Large-Scale Preparation of Giant Vesicles by Squeezing a Lipid-Coated Marshmallow-like Silicone Gel in a Buffer

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Large-Scale Preparation of Giant Vesicles by Squeezing a Lipid-Coated Marshmallow-Like Silicone Gel in a Buffer

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ABSTRACT

Giant vesicles were efficiently produced by squeezing a lipid (L-α-phosphatidylcholine from egg yolk)-coated marshmallow-like flexible macroporous silicone monolith in a buffer. The mean diameter of the obtained vesicles was 2 μm, showing a wide distribution, up to tens of micrometers, which was similar to that of vesicles formed by a natural swelling method. It was possible to prepare vesicle dispersions on a scale from several microliters to several hundred milliliters. A protein synthesis system (PURE system) contained in vesicles prepared using this method functioned effectively. Our absorbing–squeezing method is expected to help in studies that use giant vesicles such as artificial cells and drug delivery systems.

INTRODUCTION

Vesicles (liposomes), closed structures consisting of phospholipid bilayer membranes similar to cell and organelle membranes, have been used as a simple model for understanding living cell systems and their interfacial functions. Vesicles are indispensable for both elucidation of life phenomena and for basic scientific research on topics such as artificial cells, microsynthesis, drug delivery, and cosmetics.[1-3] Various processes, such as hydration, ultrasonic treatment, membrane emulsification, centrifugal sedimentation, and the use of microflow channels, have been employed to produce vesicles and to control their size distributions.[4-15] Each method has advantages and disadvantages, and thus the process most suitable for a particular purpose is selected.[16-19]

Importantly, artificial cells have been actively constructed within vesicles by reconstituting (even if partially) live cell molecular systems such as gene expression systems and microreactions.[2, 20-29] Researchers have tried to obtain direct evidence showing how to artificially create things that behave like living organisms. For example, Sato et al. have reported a special vesicle, called a "molecular robot," that autonomously changes its shape by using incorporated molecular motors and a control system.[30] To create the robot, the authors had to wrap up complex biochemical contents with a lipid bilayer membrane. Therefore, a method allowing efficient production of vesicles on a large scale would help accelerate research and development in this field.

In this paper, we report a simple, large-scale method for the preparation of giant vesicles without the need for expensive tools. Using a kitchen sponge as an inspiration, we attempted to develop a method of squeezing out giant vesicles using porous monoliths. Squeezing a soapy kitchen sponge in the air results in the formation of many bubbles. These bubbles are formed by a spherical bilayer membrane composed of surfactants. We anticipated that vesicles that have a similar hydrophilic–hydrophobic structure but with an inverted alignment could be produced by squeezing out surface-adsorbed phospholipids from a sponge into a buffer solution. However, vesicles are much smaller than bubbles in the foam, and thus the porous structure of a kitchen sponge is too coarse. A sponge with a micrometer-scale microstructure is necessary for producing micrometer-sized giant vesicles.

Therefore, we tested macroporous silicone monoliths. Hayase has investigated "marshmallow-like gels" (MGs) fabricated by a sol–gel reaction. [31] These materials, which are flexible when bent and compressed, have a macroporous structure composed of a several micrometer thick skeleton with a smooth surface and pores of several tens of micrometers in diameter. Marshmallow-like gels can separate an oil–water mixture by absorbing the mixture and then releasing the oil when squeezed like a sponge.[32] The porosity of MGs is more than 90 %, and the surface of the microstructure formed by phase separation is smooth and free of mesopores. This morphology is advantageous for the formation of giant vesicles.

EXPERIMENTAL

Materials. Acetic acid and L-α-phosphatidylcholine (PC) from egg yolk were purchased from Wako Pure Chemical Industries, Ltd. (Japan), and urea was obtained from Hayashi Pure Chemical Industry, Ltd. (Japan). Pure lipids, DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), and DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), were purchased from NOF Corporation (Japan). Methyltrimethoxysilane (MTMS), dimethyldimethoxysilane (DMDMS), and *n*-hexadecylammonium chloride (CTAC) were obtained from Tokyo Chemical Industry, Ltd. (Japan). A fluorescent dye, rhodamine B-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine B-DHPE) was obtained from Molecular Probes (USA). An *in vitro* transcription and translation system (cell-free protein synthesis kit, PUREfrex 2.0) was obtained from GeneFrontier Corporation (Japan). All reagents were used as received.

Preparation of MGs. Samples were prepared based on a previous report.[33] In particular, x mL of 5 mM aqueous acetic acid (typically, x = 150), 0.33 × x g of urea, and 10 g of CTAC were mixed in a sealed PFA bottle. Thirty milliliters of MTMS and 20 mL of DMDMS were added, and the mixture was stirred at room temperature for 20 min. The molar ratios of acetic acid:urea and MTMS:DMDMS:CTAC were $1.0{:}3.3{\times}10^{3}$ and $3.0{:}2.0{:}4.3{\times}10^{-1}$, respectively. The obtained sol was heated at 80 °C for 6 h to complete the gelation and aging processes. 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 named MG*x*.

Characterization of MGs. The microstructure of monolithic MGs was observed by scanning electron microscopy (SEM; TM3030Plus, Hitachi High-Technologies Corp., Japan). The bulk density was calculated from the mass and volume. The error was within ~5 %.

Preparation of vesicles by squeezing buffer-soaked lipidic MGs. A lipid (egg yolk PC) was dissolved to 5 mM in $CHCl_3$. A dried MG was cut into approximately 5-mm cubes, placed in a glass sample bottle, and immersed sufficiently into the phospholipid solution. The MG immediately absorbed the solution and became translucent. Subsequently, the excessive phospholipid solution was removed, and vacuum drying was performed. After drying for 20 min, a septum was attached to the glass bottle, and the samples were evacuated through a syringe needle. To the MG maintained under reduced pressure, 100 μL of the inner buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM $MgCl_2$, pH 7.0] was added through another syringe needle. For gene expression experiments, a solution containing the template DNA of the pEGFP plasmid (10 nM) was used according to the manufacturer's instructions for the PUREfrex 2.0 kit. Next, the septum was removed from the glass bottle, and the MGs were taken out and immersed in 100 μL of the external buffer (10 mM HEPES, 10 mM $MgCl_2$, pH 7.0). Finally, these wet MGs were squeezed 20 to 50 strokes with tweezers with the force of human fingers (~0.3 MPa). Under the conditions, MG gradually breaks when more intense operation is performed. These procedures are shown in Figure 1.

Flow cytometry analysis. The size distribution and the dye-loading efficiency of the generated vesicles was evaluated using a flow cytometer (Cell Lab Quanta SC-MPL; Beckman Coulter, USA). Statistical processing was performed by using the Cell Lab Quanta analysis software and R software.

Fluorescent microscopy. Observations of the generated vesicles were performed using a fluorescence microscope (IX-83; Olympus Corporation, Japan) and a laser confocal microscope (FV-1000; Olympus Corporation). For each observation, the lipid membrane was stained with the fluorescent dye rhodamine

B-DHPE. Three-dimensional images and movies were reconstructed from 30 cross-sectional images using ImageJ/Fiji.[34-36]

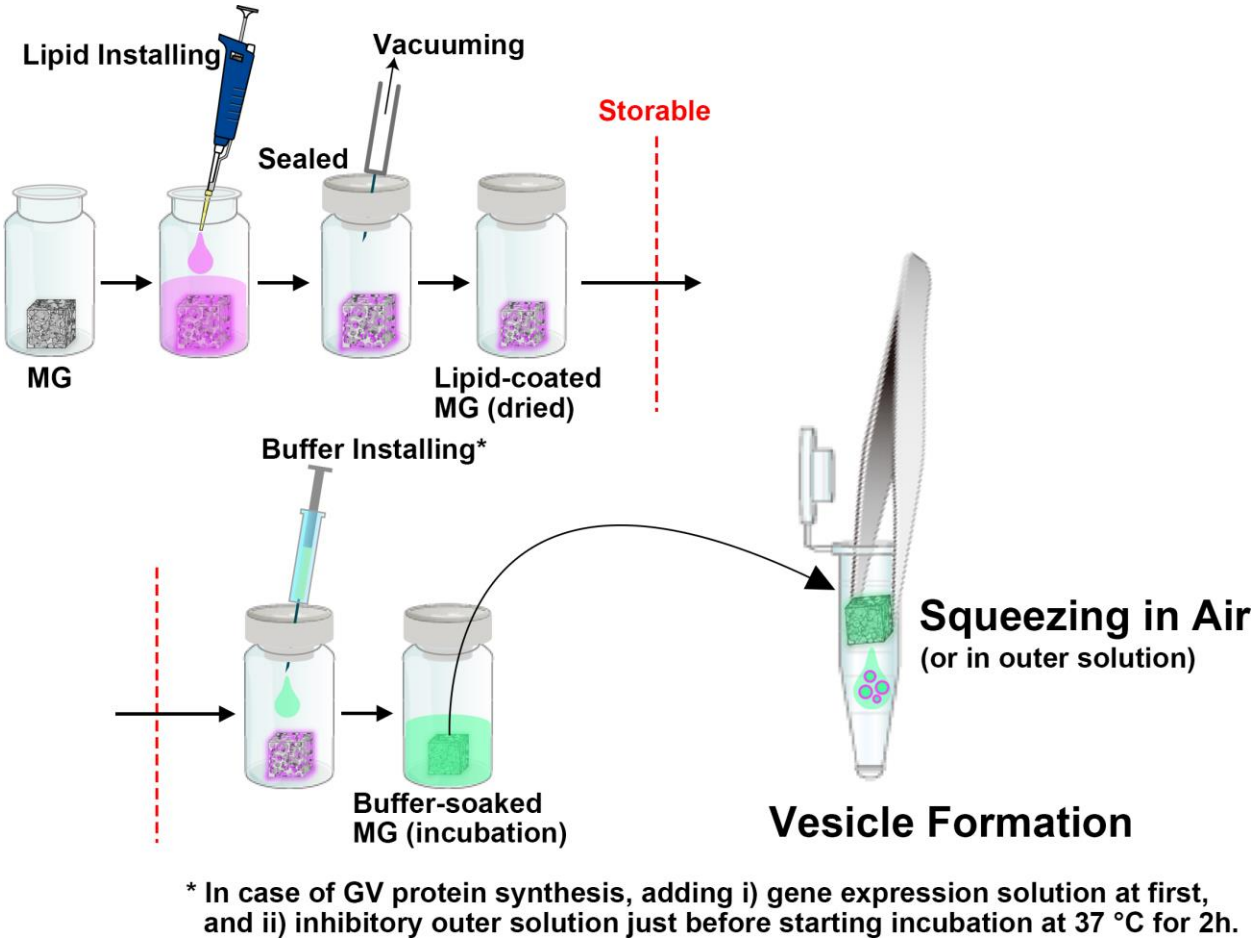

Figure 1. Procedure used to obtain giant vesicles by squeezing a marshmallow-like silicone gel (MG) coated with a lipid. After adding buffer to a dried MG, the buffer-soaked MG can be squeezed immediately, if incubation is not needed.

RESULTS AND DISCUSSION

The external appearance of MG150 and a scanning electron micrograph are shown in Figures 2a,b. Figures 2c,d and Movie S1 show laser scanning confocal microscopy images of a soaked MG150 before squeezing, and Movie S2 shows the appearance of the vesicles produced by squeezing out from the MG. These results indicated that giant vesicles could be generated by this method (Figure 2e). The vesicles prepared using MG150 were evaluated by flow cytometry. The average diameter of the vesicles was the

largest when the concentration of the lipid molecules was 5 mM (Figure 3). In any case, the observation by fluorescence microscopy showed predominant formation of multilamellar vesicles. Based on the results, this vesicle formation process can be considered a three-dimensional natural swelling method. We also attempted to control the vesicle diameter by changing the starting composition of MGs to vary the porosity and pore size by several tens of a percent. However, no significant effect was observed (Figure 4). Although further efforts are required for the precise control of the vesicle diameter, this parameter is relatively unimportant for vesicles used in artificial cell experiments.

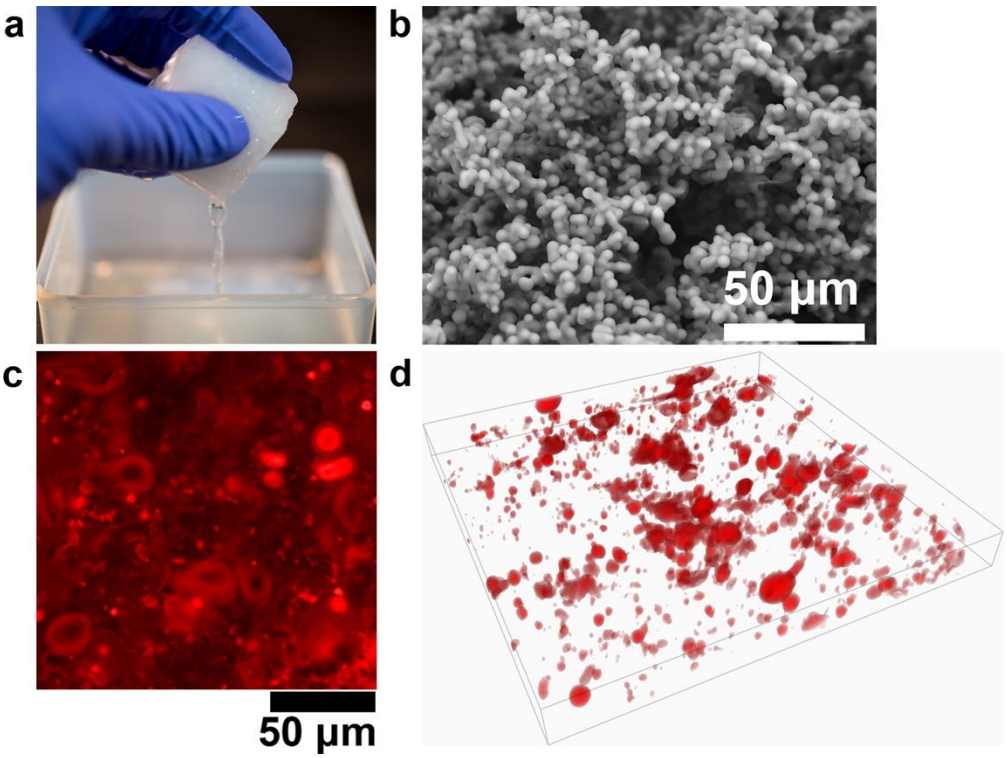

Figure 2. (a) Photograph and (b) SEM image of the marshmallow-like gel MG150. The structure was coated with a lipid, dried, and soaked in a buffer solution (before squeezing). (c) Laser scanning confocal microscopic image and (d) reconstructed image of MG150. Hydrated vesicles were stained with rhodamine B-DHPE. The size of the cuboid is 262 × 262 × 34 μm^3. See also Movie S1.

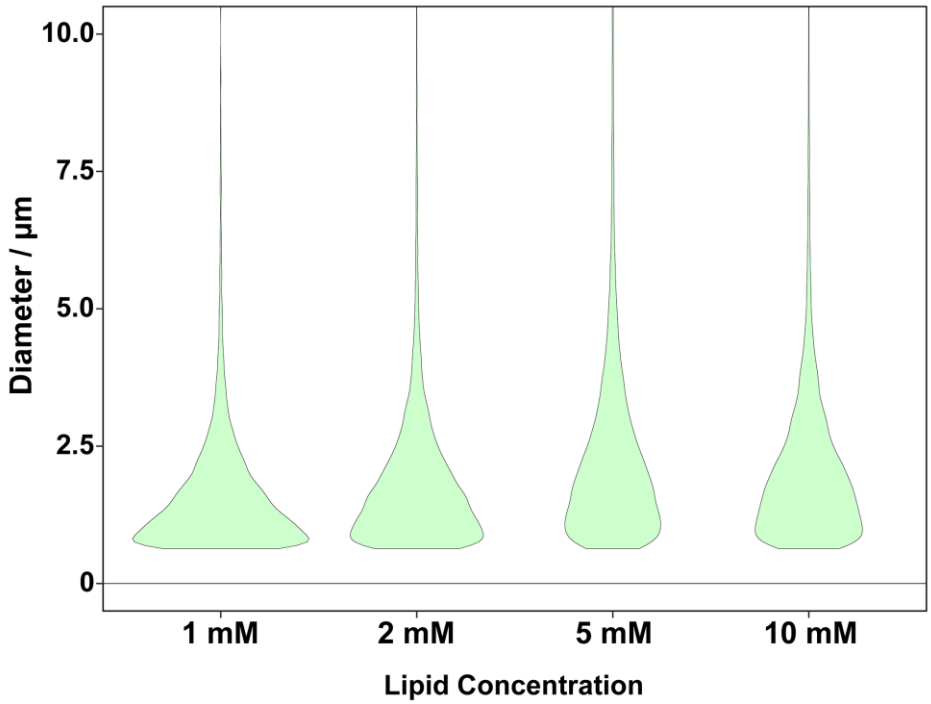

Figure 3. Distribution of vesicle diameters obtained by changing the concentration of the lipid absorbed by MG150.

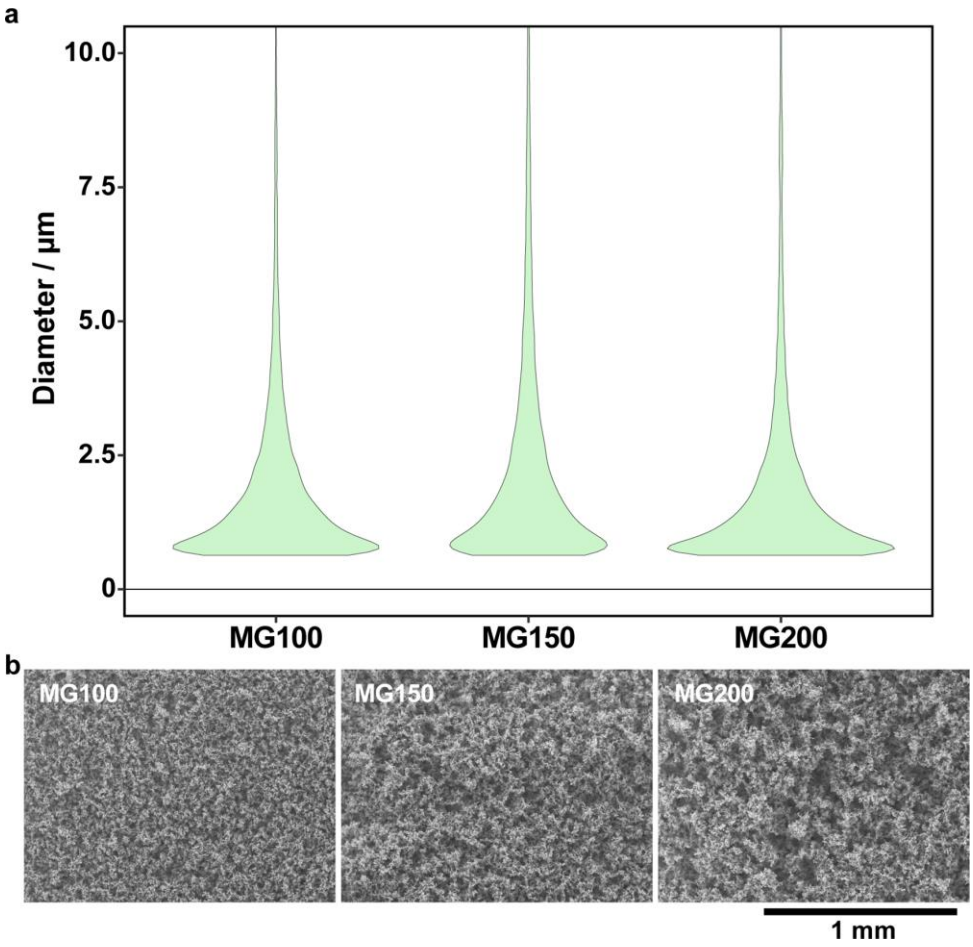

Figure 4. (a) Distribution of vesicle diameters obtained by changing the starting compositions to vary the porosity and pore size of MG*x*. (b) SEM images of the respective silicon monoliths used for the formation of the above vesicles.

Here, we discuss the size of the vesicles prepared by squeezing an MG coated with a lipid. As shown in Figure 2b, MG is composed of connected microparticles. The mean particle size of MGs was approximately 4 μm, which did not change even if porosity was varied. On the other hand, the mean size of the obtained vesicles was approximately 2 μm, regardless of the MG porosity (Figure 4). If MG microparticles were completely dispersed, the surface area of the vesicles obtained would be approximately 1/4 compared with that when lipid molecules covered the entire surface of MG particles. In practice, however, the particles that form MGs are continuously linked to each other, and the contact angle between the particles is close to 180° (see Figure 2b). This suggests that the penetration of the

buffer solution at the interface between MG particles results in lipid defects (Figure 5). In other words, the nonuniform distribution of lipid molecules on the unique structure of an MG, with an extremely large surface area, can be predicted to cause this vesicle size distribution. The typical size of vesicles produced when the planar lipid membrane is sufficiently hydrated can be estimated from the ratio of the rigidity constant of the membrane to the surface tension. The value is called a deflection length and is expressed by $\xi_\sigma = (k_c/\sigma)^{1/2}$, where k_c is the rigidity constant and σ is the lateral surface tension of the lipid bilayer membrane.[37] Assuming that the value of k_c in the case of egg yolk PC is ~2.47 × 10^{-20} J and the surface tension σ of the membrane is 10^{-5} mN·m^{-1},[38] the typical size of the vesicle is estimated to be approximately 1.5 μm. This is consistent with our current results. To optimize this squeezing method, it would be necessary to investigate the size distribution of the vesicles under other conditions, such as using MGs with a significantly different skeleton diameter (particle size) or changing the physical/chemical conditions of the membrane by adjusting the lipid composition.

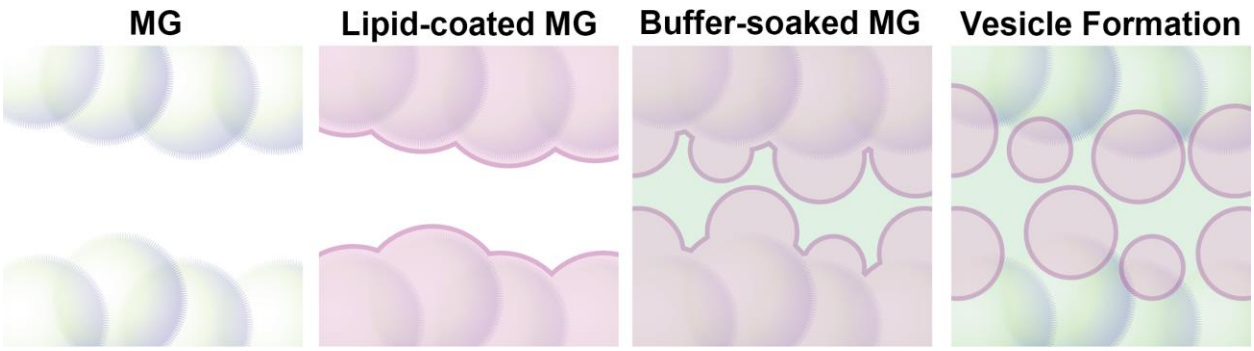

Figure 5. Potential mechanism of the generation of giant vesicles using the absorbing–squeezing method.

Subsequently, we tried to use different lipids for vesicle formation by the MG-squeezing method. The mixed lipid, egg yolk PC, and several types of pure lipids (DOPC, POPC, DMPC, DPPC, and DSPC) were applied using the present method, and vesicle formation, the encapsulation rate, and average diameter of the vesicles were evaluated using a flow cytometer. The results are shown in Table 1. It was confirmed that vesicles could be formed using several lipids with a warm buffer (60 °C); the temperature should not be lower than the lipid phase transition temperature. Interestingly, the vesicle diameters

obtained with the other lipids were larger than that obtained with egg yolk PC, which was used at room temperature, as shown in Figure 3. The effect is thought to be related to the increase in the buffer inflow volume, caused by increased lipid mobility at a higher buffer temperature. Interestingly, in the case of squeezing at room temperature, the dye-loading efficiency was not decreased (Table S1). The dried lipid on MG particle spreads more than the typical situation on the glass test tubule, because of the excess surface area. So many discontinuous membrane parts as inlets for swelling might be exposed to the water solution, even at room temperature.

Table 1. Dye-loading efficiencies and vesicle size distributions obtained using the MG-squeezing method. Each lipid concentration was 5 mM for soaking. For swelling, a warm buffer (10 μM calcein, 10 mM HEPES, 10 mM $MgCl_2$; 60°C) was used. See also Table S1.

Lipid	T_m* [°C]	Dye-loading efficiency** [%]	Average diameter*** [μm]
EYPC	3	45.6	4.3
DOPC	−17	48.3	4.4
POPC	−2	37.3	4.7
DMPC	24	46.2	4.0
DPPC	45	88.9	6.0
DSPC	55	43.7	4.1

*Transition temperature values were obtained from reference 39. **The value is based on the number of vesicles showing a fluorescence intensity $>10^1$ among a total of 15,000 vesicles counted (Figure S1). ***The shape of the size distribution curves was a monotonically decreasing function (Figure S2).

The amount of lipid molecules recovered after squeezing was estimated using the lipidic fluorescent dye rhodamine B-DHPC and a fluorospectrometer. When the vesicles were formed using MG150 with egg yolk PC, the proportion was 85% compared with the amount recovered by the natural hydration method. The result showed that there was lipid remaining in the hydrophobic silicone monolith even after squeezing.

Artificial cell-like particles, capable of autonomously synthesizing RNA and proteins based on a DNA template, would be a new platform for synthesizing functional or therapeutic proteins on-demand.[23, 27, 29] For easy preparation of such an artificial cell model, we attempted to incorporate a transcription and translation system for protein synthesis into vesicles by this squeezing method.[40] MG150 was soaked in a chloroform solution containing rhodamine B-DHPE (1:5,000 molar ratio) mixed with 5 mM egg yolk PC and then dried under vacuum overnight. The obtained lipid-coated MG150 was soaked in the solution of an *Escherichia coli* reconstituted gene expression system (PUREfrex 2.0 kit; 20 μL) mixed with an enhanced green fluorescent protein (eGFP)-encoding plasmid (73 $ng \cdot \mu L^{-1}$ final concentration) on ice. Then, an inhibitory outer buffer solution (10 mM HEPES, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, pH 7.0) was added to inhibit the external vesicular reaction. The solution was incubated at 37 °C for 2 h to synthesize the protein. After the reaction was completed, the treated MG150 was picked up and then squeezed using tweezers. Figure 6a shows a microscopic image of the MG just before squeezing. Vesicles containing the plasmid showed green fluorescence (red membrane, Figure 6b), whereas remarkably less green fluorescence was observed in samples prepared using a plasmid-free expression solution (Figure S3). Our results confirmed that this gene expression system could easily and properly perform in vesicles prepared using MGs, although the scale was in several tens of microliters. Recently, simple preparation and scale-up methods using an *E. coli* cell-free synthesis system have been reported.[41-42] Combined with the development of such cell-free protein expression system technology, the MG method would contribute to artificial cell research in the future.

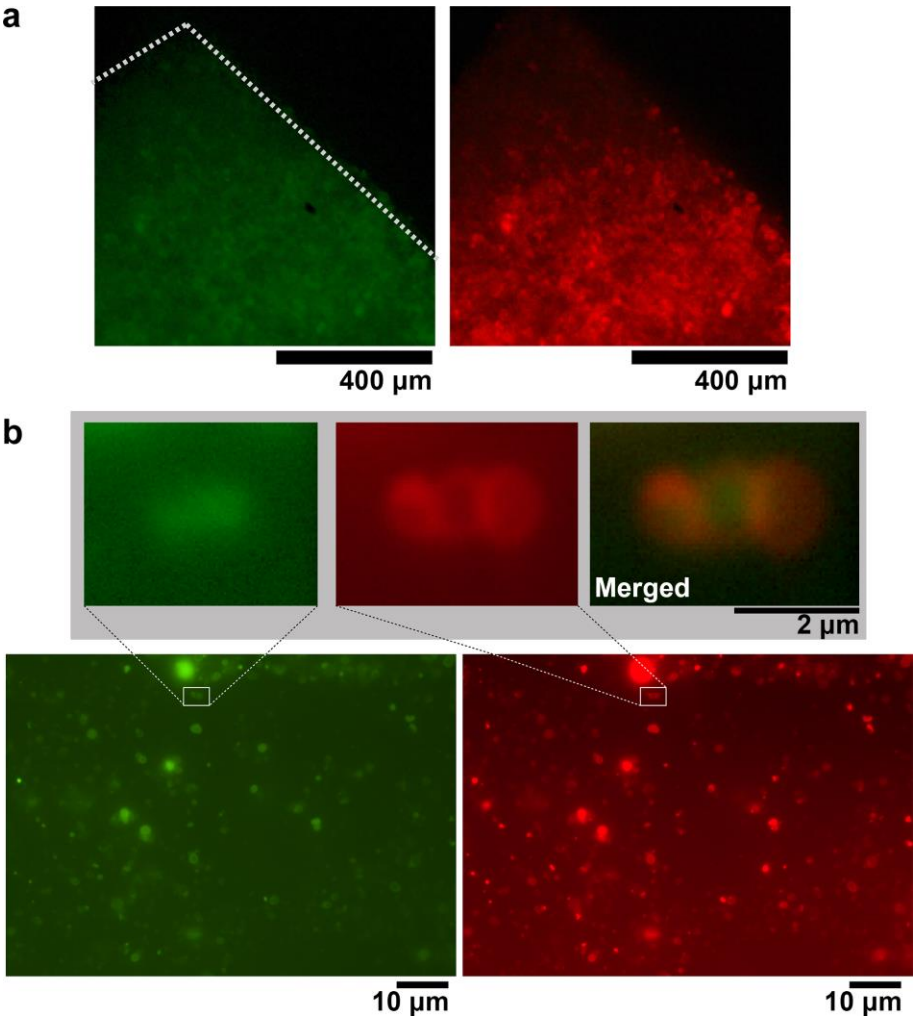

Figure 6. Microscopic images of vesicles after encapsulation of the cell-free protein synthesis solution: expressed eGFP signals (left) and membranes stained with the lipidic rhodamine B-DHPE dye (right). (a) Fluorescent signal of a corner of swelled MG150 before squeezing; (b) fluorescent image of a solution of giant vesicles squeezed from MG150.

We demonstrated the simplicity and scalability of our vesicle generation method by preparing several tens of milliliters of MG150 in a coffee plunger and by generating a large number of vesicles in one step. By this method, giant vesicles were successfully squeezed out, and a dispersion was obtained on a scale of several hundred milliliters. The quality of the vesicles was comparable to that of the vesicles prepared on a small scale (Figure 7). Although several aspects require improvement, such as the control of vesicle

size distributions, this simple method will greatly aid in both scientific research and industrial development.

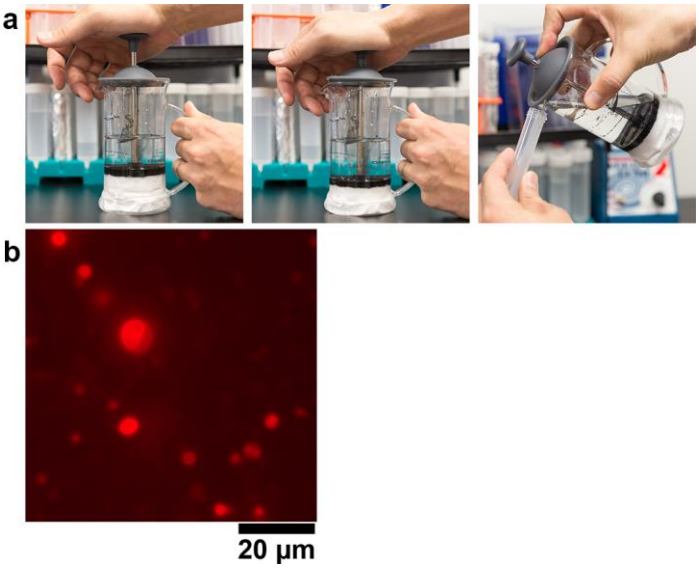

Figure 7. (a) Large-scale preparation of giant vesicles using a coffee plunger, and (b) fluorescent images of obtained vesicles.

CONCLUSIONS

We demonstrated the simplicity and scalability of vesicle generation by squeezing MGs. By this method, giant vesicles were successfully squeezed out, and a dispersion was obtained on a scale of several hundred milliliters at once. The quality of the vesicles was comparable to that of the vesicles prepared on a small scale. Since cell-free protein synthesis systems can work inside the giant vesicles, this instant generation method will be of great help in artificial cell research. Our method has advantages such as the ability to prepare in advance and stock MGs that were impregnated with a lipid. Through continuous improvements, it is also expected that vesicle generation kits can be prepared to be quickly used in the medical field.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge.

Flow cytometry analysis for hydrophilic dye loading efficiency of the pure lipid vesicles, flow cytometry analysis for the size distribution of the pure lipid vesicles, microscopic images of vesicles after encapsulation of a cell-free protein synthesis solution without a plasmid, and dye-loading efficiencies and vesicle size distributions at RT. (PDF)

A three-dimensional image showing the appearance of vesicles in MG150. (MPG)

The appearance of vesicles squeezed out from MG150. (MPG)

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Notes

The authors declare no competing financial interest.

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