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Single-step microfluidic production of W/O/W double emulsions as templates for β-carotene-loaded giant liposomes formation

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ABSTRACT

We demonstrated the microfluidic production of W/O/W double emulsion droplets aiming formation of β-carotene-incorporated giant liposomes for food and/or pharmaceutical applications. For this purpose, glass-capillary microfluidic devices were fabricated to create a truly three-dimensional flow aiming production of giant unilamellar liposomes by solvent evaporation process after W/O/W double emulsion droplet templates formation. A great challenge of microfluidic production of monodisperse and stable W/O/W double emulsion templates for this proposal is the
replacement of organic solvents potentially toxic for phospholipids dissolution. Besides, the high cost of several semi-synthetic phospholipids commonly used for giant liposome formation remains as a major technological challenge to be overcome. Thus, β-carotene-incorporated giant liposomes were generated using biocompatible solvents with low toxic potential (ethyl acetate and pentane) and non-purified soybean lecithin - a food-grade phospholipid mixture with low cost - by dewetting and evaporation of the solvents forming the oily intermediate phase of W/O/W double emulsion droplet templates. Our results showed monodisperse β-carotene-loaded giant liposomes with diameter ranging between 100 μm and 180 μm and a stability of approximately 7 days. In this way, a single-step microfluidic process with highly accurate control of size distribution was developed. This microfluidic process proposed is potentially useful for a broad range of applications in protection and delivery of active compounds.

Keywords: microfluidic; glass-capillary; soybean lecithin; solvents.

1. INTRODUCTION

Giant unilamellar vesicles (GUVs), which are aqueous volumes surrounded by single or multiple bilayers of phospholipid molecules, are ideal candidates as encapsulation systems for food and/or pharmaceutical active compounds due to the phospholipids biocompatibility. However, many of these encapsulation systems have low efficiency, making them infeasible and costly for industrial applications. For instance, the encapsulation of an active compound into GUVs through a bulk conventional emulsification method using high shear mixing conditions results in a quite low encapsulation efficiency, generally less than 35% [1]. The high cost and low efficiency make GUVs infeasible for certain applications. Moreover, the size and properties of these microparticles produced from bulk methods vary vastly. Thus, bulk methods do not yield homogeneous samples that is a desired feature for applications.
aiming at controlling release. Recently, microfluidics has made great advances in solving these problems of inefficiency and heterogeneity [2]. For example, water-in-oil-in-water (W/O/W) double emulsions have been showed great potential as templates for preparing biocompatible systems [3-6], such as solid lipid microcapsules [7], polymersomes [8] and giant liposomes [1, 9-14], for the encapsulation of food and/or pharmaceutical active compounds. Unfortunately, most of these microfluidic approaches have relied on using expensive phospholipids that are costly for food and pharmaceutical applications and involved toxic solvents in the production process that make these applications improbable. Thus, it is crucial to develop an alternative microfluidics approach that can encapsulate active compounds with high efficiency using low cost phospholipids and biocompatible solvents.

In this study, we report a high-throughput microfluidic method for fabricating GUVs using low cost, food-grade phospholipids and FDA-approved toxicological class III solvents. Firstly, we assembled a glass-capillary microfluidic device for producing ultrathin shell double emulsion templates (W/O/W) [3]. Next, lipids were dissolved in FDA-approved solvents, ethyl acetate and pentane. To further demonstrate these phospholipid vesicles as food and pharmaceutical active compound carriers, we dissolve β-carotene together with the phospholipids in the organic phase [15-16]. The aqueous cores surrounded by oil shells composed of phospholipids and organic solvents were then produced from the glass-capillary microfluidic device. After the double emulsions were collected, phospholipid vesicles were formed as the organic solvents dewet from the water-oil (W/O) interfaces and lipid bilayers self-assembled. Here, the mixture of a good lipid solvent, ethyl acetate, and a bad lipid solvent, pentane, helped the oil phase dewet fast from the water-oil interfaces and enabled bilayer formation with little amount of residual solvents. We observed that these GUVs have survived for at least 7 days,
making them great candidates as economic and biocompatible food and/or pharmaceutical carrier systems for industrial applications.

2. MATERIALS AND METHODS

2.1 Materials

The W/O/W double emulsion templates were obtained using a food-grade soybean lecithin powder (>45% w/w phosphatidylcholine, 10-18% w/w phosphatidylethanolamine, <4% w/w lysophosphatidylcholine and <3% w/w triglycerides), commercially named Lipoid S45 (Lipoid GmbH, Ludwigshafen, Germany); synthetic \(\beta\)-carotene powder (>93% w/w), pentane (99.8% v/v) and poly(vinyl alcohol) (PVA, molecular weight 13-23 kDa, 87-89% hydrolyzed) supplied from Sigma-Aldrich (St. Louis, MO, USA); sucrose (analytical-grade) and hexane (>98.5% v/v) both purchased from BDH Chemicals Ltd. (Poole, Dorset, UK); dextran (molecular weight 70 kDa, TCI Chemical Industry Co., Tokyo, Japan); chloroform (99.8% v/v, Alfa-Aesar, Ward Hill, MA, USA) and ethyl acetate (99.9% v/v, Honeywell, Muskegon, MI, USA). The microfluidic devices were obtained using cylindrical (inner and outer diameters 0.58 mm and 1 mm, respectively) and square (inner dimension 1.05 mm) glass capillaries acquired from World Precision Instruments, Inc. (Sarasota, FL, USA) and Atlantic International Technology Inc. (Rockaway, NJ, USA), respectively. Besides, polyethylene tubing of inner diameter 0.86 mm (Scientific Commodities, Inc.; Lake Havasu City, AZ, USA), stainless steel dispensing needles of inner and outer diameters 0.66 mm and 0.91 mm, respectively (McMaster-Carr, Atlanta, GA, USA) and 5-minute Epoxy\textsuperscript{®} (Devcon Corp., Danvers, MA, USA) were also used. Glass capillaries were treated using 2-

[methoxy(polyethylenoxy)6-9 propyl]trimethoxysilane (Gelest, Inc.; Morrisville, PA, USA) and trimethoxy(octadecl)ysilane (90% w/v, Sigma-Aldrich, St. Louis, MO, USA).
2.2 Methods

Fabrication of the glass capillary device

The capillary devices were built on a glass slide, and consisted of two glass cylindrical capillaries inserted into the opposite ends of a square capillary, according to reported by Utada et al. [2]. Briefly, the cylindrical glass capillaries were tapered to an inner diameter of approximately 20 µm with a micropipette puller (model P-97, Sutter Instrument, Co.; San Francisco, CA, USA), and then the tips were carefully sanded to final inner diameters approximately of 60 and 150 µm. The cylindrical tube with smaller inner diameter was treated with trimethoxy(octadecyl)silane for 1 h to render a hydrophobic surface, and the larger diameter tube with 2-[methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane for approximately 15 min to render a hydrophilic surface. The hydrophobic tube was used as the injection capillary, and the hydrophilic tube was used as the collection capillary. The device was assembled onto a glass microscope slide. For this, the square capillary was fixed to the slide with 5-minute Epoxy®. After, the cylindrical tubes were inserted into the square tubing at both ends, which enabled the alignment of the axes of the injection and collection capillaries, maintaining a separation distance between them of approximately 60 µm, according to shown in Figure 1 (a). For injection of aqueous innermost phase, a third cylindrical capillary was stretched with a burner to an outer diameter of approximately 200 µm, and inserted into the injection capillary. Finally, dispensing needles were placed at the junctions between capillaries or their ends, and fix them to the slide with 5-minute Epoxy®.

Generation of W/O/W double emulsion templates

The W/O/W double emulsion templates were obtained using an innermost aqueous phase containing 1% (w/v) PVA and 9% (w/v) dextran. The phospholipid middle phase consisted of a mixture of 0.5% (w/v) soybean lecithin and 0.125% (w/v)
β-carotene dissolved in the following organic solvent mixtures (1:1.8 v/v):
chloroform/hexane; ethyl acetate/hexane or ethyl acetate/pentane. Besides, the
continuous phase used in this study was an aqueous solution 10% (w/v) PVA. The
innermost, middle lipid, and continuous phases flowed into the microfluidic device
through connection of glass micro-syringe needles to the dispensing needles of the
device with polyethylene tubing using three syringe pumps (model PHD 2000, Harvard
Apparatus, Inc.; South Natick, MA, USA). The innermost (q₁) and middle oil (q₂)
phases were injected in stretched tube and cylindrical tube with smaller inner diameter,
respectively, at a flow rate 1000 μl/h, according to Figure 1. At the same time, the
continuous phase (q₃) flowed through the interstices between the cylindrical tapered
capillary and the square capillary, at a flow rate ranging between 3000 and 12000 μl/h.
The droplets were collected in a 50 mM sucrose solution, in order to adjust the
osmolarity between the innermost phase, continuous phase and collection solution to 50
mOsm/l evaluated by a micro-osmometer (model 3300, Advanced Instruments, Inc.;
Norwood, MA, USA). All experiments were performed at room temperature and the
process was operated in the discontinuous dripping regime, in which the formation of
W/O/W double and O/W single emulsions were monitored within the microfluidic
device using an 5× objective on an inverted microscope (model DM IRB, Leica
Microsystem; Mannheim, Germany) equipped with a high speed camera (model
Phantom v9.0, Vision Research; Wayne, NJ, USA).

Characterization of giant liposomes

Bright field and fluorescence images were obtained with a 10× objective on an
inverted fluorescence confocal microscope (model DM IRBE, Leica Microsystem;
Mannheim, Germany) at room temperature. For this, Argon (458 nm) laser was used as
excitation source and, the fluorescence emission was collected by the PMT detectors
through band pass filters between 488 and 543 nm for β-carotene. Besides, the contrast provided by the presence of dextran and PVA in the inner core of the GUVs allowed to visualize them in the bright field. Approximately 20 bright field micrographs were used to determine the particle size distribution based on diameter measurements of 200 droplets using the open-source software ImageJ (version Java 1.6.0_24, National Institutes of Health, Bethesda, MD, USA). The particle size was expressed in terms of mean diameter, while the polydispersity of the system was expressed in terms of coefficient of variation (CV), which relates standard deviation (sd) to mean diameter. Besides, the bright field images were also used to estimate relative kinetic stability by counting the GUVs number as a function of time.

3. RESULTS AND DISCUSSION

3.1 Formation of W/O/W emulsion templates

The W/O/W emulsion templates were successfully prepared using the glass-capillary device and soybean lecithin by single-step process. This process configuration forces the water droplets to become re-emulsified leading to formation of monodisperse W/O/W emulsion droplets with an ultrathin middle oil phase at the orifice of the capillary collection tube, as shown in Figure 1 (a,b) and Video 1 of the Supplementary Material. The process was operated in the discontinuous dripping regime, producing intermittently O/W single and W/O/W double emulsion. The W/O/W double and O/W single droplets were separated by density difference between them. The O/W single droplets coalesced and floated to the top of collection flask, which facilitated the oil separation. Meanwhile, the W/O/W double emulsion droplets rapidly sank because they are heavier than the collection solution. The micrograph in the Figure 1 (d) obtained from inverted microscope shows the GUVs formed in the bottom of glass flask collection. In dripping regime, the breakup of droplets is governed by the balance
between the interfacial tension that constrains the droplet to the tip of the tapered tube and the viscous forces exerted by the continuous phase that pulls the droplet downstream. Therefore, droplets detachment is proportional to the viscosity of the continuous phase, but mainly to the velocity difference between the continuous and oil phase. Thus, an accurate control of W/O/W droplet diameter generation was observed by finely tuning the flow rate of continuous phase, as shown in Figure 1 (b) and Figure 2 (a-c). The diameter of W/O/W emulsion droplets decreased with increasing flow rate of continuous phase, which ranged between approximately 100 and 180 μm for all solvent mixtures. Besides, the W/O/W emulsion exhibited high uniformity with coefficients of variation in the range of ~3.0-6.0%.

Production of W/O/W double emulsion templates with an ultrathin middle oil phase is directly associated to the design of glass-capillary devices and mainly to chemical functionalization of the glass-capillary surfaces. Such ability is not specific to a specific choice of organic phase composition or flow rates, provided that the inner and outer phases viscosity shows an adequate ratio and the device is operated in the discontinuous dripping regime [9]. The GUVs formation was only possible using W/O/W double emulsion templates from utilization of a mixture of good and bad volatile solvents of lipids with different solubility in water. Good volatile solvents, such as, chloroform and ethyl acetate, allows phospholipids to remain fully dissolved during double emulsion formation. At the same time, chloroform and ethyl acetate are more soluble in water than the bad volatile solvents, such as hexane and pentane, allowing a faster dissolution of the good solvents and a reduction of lipid solubility, triggering the oil phase to dewet from soybean lecithin more easily. This dewetting process facilitates the attractive interaction between the two layers covered by soybean lecithin at the interface of the ultrathin shell, according to shown Figure 1 (c). The phospholipids can
adsorb to the interfaces of the inner core, ultrathin shell, and the outer aqueous phase, reducing the overall interfacial energy [9]. The thickness of ultrathin shell shows generally a few micrometers, as shown in Figures 1 (b,d) and 3, much smaller than in typical double emulsion obtained by conventional methods; this enables the fabrication of giant liposomes containing minimal residual solvent within their structure.

Figure 2 (a-c) shows the particles size distribution of GUVs produced with different solvents mixture. According to Figure 2 (c) the giant liposome size obtained using ethyl acetate and pentane mixture showed a linear relationship as function of increasing continuous flow rates (9000 and 12000 μl/h). The viscosity values for pure solvents in mPa.s at 20°C (hexane: 0.33; chloroform: 0.56; ethyl acetate: 0.45 and pentane: 0.22) [17] were used to predict the viscosity of solvents mixture. Therefore, the mixtures showed the following order of viscosity: chloroform/hexane > ethyl acetate/hexane > ethyl acetate/pentane. Droplets breakup in capillary microfluidic devices occurs if the viscous forces exerted by the continuous phase - that pulls the droplet downstream - exceed the pinning forces arising from the interfacial tension - that grows and constrains the droplet at the tip of the injection capillary tube [18]. Thus, the result using ethyl acetate and pentane mixture can be related with its lower viscosity in comparison to other mixtures, reducing the viscous forces, making easier the droplets detachment and improving size control.

Thus, it was possible to confirm that single-step microfluidic production of W/O/W emulsion droplet templates aiming giant liposomes formation was efficient using solvents with lower toxicity potential to human health, such as ethyl acetate and pentane, replacing solvents as chloroform and hexane, commonly used in these processes. The choice of solvent for the industrial processing must take into account...
international regulations regarding the safety of consumers as well as the minimization of production costs. The organic solvents ethyl acetate and pentane are classified as Generally Recognized as Safe (GRAS) according to the US Food and Drug Administration (toxicological class III) and could be used in food and pharmaceutical applications [19-21]. Besides, the technical feasibility of using low-cost and non-purified food-grade phospholipids was successfully demonstrated for GUVs production.

3.2 β-carotene-incorporated giant liposomes formation

Figure 3 shows that β-carotene was successfully incorporated inside phospholipid ultrathin shell for all solvent mixtures, which was observed due to intrinsic fluorescence of β-carotene. The β-carotene location was restricted only to the lipid membrane and regulated by van der Waals interactions with the fatty acid chains, because of the absence of polar groups in its structure [15]. The confocal micrographs and particle size distributions indicated that giant liposomes were highly monodisperse. It is possible to observe that presence of β-carotene inside oil shell did not affect significantly the mean diameter and coefficients of variation (Figure 3 a-c) when compared to liposomes without β-carotene (Figure 2 a-c). Within approximately 10 days, all giant liposomes were disrupted as shown in the Figure 4 (a). Breaking kinetic profile of giant liposome rupture obtained in the presence and absence of β-carotene using ethyl acetate and pentane mixture showed similar behavior, as shown in Figure 4 (b). Relative stability can be observed in micrographs for the incorporated β-carotene-loaded GUVs (Figure 4 c). Our results indicate that solvent-type and β-carotene presence did not exert significant influence on the stability of giant liposomes obtained from soybean lecithin. Probably, the stability was achieved due to the same osmolarity of the inner, continuous and collection aqueous solutions. Such good stability can be also related to the significantly increased shear stress on the innermost droplet because
of the lubrication effect associated to a very thin width of the middle phase [11]. The lubricant effect between aqueous phases, continuous and innermost is only possible due to the ultrathin oily intermediate phase of the W/O/W double emulsion, since the drag force between aqueous phases is minimized which reduces the coalescence phenomenon. Thus, the ultrathin middle layer provides stability to the W/O/W double emulsion droplets, preventing coalescence between them. The same stability is otherwise difficult to achieve using W/O/W double emulsion templates with oil phase of wider thickness.

4. CONCLUSION

We demonstrated the high-throughput production of economic, food-grade phospholipid vesicles through a double emulsion glass-capillary microfluidic device using entirely FDA-approved class III solvents. The selected food-grade phospholipid, soybean lecithin, is cheaper than other phospholipids commonly used in food and pharmaceutical applications. Moreover, the chosen organic solvent mixtures, ethyl acetate and pentane, are more biocompatible than the solvents that have been used in previous works of microfluidic fabrication of GUVs. Furthermore, we observed controlled size distribution and good stability for 7 days. In addition, we demonstrated β-carotene incorporation in the lipid shells, confirming that GUVs, which are generally used to encapsulate hydrophilic compounds, can be used to load β-carotene and might be extended for incorporating other hydrophobic molecules. Consequently, our approach of fabricating food-grade phospholipid vesicles could also be potentially useful for a broad range of applications in protection and delivery of food and pharmaceutical active compounds.
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FIGURE CAPTIONS

Figure 1. (a) Microfluidic production of W/O/W double emulsion droplet templates with ultrathin shells containing β-carotene, according to proposed by Arriaga et al. [9]; (b) Optical microscope images of microfluidic process using different solvent mixtures at continuous flow rates ($q_3$) ranging from 3000 µl/h to 12000 µl/h at flow rate of innermost ($q_1$) and middle lipid ($q_2$) phases equal 1000 µl/h; (c) Diagram of organic solvent extraction process for GUVs formation; (d) Inverted optical microscope images of monodisperse W/O/W double emulsion droplet templates in the bottom of collection solution.

Figure 2. Influence of the continuous flow rate ($q_3$) on GUVs diameter distribution (where green, red, blue and black bars represent 3000 µl/h, 6000 µl/h, 9000 µl/h and 12000 µl/h, respectively, at flow rate of innermost ($q_1$) and middle lipid ($q_2$) phases equal 1000 µl/h using different organic solvent mixtures (1:1.8 v/v): (a) chloroform/hexane; (b) ethyl acetate/hexane and (c) ethyl acetate/pentane.

Figure 3. Confocal micrographs of β-carotene-loaded giant unilamellar liposome at flow rate of innermost ($q_1$), middle lipid ($q_2$) and continuous ($q_3$) phases equal 1000 µl/h, 1000 µl/h and 12000 µl/h, respectively, using different organic solvent mixtures (1:1.8 v/v): (a) chloroform/hexane; (b) ethyl acetate/hexane and (c) ethyl acetate/pentane.

Figure 4. Stability of GUVs obtained at flow rate of innermost ($q_1$), middle lipid ($q_2$) and continuous ($q_3$) phases equal 1000 µl/h, 1000 µl/h and 12000 µl/h, respectively. (a)
Stability time of GUVs obtained using chloroform/hexane mixture (empty bars) and ethyl acetate/pentane mixture (filled bars); (b) Fraction of unruptured GUVs as a function of time using ethyl acetate/pentane in presence of β-carotene (circle) and absence of β-carotene (square); (c) Optical microscopy as a function of time of GUVs obtained using ethyl acetate/pentane in presence of β-carotene, where the scale bar denotes 100 µm.

REFERENCES


Figure 1.

(a) Hydrophobic → Hydrophilic

(b) $q_3 = 3000 \, \mu l/h$
$q_3 = 6000 \, \mu l/h$
$q_3 = 9000 \, \mu l/h$
$q_3 = 12000 \, \mu l/h$

(c) Solvent extraction
W/O/W double emulsion template
β-carotene-incorporated giant unilamellar liposome

(d) 200 µm
Figure 2.
Figure 3.
Figure 4.

Highlights

- W/O/W emulsion templates were successfully produced using soybean lecithin;
- Ethyl acetate/pentane mixture can be used aiming at the W/O/W emulsions formation;
- β-carotene was incorporated inside phospholipid shell forming giant liposome;
- β-carotene-incorporated giant liposomes can to applied in aqueous formulations.