

Vesicles-on-a-chip: A universal microfluidic platform for the assembly of liposomes and polymersomes

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Abstract. In this study, we present a PDMS-based microfluidic platform for the fabrication of both liposomes and polymersomes. Based on a double-emulsion template formed in flow-focusing configuration, monodisperse liposomes and polymersomes are produced in a controlled manner after solvent extraction. Both types of vesicles can be formed from the exact same combination of fluids and are stable for at least three months under ambient storage conditions. By tuning the flow rates of the different fluid phases in the flow-focusing microfluidic design, the size of the liposomes and polymersomes can be varied over at least one order of magnitude. This method offers a versatile tool for future studies, *e.g.*, involving the encapsulation of biological agents and the functionalization of artificial cell membranes, and might also be applicable for the controlled fabrication of hybrid vesicles.

1 Introduction

Synthetic biology represents an emerging field of research that aims at mimicking and understanding living systems and their building blocks from a fundamental bottom-up perspective [1–11]. This approach involves the study of the physical properties and functions of biological systems from the simplest module of a living cell to more complex systems. Its success relies on the controlled realisation of tailored compartments, which are crucial for accomplishing the transition from single synthetic components to assemblies and eventually a minimal artificial cell [12]. In light of this ultimate goal, the compartments have to be capable of featuring biochemical processes that are essential for a living cell, such as, *e.g.*, the metabolism. Typically, such processes are linked to membrane-bound components, hence the cell membrane is of paramount importance for the cell's functionality and cellular life in general. In numerous studies, vesicles have been identified as ideal candidates for compartments that are capable of mimicking cellular functionalities [6, 13–16]. The fabrication of vesicles with tailored properties, however, involves significant challenges in terms of monodispersity, stability and tunability of the membrane properties [17].

Over the past decades, microfluidic devices have been developed and widely used for the controlled, high-throughput production of droplets and multiple emulsions [18–23]. Besides conventional techniques of vesicle

fabrication based on hydration and electroformation [24], microfluidics offers new routes for addressing the aforementioned challenges of the controlled realisation of vesicles. In particular, in light of their application as compartments in synthetic biology and related fields, the high encapsulation efficiency that goes along with the microfluidic approach can be considered as a major advantage. The controlled fabrication of lipid vesicles (liposomes) in a microfluidic environment using PDMS-based microfluidic designs has been demonstrated by Teh and coworkers [25], as well as very recently by Deshpande *et al.* [26]. They produced monodisperse water-in-oil-in-water (W/O/W) double-emulsions, where the lipid molecules are dissolved in the oil phase of the template. The liposomes are then formed as a result of a solvent-extraction process.

Nowadays, the reliable fabrication of polymersomes, *i.e.*, vesicles made from the self-assembly of amphiphilic block-copolymers, has received an increasing interest in basic and applied research, *e.g.*, for the encapsulation and release of drugs or other products such as proteins, enzymes and DNA. The variability of the polymer chain length and the chemical composition of the polymeric blocks allow for precisely tuning the membrane properties, such as the membrane's thickness, molecular composition and mobility [27–29]. The mechanical properties of polymersomes and their stability are considerably improved compared to vesicles made of phospholipids. As for liposomes, common techniques employed to fabricate polymersomes comprise the indirect self-assembly of block-copolymers via film hydration, electroformation and

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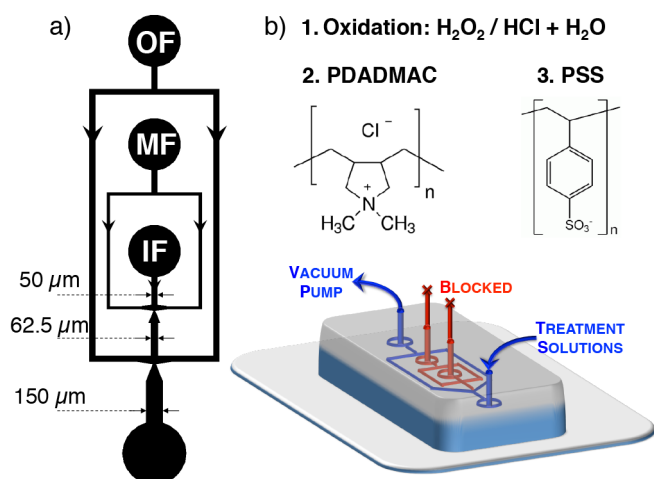


Fig. 1. a) Microfluidic design for the production of double-emulsion templates. IF, MF and OF correspond to the inner fluid, the middle fluid and the outer fluid phases, respectively. b) Illustration of the procedure for the hydrophilisation of the external (blue) channels.

pulsed jetting [17], which often yield rather polydisperse vesicles and a low encapsulation efficiency.

As a consequence of the need for using strong organic solvents (*e.g.*, toluene) in order to dissolve the block-copolymers, polymersome fabrication in microfluidic environments has so far been limited to glass-capillary microfluidic devices [30,31]. The efficiency, versatility and reliability associated with PDMS-based microfluidics, however, have fostered the efforts for realising the production of polymersomes from this well-established technology. The most recent efforts aiming at circumventing the problems inherent either in the organic solvents and their PDMS incompatibility or the fabrication of glass-capillary microfluidic devices were brought by Thiele and coworkers [32]. They employed a type of PDMS-based microfluidic device similar to the one reported by Teh *et al.* [25] and coated the channels with a glass-like layer in order to mimic the chemical properties of a glass surface and avoid swelling of the PDMS by the solvent.

In this paper, we report on the fabrication of both liposomes and polymersomes from double-emulsion templates on the exact same PDMS-based microfluidic chip. We demonstrate that a suitable combination of fluid system, channel treatment and microfluidic design provides a convenient and reliable strategy for producing both types of vesicles on the same platform without using strong organic solvents.

2 Materials and methods

2.1 Microfabrication of the PDMS-chip

The microfluidic design consists of two consecutive cross-junctions allowing for hydrodynamic flow focusing, as depicted in fig. 1a). The device was fabricated by conventional soft-lithography methods [33,34]. The photoresist

SU-8 3025 (MicroChem) was spin-cast at 2000 rpm for 30 s onto a silicon wafer (4 inches diameter, Si-Mat) in order to obtain a final layer thickness of $\sim 50 \mu\text{m}$. After a first baking, the photoresist layer was UV-exposed through a high-definition mask featuring the desired design. Subsequently, the silicon wafer was immersed in the appropriate photoresist developer in order to obtain the final positive replica of the design, the unexposed photoresist being dissolved by the developer.

After carefully cleaning the silicon wafer with isopropanol and drying with a clean nitrogen jet, a poly(dimethyl siloxane) (PDMS, Sylgard 184, Dow Corning) mixture of 10:1 ratio between base and cross-linker was poured onto the silicon wafer, degassed in a vacuum desiccator and cured at 75°C in an oven for at least 4 hours. Finally, the cured PDMS replica was peeled off, and the holes corresponding to the inlets and the outlet were punched. The PDMS replica, as well as a microscope glass slide serving as a support, were cleaned with isopropanol, ethanol and dried with a nitrogen jet in order to prepare for functionalization under air plasma treatment and irreversible bonding. The final channel widths of the PDMS chip are reported in fig. 1a). The outlet channel length was $1532 \mu\text{m}$ and the first and second cross junctions widths were $25 \mu\text{m}$ and $64 \mu\text{m}$, respectively.

2.2 Channel treatment

After the PDMS-device was fabricated, a channel treatment was performed before starting an experiment. For the successful production of W/O/W double-emulsion templates based on hydrodynamic flow focusing, the inner walls of the external channel —where the continuous outer aqueous phase is flowing— must be hydrophilic. Without such a treatment, the wetting of the continuous outer aqueous phase on the channel walls would be weak due to the intrinsic hydrophobic character of the PDMS. As a result, the shear stresses at the second junction of the PDMS-device would not be sufficiently large to initiate the double-emulsion production. For the channel hydrophilisation, we developed a specific procedure as illustrated schematically in fig. 1b): first, the channel walls were oxidised by a 1:1 mixture of hydrogen peroxide solution (H_2O_2 at 30 wt.%, Sigma-Aldrich) and hydrochloric acid (HCl at 37 wt.%, Sigma-Aldrich). This mixture was flushed from the outlet of the design to the inlet for about 2 min by using a vacuum pump system. To avoid the treatment of the other channels, the two inlets of the inner channels were blocked. After the oxidation, the channel was rinsed by flushing ultra-pure water. Subsequently, a 5 wt.% solution of the positive polyelectrolyte poly(diallyldimethylammonium chloride) (PDADMAC, Sigma-Aldrich) was flushed for about 2 min through the oxidised channel of the device (see fig. 1b), blue channels). The PDADMAC binds to the activated channel walls by ionic interactions. Eventually, a 2 wt.% solution of the negative polyelectrolyte poly(sodium 4-styrenesulfonate) (PSS, Sigma-Aldrich) was flushed for about 2 min in order to finalise the channel treatment.

2.3 Fluid phases for the production of double-emulsion templates

The double-emulsion templates were prepared with an inner aqueous fluid (IF), consisting of a mixture of the non-ionic surfactant Synperonic F108 (Sigma-Aldrich) at a concentration of 1 wt.% in ultra-pure water. The middle fluid (MF) either consisted of a phospholipid or block-copolymer solution. The phospholipid L- α -Phosphatidylcholine from egg yolk ($\sim 60\%$ TLC, Sigma-Aldrich) was dissolved at a concentration of 1 wt.% in oleic acid (derivative from olive oil, Fisher Chemical). The block-copolymer solution consisted of poly(butadiene-1,2-b-ethylene oxide) (PBD₆₅-PEO₃₅, $M_w = 4$ kDa, Polymer Standards Service, Mainz), also dissolved at a concentration of 1 wt.% in oleic acid. The outer aqueous fluid (OF) was composed of a mixture of ultra-pure water, Synperonic F108 (1 wt.%), glycerol (15 wt.%, Sigma-Aldrich), PDADMAC solution (2 wt.%, Sigma-Aldrich) and ethanol (Rotipuran, $\geq 99.8\%$, Roth) at a concentration of 28 wt.% (liposomes) or 14 wt.% (polymersomes). The role of the glycerol was to increase the viscosity of the outer solution, thus increasing the shear stresses at the second cross-junction of the microfluidic device and improving the pinch-off and double-emulsion production. Synperonic F108 was employed as a surfactant in the IF and OF in order to prevent coalescence of the double-emulsions and increase the stability of the vesicle. PDADMAC was employed to sustain the channel treatment and also to increase the viscosity of the outer solution. Finally, a sucrose solution at a concentration of 0.4 M and 0.2 M was added to the inner and the outer aqueous phase, respectively, in order to avoid bursting of the vesicles due to an osmolarity unbalance.

2.4 Experimental procedure

All experiments were observed with an inverted optical microscope (Olympus IX-81). The three fluid phases (IF, MF and OF) were dispensed in a controlled way by means of syringe pumps (neMESYS low pressure syringe pumps). Movies of the microfluidic experiments were recorded using a high speed camera (Phantom V311, Vision Research, 3200 fps at a spatial resolution of 1280×800 px²). In order to facilitate the identification of the vesicles (liposomes and polymersomes) after production, the lipophilic fluorescent dye Nile Red (Sigma-Aldrich) was added to the MF. After collecting the double-emulsions in small glass vials, the samples were observed under fluorescence microscopy (Olympus IX-73). The samples were illuminated by a high-pressure mercury lamp (Olympus U-HGLGPS) associated with a filter cube releasing a fluorescence excitation at a wavelength of 593 nm. Fluorescence microscopy images were recorded using a CCD colour camera (Canon EOS 600D, 5184×3456 px²). Finally, the images were processed and analysed using the open source software ImageJ (1.46r, National Institute of Health, USA).

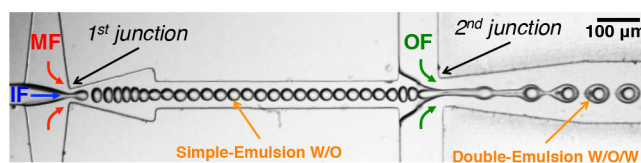


Fig. 2. Production of W/O/W double-emulsions using a PDMS-based microfluidic device, featuring two consecutive cross-junctions in flow-focusing configuration. The inner aqueous phase (IF) is sheared at the first junction by an oil phase (MF), producing a single-emulsion. This single-emulsion is in turn sheared at the second junction by an aqueous outer phase (OF) creating double-emulsion templates.

3 Results

3.1 Double-emulsion production

Liposomes and polymersomes were produced via a solvent-extraction process from W/O/W double-emulsions [25, 35]. We realised the double-emulsion templates by using the PDMS-based microfluidic device presented fig. 1a), inspired by the recent work of Teh and collaborators [25]. The basic principle of double-emulsion production is shown fig. 2. The IF was sheared at the first junction by the MF, corresponding to either the phospholipid or block-copolymer solution, and giving birth to a W/O emulsion. At the second cross-junction of the device, this simple-emulsion was in turn sheared by the OF, containing ethanol for extracting the solvent in the MF. As a result, a well-defined monodisperse W/O/W double-emulsion is produced. The hydrodynamic flow-focusing geometry of the device, together with the channel dimensions chosen here, ensure a reliable production of double-emulsion templates. By tuning the flow rates of the different fluid phases, the geometric parameters of the double-emulsions, *i.e.*, their inner diameter as well as their shell thickness, can be varied.

3.2 Oil extraction and vesicle formation

In order to obtain vesicles, the oil is removed from the MF of the double-emulsion using a solvent-extraction method [25, 35]. Here, the ethanol in the OF plays the role of a solvent-extractor for oleic acid. The formation and the stability of the vesicles is strongly dependent on the concentration of ethanol in the OF: for low concentrations (typically less than 10 wt.%) the solvent-extraction process was not sufficiently dominant and, thus, no vesicles could be obtained. For high concentrations of ethanol (more than 40 wt.%), the stability of the double-emulsion was not maintained and the inner aqueous core coalesced with the OF, leading to a simple O/W emulsion. Due to the amphiphilic character of both the phospholipids and the block-copolymers, their self-assembly upon oleic acid extraction results in the formation of vesicles in a narrow window of appropriate ethanol concentrations.

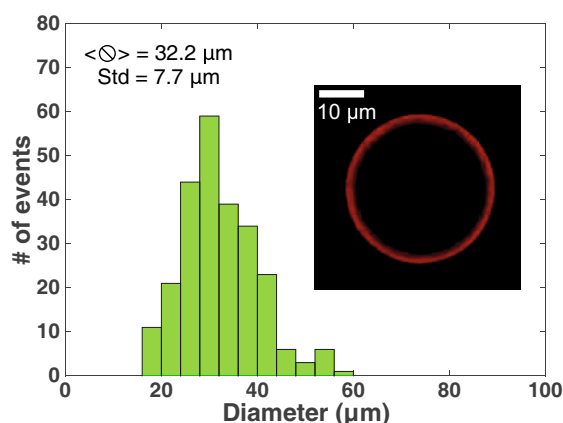


Fig. 3. Diameter distribution of liposomes: a mean diameter of $32.2\ \mu\text{m}$ with a standard deviation of $7.7\ \mu\text{m}$ is obtained from the histogram (the total number of liposomes is 247). The flow rates of the different fluid phases were: IF = $50\ \mu\text{L/h}$, MF = $100\ \mu\text{L/h}$ and OF = $650\ \mu\text{L/h}$. Inset: Fluorescence microscopy image of a liposome after solvent-extraction from the middle phase. Nile Red, a fluorescent lipophilic dye, was added to the MF for visualisation purposes.

3.2.1 Liposomes

As a first proof-of-principle of the PDMS-based microfluidic design for vesicle fabrication, we consider the formation of liposomes. The liposome formation using a microfluidic approach has been recently reported in the literature by Teh *et al.* [25]. In the present study, we demonstrate that a modified microfluidic platform for a different phospholipid and fluid system can lead to the assembly of stable liposomes that are uniform in size.

The IF, the MF (containing the dissolved phospholipids as described in sect. 2.3) and the OF were connected to the corresponding inlets of the microfluidic chip. As shown in fig. 2, stable and uniform double-emulsions were produced which contain the phospholipid molecules in their MF. Upon the extraction of the oleic acid by the ethanol (28 wt.% in the OF), the shells of the double-emulsions shrank and the self-assembling phospholipid molecules formed stable liposomes. An example is shown in the inset of fig. 3, which was taken one day after the production of the double-emulsion template. In the literature [25], it is reported that the solvent-extraction process is completed within 15 hours, in accordance with what was observed here. Furthermore, the histogram shown in fig. 3 confirms that the size distribution of the liposomes is rather monodisperse with a mean liposome diameter of $32.2\ \mu\text{m}$ (standard deviation: $7.7\ \mu\text{m}$) in this particular experiment.

3.2.2 Polymersomes

After having established the production of liposomes using the presented PDMS-based microfluidic device, our goal was to realise the fabrication of polymersomes on *the same* microfluidic platform. We demonstrate that with the

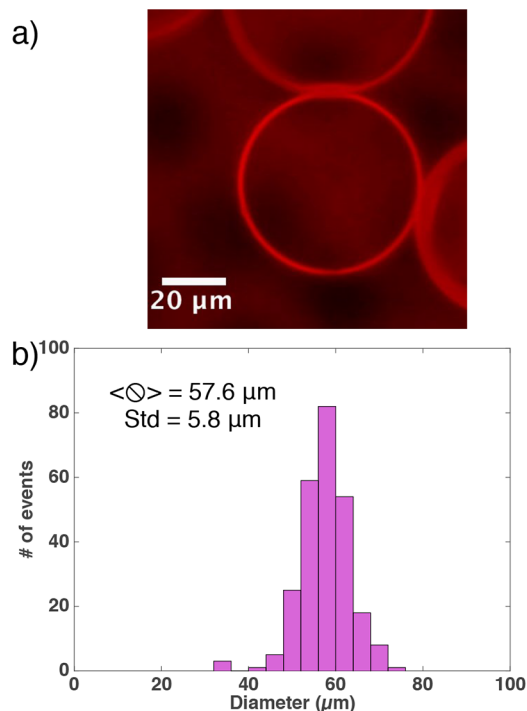


Fig. 4. a) Fluorescence microscopy image of polymersomes after solvent-extraction from the MF, taken 3 days after the double-emulsion production. b) Diameter distribution of the polymersomes: a mean diameter of $57.6\ \mu\text{m}$ with a standard deviation of $5.8\ \mu\text{m}$ is obtained from the histogram (the total number of polymersomes is 256). The flow rates of the different fluid phases were: IF = $50\ \mu\text{L/h}$, MF = $100\ \mu\text{L/h}$ and OF = $600\ \mu\text{L/h}$.

exact same system of fluids as presented before for the fabrication of liposomes, but by replacing the phospholipids in the MF by the block-copolymer PBD₆₅-PEO₃₅, the fabrication of polymersomes can be accomplished. The block-copolymer was dissolved in oleic acid (1 wt.%), while the concentration of ethanol in the OF was reduced to 14 wt.%. The composition of the IF was kept as originally used for the liposome fabrication.

After producing a stable and uniform W/O/W double-emulsion, see fig. 2, the ethanol in the OF extracts the oleic acid from the MF. The formation of polymersomes was observed a few hours after production, as illustrated by the fluorescence microscopy micrograph shown in fig. 4a). Figure 4 b) displays the diameter distribution of the polymersomes as measured 3 days after the production of the double-emulsions and demonstrates the monodispersity of the polymersomes (mean diameter: $57.6\ \mu\text{m}$, standard deviation: $5.8\ \mu\text{m}$).

3.3 Stability & size tunability

After production, all samples were collected in simple glass vials and stored under ambient lab conditions. The stability of the vesicles (liposomes as well as polymersomes) was verified for multiple samples over several months: we con-

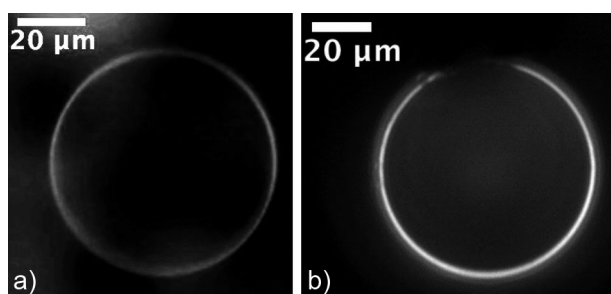


Fig. 5. Stability of the polymersomes: fluorescence microscopy images of polymersomes from the same batch taken a) 3 days and b) 3 months after production.

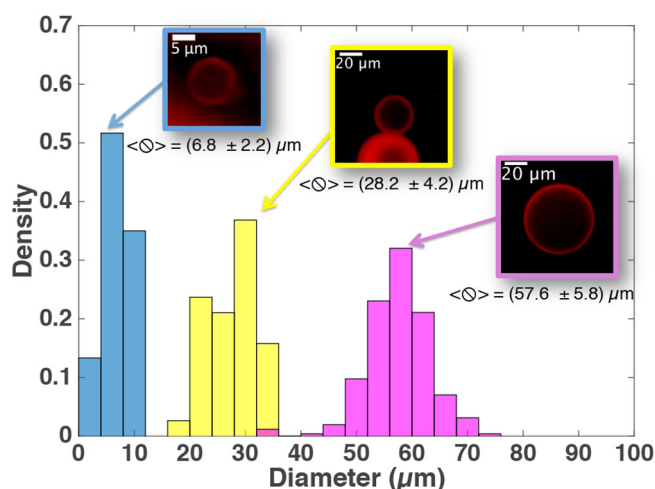


Fig. 6. Polymersome diameter distributions for different flow rates of the fluid phases. The corresponding flow rates are provided in table 1. The different histograms correspond to a total number of 403 (blue), 38 (yellow) and 256 polymersomes (magenta), respectively.

Table 1. Flow rates of the fluid phases IF, MF and OF as used for the experiments presented fig. 6.

Fluid phase/Flow rate ($\mu\text{L}/\text{h}$)	Blue	Yellow	Magenta
IF	10	20	50
MF	75	110	100
OF	400	700	600

firm that the vesicles fabricated by the present method are stable for at least 3 months after production (see fig. 5).

As previously mentioned, this microfluidic approach also allows for controlling the size of the double-emulsion templates by variation of the flow rates of the different fluid phases. In fact, the size of the vesicles can be tuned over one order of magnitude, as illustrated for three different diameter distributions of polymersomes in fig. 6. The polymersome diameter increases with increasing the ratio of the flow rate of the IF to the flow rate of the continuous aqueous phase OF. The flow rates of the experiments corresponding to the histograms in fig. 6 are reported in table 1.

4 Discussion

As discussed in the introduction, PDMS-based microfluidics was recently identified as a novel and versatile approach to fabricate liposomes [25, 26] and polymersomes [32]. However, the fact that block-copolymers typically require to be dissolved in strong organic solvents (*e.g.*, chloroform, toluene, tetrahydrofuran, cyclohexane) represents a significant limitation of this route for the fabrication of polymersomes: the low chemical resistance and swelling of the PDMS lead to an incompatibility between these organic solvents and the PDMS-based microfluidic chip. In order to circumvent this issue, glass-capillary microfluidic devices have been put forward as an alternative technology for the production of polymersomes [30, 31, 36–40].

To the best of our knowledge, so far the only successful realisation of the fabrication of polymersomes in a PDMS template has been reported by Thiele and coworkers [32]. By means of employing a glass-like coating as well as performing selective wetting treatments of the inner walls of the PDMS channels (hydrophilic for the channels with aqueous phases and hydrophobic for the channels containing organic phases), they were able to mimic some of the essential chemical surface properties of a glass-capillary microfluidic device. Nevertheless, significant modifications of the conventional design, based on two cross-junctions, were required as a result of several persistent issues and difficulties, *e.g.*, the control of the evaporation rate of the organic solvent, precipitation of the block-copolymer and, finally, clogging of the microfluidic device. Most importantly, high flow rates of the organic phase containing the block-copolymer had to be employed, which, in turn, limits the size control of the polymersomes.

The strength of the present technique for the fabrication of both liposomes and polymersomes lies in the common basis of PDMS-compatible fluid systems. The microfluidic device does not require any sophisticated design principles and treatments, other than a hydrophilisation of the external channel. We were able to produce liposomes as well as polymersomes of tunable size which are stable over several months. Thus, we might anticipate that this method has great potential for applications in synthetic biology and related fields as well as the realisation of an on-chip fabrication of hybrid vesicles with tailored properties and functionalities [41].

5 Conclusions

In conclusion, we present a versatile PDMS-based microfluidic platform together with a common fluid system for the controlled fabrication of *both* liposomes and polymersomes via a solvent-extraction process. We note that the polymersomes produced with this technique are monodisperse in size and stable over at least three months under ambient conditions. Such features are of extraordinary relevance for fundamental research on, *e.g.*, membrane mechanics and fusion. Furthermore, we show that the size of the vesicles can be tuned on a wide range by

varying the flow rates of the different fluid phases during the double-emulsion fabrication. From a synthetic biologists point of view, the present technique might offer a great potential for realising, *e.g.*, the efficient encapsulation of biological species as well as designing functionalized membranes. As a result of the universality of the concept, this novel approach might also open a promising route towards the fabrication of hybrid vesicles.

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