Supporting Information:

One-pot assembly of complex giant unilamellar vesicle-based synthetic cells

Kerstin Göpfrich,†,‡ Barbara Haller,†,‡ Oskar Staufer,†,‡ Yannik Dreher,†,‡ Ulrike Mersdorf,¶ Ilia Platzman,*†‡ and Joachim P. Spatz,*†‡

†Max Planck Institute for Medical Research, Department of Cellular Biophysics, Jahnstraße 29, D 69120, Heidelberg, Germany
‡Department of Biophysical Chemistry, University of Heidelberg, Im Neuenheimer Feld 253, D 69120 Heidelberg, Germany
¶Max Planck Institute for Medical Research, Department of Biomolecular Mechanisms, Jahnstraße 29, D 69120, Heidelberg, Germany

E-mail: ilia.platzman@mr.mpg.de; spatz@mr.mpg.de
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Text S1: Step-by-step instructions for the shaking method for GUV formation

Materials and reagents

- Micro tube with cap (e.g. Eppendorf)
- Oil-surfactant mix: HFE-7500 fluorinated oil (e.g. 3 M) with 1.4 wt% perfluoropolyether-polyethylene glycol (PFPE-PEG) block-copolymer fluorosurfactants (e.g. Ran Biotechnologies) and 10.5 mM PFPE-carboxylic acid (Krytox, MW: 7000-7500 g/mol, e.g. DuPont)
- SUV-containing aqueous phase: SUVs composed of e.g. 69% EggPC, 30% EggPG, 1% LissRhod-PE (e.g. Avanti Polar Lipids, for SUV formation protocol see Materials and Methods, main manuscript text), dissolved at a lipid concentration of 1.5 mM in e.g. 10 mM MgCl$_2$, 200 mM sucrose, 10 mM Tris and 1 mM EDTA, pH 7.4
- Release buffer: 230 mM glucose, 10 mM Tris and 1 mM EDTA, pH 7.4; potentially components for encapsulation
- Droplet-destabilizing agent: Perfluoro-1-octanol (PFO) destabilizing agent (e.g. Sigma-Aldrich)
- Vortexer or emulsificator (optional)

Note that Krytox concentration, lipid composition and buffer conditions can be adjusted as needed, but optimization of the parameters may be required to achieve the best possible GUV formation. For other tested lipid composition and buffer conditions see Table S1.
**Procedure – Timing: 10 minutes**

Note that the volumes are scalable to form 10µL to hundreds of millilitre of GUVs.

The procedure described here is for the formation of 200µl GUVs.

1. Pipette 400µl oil-surfactant mix into the microtube.
2. Add 200µl of the SUV-containing aqueous phase.
3. Form emulsion droplets by vortexing for 10s or manual shaking. Successful emulsification is indicated by the formation of a milky layer on top of the oil-surfactant mix). Note that droplet size will depend on the amount of shear stress applied to the w/o emulsion.
4. Layer 200µl release buffer on top of the emulsion droplets.
5. Slowly add 100µl of the droplet-destabilizing agent on the side of the micro tube while gently rotating.
6. As soon as the milky emulsion breaks up and a transparent aqueous layer forms on top of the oil-surfactant mix, carefully transfer the GUV-containing aqueous layer (top layer) into a separate tube or an observation chamber.
If the efficiency of GUV formation is very low, please consider the following points:

- Use SUVs within 48 h after formation.
- After approx. 6 weeks, prepare a fresh oil-surfactant mix.
- Test for Krytox contaminations of your block-copolymer fluorosurfactants, e.g. using the Rhodamine 6G assay as described in Figure S5.
- Make sure that there is no osmotic mismatch between the encapsulated aqueous phase and the release buffer.
- Screen for optimal Krytox concentrations, lipid compositions and buffer conditions for your system.
- Perform FRAP measurements with the GUVs before the release (in the droplet-stabilized state). If the diffusion coefficients are about 5 to 10 times lower than you would expect, it is possible that the SUVs have not fused at the droplet periphery. In this case, adjust the conditions (e.g. the Krytox concentration).
- To increase the yield, leave the dsGUVs at 4°C over night before the release. This will increase the yield.
- Increase the contact area between the aqueous release buffer and the droplet emulsion. If the droplets are too close to one another during the release, the GUVs may split or fuse during the process.
- The cover slides for observation of the GUVs should be coated with BSA to prevent the fusion of the GUVs with the glass surface. This is especially important if the release buffer contains Mg^{2+} ions and if the GUVs are large.
Text S2, Figure S1: Choosing an appropriate lipid concentration

Only if a sufficient amount of lipids is encapsulated inside the droplets, the GUVs can be released successfully. Therefore, we calculated the required lipid concentration as a function of GUV diameter. The lipid concentration has to be chosen such that the lipids can form a continuous bilayer at the droplet interface. It is important to consider that the surface-to-volume ratio decreases with increasing diameter. The required lipid concentration $c_{\text{Lip}}$ can be calculated as:

$$c_{\text{Lip}} = \frac{n_{\text{Lip}}}{V_{\text{Drop}}} = \frac{N_{\text{Lip}}}{N_A \cdot V_{\text{Drop}}} \quad (1)$$

where $n_{\text{Lip}}$ is the amount of lipid molecules and $V_{\text{Drop}}$ the volume of the droplet. The required number of lipid molecules, $N_{\text{Lip}}$ can be written as:

$$N_{\text{Lip}} = 2 \cdot \frac{A_{\text{Drop}}}{A_{\text{Head}}} = 2 \cdot \frac{4\pi \cdot (\frac{d}{2})^2}{A_{\text{Head}}} = \frac{2\pi d^2}{A_{\text{Head}}} \quad (2)$$

where $A_{\text{Drop}}$ is the area of the droplet and $d$ its diameter; $A_{\text{Head}}$ is the area occupied by a single lipid head group. The volume of the droplet $V_{\text{Drop}}$ is:

$$V_{\text{Drop}} = \frac{4}{3} \pi \cdot (\frac{d}{2})^3 = \frac{\pi d^3}{6} \quad (3)$$

Therefore, the required lipid concentration is inversely proportional to the droplet diameter and can be calculated as:

$$c_{\text{Lip}} = \frac{1}{N_A} \cdot \frac{2\pi d^2}{A_{\text{Head}}} \cdot \frac{6}{\pi d^3} = \frac{12}{N_A \cdot A_{\text{Head}}} \cdot \frac{1}{d} \propto \frac{1}{d} \quad (4)$$

With the Avogadro constant $N_A = 6.022 \cdot 10^{23} \; \frac{1}{\text{mol}}$ and assuming a lipid head group occupies an area of $A_{\text{Head}} = 0.7 \; \text{nm}^2$ (according to values published earlier$^1$):

$$c_{\text{Lip}} \approx \frac{28}{d} \; \text{mM} \quad (5)$$
The calculated lipid concentration as a function of GUV diameter is plotted in Figure S1.

Figure S 1: Calculated required lipid concentration as a function of GUV diameter.
Figure S2: Control experiment with uncharged surfactants

Figure S 2: Confocal fluorescent imaging of droplets stabilized by 1.4 wt% uncharged PEG-based fluorosurfactants only. The encapsulated SUVs do not fuse at the inert droplet interface and the formation of dsGUVs is inhibited.² A) Negatively charged SUVs (30 % DOPG, 34.75 % DOPC, 34.75 % POPC, 0.5 % Atto488-DOPE) in 10 mM MgCl₂, 30 mM Tris, pH 7.4.
B) Positively charged SUVs (30 % DOTAB, 34.75 % DOPC, 34.75 % POPC, 0.5 % Atto488-DOPE) in 30 mM Tris, pH 7.4. Under these conditions, fusion of the SUVs was only observed in the presence of Krytox (see Figure 1B, main manuscript text).
Figure S3: Dynamic light scattering experiments

Figure S 3: Size distribution of the SUVs after extrusion through 50 nm pores as determined by dynamic light scattering experiments (3 independent measurements, 10 runs each). The SUVs (here: 30% EggPG, 70% EggPC in 30 mM Tris) have a diameter of 58.8±4.4 nm. While vesicles can also be used without extrusion, the more homogeneous distribution after extrusion leads to an increased yield in the formation of GUVs via the shaking method.
Figure S4, Text S3: Quantification of the amount of GUVs produced via the shaking method

Figure S4: Confocal fluorescent images of free-standing GUVs representative of a less efficient (A) and a highly efficient release (B). A) Released neutral GUVs (49.75% DOPC, 49.75% POPC, 0.5% Atto488-DOPE) in 75 mM KCl, 30 mM Tris. This image is representative for a less efficient release, as it was often observed in the case of neutral lipids. The lipid debris in the background likely comes from GUVs that bursted during the release process. It can be washed away by flushing the observation chamber with buffer. B) Released negatively charged GUVs (20% EggPG, 79% EggPC, 1% LissRhod-PE) in DMEM, 20 mM MgCl₂. This image is representative for a highly efficient release, as it was often observed for negatively charged GUVs containing a mixture of EggPC/EggPG.

To quantify the amount of released GUVs, we counted the number of GUVs in at least three confocal snapshots after the GUVs settled down on the coverslide. Since the GUVs were sealed in an observation chamber with a height of 90 µm, the GUVs that settled on the coverslide were previously dispersed in a volume of 90 µm multiplied by the area of the confocal snapshots. The images below in Figure S4 are representative snapshots for a less efficient release (A) and a highly efficient release (B). By extrapolation, we estimate the number of GUVs per milliliter: 1 · 10⁶ GUVs in the first and 2 · 10⁷ GUVs in the latter case. The release rate can be approximated by comparing the number of released GUVs to the number of dsGUVs. To produce 1 mL of released GUVs, we use 500 µL of the aqueous phase for droplet formation. By measuring the mean diameter of the dsGUVs, we extrapolate the
total number of droplets (here: $4 \cdot 10^7$). This number is then divided by the number of released GUVs to estimate for the release rate – 2.5% in the first case, 50% in the latter.

Figure S5: Rhodamine 6G partitioning experiment

Figure S5: Standard series for the Rhodamine 6G partitioning experiment. Aqueous solutions of 1 mM Rho6G (Sigma Aldrich, Germany) was layered on top of the HFE-7500 oil phase containing Krytox at different concentrations between 0 and 1 mM as indicated. Due to the 1:1 chemical interaction between the Krytox molecules and Rho6G, the partitioning of Rho6G molecules into the oil phase depends on the Krytox concentration. Following 48 h of incubation, samples from the oil and the aqueous phases were carefully collected and transferred into a 96 well plate. Rho6G content was determined in a plate reader (infinite 200, Tecan) by measuring the absorbance at 500 nm. By comparison to this standard, we determined the Krytox contamination of the commercial PEG-based fluorosurfactant we used for our experiments. We found that the surfactant contained $12.4 \mu M \pm 8.8 \mu M$. Note that if the Krytox contamination lies in the millimolar range, this has to be taken into account when supplementing the surfactant with an appropriate Krytox concentration. The assay was carried out according to a previously published protocol.$^{2,3}$
Figure S6, Table S1: Variation of lipid composition and buffer conditions

| Lipid composition                                      | Buffer conditions          |
|---------------------------------------------------------|----------------------------|
| 50% DOPG, 25% DOPC, 25% POPC                           | 10 mM MgCl₂, 30 mM Tris    |
| 50% DOTAP, 25% DOPC, 25% POPC                          | 30 mM Tris                 |
| 70% EggPC, 30% EggPG                                   | 10 mM MgCl₂, 30 mM Tris    |
| 20% cholesterol, 30% DOPG, 25% DOPC, 25% POPC          | 10 mM MgCl₂, 30 mM Tris    |
| 100% Ecoli polar lipid extract                         | 10 mM MgCl₂, 30 mM Tris    |
| 30% DOTAP, 35% DOPC, 35% POPC                          | ddH₂O                      |
| 30% DOTAP, 35% DOPC, 35% POPC                          | 1 M sorbitol               |
| 30% DOPG, 35% DOPC, 35% POPC                           | 10 mM MgCl₂, 30 mM Tris    |
| 50% DOPC, 50% POPC                                     | 100 mM KCl, 30 mM Tris     |
| 50% DOPC, 50% POPC                                     | PBS                        |
| 70% EggPC, 30% EggPG                                   | cell medium (DMEM, 20 mM MgCl₂) |

Table 1: Successfully tested combinations of buffer conditions and lipid compositions for the GUV production via the shaking method. The colour code indicates the release efficiency (gray: efficient (>15%); blue: medium (5%-15%); purple: less efficient (<5%) release). In all cases, the oil phase contained HFE-7500 fluorinated oil, 1.4 wt% PEG-based fluorosurfactant and 10.5 mM Krytox; 0.5 mol% Atto488-labelled DOPE or 1 mol% of LissRhod-PE was added to the lipid mixture for visualization purposes.
Figure S 6: Confocal fluorescent imaging of GUVs produced via the shaking method with versatile lipid compositions and under diverse buffer conditions. A) GUVs were produced from negatively charged lipids (50% DOPG, 25% DOPC, 25% POPC in 10 mM MgCl$_2$, 30 mM Tris, left); neutral lipids (50% DOPC, 50% POPC in 100 mM KCl, 30 mM Tris, middle); and positively charged lipids (50% DOTAP, 25% DOPC, 25% POPC in 30 mM Tris, right). B) GUVs with different lipid compositions, including 70% EggPC, 30% EggPG (in 10 mM MgCl$_2$, 30 mM Tris, left), 20% cholesterol, 30% DOPG, 25% DOPC, 25% POPC (in 10 mM MgCl$_2$, 30 mM Tris, middle), 100% Ecoli polar lipid extract (in 10 mM MgCl$_2$, 30 mM Tris, right). C) GUVs produced in various buffers. First row: Water (30% DOTAP, 35% DOPC, 35% POPC, left), 1 M sorbitol (30% DOTAP, 35% DOPC, 35% POPC, middle), 10 mM MgCl$_2$, 30 mM Tris (30% DOPG, 35% DOPC, 35% POPC, right). Second row: 100 mM KCl, 30 mM Tris (50% DOPC, 50% POPC, left), PBS (50% DOPC, 50% POPC, middle) and cell medium (DMEM, 20 mM MgCl$_2$, 70% EggPC, 30% EggPG). In all cases, the oil phase contained HFE-7500 fluorinated oil, 1.4 wt% PEG-based fluorosurfactant and 10.5 mM Krytox; 0.5 mol% Atto488-labelled DOPE or 1 mol% of LissRhod-PE was added to the lipid mixture for visualization purposes. Scale bars: 10 µm.
Figure S7: α-hemolysin dye influx experiment

Figure S 7: Normalized fluorescence intensity inside an individual GUV as a function of time with (red) and without (blue) addition of α-hemolysin nanopores. Whereas the dye (fluorescein) enters into the GUV in the presence of the pores, no dye passage across the membrane is observed in its absence. This is a good indication for the unilamellarity of the GUVs produced via the shaking method. The experiment was conducted as described in the Materials and Methods section (main manuscript). The traces plotted here correspond to the GUVs shown in the Supporting Video S2.
Figure S8: Comparison of zeta-potentials of SUVs and GUVs

Figure S8: Zeta-potential measurements of SUVs (red) and GUVs produced by the shaking method (blue).

Figure S9: GUVs before and after centrifugation

Figure S9: Small GUVs before (A) and after (B) centrifugation at 18,000 g. While small GUVs (below 10 µm) remain intact after centrifugation, larger GUVs will burst.
**Video S1: Video protocol of the shaking method for GUV formation**

This video gives visual guidance to support first-time users of our shaking-method for the formation of GUVs. It follows the steps described in the manuscript (Figure 1 and Materials and Methods section) and the step-by-step protocol described above.

**Video S2: Dye influx experiments**

This video shows the dye (fluorescein) influx experiments in the presence and absence of α-hemolysin membrane pores. In the presence of α-hemolysin, the fluorescence intensity inside the GUVs is increasing, while GUVs remain dark in its absence. Note that the experiments were carried out at low α-hemolysin concentrations (10.7 nM heptameric pores) to avoid bursting of the GUVs. Under these conditions, inhomogeneities in the distribution of the α-hemolysin across the GUVs are expected and explain the different rates of the increase in fluorescence inside the GUVs.

**Video S3: Osmotic deflation of ‘shaken’ GUVs**

This video shows a GUV produced by the shaking method that was osmotically deflated after formation. Deflation leads to an excess membrane area and hence to budding and lipid tubulation.
References

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