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RESEARCH ARTICLE

Stability characterization of microfluidics lipid-stabilized double emulsions under physiologically-relevant conditions

[version 1; peer review: 2 approved with reservations]

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Abstract

Background: Double emulsions (DEs) are water-in-oil-in-water (or oil-in-water-in-oil) droplets with the potential to deliver combinatory therapies due to their ability to co-localize hydrophilic and hydrophobic molecules in the same carrier. However, DEs are thermodynamically unstable and only kinetically trapped. Extending this transitory state and rendering DEs more stable, would widen the possibilities of real-world applications, yet characterization of their stability in physiologically-relevant conditions is lacking.
Methods: In this work, we used microfluidics to produce lipid-stabilized DEs with reproducible monodispersity and high encapsulation efficiency. We investigated DE stability under a range of physicochemical parameters such as temperature, pH and mechanical stimulus.
Results: Stability through time was inversely proportional to temperature. DEs were significantly stable up to eight days at 4°C, five days at room temperature and two days at 37°C. When encapsulating a cargo, DE stability decreased significantly. When exposed to a pH change, unloaded DEs were only significantly unstable at the extremes (pH 1 and 13), largely outside physiological ranges. When exposed to flow, unloaded DEs behaved similarly regardless of the mechanical stimulus applied, with approximately 70% remaining after 100 flow cycles of 10s.
Conclusions: These results indicate that lipid-stabilized DEs produced via microfluidics could be tailored to endure physiologically-relevant conditions and act as carriers for drug delivery. Special attention should be given to the composition of the solutions, *e.g.* osmolarity ratio between inner and outer solutions, and the interaction of the molecules, *e.g.* carrier and cargo, involved in the final formulation.

Keywords

double emulsion, microfluidics, drug delivery, stability, temperature, pH

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Introduction

Double emulsions (DEs) are water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O) droplets frequently used in the food industry to fabricate low-fat products and improve nutrient and flavour delivery[1]. However, historically speaking, most interest derived from DEs has come from the pharmaceutical industry[1,2]. They have been studied as drug delivery systems[3–5], blood substitutes[6,7], and vaccines[8,9], with one of the earliest reported applications in the late 1960s, which aimed to enhance the absorption of insulin in the intestine[10]. DEs are usually produced in a two-step process: two immiscible solutions are mixed, forming an emulsion that is, in turn, vigorously stirred in a third solution of similar properties as the inner phase. The rotation speed of each step allows for some control over the size and number of inner droplets within the outer droplet. However, resulting populations tend to be polydisperse and vary widely in encapsulation efficiency, ranging from 10 to 98%[2,11–18]. Polydispersity has been shown to affect the release profile of drugs encapsulated in microparticles[19], while reproducible encapsulation efficiency, specifically the homogeneous concentration of drug inside each DE, is crucial for well-controlled release kinetics and therapeutic benefit[20]. Thus, these current constraints hinder more widespread commercial use of DEs.

Microfluidics is well poised to address these limitations. The microfluidic production of single, double, and even multiple emulsions has been reported[21,22], demonstrating highly monodisperse populations and encapsulation efficiencies of nearly 100%[23]. Still, complex setups, *e.g.* cleanroom microfabrication and microfluidic glass capillaries[21,24,25], have created a barrier to wider use in multiple applications. As the technology matured over the last decade, an increasing number of reports have shown the successful production of DEs in microfluidic polydimethylsiloxane (PDMS) chips, which are cheap and simple enough to fabricate and assemble[26–29].

One key characteristic of DEs, driving the recent interest in the field, is their ability to co-localize and co-transport, in a single carrier, molecules of opposing properties[30–33], such as hydrophobic and hydrophilic drugs, for example, the synergistic anticancer drugs paclitaxel and doxorubicin[34]. As a consequence, they are promising delivery systems for combinatory therapies. However, DEs remain characteristically metastable structures, *i.e.* thermodynamically unstable[29,35,36]. DEs are kinetically trapped in a transitory local energy minimum state that can move towards the global minimum at the slightest disturbance. Thus, they are prone to bursting, due to the coalescence of the inner phase with the outer phase, forming an O/W droplet[35,37,38]. In order to take advantage of their drug co-localization properties, increasing the time that they remain in this transitory state is a critical issue.

Most DEs are stabilized with surfactants, usually block copolymers[26,37,39–42], that arrange themselves at the interfaces, and other additives that increase the viscosity of the aqueous solutions[40,43]. Recent works in the field of artificial cell-like systems, which studies the origins of life and the molecular dynamics of the modern cell membrane[44], have focused on double emulsions with more biomimetic compositions, replacing synthetic surfactants and additives with molecules that could be found in the regular cellular constitution, such as lipids[25,28,29,45–47]. The exploration of these lipid-stabilized double emulsions as drug delivery systems is very recent, but promising[25]. Besides their more biologically-relevant composition, the presence of lipids confers several advantages. They allow the formation of multisomes, a network of smaller water droplets inside an oil droplet, that can be used as a multi-compartmentalized delivery system[25], and potentially enhance intestinal absorption[48]. Furthermore, it has been shown that lipids play an important role in the absorption of hydrophobic drugs[49,50], enhancing their bioavailability[51]. In 2002, the American Federal Drug Agency (FDA) issued the "Food-effect Bioavailability and Fed Bioequivalence" guidance, in which it recommended high-fat meals when taking hydrophobic drugs to improve drug absorption due to the effect of fats on the gastrointestinal tract physiology and maximization of drug transfer to the systemic circulation[48]. At least five pharmaceutical products that take advantage of these characteristics have been approved for commercial use (Intralipid, 1975; Cleviprex, 2008; Perikabiven, 2014; Smoflipid, 2016; Cinvanti, 2018)[52]. They consist of single emulsions stabilized by lipids, encapsulating only one drug (*i.e.* not combinatory therapies), and are indicated for nutritional purposes, the treatment of acute and delayed nausea and vomiting, and reduction of blood pressure. To date, no DE formulations have reached clinical stages.

A reason for the lack of commercial exploitation resides in the fact that DEs are one of the most challenging types of droplets to generate[46]. Almost every variable from formulation composition to production parameters can affect their stability. Also, a robust characterization of their stability, especially under physiologically relevant parameters, is lacking. In this work, we show that lipid-stabilized DEs can be made by microfluidics in PDMS chips fabricated under basic laboratory conditions (no cleanroom), for the generation of reproducible monodisperse populations. We characterized their stability when exposed to various stresses representing physiological conditions relevant to DE-based therapeutics, such as temperature, pH and mechanical stress. Finally, we encapsulated representative cargo in the intermediate and inner phases to assess how these additions affect the stability. The gained insights contribute to the growing body of knowledge that can advance the use of DEs in commercial applications.

Methods

Materials

Materials used were as follows: Polyvinyl alcohol (PVA), 87–90% hydrolysed, average molecular weight 31,000–50,000 (ref. 363073, Sigma Aldrich, France); 1-octanol, anhydrous, ≥99% (ref.297887, Sigma Aldrich, France.); 1,2-dioleoyl-sn-glycero-3-phosphocholine, chloroform (DOPC) (ref. 850375C, Sigma Aldrich, France); poloxamer 188 (P188) 10% (wt/vol) solution (ref. P5556, Sigma Aldrich, France); glycerol (Carlo Erba, Dutscher, ref. 453751-CER); ethanol absolute (ref. 4146012-CER, Carlo Erba, Dutscher, France); chloroform (ref. 1024451000,

Emsure, Germany); 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (ref. 42364, Sigma Aldrich, France); calcein (ref. C0004, TCI, Japan); NaOH (1M, 480717000, Carlo Erba, Dutscher, France); HCl (37%, ref. 258148, Sigma Aldrich, France); N2 (ref. GAL110350, Core Equipement, Prosynergie, France)

Microfabrication of PDMS chips

PDMS chips were produced in-house following standard soft-lithography and microfabrication protocols[53–55] without the need of a cleanroom. Briefly, SU-8 molds were spin-coated (SU-2050, Kayaku Advanced Materials, USA; 10 s at 500 rpm; 30 s at 3000 rpm) and exposed to UV-light through masks containing a previously published design[26,56], which consisted of a double-junction chip (second-junction nozzle, 50 µm; post-junction channel width: 150 µm; chip height: 50 µm). Chips were plasma bound (surface activation: 2 min) to PDMS-covered glass slides (spin-coating: 10 s at 500 rpm; 40 s at 1000 rpm).

Microfluidic production of double emulsions

PDMS chips received a surface treatment with PVA solution (2.5% (w/v)) as previously published[57]. Inner aqueous phase (IA), lipid-oil intermediate phase (LO) and outer aqueous phase (OA) solutions were prepared as described in Deshpande *et al*., 2018, with the following modifications: DOPC (6.5 mM) in the intermediate solution[29] and P188 (0.5%) in the outer solution were used (details in ESI).

The solutions were driven into the chip with a pressure-driven flow controller (OB1, Elveflow, France). The pressure ranges for each solution were: IA = 25 to 35 mbar; LO = 35 to 45 mbar; OA = 70 mbar. The pressure of the OA was kept constant throughout experiments for comparable production rates.

Image analysis

DEs were collected into a µ-Slide I Luer channel slide (height=0.4mm, Ibidi, reference 80176) attached to the exit of the production chip via a short piece of tubing. For size distribution, encapsulation, and temperature assay analyses, images were taken with an inverted AxioVert A1 microscope (Carl Zeiss, Germany) and camera (Axiocam 202 mono). For pH and flow assay analyses, an upright microscope (built in-house from 5× objective), and high-speed camera (Pixelink, reference PL-D725CU) were used. Image analysis was performed using the image software Fiji and a Python script developed in-house for automated droplet counting and sizing. The parameters of the script were adjusted to better fit the change in size over time and were verified manually through representative images. Data are reported as the mean percentage of counted DEs ± SD or SEM as specified. Statistical analysis was performed using a Student's t-test, two-sample assuming unequal variances.

The production rate was calculated using 200-frame videos of DEs exiting the chip junction captured with a high-speed camera (Pixelink, reference PL-D725CU). The total number of produced DEs in a given video was counted manually with ImageJ software and adjusted to give the production in Hertz (double emulsions per second).

Encapsulation of compounds of interest

For encapsulation assays, the inner solution was replaced with a solution containing compounds of interest: calcein (0.3 mM) in water or 100-nm diameter POPC large unilamellar vesicles (LUVs) (0.2 mM; DiI, 0.1 mM, inner phase) in glycerol (15% (v/v). LUVs were kindly provided by Prof. Peter Walde's Group (ETH Zurich, Switzerland). The experiments were performed with a hypertonic inner phase (higher concentration of solute in the inner phase than in the outer phase).

All fluorescent images were collected with an inverted AxioVert A1 microscope (Carl Zeiss, Germany) using filters (red: ex/em 546/572–640 nm; green: ex/em 470/525 nm), and respective camera (Axiocam 202 mono).

In this work, encapsulation efficiency was defined as the percent of DEs produced compared to the total number of DEs expected in a given time, *i.e.* encapsulation of an inner water phase inside an intermediate oil phase. The expected total number of DEs assumed an ideal scenario in which production was perfect and 100% of the inner solution was encapsulated inside an oil droplet, calculated as the number of DEs per frame X 200 frames. The actual number of produced DEs accounted for common defects of production which led to the escape of the inner solution to the outer solution, calculated as the total number of DEs summed for 200 frames.

Stability assays

DE temperature stability over time. DEs were generated at room temperature (RT) and collected in an Ibidi µ-Slide Luer (0.4 mm height) chip. Then, DEs were placed at the respective temperatures (4°C; room temperature (22°C, RT); 37°C) and imaged as described above at defined time points. The chip was imaged in its entirety each time and DEs were counted and measured using a Python script, as described above. Data are presented as the percentage of DEs remaining normalized to t=0 (n≥3).

DE pH stability. DEs were pipetted on a clean glass slide and imaged as described above. Aqueous solutions with pH between 1–13 were prepared by adding 1 M HCl or 1 M NaOH to a solution of water and phenol red until the desired pH was reached. Then, an equivalent volume of each solution was gently pipetted into the standing droplet. DEs were imaged 10 minutes after exposure at RT. DEs were counted using the Python script (described above). Data are presented as percent DEs remaining after exposure compared to before exposure ± SD; n ≥ 5.

Stability of unloaded and loaded DEs at different mechanical stress conditions. Different flow regimens (14, 20 and 30 mbar) were applied with pressure using an autonomous recirculation system (Cobalt, Elveflow, France) that allowed double emulsions to flow back and forth in the field of view for 100 cycles

of 10 seconds (*i.e.* 5 s forward flow, 5 s backward flow) through an Ibidi u-Slide Luer (0.4 mm height) chip at RT. Videos were captured and the number of DEs from the most populated frame was counted for every 10 cycles.

The experiment was performed with unloaded DEs and LUV-loaded DEs (0.2 mM POPC; stained with DiI, 0.1 mM). Chips under static conditions were used as controls. Images before and after flow were collected and analyzed as described above.

The pressures were converted into estimations of flow rates (mL/min) using an online calculator (Elveflow, France)[58].

Results

Microfluidic production of monodisperse DEs and size characterization

To investigate the production of DEs using our microfluidics setup, DEs were produced in double-junction PDMS chips (Figure 1.a) made with cleanroom-free standard soft lithography techniques[53–55]. The solutions were driven by a pressure-driven fluid control setup (Figure 1.a.I) from the reservoirs to the first junction, where the inner aqueous (IA) solution was enveloped by the lipid-oil (LO) solution, and then to the second junction, where the outer aqueous (OA) solution pinched the DEs with a thin layer of oil (Figure 1.a.II).

The resulting DEs were monodisperse, with an average diameter of 83.6 µm (±6.2 µm SD; total number of DEs =14,914) (Figure 1.b).

The production rate of unloaded DEs was 233 Hz which translates to more than 800,000 DEs per hour. The production efficiency (encapsulation efficiency of the inner phase in the oil shell for unloaded DEs) was 88%.

Stability assays

As aforementioned, stability is the key issue for the practical application of DEs. Emulsions larger than 0.1 µm are only kinetically stable, breaking or coalescing over time[37,59]. To investigate a potential future application as a combinatory drug delivery system, the stability of lipid-stabilized DEs was tested in different physiologically relevant conditions, being exposed to ranges of temperature, pH and mechanical stress.

DE stability at different temperatures over time. Temperature plays an important role in meta-stable states by potentially providing the necessary energy required for the molecular assembly to leave the transient equilibrium (local energy minimum) and move towards a lower energy state[59]. Temperatures were chosen according to their relevance to the lifecycle of a DE therapeutic: from physiological body temperature, 37°C, to temperatures relevant to handling and storage, RT and 4°C, respectively. Lipid-stabilized double emulsions were exposed to 4°C, RT and 37°C for a minimum of seven days, and the number of DEs and mean radius were measured (Figure 2). The highest number of DEs in a given day (*i.e.* D0 for 4°C and 37°C and D1 for RT in Figure 1) was used as the maximum value to normalize the data as a percent. At 4°C, there was a trend towards a loss of DEs over time, but a significant reduction in numbers (92%) was observed by day eight compared to day 0. At RT, the loss of DEs presented a similar profile, with a significant reduction (68%) at day 5 compared to day 0, demonstrating a positive effect of colder temperatures in prolonging the transient meta-state, as expected. DEs were considerably less resistant to warmer temperatures, with a steep decrease (79%) in numbers and statistical difference from day 2 onwards at 37°C.

Radius at D0 was 41 µm for DEs placed at 4°C, 40 µm for RT and 34 µm for 37°C. At each temperature, the mean external

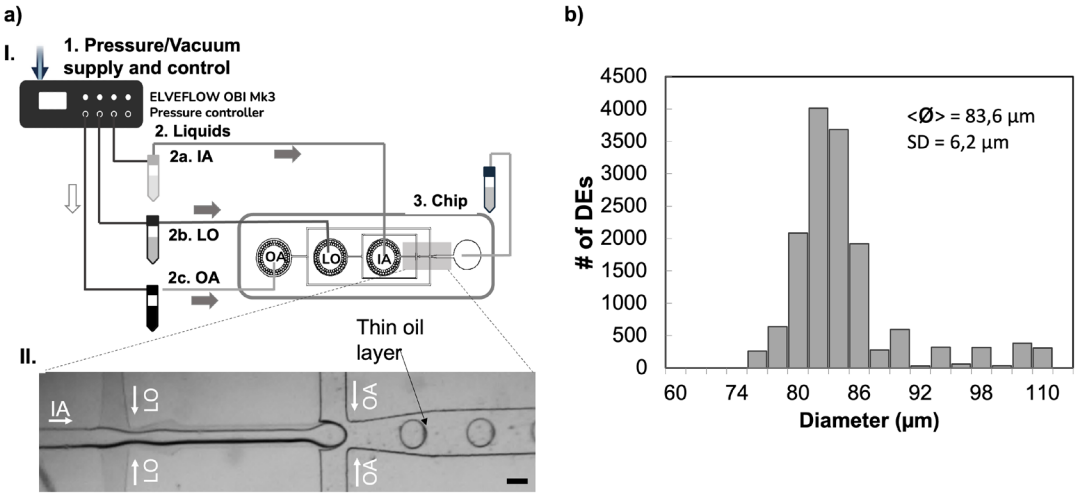

Figure 1. Microfluidic production of double emulsions (DEs) and size characterization. a) I. Microfluidic setup for production of monodisperse DEs with thin oil layers. Each reservoir (IA: inner aqueous solution; LO: lipid-oil solution; OA: Outer aqueous solution) was connected to a pressure-driven flow controller and to the respective inlet on the chip. II. DEs were pinched at the second junction, enveloped by a thin layer of oil. Scale bar is 100 µm. **b**) *Size distribution of DEs diameters in µm* (<ø>=83.6 µm, ±6.2 µm; *total number of DEs = 14,914*).

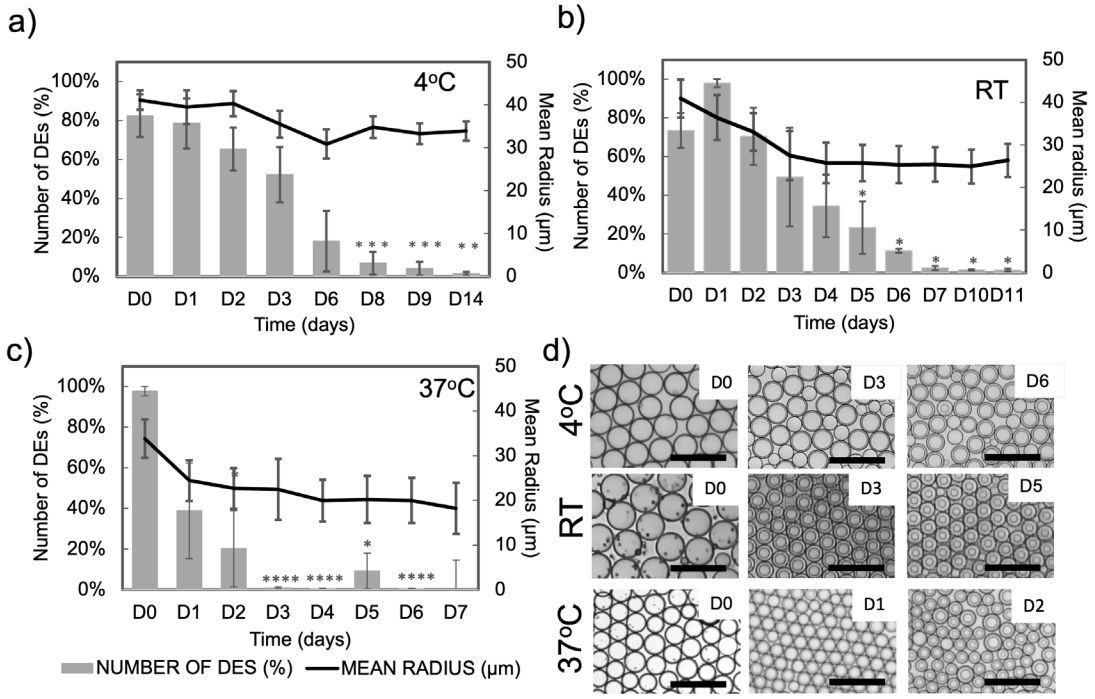

Figure 2. Stability and morphological changes of unloaded double emulsions (DEs) at different temperatures. a–**c**. Percentage of maximum value over time (left y-axis); Thick line shows DE radius (µm), +/- SD (right y-axis). **a**) DEs held at 4°C (n=4, total number of DEs at D0=16,357 DEs, +/- SEM); **b**) RT (n=3, 72,245 DEs, +/- SEM); and **c**) 37°C (n=4, 119,051 DEs, +/- SEM); **d**) Representative images at different time points highlighting the swelling of the intermediate phase; scale bars: 200 µm; Statistical significance from D0 (Student T-test): p<0.05 (*); p<0.01(**); p<0.005 (***); p<0,001 (****).

radius decreased (17%, 36% and 48%, respectively) throughout the testing period (Figure 2.d). Also, DEs showed a clear increase in the thickness of the intermediate layer over time.

Encapsulation of compounds of interest and stability of loaded DEs over time. To investigate the effect of locally encapsulated cargo on DE stability, one of the key advantages of DEs as delivery systems, the inner and intermediate phases were successfully loaded (Figure 3). DiI, a lipid-specific fluorescent dye that locates just below the lipid-water interface[60], was used as a hydrophobic cargo, and calcein or LUVs were used as hydrophilic cargo. Encapsulation efficiency was over 80% in all cases (DiI, n=5; calcein, n=2; LUVs, n=4).

Commonly, encapsulation in double emulsions produced with microfluidics is done with isotonic solutions[29,40,61], but there are reports of two-step emulsification methods using a hypertonic inner phase due to the higher stability of the resulting DEs[62,63]. Aiming to explore this characteristic and provide more flexibility to the final formulation of the outer solution for an oral drug delivery system, the DEs were loaded in the inner phase with a hypertonic solution (*i.e.* calcein or LUVs).

Encapsulation of cargo affected the post-production stability of DEs. When loaded with a hydrophobic cargo, there was a significant decrease (32%) in DE numbers by day 1 at RT (Figure 4.a), showing less stability than for unloaded DEs. Decreased DE stability was further observed as coalescence of the intermediate phase, resulting in complex structures with multiple water droplets inside a large droplet of octanol (shown stained in red). The change in morphology is also seen in the mean radius data (Figure 4.a), showing a decrease (25%) in radius up to D3. The formation of larger multi-inner droplet structures due to the coalescence of the intermediate phase caused the average radius to remain around 30 µm until D7, although most DEs that had not coalesced had burst by that time point.

Similarly, the addition of LUVs also affected post-production DE stability. The first two hours after production revealed a significant decrease (45%) in numbers, and most DEs had burst by D2 (Figure 4.b).

DE stability at different pH ranges. Another important parameter when considering an oral drug delivery system is pH[64]. DEs were exposed to a range of pH from 1 to 13, either unloaded or loaded with hydrophobic or hydrophilic cargos (DiI or LUVs, respectively) and their numbers were assessed at t=0 (before) and t=10 minutes after exposure. For unloaded DEs, 56% of DEs remained intact after exposure to pH 7. Also, there was only a significant reduction at the extremes (pH=1, 48%, and pH 13, 38%) when compared to pH 7 (Figure 5.a). The size was also not affected (Figure 5.b). A similar profile was demonstrated for DEs loaded with DiI (Figure 5.c), with only pH 1 significantly different (81% reduction) from pH 7. LUV-loaded DEs were considerably less stable, with 13–60% reduction across different pH (not significantly different from pH 7),

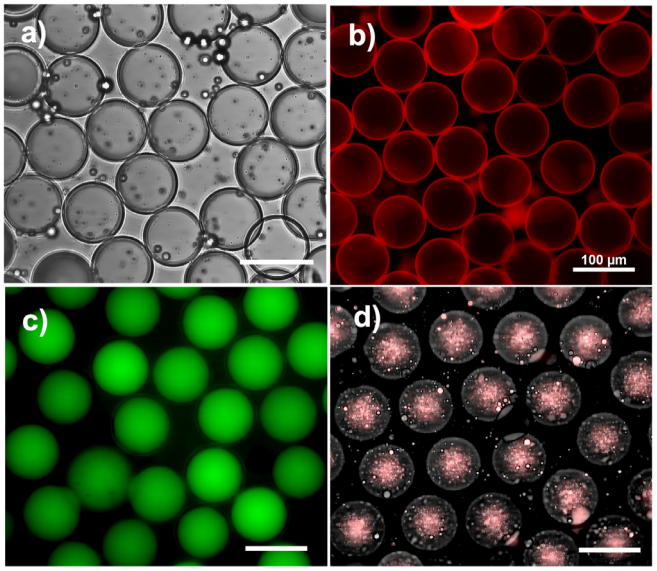

Figure 3. Encapsulation of cargo in double emulsions (DEs). a) DEs, no cargo, bright field; **b**) DEs loaded with DiI (lipid specific, intermediate phase, red fluorescent filter); **c**) DEs loaded with calcein (inner phase, green fluorescent filter); and **d**) DEs loaded with POPC LUVs pre-stained with DiI (inner phase; red fluorescent filter). Scale 100 um.

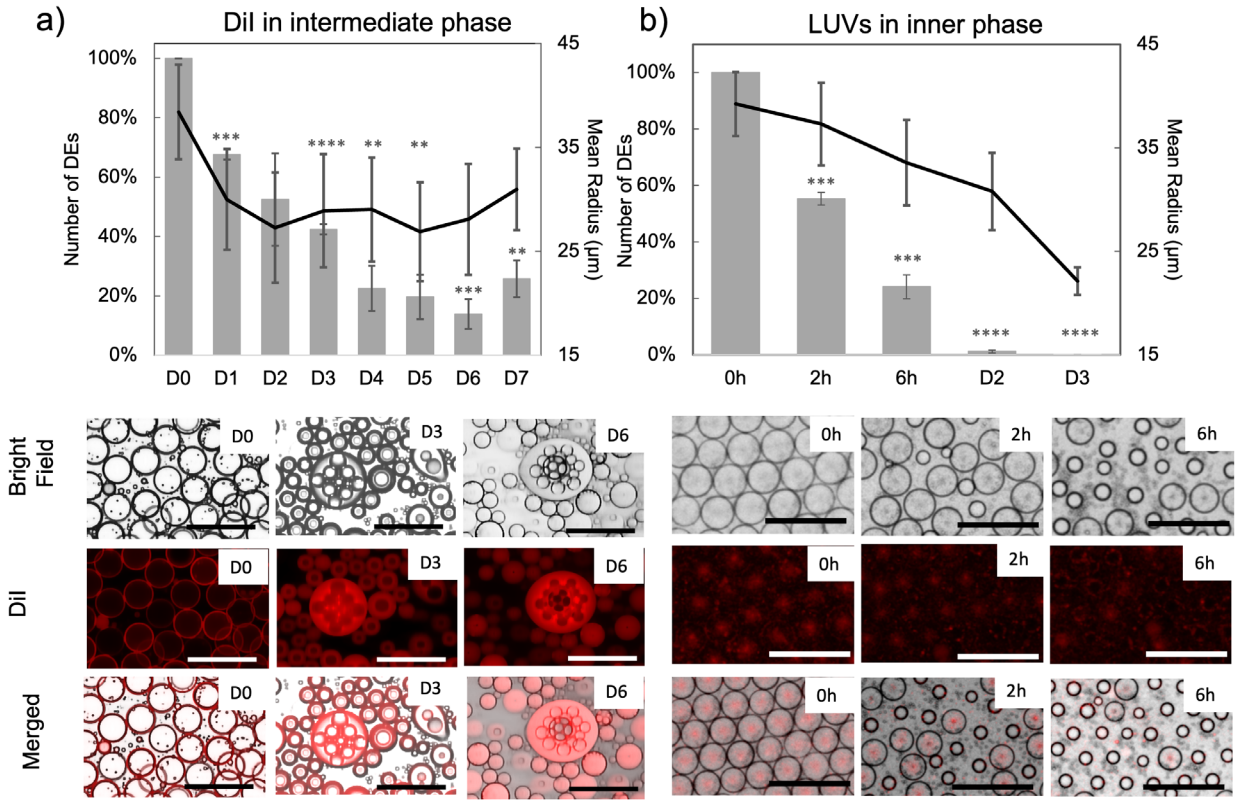

Figure 4. Stability of loaded double emulsions (DEs) over time at room temperature (RT). a) Encapsulation of hydrophobic cargo in the intermediate phase. The percentage of remaining DEs carrying DiI in the intermediate phase and the average size, in µm, observed through time at RT (n=3; total number of DEs at D0=37,492, +/-SEM; normalized to the maximum value). **b**) Encapsulation of 100nm large unilamellar vesicles (LUVs) (0,02mM POPC) in the inner phase (n=3; 10,863 DEs; +/-SEM). Insets show representative fluorescence (filter: ex 546 nm/em 572nm) and bright-field images of DEs at the indicated time points. Black line, radius (µm), +/- SD. Scale bar, 200 µm. Statistical significance from D0 (Student T-test): p<0.05 (*); p<0.01(**); p<0.005 (***); p<0,001 (****).

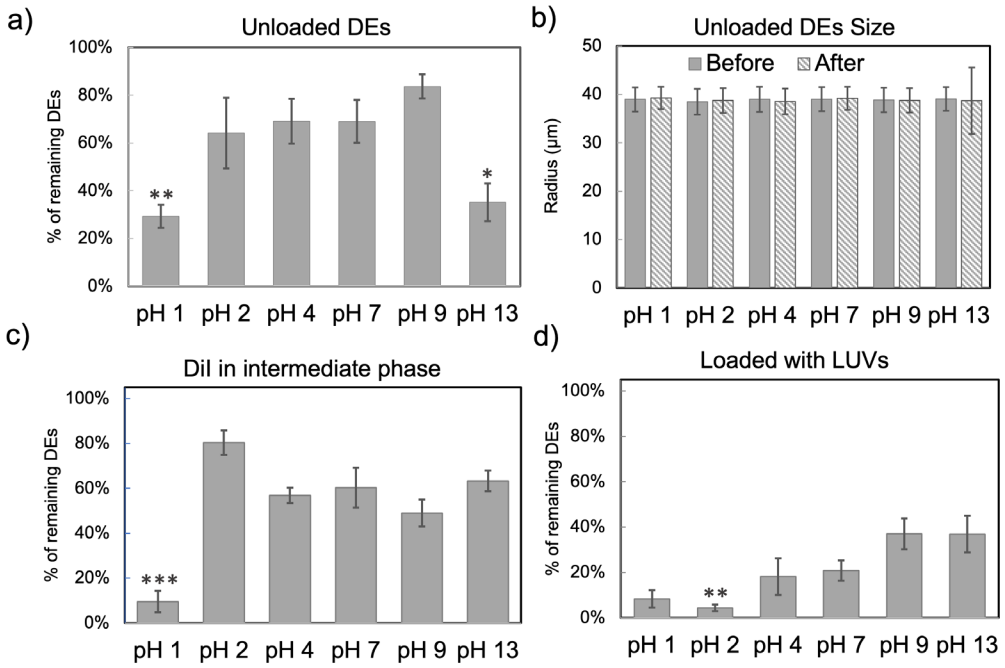

Figure 5. Double emulsion (DE) stability to different pH ranges. a) Percentage of remaining DEs after 10 min exposure to respective pH +/- SEM (pH 1, n=5, total number of DEs before exposure = 3,402; pH 2, n=5, 4,746 DEs; pH 4, n=5, 4,645 DEs; pH 7, n=10, 13,802 DEs; pH 9, n=5, 4,850 DEs; pH 13, n=5, 3,989 DEs). **b**) Radius of DEs before and after exposure to respective pH +/- SEM. **c**) Percentage of remaining DiI-loaded DEs after 10 min (pH 1, n=5, 3,402 DEs; pH 2, n=5, 4,746 DEs; pH 4, n=5, 4,645 DEs; pH 7, n=5, 6,248 DEs; pH 9, n=5, 4,850 DEs; pH 13, n=5, 3,989 DEs). **d**) Percentage of remaining large unilamellar vesicles (LUVs)-loaded DEs after 10 min (pH 1, n=5, 3,402 DEs; pH 2, n=5, 4,746 DEs; pH 4, n=5, 4,645 DEs; pH 7, n=5, 6,248 DEs; pH 9, n=5, 4,850 DEs; pH 13, n= 5, 3,989 DEs). In each panel, statistical significance from pH 7 is indicated (Student's t-test): p<0.05 (*); p<0.01(**); p<0.005 (***); p<0,001 (****).

and 79% reduction at pH 2 (significantly different from pH 7, Figure 5.d).

Stability of unloaded and loaded DEs under mechanical stimulus. The effect of flow and mechanical forces is relevant for applications involving the gastrointestinal tract due to its peristaltic properties. Mechanical stress is also known to affect the transition from a meta-stable to a thermodynamically stable state[59]. For this reason, DEs were subjected to mechanical forces caused by a stop-flow regimen made by back-and-forth cycles with an abrupt change in direction at the edges. The estimated flow rates were calculated for each pressure: 14 mbar = 0.5 mL/min; 20 mbar= 1 mL/min; 30 mbar= 2 mL/min. Up to 50 back and forth cycles, DE numbers remained similar to starting values, then numbers decreased about 30% for each of the three applied pressures by the 100th cycle (Figure 6.a). DEs loaded with LUVs presented a similar behaviour for 14 mbar and 20 mbar, but were considerably more sensitive to the pressure of 30 mbar, with only around 35% of the initial number of DEs remaining by the last cycle.

Discussion

Microfluidics as a highly suitable method for reproducible DE production

DEs with diameters in the micrometre range are well-suited for oral administration and have been reported for both pharmaceutical[4] and food[65] applications. Monodispersity is particularly crucial for drug delivery systems, because it improves reproducibility and provides increased control over encapsulation, allowing for a more homogeneous distribution of cargo[24,66].

The standard two-step emulsification production method results in considerably large size distributions, *e.g.* from 1 to 100 µm[67] or 1 to 500 µm[65]. Attempts to improve the monodispersity of two-step emulsification samples usually include extra post-production steps (*i.e.* extrusion through polycarbonate membranes[15] or a multi-purification step[68]) which adds complexity and may limit the flexibility of the formulation. The DEs presented in this work highlight the advantage of microfluidics in producing monodisperse double emulsions with a simple setup, without any post-production step, with high encapsulation efficiencies, directly in the targeted size for oral drug delivery systems.

Environmental conditions affect loaded and unloaded DEs differently

As a potential oral drug delivery system, DEs will be subjected to environmental conditions, including different pH and flow regimens of the gastrointestinal tract. For example, the pH of saliva is between 6.3 and 7.6[69]; the stomach pH is 2 or lower; and the intestine pH varies from 6.6 to 8[70].

As DEs are less dense than the outer solution and float, they could not easily be trapped to enable the exchange of the pH solution via continuous flow. Thus, pH was adjusted by the

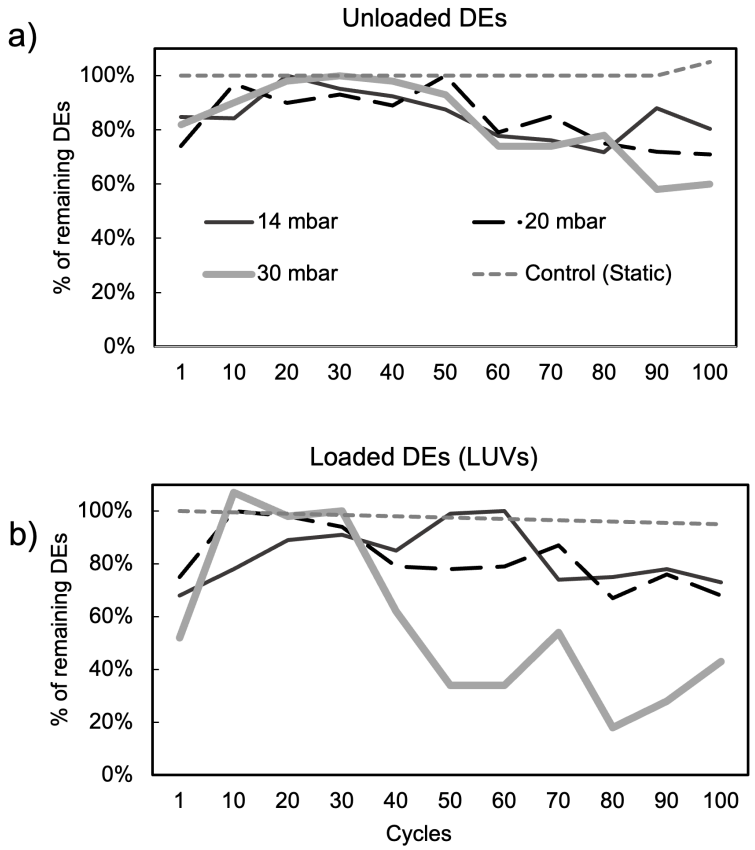

Figure 6. Stability of unloaded and loaded double emulsions (DEs) at different flow conditions. Percentage of remaining double emulsions over the highest number of DEs per cycle of 10 seconds, **a**) unloaded or **b**) loaded with 0.2mM POPC LUVs.

addition of a droplet of pH solution to a droplet of DEs. While carefully done, this provided a disruptive physical force to the DEs on the slide. Even unloaded DEs responded to this force by losing about 30% of their number at pH 7. Their reduction in number was not a consequence of doubling the volume, as all volumes were small enough that the entire content was counted in each case.

Unloaded DEs were stable across physiological pH, with increased sensitivity to the extremes. On the one hand, an optimization of the formulation, such as using a different surfactant[71] or higher concentration of glycerol in the inner phase[72], might improve stability at lower pH so DEs are better equipped to pass through the acidic environment of the stomach. However, the instability to a specific pH observed here could be leveraged as a trigger for localized drug delivery[73,74].

Loaded DEs behaved differently depending on the location and type of the cargo. When loaded with DiI in the intermediate phase, DEs were more resistant to high pH than unloaded DEs, suggesting that the properties of the cargo can be leveraged to improve stability at higher pH. On the other hand, DEs loaded with LUVs were considerably more sensitive to the increased disturbance of the addition of the pH solution and to the range of pH as well, losing more than 60% of initial numbers in all cases. Interestingly, LUV-loaded DEs presented no significant difference from pH 7 at high pH, similarly to DiI-loaded DEs, although they were more sensitive to low pH than the two other cases. These results show that this formulation is particularly sensitive to low pH, which can be accentuated by the presence of the cargo. Therefore, further optimization is needed in order to render this particular formulation apt to withstand the conditions it would face as an oral drug delivery system.

Oral drug delivery systems are subjected to the fluid flow and mechanical forces of the gastrointestinal tract. The recirculation experiments were designed to reproduce, to some extent, the environment and respective forces that would be applied to and/or felt by DEs. Here, DEs were not subjected to direct shear stress because the height of the channel was approximately four-fold larger than the diameter of the DEs, so they could flow freely with the fluid, as is expected to happen in the body. The dimensions of the gastrointestinal (GI) tract are orders of magnitude larger than the size of the DEs, so they are not expected to be constricted or to experience direct shear

stress on the surface[75]. However, DEs are expected to experience mechanical stress as they flow along the tract, such as drops, stops, turns and turbulence[75]. To reproduce this in microfluidics, we introduced a sudden change in the direction of flow, so the DEs would feel as if they were hitting a wall. The chosen pressures, and consequently, the flow rates, are in the same order of magnitude as the lowest flow rates of the GI tract (2 to 3.6 mL/min in the duodenum and jejunum[76]).

It is not surprising that the highest forces produced the biggest disruption, as LUV-loaded DEs were consistently more sensitive than the other two formulations in all tested conditions in this work. More interestingly, LUV-loaded DEs handled the two lower pressures as well as unloaded DEs, suggesting that they might withstand these external stresses as oral drug delivery systems once the formulation is optimized. In that light, it is important to consider the properties of the cargo and osmotic ratio of inner and outer solutions early in the design, so that DEs are tailored to best fulfil the intended final application.

Cargo has considerable effect on long-term DE stability

The required stability for DEs to be used as oral drug delivery systems is largely dependent on the final application, however their actual stability depends on the cargo. For perspective, the Pfizer-BioNTech mRNA vaccine (a lipid nanoparticle, not a DE) is stable for up to five days at 2–8°C and only 2–6 h at room temperature[77]. Stability of our unloaded DEs lay within this time span at fridge-based storage conditions (eight days, 4°C) and presented substantial resistance to size change or aggregation at conditions favourable to distribution and administration of a therapeutic (five days, RT). However, when loaded with cargo, the time spans at RT were significantly reduced (DiI, one day; LUVs, 2 h), demonstrating the large impact of cargo on DE stability. As exemplified by the Pfizer-BioNTech case, these time spans could still be feasible for real-world applications, although they call for complex distribution chains.

The decrease in size and swelling of the intermediate phase are expected to play an important role in the release profile of encapsulated compounds. This impact is demonstrated by studies that varied the shell thickness of core-shell microparticles, templated from double emulsions. Microparticles with thicker shells had a slower release of compounds present in the inner phase[33,78]. However, the release rate across lipid/octanol shells and the release profile when the thickness increases over time, as seen here, are less well known. The swelling of the intermediate phase is likely due to the partial miscibility of octanol in water (0.54 g/L)[79]. Octanol has recently gained relevance as a suitable intermediate phase when paired with lipids to form giant unilamellar vesicles (GUVs) after dewetting from double emulsions templates[29,40,57,80]. In our work, the octanol was intended to stay in the intermediate layer as part of the oral delivery system because it is an FDA-approved food additive[81] that has already been studied for topical delivery systems[82,83]. Therefore, the necessary conditions for the octanol dewetting, such as a given lipid concentration[84] and external stress[40,61], were purposefully not met.

To broaden the range of potential applications, the properties of the lipid monolayer and of each of the solutions can be tuned for improved stability. For example, different concentrations of lipids (*e.g.* 4% against 8% of phospholipids) have been shown to improve the long-term stability of DEs by maintaining their size and morphology constant for up to 30 days at 4°C[67], or different concentrations/combinations of surfactants[9,35,85]. On a pioneering work, Bibette *et al.*, 1998[35] demonstrated that by changing the concentration of the hydrophilic surfactant, the stability of the double emulsions could be varied from a few minutes to months. Another approach is to use DEs as a template for other types of compartments, such as GUVs[29,40,57,80,84] gels[86–88] or microparticles[33,36,51], although this increases the complexity of the process, since one or more post-production steps are required.

Localization and properties of cargo are important parameters for DE drug delivery

It is becoming well accepted that cargo plays a huge role in the physical properties and stability of encapsulated therapeutics, including micro- and nano- formulations[89,90]. This applies also to DEs, in particular because they can disrupt or enhance the overall stability of the metastable system[91]. In the case of DiI as the hydrophobic cargo, DE stability was affected in terms of morphological changes, *i.e.* coalescence of the intermediate phase.

There are various factors that can be at play. The arrangement of the hydrophobic cargo between the lipid molecules or monolayers can affect the behaviour of the membrane. DiI, for example, is located slightly below the water interface with a lipid monolayer, with the long alkyl tails parallel to the lipid molecules. Detailed studies about the location and arrangement of DiI in lipid membranes have shown that the presence of DiI increases the order around the lipid molecules, suggesting a stabilizing effect[60,92]. This effect might have played a role in avoiding the simple bursting of the DEs and promoting coalescence due to a decrease in the surface tension.

The encapsulation of LUVs in the inner phase caused a drastic decrease in the long-term stability, reducing it from days to hours. The main reason for this might be the hypertonic inner phase. The works using a hypertonic inner phase in two-step emulsification also reported a thicker oil layer around the hypertonic DEs, while microfluidics allows for a higher degree of control and thus, the possibility of combining a hypertonic inner phase with a thin oil layer. The thin oil layer is also expected to increase stability because it hinders transport across the oil layer[37,93], however the combination of both might have been detrimental. Nevertheless, the LUV-loaded DEs had stability equivalent to that of the Pfizer-BioNTech vaccine at RT, which indicates a potential for application as an oral drug delivery system. Besides, the production of hypertonic DEs opens the possibility to use osmotic triggers for regional release of drugs[94]. Further work should explore the ratio of hypertonicity between inner and outer solutions and its effect on the long-term stability of DEs.

Conclusions

Lipid-stabilized DEs were successfully produced via microfluidics in PDMS chips, fabricated without the need of a cleanroom. DEs were monodisperse and allowed for the encapsulation of molecules of different properties in specific compartments. The stability through time was inversely proportional to temperature. When encapsulating a compound of interest, the long-term stability at RT decreased substantially from several days to hours. This behaviour highlights the importance of considering the properties of cargo early on in the formulation.

When exposed to pH, unloaded DEs were only significantly unstable at the extremes (pH 1 and 13) which are outside the physiological ranges. DiI-loaded and LUV-loaded DEs were more sensitive to acidic pH, although LUV-loaded DEs were overall more unstable to the stress than the other experimental conditions. When exposed to mechanical stress, LUV-loaded DEs behaved similarly to unloaded DEs at the lower pressures and were more sensitive to higher pressures. This indicates that the high instability to the pH conditions might be linked to the osmotic unbalance more than the mechanical stress caused by the experimental setup.

Together, these results suggest that lipid-stabilized DEs produced via microfluidics could be tailored to endure physiologically relevant conditions and act as carriers for oral drug delivery. Further work should focus on the combined effect of different stresses on DE stability, *e.g.* combined effect of temperature and pH, since these conditions do not happen in isolation in real settings. Also, similar to other drug delivery systems, the cargo in DEs carriers should be actively considered as an integral part of the formulation design for better outcomes. Thus, special attention should be given to the composition and interplay of solutions and molecules involved in the final formulation from the start.

Data availability

Underlying data

Zenodo: Stability characterization of microfluidic lipid-stabilized double emulsions under physiologically-relevant conditions, https://doi.org/10.5281/zenodo.6866211[95]

This project contains the following underlying data:

- LUVs static control_summary.xlsx

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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References

1. Muschiolik G, Dickinson E: **Double Emulsions Relevant to Food Systems: Preparation, Stability, and Applications.** *Compr Rev Food Sci Food Saf.* 2017; **16**(3): 532–555.
PubMed Abstract | Publisher Full Text

2. Ding S, Serra CA, Vandamme TF, *et al.*: **Double emulsions prepared by two-step emulsification: History, state-of-the-art and perspective.** *J Control Release.* 2019; **295**: 31–49.
PubMed Abstract | Publisher Full Text

3. Sengupta S, Eavarone D, Capila I, *et al.*: **Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system.** *Nature.* 2005; **436**(7050): 568–572.
PubMed Abstract | Publisher Full Text

4. Qi X, Wang L, Zhu J: **Water-in-oil-in-water double emulsions: An excellent delivery system for improving the oral bioavailability of pidotimod in rats.** *J Pharm Sci.* 2011; **100**(6): 2203–2211.
PubMed Abstract | Publisher Full Text

5. Yang YY, Chung TS, Ng NP: **Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method.** *Biomaterials.* 2001; **22**(3): 231–241.
PubMed Abstract | Publisher Full Text

6. Lu M, Zhao C, Wang Q, *et al.*: **Preparation, characterization and in vivo investigation of blood-compatible hemoglobin-loaded nanoparticles as oxygen carriers.** *Colloids Surf B Biointerfaces.* 2016; **139**: 171–179.
PubMed Abstract | Publisher Full Text

7. Yu WP, Chang TM: **Submicron Polymer Membrane Hemoglobin Nanocapsules as Potential blood substitutes: Preparation and Characterization.** *Artif Cells Blood Substit Immobil Biotechnol.* 1996; **24**(3): 169–183.
PubMed Abstract | Publisher Full Text

8. Verma R, Jaiswal TN: **Protection, humoral and cell-mediated immune responses in calves immunized with multiple emulsion haemorrhagic septicaemia vaccine.** *Vaccine.* 1997; **15**(11): 1254–1260.
PubMed Abstract | Publisher Full Text

9. Liau JJ, Hook S, Prestidge CA, *et al.*: **A lipid based multi-compartmental system: Liposomes-in-double emulsion for oral vaccine delivery.** *Eur J Pharm Biopharm.* 2015; **97**(Pt A): 15–21.
PubMed Abstract | Publisher Full Text

10. Engel RH, Riggi SJ, Fahrenbach MJ: **Insulin: Intestinal Absorption as Water-in-Oil-in-Water Emulsions.** *Nature.* 1968; **219**(5156): 856–857.
PubMed Abstract | Publisher Full Text

11. Matsumoto S, Kita Y, Yonezawa D: **An attempt at preparing water-in-oil-in-water multiple-phase emulsions.** *J Colloid Interface Sci.* 1976; **57**(2): 353–361.
Publisher Full Text

12. Frenkel M, Shwartz R, Garti N: **Multiple Emulsions: I. stability: inversion, apparent and weighted HLB.** *J Colloid Interface Sci.* 1983; **94**(1): 174–178.
Publisher Full Text

13. Garti N, Remon GF: **Relationship between nature of vegetable oil, emulsifier and the stability of w/o emulsion.** *Int J Food Sci Technol.* 1984; **19**(6): 711–717.
Publisher Full Text

14. Omotosho JA, Law TK, Whateley AT, *et al.*: **The stabilization of w/o/w emulsions by interfacial interaction between albumin and non-ionic surfactants.** *Colloids Surf.* 1986; **20**(1–2): 133–144.
Publisher Full Text

15. Kawashima Y, Hino T, Takeuchi H, *et al.*: **Shear-induced phase inversion and size control of water/oil/water emulsion droplets with porous membrane.** *J Colloid Interface Sci.* 1991; **145**(2): 512–523.
Publisher Full Text

16. Kim CK, Kim SC, Shin HJ, *et al.*: **Preparation and characterization of**

cytarabine-loaded W/O/W multiple emulsions. *Int J Pharm.* 1995; **124**(1): 61–67.
Publisher Full Text

17. Okochi H, Nakano M: **Preparation and evaluation of w/o/w type emulsions containing vancomycin.** *Adv Drug Deliv Rev.* 2000; **45**(1): 5–26.
PubMed Abstract | Publisher Full Text

18. Benichou A, Aserin A, Garti N: **Double emulsions stabilized by new molecular recognition hybrids of natural polymers.** *Polym Adv Technol.* 2002; **13**(10–12): 1019–1031.
Publisher Full Text

19. Berchane NS, Carson KH, Rice-Ficht AC, *et al.*: **Effect of mean diameter and polydispersity of PLG microspheres on drug release: Experiment and theory.** *Int J Pharm.* 2007; **337**(1–2): 118–126.
PubMed Abstract | Publisher Full Text

20. Kita K, Dittrich C: **Drug delivery vehicles with improved encapsulation efficiency: Taking advantage of specific drug-carrier interactions.** *Expert Opin Drug Deliv.* 2011; **8**(3): 329–342.
PubMed Abstract | Publisher Full Text

21. Chu LY, Utada AS, Shah RK, *et al.*: **Controllable monodisperse multiple emulsions.** *Angew Chem Int Ed Engl.* 2007; **46**(47): 8970–8974.
PubMed Abstract | Publisher Full Text

22. Shah RK, Shuma HC, Rowat AC, *et al.*: **Designer emulsions using microfluidics.** *Mater Today.* 2008; **11**(4): 18–27.
Publisher Full Text

23. Tumarkin E, Tzadu L, Csaszar E, *et al.*: **High-throughput combinatorial cell co-culture using microfluidics.** *Integr Biol (Camb).* 2011; **3**(6): 653–662.
PubMed Abstract | Publisher Full Text

24. Utada AS, Lorenceau E, Link DR, *et al.*: **Monodisperse double emulsions generated from a microcapillary device.** *Science.* 2005; **308**(5721): 537–541.
PubMed Abstract | Publisher Full Text

25. Czekalska MA, Jacobs AMJ, Toprakcioglu Z, *et al.*: **One-Step Generation of Multisomes from Lipid-Stabilized Double Emulsions.** *ACS Appl Mater Interfaces.* 2021; **13**(5): 6739–6747.
PubMed Abstract | Publisher Full Text

26. Petit J, Polenz I, Baret JC, *et al.*: **Vesicles-on-a-chip: A universal microfluidic platform for the assembly of liposomes and polymersomes.** *Eur Phys J E Soft Matter.* 2016; **39**(6): 59.
PubMed Abstract | Publisher Full Text

27. Trantidou T, Dekker L, Polizzi K, *et al.*: **Functionalizing cell-mimetic giant vesicles with encapsulated bacterial biosensors.** *Interface Focus.* 2018; **8**(5): 20180024.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Kong L, Levin A, Toprakcioglu Z, *et al.*: **Lipid-Stabilized Double Emulsions Generated in Planar Microfluidic Devices.** *Langmuir.* 2020; **36**(9): 2349–2356.
PubMed Abstract | Publisher Full Text

29. Yandrapalli N, Petit J, Bäumchen O, *et al.*: **Surfactant-free production of biomimetic giant unilamellar vesicles using PDMS-based microfluidics.** *Commun Chem.* 2021; **4**: 100.
Publisher Full Text

30. Aditya NP, Aditya S, Yang H, *et al.*: **Co-delivery of hydrophobic curcumin and hydrophilic catechin by a water-in-oil-in-water double emulsion.** *Food Chem.* 2015; **173**: 7–13.
PubMed Abstract | Publisher Full Text

31. Choi CH, Weitz DA, Lee CS: **One Step Formation of Controllable Complex Emulsions: From Functional Particles to Simultaneous Encapsulation of Hydrophilic and Hydrophobic Agents into Desired Position.** *Adv Mater.* 2013; **25**(18): 2536–2541.
PubMed Abstract | Publisher Full Text

32. Bazylińska U: **Rationally designed double emulsion process for co-encapsulation of hybrid cargo in stealth nanocarriers.** *Colloids Surfaces A Physicochem Eng Asp.* 2017; **532**: 476–482.
Publisher Full Text

33. Li Y, Yan D, Fu F, *et al.*: **Composite core-shell microparticles from microfluidics for synergistic drug delivery.** *Sci China Mater.* 2017; **60**: 543–553.
Publisher Full Text

34. Windbergs M, Zhao Y, Heyman J, *et al.*: **Biodegradable core-shell carriers for simultaneous encapsulation of synergistic actives.** *J Am Chem Soc.* 2013; **135**(21): 7933–7937.
PubMed Abstract | Publisher Full Text

35. Ficheux MF, Bonakdar L, Leal-Calderon F, *et al.*: **Some stability criteria for double emulsions.** *Langmuir.* 1998; **14**(10): 2702–2706.
Publisher Full Text

36. Tu F, Lee D: **Controlling the stability and size of double-emulsion-templated poly(lactic-*co*-glycolic) acid microcapsules.** *Langmuir.* 2012; **28**(26): 9944–9952.
PubMed Abstract | Publisher Full Text

37. Zhao CX, Chen D, Hui Y, *et al.*: **Controlled Generation of Ultrathin-Shell Double Emulsions and Studies on Their Stability.** *Chemphyschem.* 2017; **18**(10): 1393–1399.
PubMed Abstract | Publisher Full Text

38. Leister N, Karbstein HP: **Evaluating the stability of double emulsions—A review of the measurement techniques for the systematic investigation of instability mechanisms.** *Colloids Interfaces.* 2020; **4**(1): 8.
Publisher Full Text

39. Kanouni M, Rosano HL, Naouli N: **Preparation of a stable double emulsion ($W_1/O/W_2$): Role of the interfacial films on the stability of the system.** *Adv Colloid Interface Sci.* 2002; **99**(3): 229–254.
PubMed Abstract | Publisher Full Text

40. Deshpande S, Caspi Y, Meijering AE, *et al.*: **Octanol-assisted liposome assembly on chip.** *Nat Commun.* 2016; **7**: 10447.
PubMed Abstract | Publisher Full Text | Free Full Text

41. Okushima S, Nisisako T, Torii T, *et al.*: **Controlled Production of Monodisperse Double Emulsions by Two-Step Droplet Breakup in Microfluidic Devices.** *Langmuir.* 2004; **20**(23): 9905–9908.
PubMed Abstract | Publisher Full Text

42. Yang J, Gu Z, Cheng L, *et al.*: **Effect of temperature, pH, and ionic strength on the structure and physical stability of double emulsions prepared with starch.** *LWT.* 2021; **141**: 111086.
Publisher Full Text

43. Deng NN, Yelleswarapu M, Huck WT: **Monodisperse Uni- and Multicompartment Liposomes.** *J Am Chem Soc.* 2016; **138**(24): 7584–7591.
PubMed Abstract | Publisher Full Text

44. Martino C, deMello AJ: **Droplet-based microfluidics for artificial cell generation: a brief review.** *Interface Focus.* 2016; **6**(4): 20160011.
PubMed Abstract | Publisher Full Text | Free Full Text

45. Torbensen K, Baroud CN, Ristori S, *et al.*: **Tip Streaming of a Lipid-Stabilized Double Emulsion Generated in a Microfluidic Channel.** *Langmuir.* 2021; **37**(24): 7442–7448.
PubMed Abstract | Publisher Full Text

46. Trantidou T, Regoutz A, Voon XN, *et al.*: **A "cleanroom-free" and scalable manufacturing technology for the microfluidic generation of lipid-stabilized droplets and cell-sized multisomes.** *Sensors Actuators B Chem.* 2018; **267**: 34–41.
Publisher Full Text

47. Clemente I, Torbensen K, Di Cola E, *et al.*: **Exploring the water/oil/water interface of phospholipid stabilized double emulsions by micro-focusing synchrotron SAXS.** *RSC Adv.* 2019; **9**(57): 33429–33435.
PubMed Abstract | Publisher Full Text | Free Full Text

48. Pandey V, Kohli S: **Lipids and surfactants: The inside story of lipid-based drug delivery systems.** *Crit Rev Ther Drug Carrier Syst.* 2018; **35**(2): 99–155.
PubMed Abstract | Publisher Full Text

49. Hunt JN, Knox MT: **A relation between the chain length of fatty acids and the slowing of gastric emptying.** *J Physiol.* 1968; **194**(2): 327.
PubMed Abstract | Publisher Full Text | Free Full Text

50. Khan S, Baboota S, Ali J, *et al.*: **Nanostructured lipid carriers: An emerging platform for improving oral bioavailability of lipophilic drugs.** *Int J Pharm Investig.* 2015; **5**(4): 182–91.
PubMed Abstract | Publisher Full Text | Free Full Text

51. Weng T, Qi J, Lu Y, *et al.*: **The role of lipid-based nano delivery systems on oral bioavailability enhancement of fenofibrate, a BCS II drug: Comparison with fast-release formulations.** *J Nanobiotechnology.* 2014; **12**: 39–8.
PubMed Abstract | Publisher Full Text | Free Full Text

52. Zhong H, Chan G, Hu Y, *et al.*: **A comprehensive map of FDA-approved pharmaceutical products.** *Pharmaceutics.* 2018; **10**(4): 263.
PubMed Abstract | Publisher Full Text | Free Full Text

53. Xia Y, Whitesides GM: **Soft lithography.** *Angew Chemie Int Ed Engl.* 1998; **37**(5): 550–575.
PubMed Abstract | Publisher Full Text

54. Whitesides GM, Ostuni E, Takayama S, *et al.*: **Soft Lithography in Biology and Biochemistry.** *Annu Rev Biomed Eng.* 2001; **3**: 335–373.
PubMed Abstract | Publisher Full Text

55. Kane RS, Stroock AD, Li Jeon N, *et al.*: **Soft Lithography and Microfluidics.** *Opt Biosens.* 2002; 571–595.
Publisher Full Text

56. Teh SY, Khnouf R, Fan H, *et al.*: **Stable, biocompatible lipid vesicle generation by solvent extraction-based droplet microfluidics.** *Biomicrofluidics.* 2011; **5**(4): 44113–4411312.
PubMed Abstract | Publisher Full Text | Free Full Text

57. Deshpande S, Dekker C: **On-chip microfluidic production of cell-sized liposomes.** *Nat Protoc.* 2018; **13**(5): 856–874.
PubMed Abstract | Publisher Full Text

58. Elveflow: **Microfluidic calculator - Elveflow**. (Accessed: 30th June 2022).
Reference Source

59. Kornyushin Y: **Short Communication A Model for Metastable States.** *J Non Equilib Thermodyn.* 1993; **18**: 353–358.
Publisher Full Text

60. Gullapalli RR, Demirel MC, Butler PJ: **Molecular dynamics simulations of DiI-C18(3) in a DPPC lipid bilayer.** *Phys Chem Chem Phys.* 2008; **10**(24): 3548–3560.
PubMed Abstract | Publisher Full Text | Free Full Text

61. Krafft D, Castellanos SL, Lira RB, *et al.*: **Compartments for Synthetic Cells: Osmotically Assisted Separation of Oil from Double Emulsions in a Microfluidic Chip.** *ChemBioChem.* 2019; **20**(20): 2604–2608.
PubMed Abstract | Publisher Full Text | Free Full Text

62. Kawashima Y, Hino T, Takeuchi H, *et al.*: **Stabilization of Water/Oil/Water**

Multiple Emulsion with Hypertonic Inner Aqueous Phase. *Chem Pharm Bull.* 1992; **40**(5): 1240–1246.
Publisher Full Text

63. Hino T, Kawashima Y, Shimabayashi S: **Basic study for stabilization of w/o/w emulsion and its application to transcatheter arterial embolization therapy.** *Adv Drug Deliv Rev.* 2000; **45**(1): 27–45.
PubMed Abstract | Publisher Full Text

64. Liu L, Yao WD, Rao YF, *et al.*: **pH-Responsive carriers for oral drug delivery: challenges and opportunities of current platforms.** *Drug Deliv.* 2017; **24**(1): 569–581.
PubMed Abstract | Publisher Full Text | Free Full Text

65. Yildirim M, Sumnu G, Sahin S: **Rheology, particle-size distribution, and stability of low-fat mayonnaise produced via double emulsions.** *Food Sci Biotechnol.* 2016; **25**(6): 1613–1618.
PubMed Abstract | Publisher Full Text | Free Full Text

66. Wang W, Zhang MJ, Chu LY: **Microfluidic approach for encapsulation via double emulsions.** *Curr Opin Pharmacol.* 2014; **18**: 35–41.
PubMed Abstract | Publisher Full Text

67. Qi X, Wang L, Zhu J, *et al.*: **Self-double-emulsifying drug delivery system (SDEDDS): A new way for oral delivery of drugs with high solubility and low permeability.** *Int J Pharm.* 2011; **409**(1–2): 245–251.
PubMed Abstract | Publisher Full Text

68. Bibette J: **Depletion interactions and fractionated crystallization for polydisperse emulsion purification.** *J Colloid Interface Sci.* 1991; **147**(2): 474–479.
Publisher Full Text

69. Baliga S, Muglikar S, Kale R: **Salivary pH: A diagnostic biomarker.** *J Indian Soc Periodontol.* 2013; **17**(4): 461–5.
PubMed Abstract | Publisher Full Text | Free Full Text

70. Pfeiffer JK: **Innate host barriers to viral trafficking and population diversity. Lessons learned from poliovirus.** *Adv Virus Res.* Elsevier Inc. 2010; **77**: 85–118.
PubMed Abstract | Publisher Full Text | Free Full Text

71. Pimentel-González DJ, Campos-Montiel RG, Lobato-Calleros C, *et al.*: **Encapsulation of *Lactobacillus rhamnosus* in double emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions.** *Food Res Int.* 2009; **42**(2): 292–297.
Publisher Full Text

72. van der Ark KCH, Nugroho ADW, Berton-Carabin C, *et al.*: **Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions.** *Food Res Int.* 2017; **102**: 372–379.
PubMed Abstract | Publisher Full Text

73. Malik B, Gupta RK, Rath G, *et al.*: **Development of pH responsive novel emulsion adjuvant for oral immunization and in vivo evaluation.** *Eur J Pharm Biopharm.* 2014; **87**(3): 589–597.
PubMed Abstract | Publisher Full Text

74. Cardenas-Bailon F, Osorio-Revilla G, Gallardo-Velazquez T: **Microencapsulation of insulin using a W/O/W double emulsion followed by complex coacervation to provide protection in the gastrointestinal tract.** *J Microencapsul.* 2015; **32**(3): 308–316.
PubMed Abstract | Publisher Full Text

75. Chen W, Yan G, Wang Z, *et al.*: **A wireless capsule robot with spiral legs for human intestine.** *Int J Med Robot.* 2014; **10**(2): 147–161.
PubMed Abstract | Publisher Full Text

76. Ho NFH, Merkle HP, William I: **Higuchi. Quantitative, mechanistic and physiologically realistic approach to the biopharmaceutical design of oral drug delivery systems.** *Drug Dev Ind Pharm.* 1983; **9**(7): 1111–1184.
Publisher Full Text

77. Crommelin DJA, Anchordoquy TJ, Volkin DB, *et al.*: **Addressing the Cold Reality of mRNA Vaccine Stability.** *J Pharm Sci.* 2021; **110**(3): 997–1001.
PubMed Abstract | Publisher Full Text | Free Full Text

78. Wu J, Kong T, Yeung KWK, *et al.*: **Fabrication and characterization of monodisperse PLGA-alginate core-shell microspheres with monodisperse size and homogeneous shells for controlled drug release.** *Acta Biomater.* 2013; **9**(7): 7410–7419.
PubMed Abstract | Publisher Full Text

79. PubChem Compound Database: **1-Octanol - Hazardous Substances Data Bank**. (Accessed: 30th June 2022).
Reference Source

80. Love C, Steinkühler J, Gonzales DT, *et al.*: **Reversible pH-Responsive Coacervate Formation in Lipid Vesicles Activates Dormant Enzymatic Reactions.** *Angew Chem Int Ed Engl.* 2020; **132**(15): 6006–6013.
PubMed Abstract | Publisher Full Text | Free Full Text

81. FDA: **Direct Food Substances Affirmed as Generally Recognized as Safe**. 2022. (Accessed: 30th June 2022).
Reference Source

82. Osborne DU, Ward AJI, O'neill KJ: **Microemulsions as topical drug delivery vehicles. I. Characterization of a model system.** *Drug Dev Ind Pharm.* 1988; **14**(9): 1203–1219.
Publisher Full Text

83. Osborne DW, Ward AJI, O'Neill KJ: **Microemulsions as topical drug delivery vehicles: in-vitro transdermal studies of a model hydrophilic drug.** *J Pharm Pharmacol.* 1991; **43**(6): 451–454.
PubMed Abstract

84. Bao P, Paterson DA, Peyman SA, *et al.*: **Production of giant unilamellar vesicles and encapsulation of lyotropic nematic liquid crystals.** *Soft Matter.* 2021; **17**(8): 2234–2241.
PubMed Abstract | Publisher Full Text

85. Moriano ME, Alamprese C: **Whey Protein Concentrate and Egg White Powder as Structuring Agents of Double Emulsions for Food Applications.** *Food Bioprocess Technol.* 2020; **13**: 1154–1165.
Publisher Full Text

86. Liu L, *et al.*: **Preparation of monodisperse calcium alginate microcapsules via internal gelation in microfluidic-generated double emulsions.** *J Colloid Interface Sci.* 2013; **404**: 85–90.
PubMed Abstract | Publisher Full Text

87. Flaiz L, Freire M, Cofrades S, *et al.*: **Comparison of simple, double and gelled double emulsions as hydroxytyrosol and n-3 fatty acid delivery systems.** *Food Chem.* 2016; **213**: 49–57.
PubMed Abstract | Publisher Full Text

88. Delample M, Da Silva F, Leal-Calderon F: **Osmotically driven gelation in double emulsions.** *Food Hydrocoll.* 2014; **38**: 11–19.
Publisher Full Text

89. Pilkington EH, Suys EJA, Trevaskis NL, *et al.*: **From influenza to COVID-19: Lipid nanoparticle mRNA vaccines at the frontiers of infectious diseases.** *Acta Biomater.* 2021; **131**: 16–40.
PubMed Abstract | Publisher Full Text | Free Full Text

90. Schoenmaker L, Witzigmann D, Kulkarni JA, *et al.*: **mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability.** *Int J Pharm.* 2021; **601**: 120586.
PubMed Abstract | Publisher Full Text | Free Full Text

91. O'Grady D, Barrett M, Casey E, *et al.*: **The effect of mixing on the metastable zone width and nucleation kinetics in the anti-solvent crystallization of benzoic acid.** *Chem Eng Res Des.* 2007; **85**(7): 945–952.
Publisher Full Text

92. Repáková J, Holopainen JM, Karttunen M, *et al.*: **Influence of Pyrene-Labeling on Fluid Lipid Membranes.** *J Phys Chem B.* 2006; **110**(31): 15403–15410.
PubMed Abstract | Publisher Full Text

93. Etienne G, Vian A, Biočanin M, *et al.*: **Cross-talk between emulsion drops: How are hydrophilic reagents transported across oil phases?** *Lab Chip.* 2018; **18**(24): 3903–3912.
PubMed Abstract | Publisher Full Text

94. Zhang W, Abbaspourrad A, Chen D, *et al.*: **Osmotic Pressure Triggered Rapid Release of Encapsulated Enzymes with Enhanced Activity.** *Adv Funct Mater.* 2017; **27**(29): 1700975.
Publisher Full Text

95. Giuliano B, Camila A, Jessica M, *et al.*: **Stability characterization of microfluidic lipid-stabilized double emulsions under physiologically-relevant conditions [Data set]**. *Zenodo*. 2022.
http://www.doi.org/10.5281/zenodo.6866211

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Shima Saffarionpour
University of Toronto, Toronto, Ontario, Canada

The manuscript discusses the application of microfluidics for the preparation of lipid-based double emulsions for the delivery of hydrophobic fluorescent dyes or Large unilamellar vesicles. Although the topic of the manuscript is interesting, the authors should pay specific attention to discussing their results. In some sections, the observations are just reported without the inclusion of additional discussions on the presented results and figures.

I have the following comments for the authors:

1. In the abstract section, what do authors mean here by "DEs are only kinetically trapped"? Do the authors intend to mention DEs are kinetically stable?

2. In the fourth paragraph of the introduction section, “the American Federal Drug Agency (FDA)”, FDA is the abbreviation for “Food and Drug Administration” and not federal drug agency. It should change to “the Food and Drug Administration (FDA) federal agency”

3. In the section “Encapsulation of compounds of interest”, provide the complete term for the abbreviation POPC.

4. In the section “Stability assays”, the sentence: “Emulsions larger than 0.1 μm are only kinetically stable, breaking or coalescing over time”

   Add a brief explanation to the main text for an inexpert reader, what "kinetic stability" means and why emulsions larger than 0.1 μm are not thermodynamically stable.

5. In the section, “DE stability at different temperatures over time”, comparing Figures 2a, and b, can you add an additional explanation on why a higher number of DEs were produced at RT compared with 4 degree C on Day 1 of the preparation?

6. In the last paragraph of the similar section, please add a brief explanation on why the decrease percentage was higher at 37 °C compared with the other tested temperatures.

7. In the section “DE stability at different pH”, referring to the sentence: “when compared to pH 7 (Figure 5.a). The size was also not affected (Figure 5.b). “

   Please comment and also add it to the text why a decrease in the percentage of remaining DEs was observed for pH 1 and 13 while no significant difference in the measured droplet sizes was observed in Figure 5b? It is expected that when the number of droplets decreases (reduction in remaining DE%) droplet size also enlarges.

8. Referring to the last sentences of this section, can you add an additional explanation on why a higher percentage of DE remained at acidic pH=2 for DiI in the intermediate phase compared to LUVs? Why do the percentages seem so different for DiI comparing pH 1 and 2?

9. On page 10, second paragraph, referring to the sentence:” LUV-loaded DEs were consistently more sensitive than the other two formulations in all tested conditions in this work” Can you add additional information on the probable reason?

   It is also mentioned in page 8, in the sentences:

   “DEs loaded with LUVs presented a similar behaviour for 14 mbar and 20 mbar, but were considerably more sensitive to the pressure of 30 mbar, with only around 35% of the initial number of DEs remaining by the last cycle.”

   But no specific reason is given and additional discussion is not provided. The authors should consider elaborating on the reason based on their observations.

10. In the section “Stability of unloaded and loaded DEs under mechanical stimulus”

    Please add additional information on why DEs loaded with LUV were selected for this study and not the ones with DiI. It would have been more informative if we could compare the results for both DiI and LUV loaded in DEs in Figure 6.

11. On page 10, the sentence: “Octanol was intended to stay in the intermediate layer as part of the oral delivery system because it is an FDA-approved food additive that has already been studied for topical delivery systems”

    The authors mention FDA approval of octanol as a food additive and bring examples of its application for topical delivery. Please bring examples of its applications in formulating foods.

12. On page 10, referring to the sentence: “The main reason for this might be the hypertonic inner phase.”

    Please add additional information on what does "hypertonic inner phase" mean.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Encapsulation; Emulsification; Controlled delivery; Bioactive ingredients

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 05 September 2022

https://doi.org/10.21956/openreseurope.15949.r29998

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Julien Petit
Hochschule Furtwangen University, Schwarzwald, Germany

Report for the manuscript: "Stability characterization of microfluidics lipid-stabilized double emulsions under physiologically-relevant conditions" by Giuliano *et al.*

The authors report on the fabrication and especially on the stability characterization of lipid-stabilized water-in-oil-water double-emulsions DEs (i.e. without the use of surfactant molecules). The DEs are produced using a PDMS-based microfluidic chip and an easy-to-handle method which are well-established in the literature. The authors highlight the high monodispersed character of the DEs produced with this method (which is not new) and present a detailed study on the stability of the DEs under different conditions which are not coupled (temperature, pH, mechanical stress).

Furthermore, the authors demonstrate the reliable and easy-to-perform encapsulation efficiency of such DEs using the microfluidic platform (which is also not really new). They especially report on how the DE stability is impacted depending on the type of encapsulated species and this in regards to potential future applications in drug delivery systems.

The report is well written in good scientific English and the results are interesting, especially concerning the stability study. However, I have some comments and the manuscript should be improved following these comments before being accepted for indexing. I listed my different comments/questions in the following.

- *p. 4, "Microfabrication of PDMS chips", first paragraph:* This is not really well described and written in order to allow the reader to easily reproduce what the authors did. I would suggest describing a little more in detail the process rather than referring to published articles because I know from experience that in the domain of DE production, the experimental procedure always differs a little bit from scientist to scientist or from research group to research group! A sketch of the microfluidic device with the dimensions could be helpful in order to illustrate the text.

- *p. 4, "Microfluidic production of double emulsions", second paragraph:* Did the authors measure or calculate the flow rates of the fluid phases in the channels? This information could be interesting because it is way more often employed in microfluidic experiments rather than pressures.

- *p. 4, "Image analysis", first paragraph:* What is the frame rate used? - "The parameters of the script... variances" not clear and needs re-writing.

- *p. 4, "Image analysis", second paragraph:* Why the authors are counting the DEs manually and not using the Python script they developed for image analysis?

- *p. 4, "Encapsulation of compounds of interest", first paragraph:* the authors wrote "higher concentration of solute in the inner phase than in the outer phase", what about the osmolarity issues in this case? Did the authors check that and if not this should be interesting to take into consideration.

- *p. 4, "Encapsulation of compounds of interest", third paragraph:* "The actual number of produced DEs... for 200 frames." not clear and needs re-writing.

- *p. 4, "Stability assays", first paragraph:* What is about statistics? Are the results presented later reliable and robust? How many times did the authors the measurements in order to establish an average counting? - "0.4 mm height", what volume of sample solution was analyzed? Were the DEs squeezed or mechanically deformed during the transfer to the visualization chamber? Because this could affect the final amount/concentration of DEs observed. - "n $\geq$ 3", what is "n"?

- *p. 4, "Stability of unloaded and loaded DEs at different mechanical stress conditions", first paragraph:* Here it would be helpful to have an illustration of the system in order to directly understand how the produced DEs are tested under mechanical stress.

- *p. 5, "Stability of unloaded and loaded DEs at different mechanical stress conditions", second*

paragraph: Were the DEs of comparable sizes? Because this also affects the mechanical properties of the membranes. And what is the viscosity influence of the inner phase?

- *p. 5, "Results", end of first paragraph:* In figure 1.a.II, at the first junction, a jetting regime is presented and not a dripping mode. That means that the control of the DE size is mainly done by the shear stress at the second junction of the microfluidic chip, by the OA. In this case, the reliability of the DE process could be questioned. A higher control and more versatility in terms of size distribution and oil layer thickness would be reached by controlling as well the shear stress at the first junction of the chip. Did the authors try to control the first pinch-off and if yes, what are the results?

- *p. 5, "Results", second paragraph:* Does this correspond to only one batch (i.e. one experiment) or does it represent a statistical study on different samples taken with the exact same experimental parameters (same microfluidic chip, same chemical composition of the fluid phases and flow rates)? This information should be given for more clarity.

- *p. 8, "Environmental conditions affect loaded and unloaded DEs differently", second paragraph:* It exists microfluidic designs for the trapping of DEs (or vesicles) in order to study their characteristics and stability *in-situ*. See for example the work of T. Robinson and co-workers[1]

- *p. 10, "Cargo has considerable effect on long-term DE stability", second paragraph:* "This impact is demonstrated by studies that varied the shell thickness of core-shell micropartices, templated from double emulsions." This is correlated to a previous comment from my side. Did the authors try to control the thickness of the shell in order to address such issues?

For all the reasons listed above, I cannot recommend indexing this paper in its current state.

References

1. Yandrapalli N, Robinson T: Ultra-high capacity microfluidic trapping of giant vesicles for high-throughput membrane studies. *Lab on a Chip*. 2019; **19** (4): 626-633 Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and does the work have academic merit?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Physicist with more than 4 years research experience in the domain of double-emulsion and vesicle formation in microfuidic environment.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.