



# Compartments for Synthetic Cells: Osmotically Assisted Separation of Oil from Double Emulsions in a Microfluidic Chip

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Liposomes are used in synthetic biology as cell-like compartments and their microfluidic production through double emulsions allows for efficient encapsulation of various components. However, residual oil in the membrane remains a critical bottleneck for creating pristine phospholipid bilayers. It has been discovered that osmotically driven shrinking leads to detachment of the oil drop. Separation inside a microfluidic chip has been realized to automate the procedure, which allows for controlled continuous production of monodisperse liposomes.

Giant unilamellar vesicles (GUVs) are widely used as model membranes to study the biophysical properties of phospholipid bilayers.<sup>[1–3]</sup> In parallel, they attract increasing attention as cell-like compartments in bottom-up synthetic biology, in which the long-term goal is to build a minimal cell from scratch.<sup>[4–8]</sup> Upon selecting a GUV production method for synthetic biology, the ability to encapsulate various components is essential.<sup>[2]</sup> Conventional methods for the production of liposomes comprise gentle hydration,<sup>[9,10]</sup> swelling on polymer cushions,<sup>[11,12]</sup> and electroformation.<sup>[13,14]</sup> These methods are not always optimal due to the low GUV yield in physiological buffer; poor encapsulation efficiency,<sup>[2,15,16]</sup> and, in some cases, harsh conditions to which delicate biomolecules and smaller vesicles are exposed during preparation.<sup>[17]</sup> This issue has been addressed by the phase-transfer method, which is based on preformed water-in-oil (w/o) emulsion droplets crossing a second o/w interface.<sup>[18]</sup> In recent years, several other, concep-

tually similar, methods have been developed, with the aim of providing higher productivity and better control, namely, microfluidic jetting,<sup>[19]</sup> continuous droplet interface crossing encapsulation (cDICE),<sup>[20]</sup> microfluidic formation of droplet-stabilized vesicles,<sup>[21]</sup> and microfluidic production of w/o/w double emulsions.<sup>[22]</sup> The last approach appears to be the least experimentally demanding and multiple setups for double emulsion production have been proposed. Microfluidic chips made out of glass<sup>[17,22]</sup> or polydimethylsiloxane (PDMS),<sup>[23–26]</sup> and organic phases, such as octanol,<sup>[24]</sup> chloroform/hexane,<sup>[17]</sup> and oleic acid,<sup>[27]</sup> have been used to produce stable double emulsions, which have found attractive applications for synthetic biology, such as the encapsulation of smaller vesicles, proteins, and DNA.<sup>[17,24,28]</sup> Another advantage of the double emulsion procedure is the virtual absence of losses, with respect to encapsulated solutions, and therefore, it is suitable for valuable substrates that are available in low quantities.

In addition to efficient encapsulation, mimicking nature requires a pristine bilayer, which would not compromise membrane-related phenomena, such as the folding of reconstituted membrane proteins. However, the presence of residual oil is an inherent vice of GUVs prepared from double emulsions, which necessitates removal of the organic phase. So far, a few approaches for solvent removal have been shown: evaporation,<sup>[29]</sup> spontaneous splitting off,<sup>[17,24]</sup> and squeezing.<sup>[30]</sup> Herein, we present another, simple method to separate oil from double emulsions and to generate GUVs. We show that oleic acid droplets exhibit complete dewetting from the deflating vesicles if exposed to an osmotic gradient. The latter shrinking effect has been very recently used as a tool for the manipulation of concentration and size,<sup>[31]</sup> but, to the best of our knowledge, its use to detach the oil droplet has not been demonstrated. We also integrate osmotic dewetting into a microfluidic chip to observe the process and to achieve a certain degree of modularity and automation.

We used a microfluidic chip design with two junctions for the initial formation of a double emulsion (Figure 1 and Movie S1 in the Supporting Information), similar to those commonly used.<sup>[25–27]</sup> Briefly, w/o emulsions were formed at the first junction, followed by crossing a second junction with the aqueous OF, which resulted in highly monodisperse w/o/w emulsions at 40–50 Hz. The walls of the chip after the second junction were coated with 1% poly(vinyl alcohol) (PVA) for hydrophilization to ensure proper formation of the double emulsion. The size of the w/o/w emulsions strongly depends on the size of the aqueous droplets formed at the first junction, which

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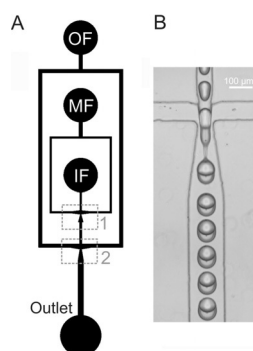
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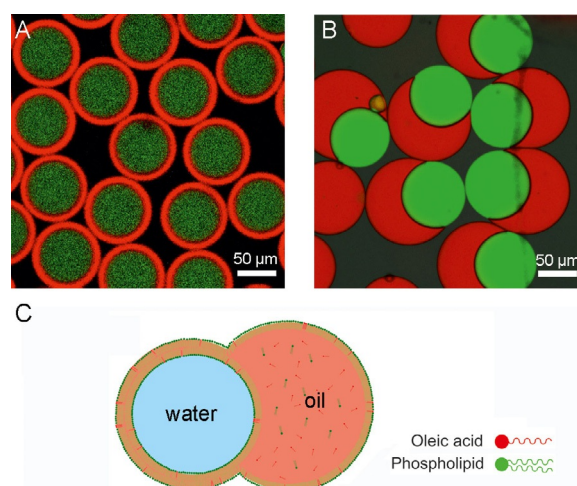
**Figure 1.** A) Microfluidic design of the PDMS chip for double emulsion formation. OF: outer fluid, MF: middle fluid, IF: inner fluid. The first and second junctions are marked as 1 and 2, respectively. B) Microscopic image of the second junction.

can be, in turn, controlled, to a certain extent, by different flow rates and the chip design (e.g., channel width). In the present case, the size of the w/o/w emulsions ranged from 40 to 70  $\mu\text{m}$ . There was no exchange between IF and OF and small volumes ( $< 500 \mu\text{L}$ ) of IF were used for the preparation. Using the setup described by Petit et al.<sup>[27]</sup> as a starting point, we reduced the number of components to create stable double emulsions with minimal composition: 200 mM sucrose as the IF, 10  $\text{mg mL}^{-1}$  L- $\alpha$ -phosphatidylcholine (soy PC) in oleic acid as the MF, and 200 mM sucrose + 1 wt% Pluronic F108 as the OF. The surfactant Pluronic F108 was added to ensure the stability of the double emulsion during its formation. Typical flow rates were  $40 \mu\text{L h}^{-1}$  for IF and MF, and  $400 \mu\text{L h}^{-1}$  for OF. The utilization of different oil phases for the production of w/o/w emulsions has led to different methods for subsequent oil removal. Extraction of oleic acid with ethanol,<sup>[26,27]</sup> as reported in the literature, was not successful in the present case because it did not result in any apparent decrease of oleic acid in the membrane (Figure S1). In addition, high ethanol concentrations might not be compatible with certain encapsulated components.<sup>[27]</sup> However, we were able to observe partial, and sometimes full, dewetting upon observing the double emulsions under a microscope on a glass slide (Figure S2). We attributed the observed phenomenon to the interplay of interfacial tensions and the osmotic imbalance between IF and OF, resulting from evaporation of the sample. To test this hypothesis, we first eliminated evaporation by using a cover slide, which resulted in partial dewetting, but no full dewetting at equal osmolarity (Figure 2). We then gradually changed the osmotic gradient between IF and OF with sucrose and sodium chloride and, eventually, reduced the IF solute concentration to one-quarter compared with that of the OF, which resulted in full dewetting (Figures S3 and S4).

In the presence of this osmotic gradient, a comparison of the interfacial tension between MF and IF ( $\gamma_{\text{MF-IF}} = 11.05 \text{ mN m}^{-1}$ ) with the value between MF and OF ( $\gamma_{\text{MF-OF}} = 0.04 \text{ mN m}^{-1}$ ) suggests that interfacial tension between IF and OF ( $\gamma_{\text{IF-OF}}$ ) is in the range of  $\gamma_{\text{MF-IF}} \pm \gamma_{\text{MF-OF}}$  ( $11.01\text{--}11.09 \text{ mN m}^{-1}$ ). Values above this range would prevent partial dewetting and lower values would result in spontaneous full dewetting, as reported by Deng et al.<sup>[32]</sup> As the osmotic gradient deflates the vesicle to match the osmolarity of the IF and OF, which might be facilitated by the presence of surfactants in the membrane, the cup-shaped bilayer (Figure 2) enwraps the reduced aqueous volume. We believe that the force balance at the interfacial three-phase contact line is not significantly changed by the osmotic gradient itself because the interface composition is not

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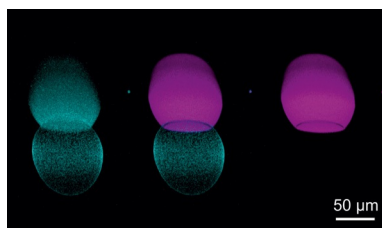
**Figure 2.** A) Confocal image of the double emulsion immediately after formation. B) Confocal image of the partially dewetted vesicle. Nile Red (red), dissolved in the MF, was used to stain the oil and lipid membrane, and fluorescein dextran (green) was encapsulated in the aqueous IF. C) Schematic representation of the partially dewetted vesicle. Notably, the orange color of the oil pocket corresponds to oleic acid and this color designation of the phospholipids does not correspond to those used in A) and B).

expected to change significantly with changes to the solute concentration in the aqueous phases. Simple geometric considerations allow the necessary volume reduction to be calculated to obtain a free vesicle. Assuming a vesicle, the surface of which is 50% dewetted, its volume should be reduced by a factor of  $2\sqrt{2}$  to be enclosed by the existing (dewetted) bilayer (see the Supporting Information).

Although in the majority of the cases more than 50% of the surface area was dewetted, we opted for a fourfold volume reduction (proportional to the osmolarity) to ensure that the dewetted surface was sufficient to enclose the reduced volume. We ascribed the final detachment of the oil pocket from the vesicle to gentle agitation during manipulation, which aided scission of the neck connecting the GUV and the droplet under transient conditions (Figure S2). Otherwise, the nearly fully dewetted vesicle (now with reduced volume) would undergo partial wetting again until reaching the initial energetically favorable equilibrium.

To test whether the dewetted membrane was a phospholipid bilayer without residual oil, we used Nile Red and a fluorescent lipid (dioleoylphosphoethanolamine-*N*-carboxyfluorescein, PE-CF) dissolved in the MF, along with the soy PC. Nile Red is a lipophilic dye, which is used to visualize intracellular lipids,<sup>[33]</sup> but is also known to be incorporated into phospholipid membranes.<sup>[34]</sup> In the present case (and for the specific imaging conditions), Nile Red was almost exclusively located in the oleic acid pocket and barely visible in the dewetted membrane, whereas PE-CF was distributed between the membrane and oil pocket (Figure 3, and fluorescence intensity profiles of partially dewetted vesicles in Figure S5).

This implies preferential partitioning of Nile Red in the oleic acid phase. We speculate that the apparent absence of Nile Red in the dewetted part suggests a negligible amount of residual oil. However, this cannot be unequivocally confirmed in



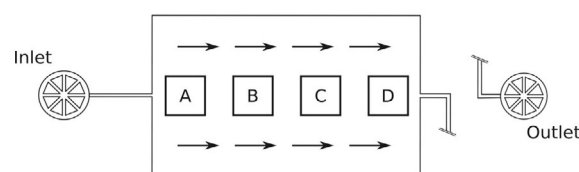
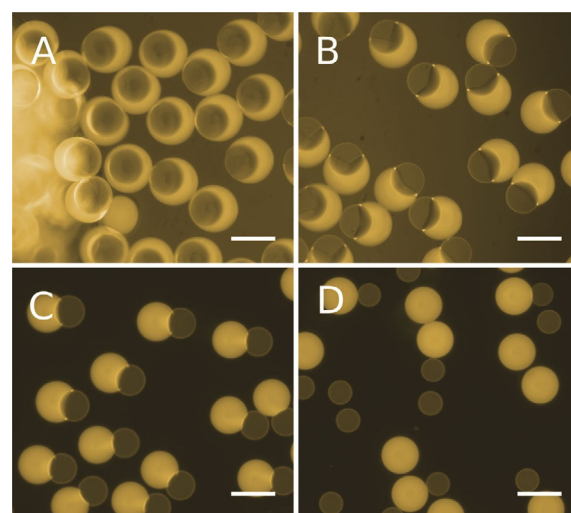
**Figure 3.** Confocal z-stacked images of the partially dewetted vesicle and the attached oil pocket. Magenta: Nile Red, cyan: PE-CF.

the limited scope of this study, especially because oleic acid is known to incorporate into the phospholipid membrane, which is, in turn, used to drive growth in protocell experiments.<sup>[35,36]</sup> From a conceptual and practical point of view, the presence of minute amounts of oleic acid should still result in a realistic mimic of natural membranes because oleic acid is a natural precursor for phospholipid synthesis. In addition, there are indications that a ligase, involved in  $\beta$ -oxidation (FadD), converts oleic acid into the coenzyme A (CoA) ester after its partitioning in the membrane, even though the protein is cytosolic,<sup>[37]</sup> this is also discussed in the context of membrane growth.

The potential presence of oleic acid would influence the properties of the bilayer, depending on its concentration, and might have an adverse influence on the stability and permeability—membranes containing oleic acid are known to be less stable<sup>[38,39]</sup>—but this effect is yet to be determined with respect to the specific application. Increased permeability for certain small molecules may actually speed up osmotic deflation and facilitate the transport of substrates for metabolic reactions encapsulated in GUVs. The oleic acid pocket of partially dewetted vesicles has also found a useful application to enable the reversible shrinking of liposomes by acting as a membrane reservoir.<sup>[31]</sup>

The osmotic gradient, sufficient for full dewetting, was determined based on observations of 10–15  $\mu\text{L}$  w/o/w emulsion suspension, applied on a microscope slide. This setup was suitable for initial screening, but it only allowed for the collection of a few  $\mu\text{L}$  of the detached vesicle suspension for further experiments. Therefore, to increase processing productivity, we automated vesicle dewetting in a simple microfluidic chip and observed the time course of the process (approximately 80 s) through microscopy (Figures 4 and S6 and Movie S2). The height of the separation chip was kept at 70  $\mu\text{m}$  (nearly matching the emulsion size) to ensure slight dragging of the oil pockets upon contact with the upper wall, and thus, aiding splitting, in addition to the beneficial influence of the hydrodynamic flow. To separate the vesicles from the detached oil pockets, it sufficed to take advantage of the density difference between oleic acid and the aqueous solution (oil droplets floated at the top of the collection tube).

We note that the dewetting chip is not essential for the splitting process. Detachment could theoretically be achieved by simply exposing the double emulsions to an osmotic gradient in an Eppendorf tube and mild centrifugation. However, use of the dewetting chip has the advantage that the process can be observed under a microscope, which, in turn, enables

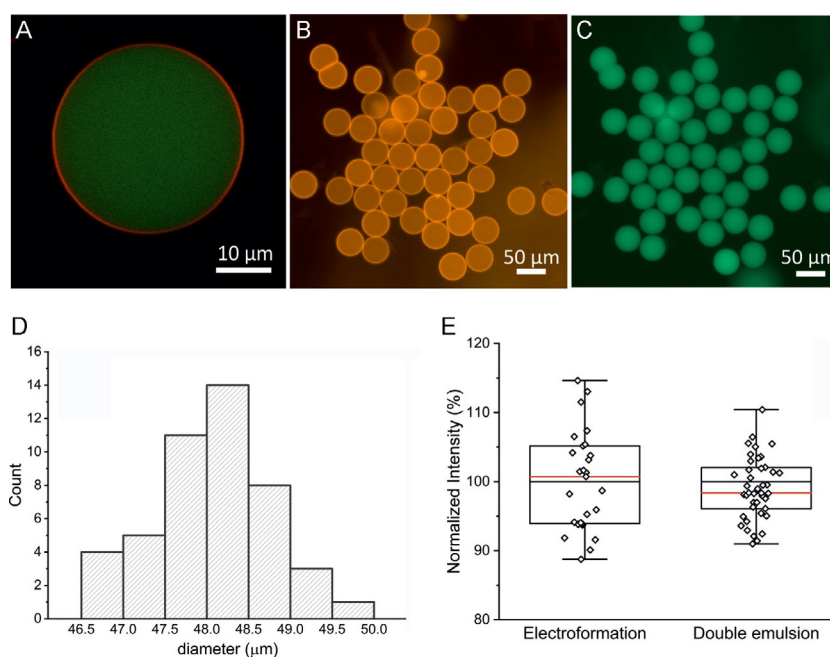


**Figure 4.** A)–D) Fluorescent images along the length of the chip, showing the dewetting and detachment of the oil pockets. Dioleoylphosphoethanolamine-*N*-lissamine rhodamine B sulfonyl (Liss-Rho-PE; yellow), dissolved in the MF was used to stain the oil and lipid membrane. Relative imaging positions are shown at the bottom. Scale bar: 100  $\mu\text{m}$ .

optimization of the detachment conditions (e.g., flow rate). Furthermore, the dewetting chip can be connected directly to the preceding double emulsion chip, which, after appropriate matching of flow rates and scaling of size, would allow for a high-throughput generation of dewetted vesicles.

The resulting GUVs (Figure 5), stained with Liss-Rho-PE in the membrane and fluorescein dextran in the IF, show no residual oil or lipid pockets in the membrane and are highly monodisperse, in contrast to electroformation (Figure S7). The distribution of fluorescein dextran is also uniform across individual liposomes (fluorescence intensity shows a standard deviation of 7.8% and interquartile range of 6%, with respect to the mean value, compared with 7.1 and 11.3%, respectively, in the case of electroformation).

Regarding the desired native state of the membrane, we should mention an issue that has not been discussed before, but is inherent to the double emulsion method. The production of stable double emulsions requires the use of a surfactant (Pluronic F108 in the present case), which may also affect the membrane properties. The influence of poloxamers has been studied in different contexts and depends on their structure—generally, hydrophobic copolymers act as permeants, whereas hydrophilic ones seal the membrane.<sup>[40]</sup> Some reported effects are mechanical stabilization<sup>[41]</sup> and the protection of vesicles against peroxidation,<sup>[42]</sup> whereas, in other cases, Pluronic F108 increases the permeability for small molecules<sup>[43]</sup> and is used for lentiviral transduction.<sup>[44]</sup> Increased permeability should not necessarily be considered as a canonical drawback for synthe-



**Figure 5.** A) Confocal image of a single vesicle. B), C) Fluorescent images of dewetted vesicles after processing in a microfluidic chip for detachment. Liss-Rho-PE (orange) dissolved in the MF was used to stain the lipid membrane; fluorescein dextran (green) was encapsulated in the aqueous IF. D) Size distribution of the dewetted vesicles. E) Fluorescein dextran intensities of vesicles produced through electroformation and double emulsion. Notably, the values have been normalized to the mean to ease comparison.

ic biology applications because this could provide a feasible mechanism for membrane transport in bioreactor-type systems (which is otherwise attained by pore-forming agents<sup>[5]</sup>), if the vesicles retain their overall structural integrity and segregate the encapsulated machinery, that is, enzymes. In addition, conventional methods for the reconstitution of membrane proteins also involve the use of surfactants, which perturb the bilayer structure and are subsequently removed.<sup>[45]</sup> In this context, the adsorption and insertion of poloxamers exhibit different timescales,<sup>[46,47]</sup> whereas desorption is as fast as adsorption,<sup>[48]</sup> which provides a feasible mechanism for surfactant displacement by washing. Yet, the influence of residual surfactant and oil has to be determined in each specific case, depending on the intended application.

In conclusion, we have shown a new and simple method for oil removal to produce GUVs from w/o/w double emulsions. The exposure of double emulsions to osmotic gradients results in shrinkage of the aqueous compartment, which causes detachment of the oil phase. Thus, we circumvent the balance of suboptimal interfacial tensions. As a result, liposomes without visible oil and lipid reservoirs are formed. The high encapsulation efficiency, experimental flexibility, and mild conditions during vesicle production potentially allow the encapsulation of complex and delicate compounds, such as proteins, DNA, and smaller vesicles, which could aid in the construction of cell-like compartments in bottom-up synthetic biology.

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** double emulsions · liposomes · microfluidics · synthetic biology · vesicles

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