachi High-Technologies Corp., Japan). Bulk density was calculated from mass and volume. The error was within ~5%. The images were shown in Figure S1.

Preparation of MCLC by simple immersion

The MGs were gently washed with EtOH three times and dried before use. Lipid (DOPC) was dissolved in $CHCl_3$ to 6.5 mM. Skin-lipid (PGPR) was dissolved in $CHCl_3$ to 20% (w/w). Dried MG was cut into approximately $2\times5\times10$ mm rectangular block shapes, placed in a glass sample bottle and immersed sufficiently into the organic solvent mixture of lipid (100 μL), skin-lipid (100 μL) and $CHCl_3$ (300μL). The MG absorbed the solution immediately and became translucent. Subsequently, the MG block was treated at 50 °C for 5 min. to remove the excessive solvent. After drying, the MG block (the color changed to white again) was soaked by adding 100 μL of the inner buffer (10 mM PBS, 100 mM NaCl, 10 mM $MgCl_2$, pH = 7.0). The wet MG block was put on the dish by tweezers, then 1,000 μL of outer buffer (PBS), was added onto the MG. These procedures are shown in Figure 1a.

Microscopy

Observation of the generated microstructures was performed using a fluorescence microscope (Axiovert 200 M, Zeiss, Germany) with a digital camera (340 M, ThorLabs, USA.). To prevent the non-specific adsorption, the slide glass surface was treated by skim-milk (10 mg/mL, 5 min, then washed by PBS) before observation. For each observation, the lipid membrane was stained using the fluorescent dye DiO (dissolved with lipids in $CHCl_3$, 0.5 μM in final) or calcein (dissolved in inner-buffer, 10 μM in final). The images and movies were obtained through the imaging software of ThorLabs Camera.

Preparation of MCLC by mechanical squeezing

A natural rubber sponge (KAIJIRUSHI, KQ3100) was cut into sheets of approximately $2\times8\times15$ mm. The sponges were soaked in ethanol and cleaned by sonication for 60 minutes before use. The cleaned sponges were placed on a 10 cm dish without overlapping, covered with a lid to prevent dust from sticking to the sponge, and left to dry at least overnight. No visual changes were observed before and after the washing treatment. Lipid (DOPC) was dissolved in $CHCl_3$ at 5%(w/w). Skin-lipid (PGPR) was dissolved in $CHCl_3$ at 20% (w/w). The dried sponges were placed in a glass sample bottle and thoroughly soaked in a mixture of Lipid (100 μL), Skin-lipid (100 μL), and Ethanol (300 μL). The sponges were then dried at 50 °C for 25 min to remove excess solvent. The dried sponge was fixed to an attachment made by a 3D printer. 300 μL of inner buffer (245 mM NaCl, 8.5 mM $MgCl_2$, 10 μM calcein/1×PBS) was dropped onto the sponge from approximately 1.5 mm above the sponge using a syringe pump (Chemyx, fusion200). Next, 1 mL of outer buffer (1×PBS) was dropped from approximately 12 mm above the sponge using a syringe pump in 1 min while the motor (Longruner stepper motor (28BYJ-48)) attached to the sponge was rotating at 6 rpm. At this time, a 35 mm dish was placed about 30 mm below the sponge and the squeezed solution was collected.

Author Contributions

Conceptualization, S.-i.M.N., T.T., A.A., R.M.; experiment, S.-i.M.N., R.S.; MG design, preparation, G.H., R.S., R.A.J.; data curation, S.-i.M.N., A.A.; writing–original draft preparation, S.-i.M.N., R.S.; writing–review and editing, A.A., R.M., G.H., T.T., R.S., R.A.J. and S.-i.M.N.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: artificial cell models · lipids · multicellular compartments · spontaneous growth · self-assembly

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