**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/cm3) 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-SealTM 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 *Nv*100/*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.