Journal of Visualized Experiments

Preparation of giant unilamellar vesicles encapsulating microspheres by centrifugation of a water-in-oil emulsion

--Manuscript Draft--

Manuscript Number:	JoVE55282R1
Full Title:	Preparation of giant unilamellar vesicles encapsulating microspheres by centrifugation of a water-in-oil emulsion
Article Type:	Corporate Submission
Keywords:	Giant unilamellar vesicle (GUV), Microspheres, Water-in-oil (w/o) emulsion centrifugation method, Model protocell, Constructive biology
Manuscript Classifications:	8.1.158.273.904: Synthetic Biology
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Abstract:	In the exploration of the origins of life and the study of biological functions, the constructive, or synthetic, biology approach, that is, the attempt to construct living entities from the bottom up, has received considerable attention. Over the past two decades, this approach has been used to construct artificial cells. In particular, giant unilamellar vesicles (GUVs) have often been used as artificial cell membranes. In this paper, we describe the preparation of GUVs encapsulating highly packed microspheres as a model of cells containing highly condensed biomolecules. The vesicles were prepared by means of a simple water-in-oil emulsion centrifugation method. Specifically, a homogenizer was used to emulsify an aqueous solution containing the materials to be encapsulated and an oil containing dissolved phospholipids, and the resulting emulsion was layered carefully on the surface of another water solution. The layered system was then centrifuged to generate the GUVs. This powerful method could be used to encapsulate materials ranging from small molecules to microspheres.
Author Comments:	This time a revised version is uploaded.
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Preparation of giant vesicles encapsulating microspheres by centrifugation of a water-in-oil emulsion

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KEYWORDS:
Giant vesicle (GV), Microspheres, Water-in-oil (w/o) emulsion centrifugation method, Model protocell, Constructive biology

SHORT ABSTRACT:
Giant vesicles containing highly packed micrometer-sized components are useful cell models. The water-in-oil emulsion centrifugation method is a simple, powerful tool for the preparation of giant vesicles with encapsulated materials.

LONG ABSTRACT:
The constructive biology and the synthetic biology approach to creating artificial life involve the bottom-up assembly of biological or nonbiological materials. Such approaches have received considerable attention in research on the boundary between living and nonliving matter and have been used to construct artificial cells over the past two decades. In particular, giant vesicles (GVs) have often been used as artificial cell membranes. In this paper, we describe the preparation of GVs encapsulating highly packed microspheres as a model of cells containing highly condensed biomolecules. The GVs were prepared by means of a simple water-in-oil emulsion centrifugation method. Specifically, a homogenizer was used to emulsify an aqueous solution containing the materials to be encapsulated and an oil containing dissolved phospholipids, and the resulting emulsion was layered carefully on the surface of another aqueous solution. The layered system was then centrifuged to generate the GVs. This powerful method was used to encapsulate materials ranging from small molecules to microspheres.

INTRODUCTION:
The constructive, or synthetic, biology approach is a fascinating avenue for exploring the boundary between living and nonliving matter. Because the cell is the minimum unit required for life as we currently understand it, various researchers have attempted to construct artificial cells from simple, well-understood chemicals so that phenomena that occur within such artificial cells can be studied, with the ultimate goal of elucidating the origins of life and studying the fundamental functions of living cells[1-3]. In particular, vesicles[4], which are spherical microcompartments made of amphiphilic molecules and can encapsulate biological molecules such as proteins[5, 6] and DNA[7-10], have often been used as models of biological membranes.

Vesicles can be classified as small (defined as having a diameter of < 100 nm), large (diameter < 1 μm), or giant (diameter > 1 μm). Giant vesicles (GVs) have been studied extensively because they are similar to living cells in size, shape, and structure. Owing to the size of GVs, morphological changes in GV membranes can easily be observed in real time under an optical microscope.

Several methods for preparing GVs have been reported[11], including the hydration method[12, 13], the freeze–thaw method[14], the electroformation method[15, 16], and the fluidic device method[17, 18]. However, encapsulating proteins and other macromolecules in GVs at high concentrations by means of these methods is difficult. In particular, it is extremely challenging to encapsulate biological materials in sufficient quantity (20–30 vol %) to mimic the crowded environment inside cells[19, 20]. To form GVs instantly, Weitz and coworkers established a water-in-oil (w/o) emulsion centrifugation method[21, 22]. This method has five important features. First, because GVs prepared by this method have low lamellarity[23, 24], their membranes are so thin that they can be deformed easily. GV membrane deformation induced by FtsZ (a bacterial cell division protein), tubulin, and other macromolecules has been studied[25-28], and we observed polyhedron-like deformation of GV membranes induced by encapsulation of microspheres[29, 30]. Second, membrane proteins can be inserted into the vesicular membrane by this method, albeit with difficulty[31]. For example, the Yomo group used this method to study the *in vitro* synthesis and pore-forming activity of the membrane protein α-hemolysin[32]. Third, it is possible to generate asymmetric GVs in which the lipid components of the inner and outer leaflets are different[22]. For example, Whittenton *et al.* generated asymmetric GVs with cationic lipids in the inner leaflet to encapsulate negatively charged polynucleotides, and with neutral lipids on the outer leaflet to decrease toxicity and nonspecific cellular uptake[33]. Fourth, the concentration and volume fraction of substances inside the GVs can be relatively high[28, 34]. Fifth, multiple types of materials can be encapsulated[35]. For example, Nishimura *et al.* encapsulated an *in vitro* transcription–translation system into GVs and used the system to express green fluorescent protein (GFP) within the GVs[36]. These five features make w/o emulsion centrifugation an indispensable method for generating cell-mimicking GVs.

In previous work, GVs generated by centrifugation were collected by means of a syringe equipped with a long 16-gauge stainless steel needle containing some of the final aqueous solution[22]. In the hands of inexperienced technicians, this collection method could easily result in contamination of the GV with some of the oil. In this study, we used the w/o emulsion centrifugation protocol developed by the Yomo group[23, 37], in which precipitated GVs are collected through a hole opened at the bottom of the centrifuge tube in which they are prepared. We prepared GVs encapsulating 1.0-μm microspheres, which are similar in size to intracellular organelles. The use of microspheres allowed us to estimate their concentration by calculating their volume fraction. Establishment of a method for preparation of GVs in which materials are densely packed is an important step for creating artificial cells. To confirm the utility of our protocol for various types of inner materials, we also demonstrated that GFP and a small water-soluble fluorescent molecule (uranine) could be encapsulated in the GVs.

PROTOCOL:

1. Preparation of GVs by the w/o emulsion centrifugation method

1.1. Prepare a stock solution of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, 25 mM) and a stock solution of Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE, 0.20 mM) in chloroform and store the stock solutions at –20 °C.

1.2. Prepare an oil solution.

1.2.1. Form a lipid film on the inside surface of a 5-mL glass vial by evaporating a mixture of the DOPC stock solution (51.0 µL) and the Texas Red DHPE stock solution (19.2 µL) under flowing nitrogen gas.

1.2.2. Incubate the film under reduced pressure overnight and then add 1.0 mL of liquid paraffin (0.86–0.89 g/cm^3) to the vial. Wrap the vial in aluminum foil and incubate it at 80 °C overnight at rest. The final concentrations of DOPC and Texas Red DHPE are 1.3 and 3.8 × 10^{-3} mM, respectively, and the DOPC:Texas Red DHPE molar ratio is 100:0.3.

1.3. Prepare the inner aqueous dispersion.

1.3.1. In a 1.5-mL lidded microtube, mix 237.5 µL of a dispersion of 1.0-µm nonfluorescent microspheres (2.5 vol %) and 12.5 µL of a dispersion of 1.0-µm fluorescent microspheres (2.5 vol %); this corresponds to a 95:5 (v/v) ratio of nonfluorescent to fluorescent microspheres.

1.3.2. Add 64 mg of sucrose followed by 125 µL of Tris-buffered solution (1 M) and 875 µL of deionized water. The final volume fraction of microspheres is 0.5 vol %, and the final concentrations of Tris-HCl (pH 7.5) and sucrose are 0.1 and 0.15 M, respectively.

1.3.3. Vortex the microtube for 30 s and then sonicate it for 10 min.

1.4. Prepare the outer aqueous solution.

1.4.1. After preparation of 10 mL of a Tris-buffered solution (0.1 M Tris-HCl [pH 7.5], 0.15 M glucose) in the same procedure as inner aqueous media, place 1 mL of the solution in a 1.5-mL lidded microtube. Vortex the microtube for 30 s and then sonicate it for 10 min.

1.5. Prepare a w/o emulsion containing microspheres.

1.5.1. Mix 1 mL of the oil solution (liquid paraffin containing DOPC and Texas Red DHPE) with 300 µL of the inner aqueous solution in a 1.5-mL microtube.

1.5.2. Emulsify the two components in the microtube by using a mechanical homogenizer (an agitator with blades that rotate at high speed) operated at 10,000 rpm for 2 min at room temperature.

1.6. Precipitate the GVs.

1.6.1. Gently layer 300 µL of the w/o emulsion on the upper surface of 1 mL of the outer aqueous solution at 4 °C in a 1.5-mL lidded microtube. Chill the microtube for 10 min at 4 °C.

1.7. Collect the GVs.

1.7.1. Immediately after chilling the microtube, centrifuge it at 18,000 x *g* for 30 min. Obtain the precipitated GVs by piercing the bottom of the microtube with a pushpin and collecting one droplet in a sterilized 1.5-mL microtube.

1.7.2. Dilute the precipitated GV droplet 10–100 fold by volume with the outer aqueous solution if the obtained GVs are obtained in quantities large enough to make observation difficult.

2. Microscopy observation of GVs

2.1. Prepare microscopy specimen.

2.1.1. Place an adhesive incubation chamber for *in situ* polymerase chain reaction and hybridization (chamber size 9 mm × 9 mm × 0.3 mm thick) on top of a microscope cover glass.

2.1.2. Using a micropipette, deposit 25 µL of the diluted precipitated GVs on the specimen area and immediately place another cover glass (approximately 0.15 mm thick) on top of the incubation chamber.

2.2. Record differential interference contrast microscopy images of the GVs.

2.2.1. Record microscopy images of the vesicles with a microscope (10×, 20×, and 40× objectives) equipped with a 12V100WHAL-L halogen lamp.

2.3. Conduct fluorescence microscopy observations.

2.3.1. Insert U-FBNA and U-FMCHE fluorescence mirror units into the microscope. Fit the units with 470–495-nm and 565–585-nm excitation filters, respectively, and with emission filters that transmit 510–550-nm and 600–690-nm light, respectively.

REPRESENTATIVE RESULTS:

The w/o emulsion centrifugation method is illustrated photographically and schematically in **Figure 1**. The schematic image in **Figure 1i** suggests that the most important determinant of the success of this method is that the specific gravity of the inner aqueous solution must be larger than that of the outer aqueous solution, so that the GVs will precipitate during centrifugation. In addition, the formation of a lipid monolayer at the w/o interface requires that the system be chilled for 10 min after the emulsion is layered on the outer aqueous solution. Because the GVs form by transfer of emulsion droplets across the w/o interface, the osmotic pressures in the inner and outer aqueous layers must be the same. As a control experiment, we also prepared GVs

containing no microspheres by means of the process shown in **Figure 1** and step 1.3, except that the inner aqueous solution was prepared without microspheres.

Collection of the precipitated GVs after centrifugation is shown in **Figure 2**. In addition to the semitransparent phase at the very bottom of the microtube, we also observed a white, turbid intermediate phase in the outer aqueous solution (**Figure 2a**). This intermediate phase was rich in aggregations of microspheres and oil, whereas the bottom phase contained the GVs. Therefore, after piercing the bottom of the microtube with a pushpin (**Figure 2b**), we collected only the first drop (**Figure 2c**), which contained massive amounts of GVs. It is important to make sure that no more than two drops are collected; any additional drops may contain aggregations of microspheres and lipids, which will result in a lower density of GVs. The obtained vesicular dispersion often contained encapsulated materials outside of the GVs because the GVs often rupture during centrifugation. To obtain only GVs, a sorting method such as dialysis, gel filtration, or fluorescence-activated cell sorting should be chosen, owing to the sizes of the encapsulating materials. If necessary for the purpose for which the precipitated GVs are to be used, they can be diluted with the outer aqueous solution. In some cases, the intermediate phase extended to the bottom of the tube, suggesting that any vesicles that formed were held together by the oil.

We obtained differential interference microscopy and fluorescence microscopy images of the GVs without microspheres (**Figure 3a, b**) and with microspheres (**Figure 3c–f**). Consistent with previous reports[23, 37], GVs with low lamellarity were formed by our protocol. Lipids conjugated with Texas Red DHPE, which emits red fluorescence, were used so that vesicle formation could be directly confirmed by visualization of the thin membrane. Of the 160 GVs that we obtained, 55 encapsulated microspheres and 105 were empty, giving a ratio of encapsulation of 34%.

We determined the volume fraction (φ, vol %) of microspheres in the GVs by means of the following method. Because each GV contains dozens to several hundred microspheres, counting all the microspheres under an optical microscope is challenging. Therefore, we mixed the nonfluorescent 1.0-μm microspheres with a small amount of fluorescent microspheres, which were manually counted under the fluorescence microscope. The total number (N) of encapsulated microspheres was calculated by multiplying the number (n) of manually counted fluorescent microspheres by 20 (based on the original 95:5 [v/v] ratio of nonfluorescent to fluorescent microspheres). The value of φ was then estimated as $Nv100/V$, where v is the volume of the microspheres and V is the volume of the individual GV. Note that estimation of N from n gives rise to counting errors, and these errors must be taken into account when calculating φ. The value of φ was estimated from N, which was directly calculated as $20n$, and this in turn resulted in the probability that φ accurately represents the true value is <50%. In fact, N fluctuates to some extent around $20n$, so we need to consider it as $20(n \pm i)$, where i is the error in n. We estimated i in order that a probability of $n \pm i$ could be more than 50% obtained on the basis of a Poisson distribution. We estimated i, which in turn allowed us to calculate $20(n \pm i)$ and values of φ that included counting errors for GVs with diameters of 10 and 15 μm (**Table 1**). According to this procedure, the volume fraction of microspheres in the GV shown in **Figure 3c** was estimated to be 11 ± 3 vol %. The precision of the calculated volume fraction was 10–30%.

Our results indicate that we successfully encapsulated 1-μm microspheres in GVs at a high volume fraction. We were also able to encapsulate other materials into 100 mol % DOPC GVs using the same outer aqueous solution and the same protocol (**Figure 4**). Specifically, GVs containing 0.1-μm microspheres were prepared from a Tris-buffered solution containing 0.1 M Tris-HCl (pH 7.5), 0.15 M sucrose, and 0.5 vol % fluorescent microspheres by means of the protocol described for GVs containing 1.0-μm microspheres (**Figure 4a**). Following the protocol described above, DOPC GVs containing GFP (0.1 M Tris-HCl [pH 7.5], 0.15 M sucrose, and 100 μg/mL GFP; **Figure 4b**) and GVs containing uranine (0.1 M Tris-HCl [pH 7.5], 0.15 M sucrose, and 30 μM uranine; **Figure 4c**) were also prepared.

Table 1. Numbers and volume fractions (φ, vol %) of microspheres[a]

[a] Errors were determined as described in the text; n = number of manually counted microspheres; N = total number of encapsulated microspheres.

Figure 1: Flow chart and schematic depiction of w/o emulsion centrifugation method.

(a) Oil solution consisting of DOPC and Texas Red DHPE (100:0.3 molar ratio) in liquid paraffin. (b) Inner aqueous dispersion consisting of sucrose and microspheres in Tris-HCl buffer. (c) Outer aqueous solution consisting of glucose in Tris-HCl buffer. (d) Mixture of 1 mL of oil solution and 300 μL of inner aqueous dispersion. (e) Emulsification with a homogenizer (10,000 rpm, 2 min). (f) The w/o emulsion. (g) Layering of 300 μL of the emulsion on 1 mL of the outer aqueous solution. (h) Precipitated GVs just after centrifugation. (i) Schematic depiction of the principle of the w/o emulsion centrifugation method. The black arrow indicates the direction of centrifugal acceleration.

Figure 2: Piercing of the microtube.

(a) Precipitated GVs just after centrifugation (also depicted in **Figure 1h**). The red ellipse indicates the droplet of the precipitated GV dispersion. (b) Piercing of the microtube with a pushpin. The pellet containing the GVs was obtained by piercing the microtube near the bottom with a pushpin and collecting droplets from the hole. (c) Droplet of the precipitated GVs (indicated by the yellow arrow) dispersion diluted with the outer aqueous solution.

Figure 3: Microscopy images of GVs.

(a) Differential interference contrast microscopy image of a GV without microspheres. The diameter of GV was about 10 μm. Scale bar = 10 μm. (b) Fluorescence microscopy image of a GV without microspheres. The red fluorescence was emitted by Texas Red DHPE. (c) Differential interference contrast microscopy image of a GV containing microspheres. (d) Fluorescence microscopy image of 1.0-μm microspheres (YG carboxylate microspheres) inside a GV. The number of counted fluorescent microspheres (n) was 6. We estimated the errors (i = 2) based on Poisson distribution. Then we estimated total number of inner microspheres (N = 20 ($n \pm i$)) was 120 ± 40. It was calculated that the GV contained 1.0-μm microspheres at a volume fraction ($\varphi = Nv100/V$) of approximately 11 ± 3 vol %, where v is the volume of the microspheres and V is the volume of the GVs. (e) Fluorescence microscopy image of a GV membrane. The red fluorescence was emitted by Texas Red DHPE. (f) Merged image of the images in panels **d** and **e**.

Figure 4: Differential interference contrast and fluorescence microscopy images of GVs with different encapsulated materials.
Differential interference contrast microscopy (top) and fluorescence microscopy (bottom) images of GVs containing (a) 0.1-µm microspheres, (b) GFP, and (c) uranine. Scale bars = 10 µm.

DISCUSSION:
The specific gravities of the inner aqueous dispersion medium and the outer aqueous solution must be chosen carefully. For the w/o emulsion to precipitate into the outer aqueous solution during centrifugation, the specific gravity of the inner aqueous dispersion medium must be larger than that of the outer aqueous solution. We tried to prepare GVs using inner and outer solutions without sugars, but we obtained no GVs under these conditions, because the inner aqueous solution did not have enough mass to cross the interference between the two phases. If there is a large osmotic pressure difference between the two solutions, GVs that precipitate into the outer aqueous solution may shrink or rupture. Therefore, the osmotic pressure inside and outside the GVs must be equal. To accomplish this, we used sucrose as a solute in the inner aqueous dispersion and glucose as a solute in the outer aqueous solution; both sugars were at the same concentration. Both salt[22] and sugar[23, 35, 38] have been used for such purposes, but sugar is usually employed because it is less toxic and more soluble than salt. However, if too much sugar is added, the GVs may come into contact with the bottom of the cover glass and collapse. There are several strategies for avoiding this. One strategy is to reduce the specific gravity difference between the inner and outer aqueous solutions so that the GVs precipitate under mild conditions; specifically, the supernatant of the precipitated GV dispersion can be exchanged with a solution that is identical to the inner aqueous solution to reduce the possibility of vesicular rupture by adhesion to the cover glass as a result of buoyancy. Another strategy is to precoat both of cover glasses with a thin lipid film[29].

Proper preparation and incubation of the emulsion are important. We used liquid paraffin to prepare the oil solution. Mineral oil[26, 31], dodecane[21, 33], and squalane[33, 39] are also often used as solvent oils. Because these materials vary in specific gravity, viscosity, and surface tension, different numbers of vesicles form even when the same centrifugation conditions are used[33]. To obtain the vesicles with the target properties, it is essential to optimize the specific gravities and viscosities of the inner and outer aqueous solutions as well as the centrifugal acceleration. For preparation of the oil phase, incubation must occur at high temperature and in a dry environment such as an incubator or a dehydrator. In this study, we heated the liquid paraffin to 80 °C to completely dissolve the lipid molecules. In addition, the emulsion should be prepared only as needed and should be immediately subjected to centrifugation because it is unstable just after it is prepared and the w/o droplets readily fuse to one another. The emulsion can be prepared in large quantities by sonication, vortexing, or tapping. However, using a homogenizer allows for rapid preparation of large amounts of emulsion and easier emulsification in oil with a high viscosity. It is also important that the emulsion be layered on the outer aqueous solution gently and rapidly and then chilled at 4 °C. To shorten the time between emulsification and centrifugation, the oil–outer aqueous solution system can be prechilled before the emulsion is layered on it, and the whole system can then be centrifuged immediately. If the white turbidity appears faster or slower than usual, the mechanical homogenizer must be thoroughly rinsed to

remove cleaning detergents. In addition, before emulsification, the oil solution, inner aqueous solution, and emulsifier must be returned to room temperature.

Proper centrifugal acceleration is also important. By centrifuging at 18,000 x *g*, we were able to prepare GVs with a single interior material encapsulated at a high concentration. When the encapsulation of multiple materials is required, it is better to reduce the centrifugal acceleration. For example, an actin assembly system encapsulating seven compounds was achieved with centrifugation at less than 350 x *g*[35]. In cases in which centrifugation is undesirable, GVs can be obtained by adjusting the sugar concentration or by precipitating the emulsion under the influence of its own mass[38, 40, 41].

The method reported herein has two major limitations. One is that oil molecules (paraffin, in this case) can be solubilized in the GV membrane, as has been pointed out by the Weitz group[21]. When insertion of a membrane protein into the GV membrane is desired, the effects of co-existing oil molecules on the protein must be considered. The other limitation is the variation of volume fraction. In this study, we estimated that the volume fractions of microspheres in the GVs ranged from 4 to 30 vol %; the volume fractions were not identical to the volume fraction of the inner aqueous solution used for GV preparation. Although we were able to encapsulate microspheres in the GVs at a volume fraction high enough for microscopy observation, this method is not suitable for the preparation of GVs with a uniform volume fraction distribution. It has been reported that the distribution of microsphere volume fractions changes during centrifugation[34].

The w/o emulsion centrifugation method is commonly used for the formation of GVs containing encapsulated materials. However, few reports have described the preparation of GVs encapsulating microscale materials[34, 42]. Recently, molecular robots containing an encapsulated DNA device or a molecular device have been constructed[43, 44]. GVs with compartments are the first choice for these kinds of applications; therefore, techniques, such as ours, that could be used for encapsulating magnetic microspheres and microspheres with diverse surface functionalization can be expected to be useful[44].

ACKNOWLEDGMENTS:
We thank Tomoko Yamaguchi for drawing the schematic image in Figure 1. This study was supported by the Okazaki ORION Project of the Okazaki Institute for Integrative Bioscience of the National Institutes of Natural Sciences (NINS); by the Astrobiology Center Project (no. AB281010) of NINS; by a Grant-in-Aid for Scientific Research on Innovative Areas (Dynamical ordering of biomolecular systems for creation of integrated functions) (no. 25102008) from the Japan Society for the Promotion of Science (JSPS); by a Grant-in-Aid for Young Scientists (B) (to K.K., no. 15K17850) from JSPS; and by a Grant-in-Aid for Research Activity Start-Up (to Y.N., no. 15H06653) from JSPS. Additional support was provided by a grant from the Noguchi Institute, a grant from the Kao Foundation for Arts and Sciences, and a grant from the Kurita Water and Environment Foundation.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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revised Figure

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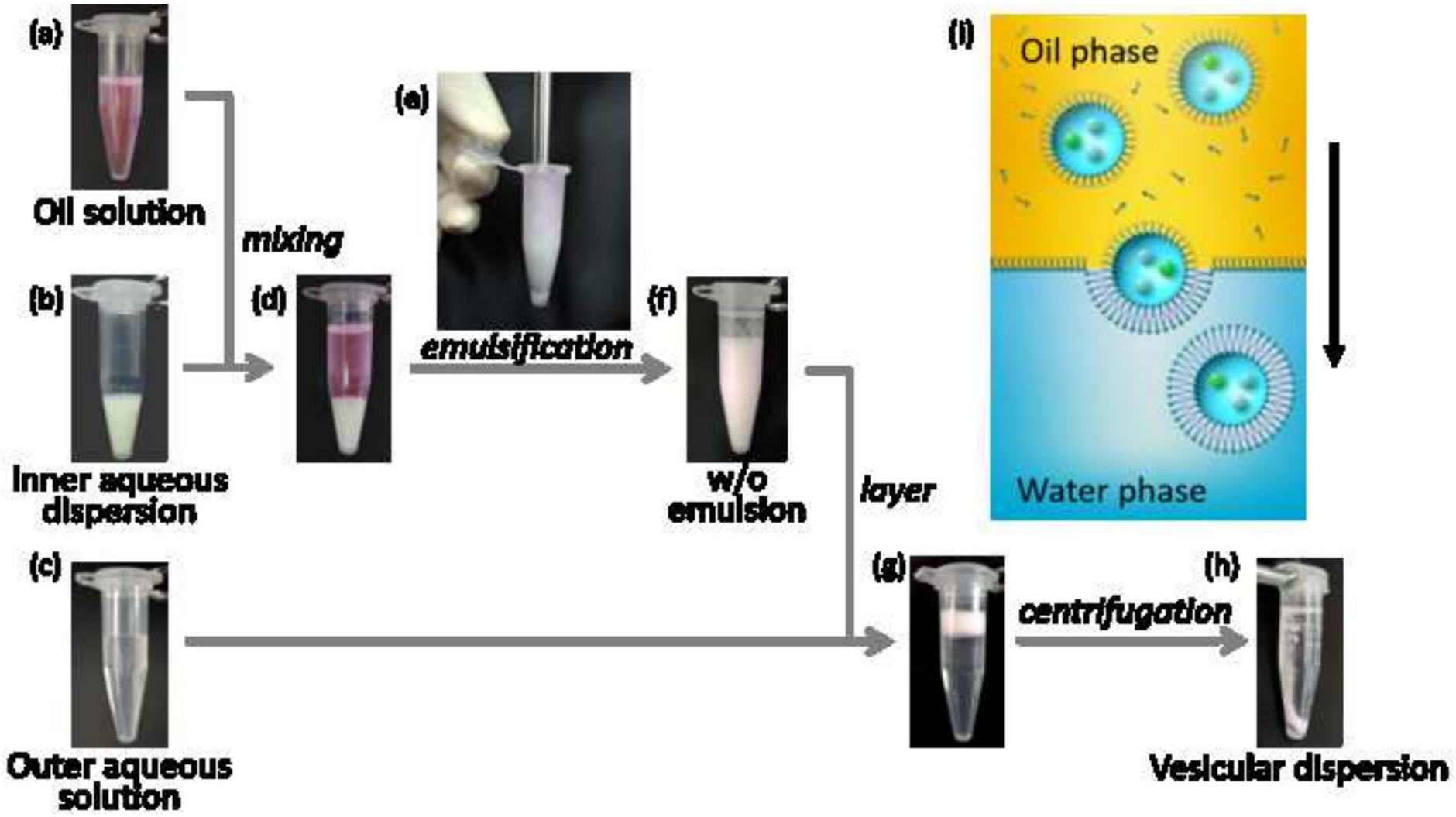

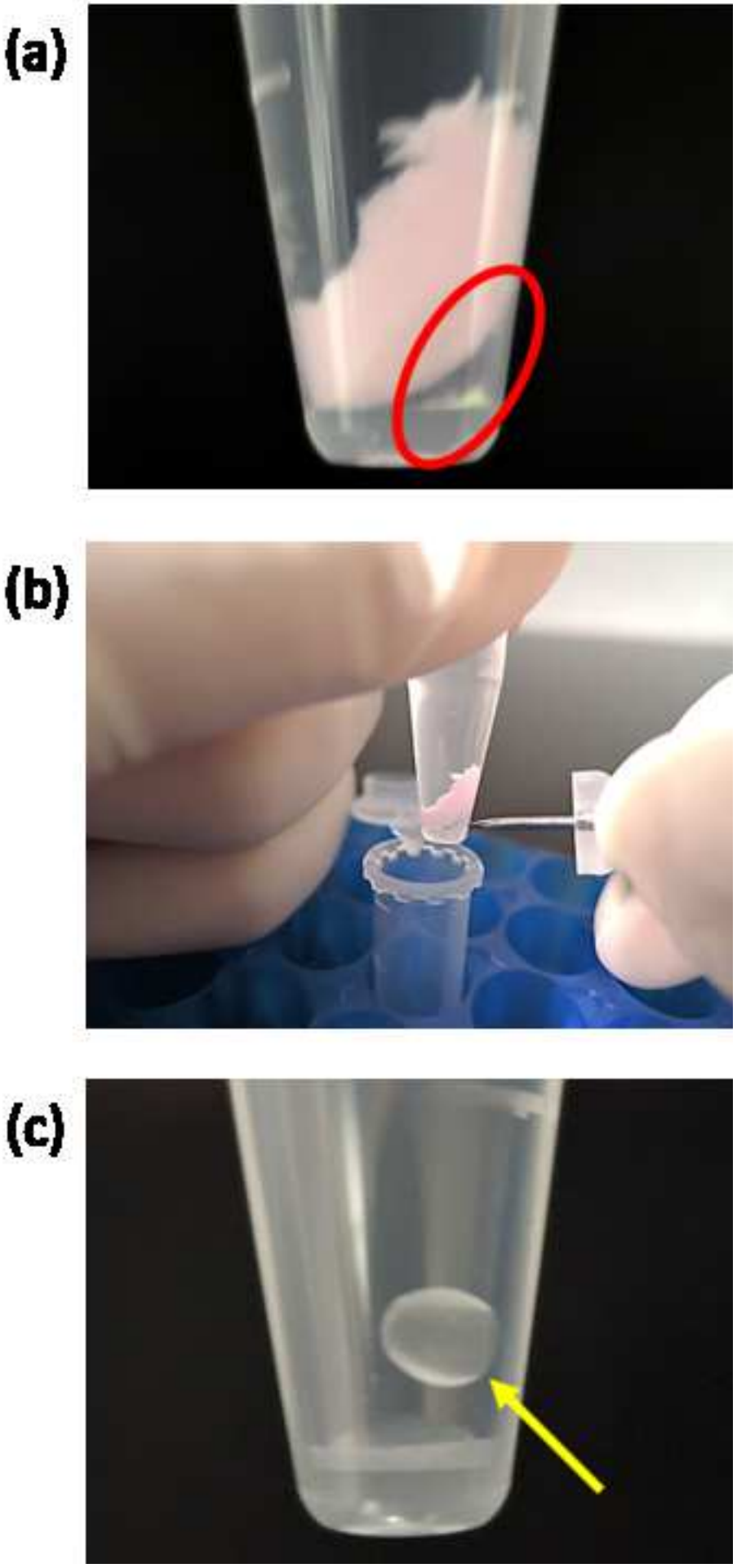

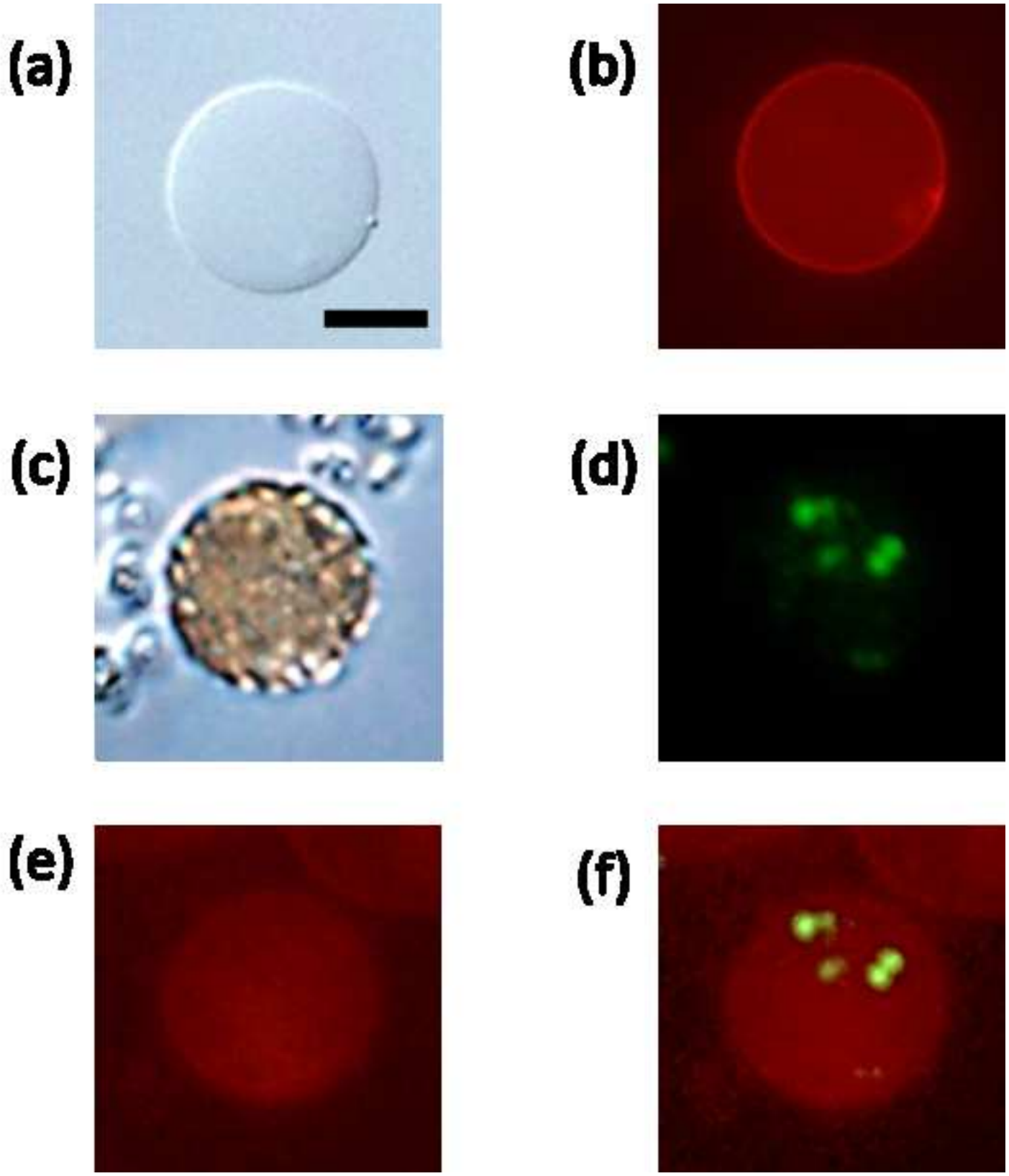

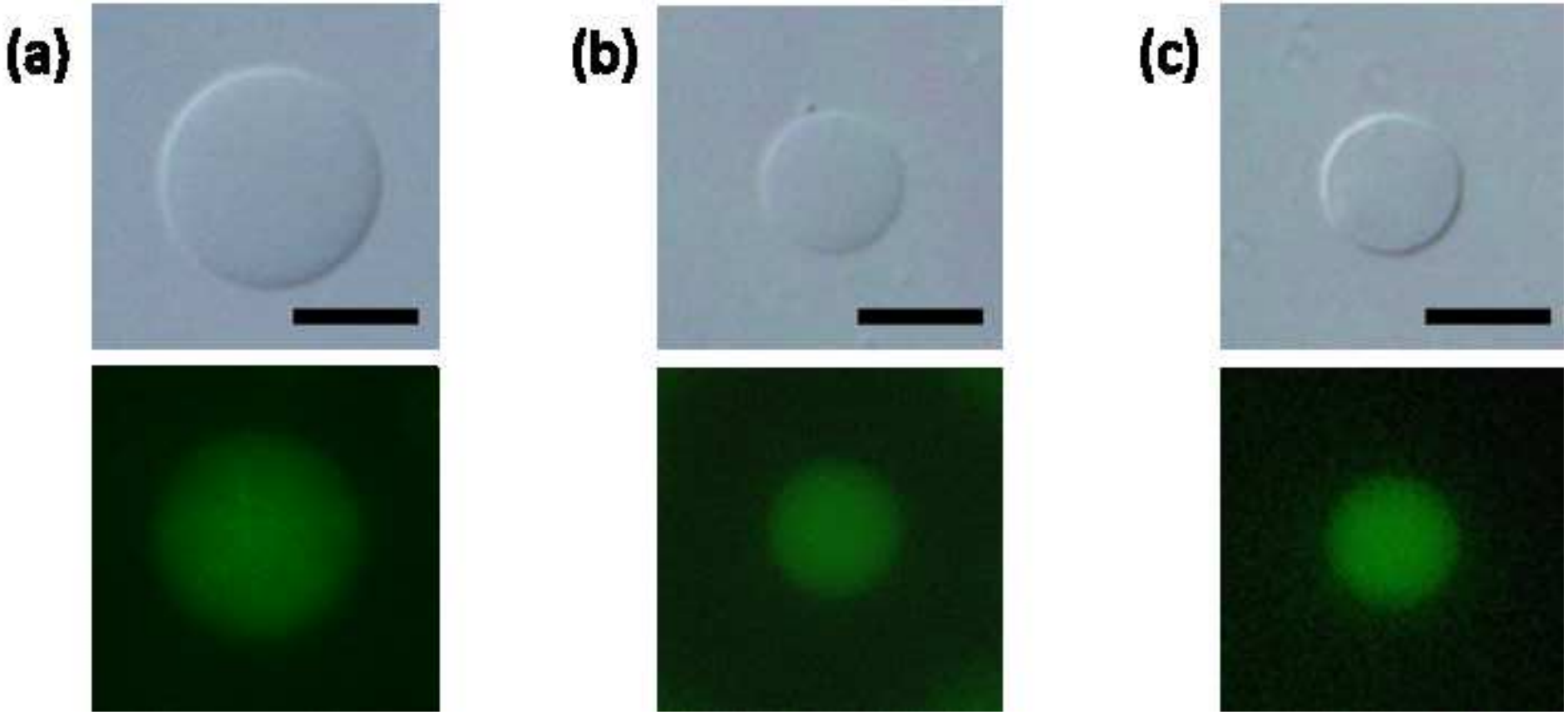

Table 1. Numbers and volume fractions (φ, vol %) of microspheres[a]

n	*N*	φ (10-µm diameter)	φ (15-µm diameter)
3	60 ± 20	6.0 ± 2.0	1.8 ± 0.6
4	80 ± 20	8.0 ± 2.0	2.4 ± 0.6
5	100 ± 20	10 ± 2	3.0 ± 0.6
6	120 ± 30	12 ± 4	3.6 ± 1.2
10	200 ± 32	20 ± 3	5.9 ± 0.9
20	400 ± 45	40 ± 5	12 ± 2
30	600 ± 55	-	18 ± 2

[a] Errors were determined as described in the text; n = number of manually counted microspheres; N = total number of encapsulated microspheres.

Excel Spreadsheet- Table of Materials/Equipment

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REAGENTS:

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)	Avanti Polar Lipids	850375C	Vesicular membrane molecule
Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE)	Thermo Fisher Scientific	T1395MP	
Liquid paraffin	Wako Pure Chemical Industrie	128-04375	Oil, Density (20℃) 0.86 −0.89 g/mL
1 M Tris-HCl (pH 7.5)	Nippon Gene Co.	318-90225	Buffer solution
D(+)-Glucose	Wako Pure Chemical Industrie	049-31165	For outer water solution
Sucrose	Wako Pure Chemical Industrie	196-00015	For inner water solution
Polybead carboxylate microspheres 1.0 μm	Polysciences	08226-15	Nonfluorescent, 2*R* = 1.0 μm
Fluoresbrite YG carboxylate microspheres 1.0 μm	Polysciences	15702-10	Fluorescent, 2*R* = 1.0 μm
Fluoresbrite YG carboxylate microspheres 0.10 μm	Polysciences	16662-10	Fluorescent, 2*R* = 0.10 μm
Fluorescein sodium salt (uranine)	Sigma Aldrich Japan	F6377-100G	
GFP standard (recombinant)	Vector Laboratories	MB-0752	

EQUIPMENT:

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Centrifuge 5427R	Eppendorf	5409000233	Centrifuge rotor: FA-45-48-11
Physcotron	Microtec Co.	NS-310EIII	Handy micro-homogenizer
Generator shaft	Microtec Co.	NS-4	Homogenizer attachment
Frame-Seal incubation chambers for *in situ* PCR and hybridization	Bio-Rad Laboratories	SLF0201	Hybridization chamber volume: 25 μL Chamber size: 9 × 9 × 0.3 mm
Frame-Seal incubation chambers for *in situ* PCR and hybridization	Bio-Rad Laboratories	SLF0601	Hybridization chamber volume: 65 μL Chamber size: 15 × 15 × 0.3 mm
Microman E	Gilson	FD10006	Pipette for viscous liquid. We measured liquid paraffin by using this.
Inverted microscope	Olympus Co.	IX73	
Mirror unit	Olympus Co.	U-FBNA	Excitation filter: 470-495 nm Emission filter: 510-550 nm
Mirror unit	Olympus Co.	U-FMCHE	Excitation filter: 565–585 nm Emission filter: 600-690 nm

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Author(s): Yuno Natsume, Tong Zhu, Hsin-i Wen, Kazumi Itoh, Li Sheng, Kensuke Kurihara

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Editorial comments:

Changes to be made by the Author(s):

2. For more than 6 authors, list only the first author then et al.

Answer

We modified the references as specified.

3. Please abbreviate all journal titles.

Answer

We revised accordingly.

4. Grammar

-Although not incorrect, the grammar in the first sentence of the Long Abstract is somewhat confusing. "In the exploration of the origins of life and the study of biological functions, the constructive, or synthetic, biology approach, that is, the attempt to construct living entities from the bottom up, has received considerable attention."

Answer

We broke this long sentence into two shorter sentences to make the meaning clearer. The revised sentences are as follows (lines 66-69):

"The constructive biology and the synthetic biology approach to creating artificial life involve the bottom-up assembly of biological or nonbiological materials. Such approaches have received considerable attention in research on the boundary between living and nonliving matter and has been used to construct artificial cells over the past two decades."

-Introduction, lines 90-93: "Giant unilamellar vesicles (GUVs) have been studied extensively not only because they are similar to cells in size and shape but also low lamellarity because they can be used in fields in which incorporation of proteins into the vesicle membrane is needed."

Answer

In response to the comments of Reviewer #1, we changed the term "giant unilamellar vesicles" to "giant vesicles." In addition, we modified the relevant text to make it clear and grammatical (lines 90-93).

"Giant vesicles (GVs) have been studied extensively because they are similar to living cells in size, shape, and structure. Owing to the size of GVs, morphological changes in GV membranes can easily be observed in real time under an optical microscope."

-2.2 - The second sentence is missing a period.

Answer

We added the missing period.

-In the second paragraph of the discussion, this sentence could be cleaned up: "If the sample appears abnormal during emulsification (for example, if the white turbidity appears faster or slower than usual), the equipment for emulsification must be thoroughly rinsed to remove cleaning detergents because an abnormal appearance can be caused by cleaning detergents."

Answer

We revised the sentence as follows (lines 331-334):

"If the white turbidity appears faster or slower than usual, the mechanical homogenizer must be thoroughly rinsed to remove cleaning detergents. In addition, before emulsification, the oil solution, inner aqueous solution, and emulsifier must be returned to room temperature."

5. The protocol is quite short, barely over one page in length, with ample room to provide additional detail.

Answer

We added additional details.

-1.2 - The second sentence says to wrap "the microtube" in foil, but no microtube has been introduced yet. The first sentence says to prepare an oil solution but does not specify the type or volume of container.

Answer

This problem has been corrected as follows (lines 139-143):

"Prepare an oil solution. Form a lipid film on the inside surface of a 5-mL glass vial by evaporating a mixture of the DOPC stock solution (51.0 μL) and the Texas Red DHPE stock solution (19.2 μL) under flowing nitrogen gas. Incubate the film under reduced pressure overnight and then add 1.0 mL of liquid paraffin (0.86–0.89 g/cm^3) to the vial. Wrap the vial in aluminum foil and incubate it at 80 °C overnight at rest."

-1.3, 1.5 - Same general comment as above.

Answer

We revised as follows:

Step 1.3 (lines 146-152)

"Prepare the inner aqueous dispersion. In a 1.5-mL lidded microtube, mix 237.5 µL of a dispersion of 1.0-µm nonfluorescent microspheres (2.5 vol %) and 12.5 mL of a dispersion of 1.0-µm fluorescent microspheres (2.5 vol %); this corresponds to a 95:5 (v/v) ratio of nonfluorescent to fluorescent microspheres. Then add 64 mg of sucrose followed by 125 µL of Tris-buffered solution (1 M) and 875 µL of deionized water. The final volume fraction of microspheres is 0.5 vol %, and the final concentrations of Tris-HCl (pH 7.5) and sucrose are 0.1 and 0.15 M, respectively. Vortex the microtube for 30 s and then sonicate it for 10 min."

Step 1.5 (lines 159-162)

"Mix 1 mL of the oil solution (liquid paraffin containing DOPC) with 300 µL of the inner aqueous solution in a 1.5-mL microtube. Emulsify the two components in the microtube by using a mechanical homogenizer (an agitator with blades that rotate at high speed) operated at 10,000 rpm for 2 min at room temperature."

-Section 3 needs to be fleshed out. What type of glass plate? How is an incubation chamber used as a spacer? What type of DIC and fluorescent microscopes? How should images be captured and recorded? Step-wise detail should be provided for this section.

Answer

We added some additional details, and in response to editor's comment #6, we moved what was formerly Section 2 to the results section. Section 3 in the original manuscript is Section 2 in the revised manuscript. The revised passages are as follows:

Step 2.1 (lines 175-179)

"Place an adhesive Frame-Seal™ incubation chamber for *in situ* polymerase chain reaction and hybridization (chamber size 9 mm × 9 mm × 0.3 mm thick, Bio-Rad Laboratories) on top of a microscope cover glass. Using a micropipette, deposit 25 µL of the diluted precipitated GVs on the specimen area and immediately place another cover glass (approximately 0.15 mm thick) on top of the incubation chamber."

Step 2.2 (lines 181-185)

"Record differential interference contrast microscopy images of the GVs. We recorded microscopy images of the vesicles with an Olympus IX73 microscope (10×, 20×, and 40× objectives) equipped with a 12V100WHAL-L halogen lamp. The images were recorded with an Olympus DP73 camera. Observation and analysis were performed using the Olympus cellSens® image-recording system."

6. As a note, Section 2 consists of two steps of calculations, which will not be filmable.

Answer

We moved Section 2 of the original manuscript to the results section of the revised manuscript (lines 226-239). Section 3 in the original manuscript is Section 2 in the revised manuscript.

Reviewer #1:

Manuscript Summary:

The manuscript presented by Dr. Natsume and Dr. Kurihara deals with the preparation of giant vesicles (GVs) which contain microbeads. The preparation method, here called "water-in-oil emulsion centrifugation" is a novel method of vesicle preparation that attracts the attention of several researchers, who often get tricked by the experimental details of this method.

The manuscript describes in details the method, with clear image and text. Readers will enjoy reading this work as it is quite useful for practical viewpoint. The idea of encapsulating microbeads is just an example of applications, because - as the authors correctly say - many other particles can be encapsulated by this method inside GVs. This is perhaps the most important point to be emphasized by the authors when they speak about the features of the method. The possibility of encapsulating macromolecular solutes and small particles inside vesicles. This is indeed difficult by other methods. Moreover, this method can be applied with high ionic strength buffer, whereas other methods often do not (some can).

Therefore, a JoVE publication on this subject is very timely and interesting for the scientific community working on giant vesicles.

In this respect, the topic presented here is valuable for publication.

There are however a number of Major and Minor issues that prevent the publication of the manuscript in its current form. In particular:

Major & Minor Concerns:

1) English language needs an in-depth revision from native English speakers. In too many points, the text is not well readable and it can be improved a lot.

Answer

We appreciate your useful comments and suggestions, which have helped us to make our manuscript more convincing. Our resubmitted manuscript has been checked by two native-English-speaking professional science editors.

2) The Authors from the very beginning use the term GUV (giant unilamellar vesicles) to refer to the vesicles studied here and obtained by the method. However, they do not provide any evidence that the vesicles are really unilamellar. The investigation of vesicle lamellarity is not easy, and the authors should be more careful in defining their GVs as GUVs. It can be said, however, that a recent work on the lamellarity of GVs produced by this method has been detailed investigated, as reported by the paper of Chiba et al. in 2014.

Unfortunately, the Authors did not cite this paper, whereas I believe that it would be useful to cite it and comment it, with a careful note on the extrapolation of their result (most of GVs are unilamellar) to the present study. In other words, I would be more cautious about using the term 'unilamellar' without an experimental proof. A citation - and discussion with respect to the present paper - about previous studies on very similar system is probably very useful to the readers.

Answer

On the basis of your comments, we eliminated the term "unilamellar" from the manuscript and added a statement about the low lamellarity of vesicles prepared by the w/o emulsion centrifugation method (lines 101–102). In addition, we added a reference to the Chiba et al. 2014 paper (ref. 24 in the revised manuscript).

3) On page 2, line 85, I would first explain better what vesicle are. For example: vesicles, which are generally spherical microcompartments made of …

Answer

On the basis of your advice, we modified the sentence in lines 85-86.

4) Page 3 line 89, is '2R' the diameter?

Answer

Yes. We revised to address this comment.

5) Page 3, line 92. I partially disagree with the authors about the fact that GVs are useful especially because proteins can be inserted in their membrane. Actually, the large majority of membrane protein reconstitution has been done with SUV, not with GVs. The main relevance of GVs in my opinion is their size, which allows direct observation in real time by light microscopy. This remark is also valid against property #1 enunciated by the Authors few lines below (page 3, line 103). Ref. 23 and Yomo's references refer to the alpha-haemolysin case, a somehow very special case of membrane insertion. The work of Yoshikawa et al. on a K-channel protein, which would have been more pertinent here, has not been cited.

Answer

We modified the relevant text to address this comment. We eliminated the classification of vesicles by membrane lamellarity and instead concentrated on the size classification. We agree that insertion of membrane proteins into vesicular membranes is difficult. Therefore, we revised "easily be incorporated into the membrane" to "membrane proteins can be inserted into the vesicular membrane by this method, albeit with difficulty" (lines 106-107). In addition, we

replaced the first and second features in the original manuscript with two different features in the revised manuscript, because research on the insertion of proteins into the membrane is rare. Thank you for pointing out the Yoshikawa et al. reference. Because the method of these investigators does not involve centrifugation, we did not cite the reference in this part of the paper. Nevertheless, because it is important for the readers of our paper, we cited it in the discussion section (ref. 41) of the revised manuscript).

6) When the authors, on page 3, speak about Z-ring, they refer to papers where vesicles were not prepared by the water-in-oil emulsion centrifugation method. I am not sure these references are really pertinent here. But they miss instead a work where FtsZ has been inserted in GVs prepared by this method (J. Biol. Chem. 2013, 288, 26625-26634).

Answer

Our check of ref. 26 (Osawa M. et al.) in the original manuscript indicates that these investigators also prepared GVs by means of the water-in-oil emulsion centrifugation method. However, we agree that the recommended reference (*J. Biol. Chem.* 2013, **288,** 26625-26634) is important, and we added it to the revised manuscript as ref. 25.

7) In conclusion, when in line 102 (page 3) the authors write "This method has five important features" actually their arguments are not really always based on papers using the method, as they cite papers based on different vesicle preparation method, and do not cite instead papers based on the method. My overall suggestion is that the Authors make a literature check for inserting only those references, which are pertinent here. Other references can be also used, but they must be properly placed in the text.

Answer

We checked all references in the relevant parts of the manuscript. As a result, we deleted ref. #31 (original ref. numbering; Hamada et al., 2008); Hamada et al. used the w/o emulsion transfer method without centrifugation. We believe that the other references are relevant to the revised manuscript.

8) "unfamiliar manufacturer" sounds a bit strange (page 3, line 119)

Answer

We changed “unfamiliar manufacturer” to “inexperienced technicians” (line 120).

9) Page 4, line 137-141. It is not clear whether after addition of paraffin the sample is kept under stirring or just incubated at rest. Moreover I am not fully sure about the meaning of the final lipid concentration obtained. The Authors refer to 1.27 mM and 3.81 uM. Do they

mean that they get always the same final concentration? Do they did repetitions? What the variations from sample to sample?

Answer

We always obtained the same final lipid concentrations. Therefore, we modified the concentrations to 1.3 mM (line 143) and 3.8 μM (line 143), respectively. The final concentrations were always the same to two significant figures because we always dispensed the DOPC and Texas Red DHPE stock solutions using micropipettes (51.0 and 19.2 μL, respectively).

10) For readers, it would be useful to specify better what is the "mechanical homogenizer" (Page 4, line 154). Please use the same term also in the final Table (list of instruments)

Answer

In the revised manuscript, we included more information about the homogenizer (lines 161-162). In addition, we modified the reagents/equipment table.

11) Page 4, line 164, Correct English of "… if massive of…"

Answer

We modified this as follows: "if the obtained GVs are obtained in quantities large enough to make observation difficult." (lines 1721-172)

12) Page 4, line 170, please explain better that one counts the fluorescent beads and multiply by 20 in order to count also the non-fluorescent beads

Answer

We moved this discussion to the result section and modified it as follows (lines 228-236):

"Therefore, we mixed the nonfluorescent 1.0-μm microspheres with a small amount of fluorescent microspheres, which were manually counted under the fluorescence microscope. The total number (N) of encapsulated microspheres was calculated by multiplying the number (n) of manually counted fluorescent microspheres by 20 (based on the original 95:5 [v/v] ratio of nonfluorescent to fluorescent microspheres). The value of φ was then estimated as $Nv100/V$, where v is the volume of the each microspheres and V is the volume of the individual GV. Note that estimation of N from n gives rise to counting errors, and these errors must be taken into account when calculating φ."

13) Results. I suggest the authors to present first the results of GVs formation in the absence of microbeads. First, this is a useful control. Second, this is really what many people are looking for because they are interested in the water-in-oil emulsion

centrifugation method. Please also refer that other images about the method are available in the Yomo's work (Fujii et al. Nature Protocols 2014)

Answer

We conducted the requested control experiments and added a description of the results to the results section (Fig. 3) of the revised manuscript.

14) Page 5, line 206, I suggest to change the word "water" with "aqueous"

Answer

Thank you for this suggestion; we replaced "water" with "aqueous."

15) Page 5, line 218: it is not clear what the Poisson distribution refer to. Please explain better to the readers

Answer

We added explanatory text (lines 233-241).

16) The sentence starting on page 5 (last line) and ending on page 6 is not clear (from "By this mean…" to " 15 um (Table 1)")

Answer

We modified the sentence as follows to clarify (lines 240-241):

"We estimated i, which in turn allowed us to calculate 20(n ± i) and values of ϕ that included counting errors for GVs with diameters of 10 and 15 μm **(Table 1).**"

17) References 33-35 have been used to discuss the use of the sugar gradient. But again the authors miss the original reference, which dates back to 2008 by Hamada (J Phys Chem B 112:14678). Please refer to this key work that allowed a facile GVs formation in all next studies.

Answer

We added the work by Hamada et al. to the revised manuscript as ref. 38.

18) Page 7 line 280-281 not clear to most of the readers. Please specify

Answer

We modified these lines as follows:

"specifically, the supernatant of the precipitated GV dispersion can be exchanged with a solution that is identical to the inner aqueous solution, to reduce the possibility of vesicular rupture by adhesion to the cover glass as a result of buoyancy" (lines 309-312).

19) Page 7 line 290. What is the meaning of "low humidity" here? Please explain

Answer

We added the following text: "incubation must occur at high temperature and in a dry environment such as an incubator or a dehydrator" (lines 321-322).

20) Page 7 line 293. It is not the emulsion that fuse, but the water-in-oil droplets. Please revise

Answer

We revised to address this comment.

21) Importantly, the authors do not mention one of the main limitation of the method, namely the possibility that some paraffin is solubilized in the GVs membrane. This has been discussed in only few articles, possibly to be mentioned. But it is a good idea to give the reader this information, and specify that for some applications (especially those mentioned in the introduction, namely in constructive biology) this does not represent a serious limitation, at least in first approximation. Clearly for membrane proteins one should consider the possible presence of oil in the membrane, which can affect the protein function.

Answer

In response to this comment, we added another limitation, which is the possibility that paraffin may be present in the vesicular membrane. In addition, because we think that this limitation is more important for readers than the variation of the volume fraction of encapsulates, we changed the order in which the two limitations are described (lines 344-347).

Reviewer #2:

Manuscript Summary:

In this paper the authors speak about giant unilamellar vesicles (GUVs) used as artificial cell membranes. They describe the preparation of GUVs encapsulating highly packed microspheres as a model of cells containing highly condensed biomolecules. The vesicles were prepared by the water-in-oil emulsion centrifugation method. An aqueous solution containing the materials to be encapsulated was emulsified in an oil containing dissolved phospholipids, and the resulting emulsion was layered carefully on the surface of another water solution. The layered system was then centrifuged to generate the GUVs. This powerful method could be used to encapsulate materials ranging from small molecules to microspheres.

The title and the abstract are appropriate for this article since are related to a simple method with which is possible to encapsulate micrometric particles within giant unilamellar lipid vesicles. Of course that microspheres are considered a model of any kind of solute that can be entrapped but, since they are totally inert, it does not take into account possible interaction of chemical species in a so crowded environment.

All the reagents and equipment are listed in the table and the experimental procedure is well described in text and in figure 1 and figure 2.

Major Concerns:

-No experiments control are shown in the paper, i.e. GUVs preparation without microspheres and GUVs preparation with microspheres in absence of sugars.

Answer

Thank you for your helpful comments. We conducted control experiments involving empty GVs and have described the results of these experiments in the results section of the revised manuscript (Figure 3, lines 200-202 and 277-279). We also conducted experiments involving GV preparation with microspheres in the absence of sugars. Under these conditions, we did not obtain any GVs, likely because the specific gravity was too low for GV formation. We also described this experiment in the revised manuscript (lines 298-300).

-Since a normal optical microscope has been used and not a confocal one, what is the evidence that the microspheres are in the water core of GUVs and not absorbed on the lipid membrane?

Answer

The microspheres in the GVs moved around under the optical microscope, which indicates that they did not adhere to or absorb on the membrane. Because the diameter of the microspheres

used in the current study was larger than the thickness of the vesicular membrane, the microspheres were not absorbed in the membrane.

-No informations about the yield of GUVs obtained with and without microspheres

Answer

We conducted additional experiments to address this comment. We counted the number of GVs with microspheres (N = 55) and the number of GVs without microspheres (N = 105), we calculated the ratio of encapsulation (P_{enc} = 34%), and we added these values to the manuscript (lines 223-224).

Minor Concerns:

-At row 89, there is an error radius/diameter

Answer

We corrected this error.

-The manuscript must be corrected by an English mother tongue

Answer

We had the manuscript edited by two native-English-speaking professional science editors.

Additional Comments to Authors:

my suggestion is that the paper is suitable for publlication if the authors revise the manuscript according to my remarks above.

Answer

Thank you for your kind words. We hope that we have responded satisfactorily to the comments.

Reviewer #3:

Manuscript Summary:

Artificially prepared GUV containing highly concentrated microspheres is a promising cell model. In this article, the authors describe a simple preparation method of GUVs encapsulating microparticles such as fluorescent beads. The proposed method will contribute to the researches based on artificial cell model.

Major Concerns:

-Line 145: Does the value (0.5 vol %) mean that the volume fraction of nonfluorescent and fluorescent microspheres in Tris-buffered solution was 0.5 vol %? If so, why was the value (0.5 vol %) greatly different from the volume fractions of microspheres in GUVs (4-30 vol %, in Line 315)?

Answer

Thank you for your helpful comments. We modified the text to address this comment (lines 146-152).

As described in a previous study (Y. Natsume et al., *Chem Lett* 2013; ref. 34), we speculated that the difference in volume fraction between the microspheres in the dispersion and the microspheres encapsulated in GVs was derived from the centrifugation. Because the microspheres in the current study also have diameters in the micrometer range, they tend to be strongly influenced by centrifugation. This influence leads to localize the microspheres in the GVs, and the GVs divided into GVs containing numerous microspheres and empty GVs. In fact in this study, we observed empty GVs as well as GVs containing microspheres.

-Line 202-203: Did the first drop contain not only GUVs but also unencapsulated microspheres? If so, is it possible to separate the GUVs and unencapsulated microspheres by centrifugation or other processes?

Answer

We added a list of methods that can be used to separate GVs that contain materials from materials outside GVs; these methods include dialysis and FACS sorting. See lines 211-215.

Minor Concerns:

-Line 175: count errors -> count errors.

Answer

We made this correction.

-Line 257: The authors should explain how the value (11 vol %) was obtained. The values n, v, and V in Figure 3 should be described.

Answer

We modified the text to address this comment (lines 282-286).

-Line 322: have been described -> have described

Answer

We corrected the sentence.

-Figure 1: It is a little difficult to read the characters in Figure 1. The authors should improve the resolution.

Answer

We modified Figure 1 in response to this comment.

-EQUIPMENT: It is difficult to read the comments/description of Microman E.

Answer

We revised to address this comment.