On-chip microfluidic production of cell-sized liposomes

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In this protocol, we describe a recently developed on-chip microfluidic method to form monodisperse, cell-sized, unilamellar, and biocompatible liposomes with excellent encapsulation efficiency. Termed octanol-assisted liposome assembly (OLA), it resembles bubble-blowing on a microscopic scale. Hydrodynamic flow focusing of two immiscible fluid streams (an aqueous phase and a lipid-containing 1-octanol phase) by orthogonal outer aqueous streams gives rise to double-emulsion droplets. As the lipid bilayer assembles along the interface, each emulsion droplet quickly evolves into a liposome and a 1-octanol droplet. OLA has several advantages as compared with other on-chip techniques, such as a very fast liposome maturation time (a few minutes), a relatively straightforward and completely on-chip setup, and a biologically relevant liposome size range (5–20 μm). Owing to the entire approach being on-chip, OLA enables high-throughput liposome production (typical rate of tens of Hz) using low sample volumes (~10 μl). For prolonged on-chip experimentation, liposomes are subsequently purified by removing the 1-octanol droplets. For device fabrication, a reusable silicon template is produced in a clean room facility using electron-beam lithography followed by dry reactive ion etching, which takes ~3 h. The patterned silicon template is used to prepare polydimethylsiloxane (PDMS)-based microfluidic devices in the wet lab, followed by a crucial surface treatment; the whole process takes ~2 d. Liposomes can be produced in ~1 h and further manipulated, depending on the experimental setup. OLA offers an ideal microfluidic platform for diverse bottom-up biotechnology studies by enabling creation of synthetic cells, microreactors and bioactive cargo delivery systems, and also has potential as an analytical tool.

INTRODUCTION

Liposomes are stable, aqueous, three-dimensional microconfinements that are separated by a lipid bilayer from their aqueous environment. Owing to their self-assembling nature and biomimicking properties, liposomes have become an important and versatile tool in basic as well as applied sciences. One particularly fascinating and rapidly developing field is synthetic biology, in which the grand challenge is to form, from the bottom up, an artificial cell that will mimic the fundamental characteristics of living systems[1–3]. Liposomes are one of the most promising basic carriers for such synthetic cells for several reasons: their ability to change shape, the availability of several production methods, and, in particular, because their membrane bears a great resemblance to the plasma membrane of cells[4,5]. In recent years, microfluidics has emerged as an important technology in the development of novel methods to produce liposomes in a highly controlled manner[6,7]. Indeed, the importance of this technology and its potential for the manufacture of vesicles with reproducible size and content have been recognized for years[8]. In this protocol, we provide a detailed description of a recently developed microfluidic method to form monodisperse, unilamellar, cell-sized (5–20 μm) liposomes[9] that remain stable for prolonged periods (>24 h). We further describe an extension of the method to purify liposomes from 1-octanol droplets (a by-product formed in the process) on the same chip to facilitate further experimentation, yielding up to 95% pure samples[10].

Development of the protocol

Our protocol for making liposomes has been inspired by the elegant use of microfluidics to form double-emulsion droplets, i.e., water-in-organic-solvent-in-water emulsions[11,12]. Such double-emulsion droplets were first produced using glass microcapillary devices by hydrodynamically focusing immiscible fluids with a high degree of control over their size[13]. By dissolving lipids in a volatile organic phase (such as toluene and chloroform), liposomes could be produced by evaporating the solvent from double-emulsion droplets[11]. This idea was further adapted to PDMS-based microfluidic devices, in which oleic acid was used as the organic phase and was slowly removed (over >10 h) by the addition of ethanol in the external phase, in order to form liposomes[12]. We set out with the idea to use a PDMS-based microfluidic system and an organic phase that is partially miscible in water, in order to speed up the solvent-extraction process after double-emulsion droplet formation. As alcohols meet this criterion and are good lipid solvents, we systematically checked the suitability of different alcohols as the organic phase[9].

We unsuccessfully tried to use several different alcohols (from ethanol to 1-heptanol) to form double-emulsion droplets and further transform them into liposomes[9]. Use of 1-octanol as the organic phase, however, led to substantial and advantageous improvements. Stable and monodisperse double-emulsion droplets were formed that immediately transformed into an intermediate phase of a double-emulsion droplet with a side-attached 1-octanol pocket containing excess lipids. Importantly, as the droplets flowed downstream of the channel, the pocket completely detached to yield stable, unilamellar liposomes. This entire process happens within minutes and is biocompatible and effective for a variety of lipid compositions. Thus, instead of relying on the time-consuming solvent-extraction process, we devised a much quicker process to physically separate the organic solvent and form liposomes. We named this process 'octanol-assisted liposome assembly (OLA)'[9].

With the aim to perform the entire experiment on-chip, we subsequently developed a straightforward and passive method to remove unwanted 1-octanol droplets from the system and thus obtain a pure yield of liposomes[10]. We used a density-based separation technique to make less-dense 1-octanol droplets float to the top of a separation hole, while the liposomes were gently allowed to re-enter the microfluidic channel. This simple technique allowed us to obtain pure liposome samples for further experimentation.

Applications of the method

As OLA is a method that produces giant (>1 μm) unilamellar vesicles (GUVs), it has potential applications in several diverse fields[14]. As lipid bilayer is the major universal component of cellular membranes, liposomes serve as an excellent model system for cells. Thus, one of the key applications of OLA is as a tool to study *in vitro* bottom-up biology. Extrapolating this idea further, it may serve as an ideal tool to create synthetic cells, which represent one of the ultimate challenges in synthetic biology[1,15,16]. One of the major obstacles that is currently faced in this field is the lack of a controlled and high-throughput way to generate membrane-bound containers with well-defined size, composition, and organization, and this is where the microfluidic-based OLA system can lead to substantial improvements, mainly due to the improved production control and low sample volumes required[17]. For example, OLA-based liposomes can be used to investigate cell division by reconstituting a minimal bacterial divisome machinery[18–21]. Similarly, they can be used to assemble eukaryotic cytoskeletal elements, such as actin or microtubules, to study their dynamics in a deformable container, and, ultimately, to obtain minimal motility machinery[22–24]. Using diffusive transport across the membrane to set up a constant flux of nutrient and waste products, liposomes can serve to reconstitute complex genetic and metabolic networks, and to *ex vivo*–synthesize cytosolic and membrane proteins, nucleic acids, and lipids[25–27]. Going one step further, the technique could assist in producing artificial cells that may someday even be capable of communicating with each other or with natural living cells, for example, by adopting a quorum-sensing mechanism or by achieving modular compartmentalization[28–30].

Another major field in which OLA may serve as a useful tool is that of membrane biochemistry/biophysics. For example, OLA-generated liposomes will be useful in reconstituting membrane proteins and investigating protein–lipid interactions, and in the study of membrane permeability of drug molecules[31–36]. GUVs confined in a controllable environment are also highly suitable for studying vesicle dynamics such as membrane fluctuations, phase separation of lipids, pore formation, and membrane fission–fusion processes[37–40].

Liposomes have been widely used in the pharmaceutical and cosmetic industries[41,42]. Owing to its excellent encapsulation efficiency and the monodisperse samples produced, OLA-based liposomes have the potential to act as controlled drug and DNA/siRNA delivery systems for *ex vivo* and *in vivo* applications. However, the size of the liposomes must be reduced (preferably to the submicrometer range) and the throughput must be increased[41,43]. In general, liposomes will be useful as carriers to encapsulate bioactive cargo, ranging from biomolecules to entire living cells[44]. They may also prove useful in bioanalytical systems, e.g., in immunoassays, or as biosensors, particularly in integrated microfluidic devices[45,46]. Finally, massive parallelization of the method may make it suitable for cell-free biotechnology using *in vitro* transcription–translation machinery[47].

Comparison with other methods

With a wide usage of liposomes in diverse disciplines, a broad range of bulk as well as microfluidic methods have been developed to make liposomes. An ideal production method should be able to form unilamellar, monodisperse liposomes over a wide size range (small unilamellar vesicles (SUVs; <100 nm), large unilamellar vesicles (LUVs; 100–1,000 nm), GUVs) at a high production rate and with an excellent encapsulation efficiency. Traditionally, various batch methods such as thin-film hydration[48], extrusion[49], and electroformation[50] have been successfully implemented to form liposomes in bulk solutions. These methods are relatively simple to implement, as they do not require sophisticated instrumentation and are free of any solvent traces in the bilayer[7]. Nonetheless, they suffer from major drawbacks such as polydispersity in the liposome size, variability in the encapsulated content, and low encapsulation efficiency (<10–20%)[7,8]. In recent years, several microfluidic techniques (either continuous-flow-based or droplet-based) have been developed that provide much better control over the production process because of the characteristic laminar flow and tunable mixing that microfluidics offers[51]. The main drawbacks of these methods, however, have been the traces of solvent (which is used to dissolve the lipids) in the lipid bilayer and the time required for liposome maturation. In addition, for microcapillary-based approaches, the setups are relatively complicated, requiring a considerable level of expertise, and the size of the formed liposomes is relatively large, ~50 μm (refs. 11,52). OLA overcomes these drawbacks, as it uses a much more biocompatible solvent as compared with traditionally used ones[53,54], applies a straightforward PDMS-based device preparation, and can make liposomes over a biologically relevant size range (5–20 μm)[9]. The liposome size is mainly governed by the flow velocities of the inner aqueous phase (IA) and outer aqueous phase (OA), as a result of which, the obtained liposomes are highly monodisperse with a small coefficient of variation (ranging between 4 and 11%; ref. 9). The liposomes are obtained within minutes for further experimentation, which is advantageous when handling biomolecules. In addition, as an exclusively on-chip technique, there is no loss of liposomes while transferring them from a production device to the observation chamber, resulting in a adept post-production control. The liposomes are stable and can be stored on-chip for a prolonged time period (>24 h). Finally, the volumes required for the protocol are very low; one can use a mere 10-μl solution to form liposomes when valuable materials must be encapsulated. **Table 1** compares salient features of a number of prominent liposome-production techniques[7,55].

Overview of the procedure

The OLA protocol is a microfluidic-based method designed to produce and purify liposomes within a PDMS-based chip (**Fig. 1**). The basic design of the entire microfluidic device is shown in **Figure 1a** and is provided as a computer-aided design (CAD) file in the **Supplementary Data**. We use electron-beam (e-beam) lithography and dry reactive ion etching to render the designs on a silicon template to obtain a master (Steps 1–18).

TABLE 1 | Comparison of various liposome-forming methods.

Method	Production approach	Size range, distribution	Lamellarity	Encapsulation efficiency	Solvent traces in membrane
Thin-film hydration[48]	Bulk	GUV, polydisperse	Multilamellar	Low	No
Extrusion[49]	Bulk	SUV–LUV, monodisperse	Unilamellar	Low	No
Electroformation[50]	Bulk	GUV, polydisperse	Uni- + multilamellar	Low	No
Inverted emulsion transfer[58,67]	Bulk + chip (optional)	GUV, can be monodisperse	Unilamellar[a]	High	Yes
cDICE[68]	Bulk	GUV (>10 µm), monodisperse	Unilamellar[b]	High	Yes
Flow focusing[69,70]	Chip	SUV–LUV, monodisperse	Unilamellar	Low	Yes
Layer-by-layer assembly[60]	Chip	GUV (>50 µm), monodisperse	Unilamellar[b]	High	Yes
Solvent extraction[11,12]	Bulk + chip	GUV (>10 µm), monodisperse	Unilamellar	High	Yes
Pulsed jetting[71,72]	Chip	GUV (>50 µm), monodisperse	Unilamellar[b]	High	Yes
Double-emulsion dewetting[52]	Chip	GUV (>25 µm), monodisperse	Unilamellar[b]	High	Yes
OLA[9]	Chip	GUV (>5 µm), monodisperse	Unilamellar[b]	High	Yes

[a]Unilamellarity inferred from fluorescence-quenching assay. [b]Unilamellarity inferred from successful insertion of α-hemolysin protein pores into the lipid bilayer.

The microfluidic devices are prepared using a standard soft-lithography process (Steps 19–43). The crucial part of the design consists of a six-way junction (**Fig. 1b**), where one IA channel, two lipid-carrying organic phase (LO) channels, and two OA channels fuse and branch out into a wider post-junction channel. At some distance from the junction, liposomes are formed. After that point, a separation hole is punched, to allow for a density-based separation of 1-octanol droplets from the liposomes (**Fig. 1c**).

A vital step in the protocol is to render the post-junction part of the device hydrophilic. This is done in order to prevent the LO from wetting the inherently hydrophobic surface of the device. The hydrophilic treatment is achieved by injecting a 2.5% (wt/vol) polyvinyl alcohol (PVA) solution through the OA channels and letting the PVA molecules adhere to the surface (**Fig. 2**). Positive air pressure on the IA and LO channels prevents the solution from entering these channels (**Fig. 2c**). Finally, the PVA solution is removed by applying partial vacuum at the device inlets and outlets (Steps 44–60; and **Supplementary Video 1**).

A schematic flow diagram summarizing the key steps involved in preparing a master wafer (Steps 1–18) and manufacturing a ready-to-use microfluidic device (Steps 19–60) is presented in **Supplementary Figure 1**.

To make liposomes, the IA stream and the surrounding LO streams are hydrodynamically focused and subsequently pinched off by the two OA streams (Steps 61–71; **Fig. 3a**, **Supplementary Video 2**). The IA and LO fluid streams are immediately broken into stable and monodisperse double-emulsion droplets by the continuous phase (OA), a process commonly known as the dripping regime[56]. These double-emulsion droplets are formed with a typical rate of tens of Hz and are carried downstream of the channel. Within a few seconds after their formation, these droplets undergo a partial dewetting process and form two distinct phases: a prominent pocket of 1-octanol that contains excess lipids, and an inner aqueous lumen surrounded by an in-the-making lipid bilayer (**Fig. 3b**, **Supplementary Video 3**). This partial dewetting happens spontaneously in order to minimize the interfacial energies between the three phases. In terms of involved interfacial tensions (γ_{IA-OA}, γ_{IA-LO}, and γ_{OA-LO}), it indicates that the spreading coefficient of the LO ($S_{LO}=\gamma_{IA-OA}-\gamma_{IA-LO}-\gamma_{OA-LO}$) has a negative value[57]. The dewetting process continues, i.e., the 1-octanol pocket continues to protrude outward, as the bilayer zips along the entire interface. Within a few minutes, the 1-octanol pocket buds off completely, in the form of a droplet, and gives rise to a liposome. The liposomes and 1-octanol droplets coexist in the device (**Fig. 3c**, **Supplementary Video 4**). This complete dewetting of the 1-octanol pocket from the

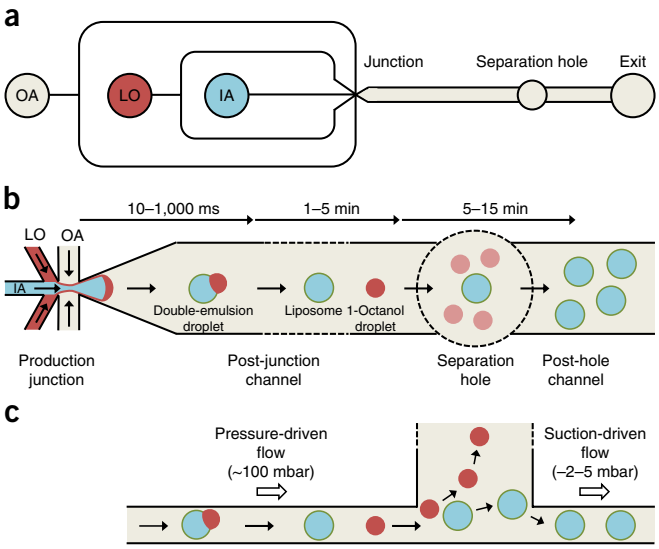

Figure 1 | Schematics showing OLA-based liposome production and purification. (**a**–**c**) Overall layout of the microfluidic device and the post-junction channel (**a**), as well as a top view (**b**) and side view (**c**) of the OLA junction, showing liposome production and subsequent liposome purification. The IA and the LO are hydrodynamically focused and get pinched off by the two OA streams to form a double-emulsion droplet. As the double-emulsion droplet flows downstream, lipids assemble at the interface to form a bilayer, while 1-octanol molecules (and excess lipids) spontaneously phase-separate to form a prominent pocket. The 1-octanol pocket eventually separates in the form of a droplet to form a unilamellar liposome. The liposomes and 1-octanol droplets then enter the separation hole, where the droplets drift upward, whereas the liposomes are slowly sucked into the post-hole channel. The three inlet holes and the exit hole are 0.77 mm wide, whereas the separation hole is 0.33 mm wide. IA, inner aqueous phase; LO, lipid-carrying organic phase; OA, outer aqueous phase.

liposome suggests that the spreading coefficient of the OA, $S_{OA} = \gamma_{IA-LO} - \gamma_{IA-OA} - \gamma_{OA-LO}$, is > 0 (ref. 52). Note that, apart from purely energetic considerations, the continuous flow of the external fluid and possibly interactions with the channel walls are important for the process as well, as lack of flow or fewer interactions with the walls may lead to the absence of complete budding off.

The liposomes are further purified from 1-octanol droplets through the use of a separation hole that is punched into the middle of the post-junction channel at a sufficient distance from the production junction (**Fig. 4a**, **Supplementary Video 5**). Lighter 1-octanol droplets, as compared with the surrounding aqueous phase, move upward, whereas the liposomes are forced to enter the post-hole channel by applying gentle suction at the exit hole (**Fig. 4b**, **Supplementary Video 6**).

Level of expertise needed to implement the protocol

The initial lithography process to obtain the designs on a silicon master wafer requires a clean room facility and appropriate training. Alternatively, one can obtain such a custom-designed master commercially (for instance, from NIL Technology). Once a master is prepared, the rest of the protocol can be performed in a standard wet lab without any particular level of expertise (beyond bachelor of science level or equivalent), with proper instructions. In our experience, a person without any previous experience in microfluidics can master the protocol within 2 weeks.

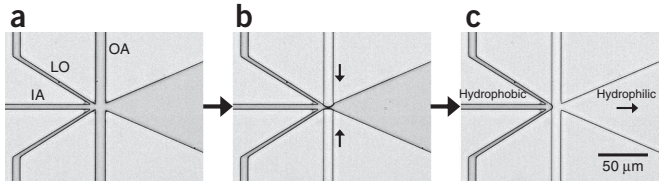

Figure 2 | Hydrophilic surface treatment of the microfluidic device. (**a**) Bright-field image of the production junction before the treatment. (**b**) Aqueous PVA solution is injected into the two OA channels and reaches the junction. Positive air pressure is maintained in the IA and LO channels, which prevents the solution from entering these channels. (**c**) The two fluid streams merge and flow into the post-junction channel. After an incubation time of ~3–5 min, the PVA solution is removed by applying vacuum at the exit hole and simultaneously increasing the air pressure on the IA and LO channels. This renders the post junction channel hydrophilic.

Limitations

An inherent limitation of OLA is its inability to form asymmetric lipid bilayers, i.e., bilayers with a different lipid composition for the inner and outer leaflet. As lipids are dissolved in 1-octanol and subsequently form a bilayer, the lipid composition is the same for both the leaflets. Methods that can form liposomes with asymmetric bilayers (e.g., inverted emulsion transfer[58], pulsed jetting[59], and layer-by-layer assembly[60]) do so with a two-step procedure in which the inner leaflet and outer leaflet are not formed simultaneously. This allows for the use of different lipid compositions for the two leaflets.

As 1-octanol is used as the organic solvent to dissolve lipids and form the initial double-emulsion droplet, one cannot discard the possibility that traces of 1-octanol will be left in the liposome bilayer. These traces are present, however, only in minute quantities, as inferred from the lack of any obvious inclusions in the bilayer as observed via fluorescence microscopy, as well as from the ability to insert protein (α-hemolysin) pores into the bilayer[9]. In addition, because of the low but finite solubility of 1-octanol in water (0.46 g/liter)[61], trace amounts may dissolve in the aqueous lumen and the external environment. Finally, we note that 1-octanol has been shown to be one of the more biocompatible solvents, as compared with other commonly used organic solvents such as hexane, decane, and chloroform[54]. Nonetheless, as with any other method that uses organic solvents (e.g., inverted emulsion transfer), one must be aware that the membrane dynamics can be affected even by a low fraction of contaminating solvent[62]. 1-Octanol droplets are an inevitable side product of OLA. For long-term on-chip experimentation, it is highly recommended to separate the liposomes from this side product, which adds an additional step to the protocol.

The presence of glycerol, in both the IA and OA, is highly beneficial for the production of double-emulsion droplets, as glycerol increases the viscosity of the OA, improving the shearing of the viscous LO[12]. Glycerol also helps in stabilizing the double-emulsion droplets and liposomes as it has been shown to decrease membrane fluidity and increase membrane stability[12,63]. If strict glycerol requirements (lower concentration or complete absence) are needed, some impact on the production process may be expected.

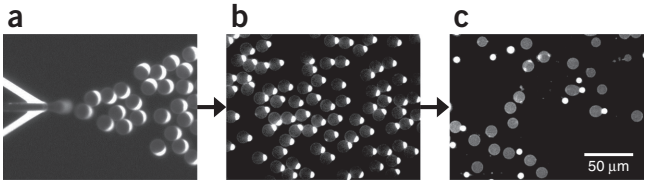

Figure 3 | Octanol-assisted liposome assembly. Fluorescence images showing the key steps leading to liposome formation. (**a**) Formation of double-emulsion droplets at the junction, resembling a bubble-blowing process. (**b**) 1-Octanol, along with excess lipids, forms a prominent pocket, as a lipid bilayer begins to assemble at the interface. (**c**) The pockets eventually separate to form a mixture of liposomes and 1-octanol droplets. The fluorescence is emitted by fluorescent Rh-PE lipid molecules.

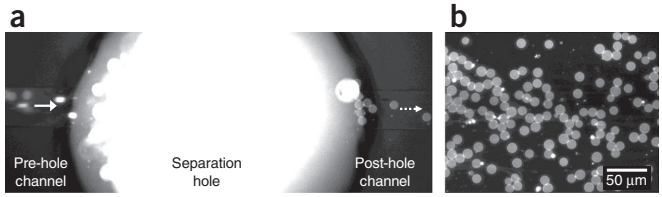

Figure 4 | On-chip purification of liposomes. Fluorescence images showing the key steps leading to the separation of liposomes from 1-octanol droplets. (**a**) A view across the separation hole, where the impure mixture can be seen entering the hole at high flow velocity (indicated by solid arrow), whereas only liposomes enter the post-hole channel at a low flow velocity (indicated by dashed arrow). The separation hole appears highly fluorescent because of the accumulation of 1-octanol droplets. (**b**) The post-hole channel is predominantly filled with liposomes and some occasional contaminants. The fluorescence is emitted by fluorescent Rh-PE lipid molecules.

Experimental design

Master wafer design and fabrication. Although we have used e-beam lithography to create the microstructures on a silicon template, one should be able to use optical lithography, as long as the pattern, especially the production junction, is well defined, i.e., with smooth walls and sharp corners. For users who do not have access to a clean room facility, custom-designed silicon templates can also be commercially obtained (e.g., from NIL Technology). In addition, one can think about scaling up the device to obtain liposomes at substantially higher throughput (>100 Hz). This can be particularly useful if one intends to harvest liposomes for off-chip experimentation. Along with the relative flow velocities of the IA and OA, the dimensions of the production junction determine the lower limit on the size of the liposomes that can be produced using OLA. The use of a PDMS-based microfluidic system, as well as nonlinear increase in the hydraulic resistance of the device, puts a lower limit on the dimensions of the flow channels and the production junction. This sets the lower limit on the liposome size obtained using standard OLA technique to ~5 µm. Using a multiheight approach (different heights for different parts of the device), one can further lower the limit on liposome size. However, such devices are less robust, as they are highly prone to clogging. Regarding on-chip purification of liposomes from 1-octanol droplets, if one wants to instead proceed with off-chip experiments, one can consider a similar density-based separation outside the chip.

Lipid compositions and liposome contents. The OLA technique lends itself to forming liposomes of various lipid compositions. Although we have used 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as the lipid of choice to form liposomes for this protocol, we have also successfully tested several different lipid compositions. Examples include DOPC:1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DOPG), molar ratio 2:1; DOPC:1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), molar ratio 7:3; DOPC:cholesterol (Chol), molar ratio 7:3; DOPC:1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso PC), molar ratio 7:3; DOPC:1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DGS-NTA-Ni), molar ratio 9:1; and DOPC:oleic acid, molar ratio 1:1. Note that these are not the only possibilities, and that users should test the lipid composition of their choice to check its compatibility with OLA.

Surfactants. OLA production requires the use of the nonionic triblock copolymer surfactant Poloxamer 188 (P188) in the OA, in order to form stable double-emulsion droplets at the production junction. In the absence of P188, the droplets immediately fuse with each other and also burst very easily, leading to uncontrolled production. This is similar to water-in-oil droplet systems, in which—in the absence of potent surfactants—the droplets fuse with each other because the oil–water interface is not stabilized. P188 also substantially decreases the interfacial tension between the two zipping lipid monolayers, and thus assists the dewetting process[52]. P188 does not insert itself into the lipid bilayer, but it weakly adsorbs on the liposomal membrane surface[64]. If there is a critical need to eliminate the P188 from the system, one may purge the system with a P188-free solution after the liposomes are formed, using an extra channel.

Controlling fluid flow. We use pressure pumps to control fluid flow because of the fast response time and high sensitivity that they offer. Alternatively, one should be able to use syringe pumps. The performance (e.g., efficiency of the surface treatment and monodispersity of the sample) may, however, be affected by the sensitivity and the response time of the pump in use.

MATERIALS

REAGENTS

- Silicon wafer, e.g., 4 inches in diameter, ~500 µm in thickness, one side polished, type/orientation NP<100>, PB<100>, resistivity 1–10 Ω-cm (International Wafer Service)
- 1,1,1,3,3,3-hexamethyldisilazane (HMDS; VWR, cat. no. 51152885)
  ! CAUTION This compound is highly flammable and is toxic upon contact with skin or if inhaled. Keep away from heat and wear protective gloves, protective clothing, eye protection, and face protection.
- A negative resist, e.g., NEB22A (Sumitomo Chemical, cat. no. WY8041-GB-004)
- Developer, e.g., Microposit MF-322 (micro resist technology, cat. no. B1.08.001-0003)
- Acetone (Honeywell, cat. no. 32201)
- (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (abcr, cat. no. AB111444)
  ! CAUTION This compound causes severe skin burns and eye damage. It reacts explosively with water. Wear protective gloves, protective clothing,

eye protection, and face protection. Work under a dry, inert gas atmosphere while handling it. Once aliquoted, gently flush the stock bottle with argon, and seal the lid with Parafilm.
- 2-Propanol (Merck, cat. no. 109634)
- Pure water obtained through an ultrafilter, e.g., Biopak Polisher (Merck, cat. no. CDUFBI001)
- Polyvinyl alcohol (PVA), molecular weight 30,000–70,000, 87–90% hydrolyzed (Sigma-Aldrich, cat. no. P8136)
- Glycerol (Sigma-Aldrich, cat. no. G2025)
- Poloxamer 188 (P188) solution, 10% (wt/vol) (Sigma-Aldrich, cat. no. P5556)
- Polydimethylsiloxane (PDMS) and curing agent (Dow Corning, Sylgard 184 silicone elastomer kit)
- Lipids, e.g., DOPC (Avanti Polar Lipids, cat. no. 850375C)
- Fluorescent lipids for visualization, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt; Rh-PE; Avanti Polar Lipids, cat. no. 810150C)
- Ethanol absolute (VWR, cat. no. 20821)
- 1-Octanol (Sigma-Aldrich, cat. no. 297887)
- Dextran, Alexa Fluor 647 (Thermo Fisher Scientific, cat. no. D22914)

EQUIPMENT

Clean room equipment
- Spin-coating system (SUSS MicroTec)
- Hot plates (Harry Gestigkeit, cat. no. 2860EB)
- Electron-beam lithography system (Raith, model no. EBPG5200)
- Upright microscope (Olympus, model no. BX51M)
- Dry reactive ion etching system (Adixen; AMS, model no. 100 I-speeder)
- Stylus profiler (e.g., Bruker, DektakXT model)

Wet lab equipment
- Hot plate stirrer (e.g., AccuPlate, model no. D0420)
- Syringe filter unit, 0.22 µm (Merck, cat. no. SLGV033RS)
- Syringe, capacity 1 ml (BD Plastipak, cat. no. 300013)
- Inert gas (e.g., argon) supply
- Vacuum desiccator (Kartell)
- Glass syringe, 250 µl (gas-tight; Hamilton, cat. no. 1725N)
- Glass syringe, 10 µl (Microliter; Hamilton, cat. no. 701ASN)
- Clean air/nitrogen supply with a spray gun (Integris)
- Glass vials (VWR International, cat. no. 548-0385) with screw caps (VWR International, cat. no. 548-0389)
- Transfer pipette (Sigma-Aldrich, cat. no. Z350796)
- Knife (Stanley, cat. no. 10-418) and blades (Stanley, cat. no. 11-301T)
- Biopsy punch, inner diameter (i.d.) = 0.77 mm (World Precision Instruments, cat. no. 504529)
- Biopsy punch, i.d. = 0.33 mm (World Precision Instruments, cat. no. 504638)
- Dissecting microscope with up to 32× magnification (e.g., Zeiss, model no. Stemi DV4 SPOT)
- Glass slides, 24 mm × 32 mm, thickness no. 1 (VWR, cat. no. 631-0143)
- Flat-tip tweezers (Ideal-tek, cat. no. 35A.SA)
- Plasma-generating system (e.g., Plasmatic Systems, model no. Plasma-Preen I)
- Inverted microscope (e.g., Olympus, model no. IX81)
- Low-magnification air objectives, 10× (numerical aperture = 0.30) and 20× (numerical aperture = 0.75) (Olympus)
- Fluorescence detection system with a light source (e.g., Excelitas Technologies, model no. X-Cite 120Q)
- Digital camera (e.g., Neo sCMOS; Andor Technology)
- Tubing (thicker), i.d. = 0.51 mm, outer diameter (o.d.) = 1.52 mm (Tygon Microbore Tubing, cat. no. EW-06419-01)
- Tubing (thinner), i.d. = 0.25 mm, o.d. = 0.76 mm (Tygon Microbore Tubing, cat. no. EW-06419-00)
- Needles, o.d. = 0.6 mm (BD Microlance, cat. no. 300800) and o.d. = 0.4 mm (Terumo, cat. no. NN-2719R)
- Metal connectors (thicker, o.d. = 0.6 mm), custom-made by cutting needles (BD Microlance, cat. no. 300800) into 1-cm pieces
- Metal connectors (thinner, o.d. = 0.4 mm), custom-made by cutting needles (Terumo, cat. no. NN-2719R) into 1-cm pieces
- Fittings for the tubing (Idex, cat. no. F-120)
- Microfluidic flow control system (e.g., system bases with three low-pressure channels (0–1,000 mbar), one negative-pressure channel (0–800 mbar), and four high-pressure channels (0–7,000 mbar), Fluigent, cat. no. V00000501)
- Interface between the microfluidic flow control system and the microfluidic system (e.g., Fluiwell; Fluigent)
- Disposable plastic vials, capacity = 0.5 ml (Fluigent)
- Vacuum pump, volume flow rate = 8 m^3/h, ultimate pressure < 10^{-4} (e.g., Leybold Trivac, model no. D8B)
- Dry wipes (Kimtech, cat. no. 7552)
- Micropipettes

Software
- Computer-aided design (CAD) software (e.g., AutoCAD, https://www.autodesk.eu/products/autocad/overview)
- MAESFLO software, v3.2.1 (Fluigent, https://www.fluigent.com/product/software-solutions/)
- Image acquisition software (Micro-Manager, v1.4.14, https://micro-manager.org)

REAGENT SETUP

PVA solution Take water in a beaker and place a clean magnetic stirrer inside. Place the beaker on a hot plate stirrer and start stirring (~500 r.p.m.). Gradually dispense the required amount of PVA powder (a 2.5% (wt/vol) stock solution in a volume of 10 ml is convenient to make and use) into the water so that it does not form big clumps. Gradually increase the temperature to 85 °C and wait until the solution becomes clear and no clumps are seen. As an additional precautionary step, one can filter the PVA solution. Store at room temperature (20–25 °C) and use as long as no precipitation or aggregates are seen, usually up to a few months.

IA solution Dispense the appropriate amount of glycerol into water, so that the final concentration is 15% (vol/vol) glycerol. 100 µl of solution is more than sufficient for a typical experiment. Pipette glycerol, which is highly viscous, slowly, in order to avoid air bubbles entering the pipette tip. One can also cut the end of the pipette tip to facilitate the procedure. Shake the tube in order to mix the contents. Glycerol is added in order to stabilize the double-emulsion droplets and should be removed (or used in lower concentration) only if it is mandatory. IA solution without any additional substances can be stored at room temperature indefinitely; the stability will vary greatly, depending on what other chemicals or biomolecules are added. A fluorescent molecule (e.g., 0.4 µM of Dextran, Alexa Fluor 647, molecular weight 10,000) can be added in order to visualize the lumen of the liposome. We recommend adding any further chemicals or biomolecules that must be encapsulated inside the liposome directly before use.

OA solution Dispense the appropriate amount of glycerol and P188 in water. The final concentrations are 15% (vol/vol) glycerol and 5% (wt/vol) P188. 100 µl of solution is more than sufficient for a typical experiment. Pipette glycerol, which is highly viscous, slowly, in order to avoid air bubbles entering the pipette tip. One can also cut the end of the pipette tip to facilitate the procedure. Shake the tube gently in order to mix the contents but avoid foam formation due to the presence of the surfactant. OA solution without any additional substances can be stored at room temperature indefinitely; the stability will vary strongly depending on what other chemicals or biomolecules are added.

Lipid stock solution Dispense the lipid solutions (e.g., DOPC and Rh-PE) into a round-bottom flask using a glass syringe. Use separate syringes for fluorescent and nonfluorescent lipids in order to avoid contamination, and also because different volumes will be required. One fluorescent lipid molecule for every thousand nonfluorescent lipid molecules is a good ratio for fluorescence imaging. Evaporate the chloroform by passing a gentle stream of nitrogen into the round-bottom flask so that a dry lipid film forms at the base of the flask. Get rid of any remaining chloroform by placing the flask in the desiccator for at least 2 h in partial vacuum (~10 mbar). Add ethanol to form a 10% (wt/vol) stock solution and cover the flask with Parafilm. Rotate the flask so that the entire lipid film is dissolved. In the case that the lipids do not dissolve in pure ethanol, add equal volumes of ethanol and chloroform to a final concentration of 5–10% (wt/vol). Usually, 50–100 µl of lipid stock solution is convenient to prepare and use. Pipette the solution into a glass vial. Replace the air in the vial by gently flushing with an inert gas. Seal the lid with Parafilm and store at −20 °C. The stock solution can be used as long as the solution looks clear and without any aggregates, usually up to a few months.

LO Dispense 1-octanol into a plastic vial. Pipette the lipid stock solution in 1-octanol, to a final concentration of 0.2% (wt/vol). Usually, 50 µl of solution (1 µl of lipid stock solution + 49 µl of 1-octanol) is sufficient for several experiments. Mix by slowly pipetting up and down, and take care to not introduce any air bubbles into the solution. Make the solution as fresh as possible and use it on the same day.

PROCEDURE

Preparation of a master wafer ● TIMING ~3 h

▲ CRITICAL Steps 1–12 should be performed in a clean room facility (for this work, a class 10,000 (ISO 7) facility with a class 100 (International Organization for Standardization (ISO) 5) work area was used). A schematic flow diagram summarizing the key steps involved in preparing the master wafer (Steps 1–18) is shown in **Supplementary Figure 1a**.

1| Prime a 4-inch-diameter clean silicon wafer by placing ~3 ml of HMDS on it, and spin-coating it at 1,000 r.p.m. for 1 min. Immediately bake the wafer at 200 °C for 2 min. This will enhance subsequent resist adhesion.

2| Spin-coat the wafer with ~3 ml of NEB22A at 500 or 1,000 r.p.m. for 1 min. Immediately bake the wafer at 110 °C for 3 min. Any other suitable negative resist may also suffice, following a standardized protocol.
▲ CRITICAL STEP If deeper structures are required, spin-coat NEB22A at 500 r.p.m. for ~5 s, and let the wafer rest for 3 min. This way, a thicker (although not very uniform) layer of NEB22A is obtained.
? TROUBLESHOOTING

3| Write the desired pattern (see the **Supplementary Data** for the standard OLA design) on the coated wafer using an electron-beam lithography system following the manufacturer's instructions. We use an EBPG 5200 system with the following parameters: acceleration voltage = 100 kV, aperture = 400 μm, and dose = 16–18 $\mu C/cm^2$.

4| Bake the wafer immediately (within minutes) at 105 °C for 3 min.
■ PAUSE POINT The baked wafer can be kept at room temperature in a dust-free environment indefinitely, before proceeding.

5| Develop the wafer by dipping it into MF322 for 40 s, then dipping it in diluted MF322 solution (10% (vol/vol) MF322, diluted in water) for 20 s, and finally in water for 20 s. Dry the wafer.

6| Check the wafer under the microscope to see whether the patterns have developed properly.
? TROUBLESHOOTING
■ PAUSE POINT The developed wafer can be kept at room temperature in a dust-free environment indefinitely, before proceeding.

7| Clean the chamber of the inductively coupled plasma (ICP) reactive ion etcher (we use an AMS 100 I-speeder) using O_2 gas at 200 standard cubic centimeters per min (SCCM) for ~15 min, with the ICP power set to 1,800 W and a biased power set to 60 W.
▲ CRITICAL STEP If the chamber is not cleaned before etching the wafer, unwanted substances (depending on the previous etching process) can be deposited on the wafer surface, making it unusable.

8| Place the wafer obtained from Step 6 in the AMS 100 I-speeder. Keep the pressure ~0.04 mbar. Keep the temperature of the wafer at 10 °C and maintain the temperature of the main chamber at 200 °C. Keep the sample holder at 200 mm from the plasma source.

9| Etch the wafer using a Bosch deep reactive ion etching process. The process consists of alternate etching (SF_6) and passivation (C_4F_8) cycles. The etching step is 200 SCCM SF_6 for 7 s with the ICP power set to 2,000 W while the capacitive coupled plasma (CCP) power (biased power) is switched off. The passivation step is 80 SCCM C_4F_8 for 3 s with the ICP power set to 2,000 W and the CCP power in chopped low-frequency bias mode: 80 W for 10 ms and 0 W for 90 ms. The etching time depends on the desired structure height (typically 7–11 μm) and the etching rate (typically 2–3 μm/s), which is thus typically 3–4 min.
▲ CRITICAL STEP If the etching time is too long, the protective layer of NEB22A will be completely removed and will result in rough structures with undesired height. To avoid this, do not etch longer than ~3 min 30 s when NEB22A is spin-coated at 1,000 r.p.m., or longer than ~5 min when NEB22A is spin-coated at 500 r.p.m. When NEB22A is spin-coated at 500 r.p.m. for only 5 s, etching time can be much longer (>10 min).
? TROUBLESHOOTING

10| Clean the wafer in the AMS 100 I-speeder (pressure of 0.04 mbar, ICP power set at 2,500 W with a biased power of 60 W, a source-target distance of 200 mm, and the temperature of the wafer set at 10 °C) using O_2 gas at 200 SCCM for 3–5 min.
? TROUBLESHOOTING

11| Measure the height of the etched structures using a DektakXT stylus profiler.

12| Clean the wafer (now called a 'master') with acetone, rinse in deionized water, and finally dry it.
■ **PAUSE POINT** The master can be kept at room temperature in a dust-free environment indefinitely, before proceeding.

Surface treatment of the master ● TIMING ~12 h
13| Replace the air in the desiccator with argon.

14| Pipette ~20 µl of (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane into a vial and place it in the desiccator. Do not close the tube.

15| Place the master inside the desiccator and turn on the vacuum. Wait until the pressure reaches a value of <10 mbar.

16| Close the valve on the desiccator and turn off the vacuum. Keep the desiccator in a safe place, e.g., a fume hood.

17| Leave the master under partial vacuum for ~12 h. The master is now ready to use.
■ **PAUSE POINT** The master can be stored at room temperature indefinitely. It can be used repeatedly to make microfluidic devices, as long as it is kept clean, by covering it to avoid accumulation of dust particles on its surface, and is handled carefully.

18| Similarly, silanize a clean blank silicon wafer, following Steps 13–17. This will be used later for making PDMS-coated glass slides.

Preparation of the microfluidic device ● TIMING ~6 h
▲ **CRITICAL** A schematic flow diagram summarizing the key steps involved in manufacturing a ready-to-use microfluidic device (Steps 19–60) is shown in **Supplementary Figure 1b**.

19| *Preparation of the PDMS blocks bearing the required pattern (Steps 19–28)*. Place the master on a square piece of aluminum foil. Fold the foil around the master so as to create a well-like structure (**Supplementary Fig. 2a**).
▲ **CRITICAL STEP** While folding a foil around the master, handle the master gently to avoid breaking it. Preferentially, keep the master on a solid surface to aid the process. Make sure that no gaps are left between the edge of the master and the foil beneath.

20| Prepare an appropriate amount of PDMS in a weighing cup. Using a transfer pipette, add PDMS and the curing agent at a mass ratio of 10:1 (alternatively, 15:1 also suffices). Generally, 30 g of PDMS + 3 g of curing agent are enough for one master.

21| Using the same pipette, mix the two components vigorously, for ~2 min.
▲ **CRITICAL STEP** It is essential to mix the components homogeneously, as they will not mix spontaneously because of the high viscosity of PDMS. If not mixed homogeneously, the curing process will not be uniform, and this will affect further processing, such as peeling off the PDMS layer from the master.

22| Desiccate the mixture in a vacuum desiccator at room temperature until most of the air bubbles are removed (this usually takes ~15–30 min).
▲ **CRITICAL STEP** Desiccation will expand the trapped air bubbles before they escape into the atmosphere. This can cause spilling of the mixture, reducing the quantity substantially. To avoid this, bring the pressure in the desiccator to atmospheric pressure by slowly opening up the valve. Only partially increasing the pressure can also suffice. Repeat the process (usually four to six times) until no more bubbles emerge from the PDMS–curing agent mixture.
? TROUBLESHOOTING

23| Slowly pour the mixture onto the master and desiccate again until any remaining air bubbles are removed (this usually takes ~10–15 min).

24| Bake the master in an oven at 80 °C for at least 4 h.

25| Take out the master from the oven and allow it to cool to room temperature. Carefully remove the aluminum well surrounding the master. Slowly peel off the PDMS layer from the top of the master.
▲ **CRITICAL STEP** Do not hurry while peeling off the PDMS layer, as there is a chance that small chunks of PDMS, in close contact with the etched structures, might be left behind. Such small stuck-on parts are difficult to remove and can damage the master itself.
? TROUBLESHOOTING

26| With the patterned structures facing upward, cut separate PDMS blocks, each containing a single microfluidic device, using a sharp blade.
▲ **CRITICAL STEP** Always keep the PDMS blocks with the patterned surface exposed to air. This ensures minimal contact of the patterned surface with any solid surface and reduces the chance of dirt particles sticking to it. It also avoids possible damage to the device.

27| Place the PDMS block on a solid surface, preferably of dark color, and illuminate it from an angle so that the patterned structures are visible. Punch holes for the three inlets and an exit, using a biopsy punch (i.d. = 0.77 mm).
▲ **CRITICAL STEP** Use a sharp puncher and make sure that the hole is as vertical as possible. In addition, make sure that the puncher has penetrated all the way to the other side of the block before you try to remove the punched part. At the same time, do not push too hard, as that will damage the puncher. Retract the puncher slowly, while slightly rotating it (alternating between clockwise and anticlockwise directions). If the puncher is retracted too quickly or rotated too much, the PDMS surface of the hole can crack, leading to leakage during experimentation.
? TROUBLESHOOTING

28| Punch the separation hole using a biopsy punch with a smaller i.d. (0.33 mm). The process is substantially facilitated by observing the microfluidic channel through a dissecting microscope, using a 35× magnification.
▲ **CRITICAL STEP** It is crucial that the separation hole be punched as symmetrically as possible across the channel. An asymmetrically punched separation hole can affect liposome purification. Punching a symmetric hole is facilitated by narrowing the microfluidic channel and placing two symmetrically placed symbols (fork-like symbols in the design provided in the **Supplementary Data**) across the channel. In addition, the separation hole should be punched at a sufficient distance from the production junction so that 1-octanol droplets are budded off from liposomes before reaching the separation hole.
■ **PAUSE POINT** The punched PDMS blocks can be stored in a covered container (e.g., Petri dish) at room temperature for at least a few months (**Supplementary Fig. 2b**).

29| *Preparation of PDMS-coated glass slides (Steps 29–38)*. Place the blank, silanized wafer on a piece of aluminum foil.

30| Mix ~10 g of PDMS with 1 g of curing agent and desiccate to remove the air bubbles (Step 22).

31| Pour ~80% of the mixture onto the blank wafer evenly. Avoid forming air bubbles.

32| Blow away dirt particles from a 24-mm × 32-mm glass slide using a nitrogen spray gun, with the gun directed tangentially along the surface.

33| Slowly press the cleaned glass slides, using the back side of the tweezers, onto the PDMS layer until they are completely immersed in it (**Supplementary Fig. 2c**). Five slides fit comfortably on a 4-inch wafer.
▲ **CRITICAL STEP** If the slides are not pressed hard enough, they will have a thick layer of PDMS underneath, which may prevent imaging with higher-magnification objectives (>40×). However, if pressed too hard, the slide can break. Try to find the right amount of force needed to achieve the required result. Adequately pressed glass slides have a thin (~50 μm) PDMS layer attached to them. In general, pressing with the thumb (preferably with gloves) is much more effective and easier. However, this can leave a print on the glass surface, which can cause difficulty in further processing, i.e., when peeling off the top PDMS layer.
? TROUBLESHOOTING

34| Pour the remaining PDMS mixture where the upper surface of the glass slides is still not covered with PDMS.
▲ **CRITICAL STEP** If the whole wafer is covered with a thin layer of PDMS, it will be much easier to peel it off once it is cured. On the other hand, pouring too much PDMS will cause it to spill onto the aluminum foil, which is disadvantageous for the peeling-off process.

35| Bake the wafer in an oven at 80 °C for at least 2 h.

36| Peel off the cured PDMS layer from the top of the wafer so that only the glass slides, with their undersides bearing a thin PDMS layer, are left on the wafer (**Supplementary Fig. 2d**).
▲ **CRITICAL STEP** While peeling off the PDMS layer, support the glass slides with your thumb. Slowly roll away the PDMS layer, starting from the corners of the slides. Make sure that the glass slides do not detach from the wafer during the process, as if this happens, they will likely break, because of the high Young's modulus of glass.
? TROUBLESHOOTING

37| Using a blade, carefully remove the glass slides. Insert the blade underneath the glass slide, along the long axis. Gently tilt the blade so that the glass slide starts to peel off from the wafer. Continue the process until the entire glass slide is detached.
? TROUBLESHOOTING

38| Store the glass slides in a container, with the PDMS-coated layer exposed to air.
■ **PAUSE POINT** The PDMS-coated glass slides can be stored at room temperature for at least a few months.

39| *Bonding of the microfluidic device (Steps 39–43)*. Using tweezers, thoroughly rinse the PDMS block with 2-propanol and, using the nitrogen spray gun, immediately dry it with a strong nitrogen flow (4–6 bar).
▲ **CRITICAL STEP** Make sure that the tweezers are gripping the nonpatterned part of the PMDS block. Tightly holding the patterned surface can damage it, compromising device functionality. It is more effective to use the spray gun without any filter in order to have a stronger nitrogen flow.

40| Expose the patterned side of the PDMS block and the PDMS-coated glass slide to oxygen plasma for 10 s (3–4 standard cubic feet per h of O_2, 200 W) using a Plasma-Preen system, in order to activate the surfaces.
▲ **CRITICAL STEP** Check that the plasma is generated properly, by observing a purple glow in the plasma chamber. It can take a few seconds before the plasma is generated, so adjust the exposure time accordingly. If the surface does not become activated, bonding will not take place and the devices will leak. Flush the plasma-generating chamber with oxygen before activating the plasma, in order to ensure good plasma quality.

41| Immediately after the plasma treatment, remove any possible dirt particles from the PDMS block and the glass slide, using the nitrogen spray gun.

42| Place the PDMS block on a well-lit surface with the pattern side facing upward. Gently lay the glass slide, with the PDMS-coated side facing downward, on the PDMS block in order to form a bond between the two surfaces (**Supplementary Fig. 2e**).
▲ **CRITICAL STEP** When bonding, touch one end of the glass slide to the block, and then slowly lower the other end so that bonding takes place from one end to the other. This prevents trapping of any air bubbles between the two surfaces. To eliminate possible trapped air bubbles and to reinforce the bonding, gently tap the glass slide with the tweezers.
? TROUBLESHOOTING

43| Bake the bonded devices in an oven at 80 °C for ~20 min.
■ **PAUSE POINT** The bonded devices should be stored at room temperature before commencing the surface treatment.

Surface treatment of the microfluidic device ● TIMING ~20 min per device
▲ **CRITICAL** Surface treatment using PVA is the most critical step in the OLA device preparation. The treatment is difficult to perform on freshly bonded devices, as the surfaces are too hydrophilic to control the fluid flows. Four hours was found to be an effective resting duration before commencing the surface treatment. However, a prolonged overnight resting period can also provide comparable results.

44| Equilibrate the MFCS-EZ flow-control system (positive pressure: 0–7,000 mbar) for 10 min. Prepare the microscope for bright-field imaging with a 10× objective and set the camera to live mode.
▲ **CRITICAL STEP** Even though a high-pressure channel is used, ~2 bar of pressure is needed for a successful PVA treatment. Depending on the design, this can be decreased to 1 bar, in which case a low-pressure MFCS-EZ flow control system can be used instead.

45| Fix the device onto the microscope stage so that the post-junction channel is as horizontal as possible. Inspect the device for any potential problems, such as dirt particles clogging the channels, collapse in the channels, and blocked inlet/exit holes.
? TROUBLESHOOTING

46| Cut three pieces of thicker tubing (i.d. = 0.51 mm) and one piece of thinner tubing (i.d. = 0.25 mm), and insert them into separate fittings. Use a fitting with a tube sleeve, in the case of the thinner tubing. Insert the metal connectors (o.d. = 0.6 mm for thicker tubes and o.d. = 0.4 mm for thinner tubing) at the right end of the tube pieces (**Supplementary Fig. 2f**). Loosely screw the fittings into the Fluiwell system.
▲ **CRITICAL STEP** Make sure that the ends of the cut pieces have an ~45° bevel. This helps in inserting the metal connector into the tubing. Make sure that the metal connectors are cleaned with ethanol beforehand.

47| Prepare another piece of thicker tube (with a metal connector) and connect it to the vacuum pump, through appropriate fittings.

48| Dispense an appropriate amount of 2.5% (wt/vol) PVA solution into the vial. 100 µl of PVA solution is enough for surface-treating four to six devices.

49| Fix three empty 'air' vials and the PVA-containing vial to the Fluiwell system. Connect one of the thicker tubes to the PVA vial and connect two additional thicker tubes and one thinner tube to the empty 'air' vials.

50| Adjust the length of the PVA tube such that it just reaches the bottom of the vial. Tighten all the fittings.
▲ **CRITICAL STEP** If the tubing does not reach the bottom of the vial, there is a high probability of air bubbles being introduced into the microfluidic device because of insufficient solution volume, which will hamper the surface treatment. At the same time, make sure that the tube is not distorted, in order to ensure stable, continuous flow.

51| Insert the other two thicker 'air' tubes into the IA and LO holes of the device.
▲ **CRITICAL STEP** Insert the metal connectors gently into the holes, in order to avoid breaking the glass and severely affecting device functionality. If one wants to be extra careful, keep the device on a solid surface while inserting the metal connector so that the device can tolerate much more pressure.
? TROUBLESHOOTING

52| Increase the pressure on the PVA channel to ~10 mbar, so that the solution starts flowing steadily through the tube. Once the solution comes out of the metal connector in the form of a droplet, insert the connector into the OA hole without lowering the pressure. Wipe off any liquid in the vicinity of the hole with a dry wipe.

53| Increase the pressure to ~80 mbar so as to have a rapid, yet controllable flow of the PVA solution into the OA channel. Follow the progress of the solution through the live feed of the camera.
? TROUBLESHOOTING

54| Once the PVA solution is close to the junction, increase the air pressure for both the IA and LO channels (~120 mbar) (**Fig. 2a**,**b**).
▲ **CRITICAL STEP** The exact pressures needed on the PVA and air channels will depend on the actual device design, the hydrophilicity of the device, and the concentration of the PVA solution. As a rough guideline, for the design provided in the **Supplementary Data**, ~80 mbar of pressure on the PVA channel (containing 2.5% (wt/vol) PVA solution) and ~120 mbar of pressure on the air channel leads to a stable interface at the junction.

55| As the PVA solution arrives at the junction from both the OA channels, maneuver the pressures in such a way that the PVA solution forms a concave meniscus at the junction (**Fig. 2c**).
▲ **CRITICAL STEP** While maneuvering the pressures, avoid pushing the PVA solution into the junction, i.e., into the IA and LO channels, at any cost. This will make the exposed area hydrophilic, strongly compromising the device functionality. To have some buffering capacity, one can design small 'bumpers' at the junction (**Supplementary Fig. 3**). In this case, even if the meniscus of the PVA solution moves inward until the start of the bumpers, the air pressure can be quickly increased to move the meniscus to the end of the bumpers, without affecting the device functionality. Such a design, however, increases the probability of the PVA clogging at the junction.
? TROUBLESHOOTING

56| Incubate for 3–5 min, to allow the PVA polymers to adsorb to the surface. Make sure that the PVA solution has flowed past the separation hole and reached the exit hole.
▲ **CRITICAL STEP** Keep an eye on the PVA–air interface throughout the incubation time, as it is very sensitive to pressure fluctuations. The interface is also affected by the progress of the PVA solution along the channel, for example, when it reaches the exit or the separation hole. Even a small increase in the pressure (e.g., an air bubble escaping into the exit hole) can move the interface slightly into the IA and LO channels, which may make the device unusable. Only when the entire post-junction area is completely filled with PVA solution and devoid of bubbles, does the PVA–air interface becomes stable. In general, longer incubation times will result in better surface treatment and device performance.

57| Decrease the pressure on the PVA channel to zero, quickly increase the air pressure on the IA and LO channels to ~2 bar, and remove the PVA tube from the microfluidic chip.
▲ **CRITICAL STEP** Remove the PVA tube by holding the portion of the tube containing the metal connector, in order to avoid detachment of the tube from the connector. Simultaneously, support/push the PDMS surface of the device in order to avoid lifting up the device and as a result, potentially cracking the glass slide.
? TROUBLESHOOTING

58| Turn on the vacuum pump and apply vacuum to the exit hole, and then to the OA inlet, in order to remove the PVA solution from the device.

59| To remove PVA from the separation hole, insert the thin tube into the hole and apply sufficient positive pressure (up to 2 bar).
▲ **CRITICAL STEP** At the end of the treatment, make sure that the OA inlet, the exit, and the separation hole are not clogged by PVA. Whether that is the case can be checked by pressurizing them with air and observing that the microfluidic channels slightly expand.

60| Bake the device in an oven at 120 °C for roughly 15 min to heat-immobilize the PVA polymers onto the surface.
■ **PAUSE POINT** PVA-treated devices can be stored at room temperature in a dust-free environment for at least a month.

Liposome production (OLA) and purification ● TIMING 1 h, can vary widely according to the experiment
61| Equilibrate the MFCS-EZ flow control system (positive pressure: 0–1,000 mbar, negative pressure: 0–800 mbar) for 10 min. Prepare the microscope for bright-field imaging with a 10× objective and set the camera to live mode.

62| Fix the device on the microscope stage so that the post-junction channel is as horizontal as possible. Inspect the device for any potential problems, such as dirt particles clogging the channels, collapse in the channels, and blocked inlet/exit holes.
? TROUBLESHOOTING

63| Cut four pieces of thicker tubing and insert them into separate fittings. Insert the thicker metal connectors at the other ends of the tube pieces. Loosely screw the fittings into the Fluiwell system (three into positive-pressure channels and one into the negative-pressure channel).
▲ **CRITICAL STEP** Make sure that one end of the cut pieces has an ~45° bevel. This helps in inserting the metal connector into the tubing.

64| Dispense sufficient amounts of the IA and OA into two clean vials. 100 µl of solution is more than sufficient for a typical experiment. Avoid trapping any air bubbles between the vial walls and the solution. Note that the LO is directly prepared in the vial, just before the experiment (Reagent Setup). Tightly fix the three vials to the positive-pressure channels and one empty 'air' vial to the negative-pressure channel.

65| Adjust the length of each tube such that it just reaches the bottom of the vial. Tighten the fitting.
▲ **CRITICAL STEP** If the tubing does not reach the bottom of the vial, there is a high probability of air bubbles being introduced into the microfluidic device because of insufficient solution volume, which will severely hamper liposome production. At the same time, make sure that the tube is not distorted, in order to ensure stable, continuous flow.

66| Insert the 'air' tube into the exit hole of the device.

67| Increase the pressure on the OA tube to ~10 mbar so that the solution starts flowing steadily through the tube. Once the solution comes out of the metal connector in the form of a droplet, insert the connector into the OA hole without lowering the pressure. Note that this can result in loss of a small amount of solution (1–3 µl). Although this amount is usually negligible, it may be necessary to take this loss into account when working with small sample volumes (~10 µl). Repeat the procedure for the LO and IA channels, so that all the three phases are on the verge of entering the microfluidic device. Wipe off any liquid in the vicinity of the hole with a dry wipe.

68| Increase the pressure to ~50 mbar on the OA channel so that the OA starts flowing steadily through the channels. Do the same for the LO and finally for the IA. The exact pressures needed to push the solutions into the channels may vary substantially depending on how deep the metal connector has been inserted inside the hole and the position of other fluids inside the device. Once all three phases are steadily flowing inside the channels, maneuver the pressures such that they arrive at the junction in the following order: OA, IA, and LO.
▲ **CRITICAL STEP** It is highly recommended that the OA arrive first at the junction and flow downstream into the post-junction channel. The IA should preferably arrive next, and finally the LO, to commence double-emulsion production. In addition, it is important that all three inlet holes be already filled with their respective solutions before the OA flows downstream; otherwise, too much air will have to be removed, either slowly through the channel walls or rapidly by blowing air bubbles. Production of air bubbles can adversely affect the liposome purification stages. Make sure that the LO is continuously flowing, as it is prone to clogging.
? TROUBLESHOOTING

69| Once the three phases arrive at the junction, switch to the 20× objective, and maneuver the pressures such that double-emulsion droplets begin to form (**Fig. 3a**). The size of the droplets is mainly governed by the relative flow velocities of the IA and the OA. Although the IA stream velocity determines how much volume is encapsulated within the droplet, OA stream velocity determines the pinching-off rate[9].
▲ **CRITICAL STEP** The exact pressures needed to form stable double-emulsion droplets will vary from device to device. For the design given in the **Supplementary Data**, a typical range for the three phases is between 50 and 150 mbar. If the double-emulsion droplet production does not begin immediately, lower the pressure on the three phases gradually (20–50 mbar) such that the LO starts forming a film at the junction but breaks as it is pushed by the IA. Then gradually increase the pressure on the OA, so as to facilitate the pinch-off process, until stable emulsion droplets start forming. It is recommended to keep an eye on the production process from time to time, even in case of stable liposome production. This is to avoid any severe clog formation in the LO channels. If one of the LO channels becomes severely clogged, it is often still possible to continue with the liposome production, although in a less-than-optimal way. In general, having a comparatively smaller 1-octanol pocket with respect to the size of the liposome will lead to quicker budding off. As a general rule, 1-octanol droplets that are less than half of the size of the corresponding liposome is optimal. Having a considerably larger 1-octanol pocket (i.e., larger than the liposome it is attached to) will not lead to budding off, at least not within the usual time line of a few minutes. A typical size range for the 1-octanol droplets obtained during liposome production is 4–8 µm (ref. 10). Make sure that the pockets are separating from the liposome before they reach the separation hole, otherwise, double-emulsion droplets may be sucked into the post-hole channel. In addition, maintain sufficient flow velocity in order to avoid the formation of any possible aggregates of liposomes and/or droplets.
? TROUBLESHOOTING

70| Follow the double-emulsion droplets downstream of the channel to see whether they are stable (**Fig. 3b**) and whether the pockets are eventually separating to form a mixture of liposomes and 1-octanol droplets (**Fig. 3c**).
? TROUBLESHOOTING

71| Simultaneously, start the suction pressure so that the liposomes begin entering the post-hole channel, providing a purified yield of liposomes (**Fig. 4**).
▲ **CRITICAL STEP** The purification will be much more effective if the OA already fills the hole and occupies the post-hole channel before the very first 1-octanol droplets or liposomes reach the separation hole. If the hole is not yet filled, the particles will simply follow the fluid flow into the post-hole channel, and no separation will occur. One can alternatively fill the separation hole externally using a syringe. However, this has a high risk of introducing air bubbles into the device and temporarily disrupting proper device functioning. For the design given in the **Supplementary Data**, 2–4 mbar of suction was most effective for carrying out liposome purification[10].
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	The wafer is not uniformly covered with the resist	The amount of resist used for the spin-coating was not enough	Remove the resist layer by washing the wafer with acetone and repeat the spin-coating procedure (Steps 1 and 2)
6	The pattern is not written properly and shows inhomogeneity	The e-beam settings were not optimal, e.g., dose, current	Clean the wafer with acetone and repeat the spin-coating and e-beam procedure (Steps 1–4)
	There is still some unwanted resist left, especially in sharp corners	The wafer was not developed long enough	Repeat the development procedure (Step 5)
9	The wafer seems to be coated with some unwanted substance	The chamber was not cleaned properly	Try to get rid of the unwanted deposition by following Step 10
	No resist can be seen over the patterns	The wafer has been etched too long	Discard the wafer. Repeat the procedure with a shorter etching time and check the etching progress as described in Step 11
10	The wafer is still coated with some unwanted substance	The chamber was not cleaned properly	Discard the wafer and clean the chamber (Step 7)
22	The PDMS-curing agent mixture spills over during desiccation	The vacuum was not released frequently enough	If the spill is small, continue with the procedure. Otherwise, prepare a new mixture (Steps 20 and 21)
		The mixture amount is inappropriate for the cup volume	Decrease the mixture volume (use two batches) or use a bigger cup
25	The cured PDMS layer is not removed properly from the master, leaving residues behind	Silanization was not done properly	Try to carefully remove the residues and repeat the silanization (Steps 13–17). If this proves too difficult, discard the wafer
		PDMS-curing agent was not properly mixed	
27	Walls of the punched hole look rough and cracked	The puncher is damaged	Discard the PDMS block and replace the puncher
		The puncher was not all the way through the PDMS block before the hole was cut	Discard the PDMS block. In addition, check that there are no PDMS residues left in the puncher
		The puncher was removed too rapidly	Discard the PDMS block
33	The glass slide breaks while being pressed into the uncured PDMS layer	The slide was pressed too hard, or too quickly	Using tweezers, carefully remove the slide, and press a new slide in
36	The glass slide breaks while peeling off the upper cured PDMS layer	The slide was not held with a strong enough force, or the PDMS layer was peeled off too quickly	Discard the broken slide, unless only a small, inconsequential portion is broken
37	The glass slide breaks while being detached from the wafer	The slide was peeled off too quickly, and/or along the width and not along the length of the glass slide	Discard the broken slide, unless only a small, inconsequential portion is broken
42	The glass slide and the PDMS block are not becoming bonded	Plasma was not generated properly	Repeat the plasma treatment (Steps 39–42)

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
45	Some particulate matter is blocking a channel(s) or the junction	The PDMS block and the glass slide were not cleaned properly before bonding	If the blockage is in the channel(s), try to perform the surface treatment. In the case of blockage at the junction, discard the device and next time make sure that the PDMS block and the glass slide are clean (Steps 39 and 41)
	One or more channels are collapsed	Too much pressure was applied during device bonding	For a small collapse, try to perform the surface treatment. If the collapse is too big, discard the device
		Aspect ratio (width/height) of the channel is too high, usually >100	Redesign the device (decrease channel width or introduce support pillars) or increase channel height, in order to reduce the aspect ratio Increase the PDMS/curing agent ratio, in order to decrease the flexibility of the cured PDMS block
51	The device cracks while the metal connector is being inserted	Too much pressure was applied while inserting the metal connector	If the crack is very small or not across the channel, continue with treatment. If the crack is prominent or across the channel, discard the device
	The channel collapses after the metal connector is inserted	Too much pressure was applied while inserting the metal connector	For a small collapse, continue with the treatment. If the collapse is too big, discard the device
53	The PVA solution does not enter the channel, even after applying high pressure	The metal connector is not inserted deep enough into the device	Remove and re-insert the tube into the device
		The fitting is not screwed tight enough into the Fluiwell system	Tighten the fitting
		The inlet hole is leaking	Discard the device
		The PDMS block is improperly bonded to the glass slide	Discard the device
55	PVA solution arrives only from one side	PVA solution is stuck somewhere upstream of the channel because of unwanted channel obstruction	Increase the pressure on the PVA and air channels until the solution arrives from the other side
	PVA solution does not flow and respond to the increase in pressure	The low flow velocity has led to the formation of a clog in the channel	Try increasing the air pressure. If the clog does not go away, discard the device
	PVA solution goes into the IA and/or LO channels	The air/PVA pressure was not controlled properly	If the extent is very small (a few microns inside the junction for a few seconds), continue with the treatment. Otherwise, discard the device
57	The metal connector remains in the device while the tube is removed	The tube was not removed properly	Try to remove the metal connector by hand or re-insert the tube into the connector and then remove the tube properly, with the metal connector
	The device cracks while the tube is removed	The device was not supported properly	If the crack is very small or not across the channel, continue with treatment. If the crack is prominent or across the channel, discard the device
	PVA solution is not removed after applying vacuum and positive air pressure	Incubation time was too long and/or the concentration of the PVA solution was too high, leading to a clogged channel	Try increasing the air pressure even further (up to 2.5 bar). If not successful, discard the device
	The air tubes pop out of the device	The air pressure was too high	Decrease the air pressure and re-insert the air tubes
		The metal connector is not inserted deep enough into the device	Decrease the air pressure and re-insert the air tubes

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
62	Some particulate matter is blocking a channel(s)	The PDMS block and the glass slide were not cleaned properly before bonding	Try to perform the experiment to see whether the blockage is causing a major issue. If the blockage is too big, discard the device
	One or more channels are collapsed	Too much pressure was applied while retracting the metal connectors during the surface treatment	For a small collapse, try to perform the experiment. If the collapse is too big, discard the device
	OA inlet/separation hole/exit is clogged	PVA was not successfully removed during surface treatment	Try to perform the experiment (see troubleshooting advice for Steps 68 and 71)
68	The OA/LO/IA is not entering the channel, even after applying high pressure	The metal connector is not inserted deep enough into the device	Quickly remove and re-insert the tube into the device
		The hole is leaking	Discard the device
		The fitting is not screwed tight enough	Tighten the fitting
		The OA channel entrance or the device exit is clogged with PVA	Temporarily increase the pressure in order to force the solution to enter the channel and dissolve the clog
			Try to break open the clog with the help of a needle, by inserting it into the hole
		The metal connector is clogged because of insufficient cleaning	Clean the metal connector using ethanol, and if the problem persists, replace the connector
69	LO does not form a film across the junction but forms 1-octanol droplets instead LO is clogged	PVA treatment was not done properly	Discard the device
		Flow velocity of LO is too low, or the IA/OA initially went into the LO channel	Increase the pressure on the LO channel (with concomitant pressure increase on IA and LO channels) to flush away the clog
	There is a clog right at the junction	Unwanted material has accumulated, blocking the junction	Temporarily increase the pressure on the IA/LO/OA channels to eliminate the clog
	IA merges with the OA by forming a channel through the LO film	Some particulate matter or the junction corner is facilitating the merging of the two aqueous phases	Draw back the IA by quickly reducing the pressure and bring it back to the original value
	The LO forms a film across the junction but then immediately breaks	The pinch-off is not efficient enough	Increase the pressure on the OA channel
	The double-emulsion droplets break immediately after their formation	The production is not yet stabilized	Wait (1–2 min) to see whether there is an improvement
		Post-junction channel walls are contaminated with 1-octanol residues	Flush the surface by gradually increasing the pressure on the OA channel
		The pinch-off is not efficient enough	Increase the pressure on the OA channel
70	The pockets do not separate to give rise to liposomes	The size of the pocket is too large	Decrease the pressure on the LO channel and/or increase the pressure on the IA channel
		The flow velocity is too low	Increase the pressure on the OA channel

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
71	There is no flow in the post-hole channel, even after applying enough suction	The exit is clogged with PVA	Re-insert the suction tube or use a syringe to try to fill the exit hole with OA, in order to dissolve the clog
			Try to break open the clog with the help of a needle, by inserting it into the hole
	There is high flow in the post-hole channel in spite of having low/no suction pressure	The separation hole is partially/fully blocked	Try to eliminate the clog by carefully inserting a syringe and drawing out or injecting some solution
	1-Octanol droplets also enter the post-hole channel	The suction is too high	Reduce the suction
	Double-emulsion droplets enter the post-hole channel	Liposome formation process is not efficient	Fix liposome production before starting the purification process
	Aggregates of liposome/1-octanol droplets enter the post-hole channel	The flow velocity in the post-junction channel is too low	Fix liposome production before starting the purification process

● TIMING

Steps 1–12, preparation of a master wafer: ~3 h
Steps 13–18, wafer surface treatment: ~12 h
Steps 19–43, preparation of the microfluidic device: ~6 h
Step 44–60, device surface treatment: ~20 min per device
Steps 61–71, OLA and liposome purification: ≥1 h (can vary widely according to the experiment)

ANTICIPATED RESULTS

We have provided a detailed protocol for optimal production and purification of micron-sized, unilamellar liposomes using a microfluidic method, OLA (see **Fig. 1** for the schematic). We have highlighted the key steps to the success of OLA: (i) hydrophilic surface treatment of the microfluidic device; (ii) start of the liposome production, particularly the sequence in which the different phases arrive at the production junction; (iii) filling of the separation hole sufficiently before starting the purification process.

Surface treatment of the microfluidic device

The key steps resulting in successful surface treatment are shown in **Figure 2** (see also **Supplementary Video 1**). By keeping a stable interface between the air and the PVA solution, the device will be rendered partially hydrophilic, which is a highly essential step in device preparation before performing OLA.

Figure 3a and **Supplementary Video 1** show an optimal double-emulsion droplet production. A typical production rate can be varied between 1 and 100 Hz. A less-than-optimal production can be seen in **Supplementary Video 7**, in which the formation of the lipid film across the junction is asymmetric because of the presence of a small IA bubble in the LO. This results in the formation of a mixture of double-emulsion droplets with two different pocket sizes, and occasional 1-octanol droplets. There is also some loss of the IA into the OA. Despite having problems at the junction, one can still obtain adequate liposome production, which demonstrates the robust nature of the method. To further highlight the robust nature of the method, one can obtain liposome production in spurts, even when one of the LO channels becomes completely blocked.

Separation of the liposome and 1-octanol pocket

Double-emulsion droplets with prominent pockets will form immediately after the production junction and will flow downstream of the channel (**Fig. 3b** and **Supplementary Video 3**). Within a few minutes, the pockets will start resembling droplets, having a very small contact area with the liposome. These droplets will eventually separate from the liposomes (**Fig. 3c** and **Supplementary Video 4**). The distance after the junction where the separation occurs depends on the flow velocity. The separation also depends on the size of the pocket. In general, we have observed that smaller pockets separate

more efficiently than larger ones. Sufficient flow velocity must be maintained in order to avoid the formation of possible aggregates of liposomes and/or droplets before they reach the separation hole and formation of tube-like structures probably formed from remnants of burst liposomes. An example of such a suboptimal scenario in the post-junction channel is given in **Supplementary Video 8**, in which aggregation of particles and extensive tubular structures can be clearly seen. Increasing the flow velocity in the channel can bring the production back to normal.

On-chip purification of liposomes

Once in the separation hole, both 1-octanol droplets and liposomes will move upward because of the low resistance of the hole as compared with that of the post-hole channel. However, when enough suction is applied to the exit hole, predominantly liposomes will enter the post-hole channel (**Fig. 4a** and **Supplementary Video 5**). This will substantially increase the sample purity, resulting in up to 95% purified samples[10] (**Fig. 4b** and **Supplementary Video 6**). However, if there is aggregation of the 1-octanol droplets or if the suction is too strong, such density-based purification will not work optimally, as can be seen in **Supplementary Video 9**. After the purification, liposomes will be stable over multiple hours, and can be further manipulated depending on the needs of a particular experiment. For example, microfluidic traps can be designed to immobilize the liposomes, or side channels can be added in order to add/remove particular chemicals[65,66].

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING INTERESTS
The authors declare no competing interests.

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