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Mariano Michelon, Yuting Huang, Lucimara Gaziola de la Torre, David A. Weitz, Rosiane Lopes Cunha

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

3 Mariano Michelon[1,§], Yuting Huang[2,§], Lucimara Gaziola de la Torre[3], David A. Weitz[2]
4 and Rosiane Lopes Cunha[1]

5 [1] Department of Food Engineering, School of Food Engineering, University of
6 Campinas, Campinas 13083862, SP, Brazil

7 [2] Department of Physics, John A. Paulson School of Engineering and Applied Sciences,
8 Harvard University, Cambridge 02138, MA, USA

9 [3] Department of Materials and Bioprocess Engineering, School of Chemical
10 Engineering, University of Campinas, Campinas 13083852, SP, Brazil

11 [§] Both authors contributed equally to this work

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13 Corresponding author (Mariano Michelon):
14 *E-mail*: michelon@lmmp.mec.puc-rio.br fax: +55 (19) 35214047
15 Yuting Huang: yutingh@seas.harvard.edu
16 Lucimara Gaziola de la Torre: latorre@feq.unicamp.br
17 David A. Weitz: weitz@seas.harvard.edu
18 Rosiane Lopes Cunha: rosiane@unicamp.br

19

20 **ABSTRACT**

21 We demonstrated the microfluidic production of W/O/W double emulsion droplets
22 aiming formation of β-carotene-incorporated giant liposomes for food and/or
23 pharmaceutical applications. For this purpose, glass-capillary microfluidic devices were
24 fabricated to create a truly three-dimensional flow aiming production of giant
25 unilamellar liposomes by solvent evaporation process after W/O/W double emulsion
26 droplet templates formation. A great challenge of microfluidic production of
27 monodisperse and stable W/O/W double emulsion templates for this proposal is the

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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

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53 aiming at controlling release. Recently, microfluidics has made great advances in
54 solving these problems of inefficiency and heterogeneity [2]. For example, water-in-oil-
55 in-water (W/O/W) double emulsions have been showed great potential as templates for
56 preparing biocompatible systems [3-6], such as solid lipid microcapsules [7],
57 polymersomes [8] and giant liposomes [1, 9-14], for the encapsulation of food and/or
58 pharmaceutical active compounds. Unfortunately, most of these microfluidic
59 approaches have relied on using expensive phospholipids that are costly for food and
60 pharmaceutical applications and involved toxic solvents in the production process that
61 make these applications improbable. Thus, it is crucial to develop an alternative
62 microfluidics approach that can encapsulate active compounds with high efficiency
63 using low cost phospholipids and biocompatible solvents.

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

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78 making them great candidates as economic and biocompatible food and/or
79 pharmaceutical carrier systems for industrial applications.

80 **2. MATERIALS AND METHODS**

81 **2.1 Materials**

82 The W/O/W double emulsion templates were obtained using a food-grade
83 soybean lecithin powder (>45% w/w phosphatidylcholine, 10-18% w/w
84 phosphatidylethanolamine, <4% w/w lysophosphatidylcholine and <3% w/w
85 triglycerides), commercially named Lipoid S45 (Lipoid GmbH, Ludwigshafen,
86 Germany); synthetic β-carotene powder (>93% w/w), pentane (99.8% v/v) and
87 poly(vinyl alcohol) (PVA, molecular weight 13-23 kDa, 87-89% hydrolyzed) supplied
88 from Sigma-Aldrich (St. Louis, MO, USA); sucrose (analytical-grade) and hexane
89 (>98.5% v/v) both purchased from BDH Chemicals Ltd. (Poole, Dorset, UK); dextran
90 (molecular weight 70 kDa, TCI Chemical Industry Co., Tokyo, Japan); chloroform
91 (99.8% v/v, Alfa-Aesar, Ward Hill, MA, USA) and ethyl acetate (99.9% v/v,
92 Honeywell, Muskegon, MI, USA). The microfluidic devices were obtained using
93 cylindrical (inner and outer diameters 0.58 mm and 1 mm, respectively) and square
94 (inner dimension 1.05 mm) glass capillaries acquired from World Precision
95 Instruments, Inc. (Sarasota, FL, USA) and Atlantic International Technology Inc.
96 (Rockaway, NJ, USA), respectively. Besides, polyethylene tubing of inner diameter
97 0.86 mm (Scientific Commodities, Inc.; Lake Havasu City, AZ, USA), stainless steel
98 dispensing needles of inner and outer diameters 0.66 mm and 0.91 mm, respectively
99 (McMaster-Carr, Atlanta, GA, USA) and 5-minute Epoxy® (Devcon Corp., Danvers,
100 MA, USA) were also used. Glass capillaries were treated using 2-
101 [methoxy(polyethyleneoxy)6-9 propyl]trimethoxysilane (Gelest, Inc.; Morrisville, PA,
102 USA) and trimethoxy(octadecyl)silane (90% w/v, Sigma-Aldrich, St. Louis, MO, USA).

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103 **2.2 Methods**

104 **Fabrication of the glass capillary device**

105 The capillary devices were built on a glass slide, and consisted of two glass
106 cylindrical capillaries inserted into the opposite ends of a square capillary, according to
107 reported by Utada et al. [2]. Briefly, the cylindrical glass capillaries were tapered to an
108 inner diameter of approximately 20 μm with a micropipette puller (model P-97, Sutter
109 Instrument, Co.; San Francisco, CA, USA), and then the tips were carefully sanded to
110 final inner diameters approximately of 60 and 150 μm. The cylindrical tube with
111 smaller inner diameter was treated with trimethoxy(octadecyl)silane for 1 h to render a
112 hydrophobic surface, and the larger diameter tube with 2-[methoxy(polyethyleneoxy)6-
113 9 propyl]trimethoxysilane for approximately 15 min to render a hydrophilic surface.
114 The hydrophobic tube was used as the injection capillary, and the hydrophilic tube was
115 used as the collection capillary. The device was assembled onto a glass microscope
116 slide. For this, the square capillary was fixed to the slide with 5-minute Epoxy®. After,
117 the cylindrical tubes were inserted into the square tubing at both ends, which enabled
118 the alignment of the axes of the injection and collection capillaries, maintaining a
119 separation distance between them of approximately 60 μm, according to shown in
120 Figure 1 (a). For injection of aqueous innermost phase, a third cylindrical capillary was
121 stretched with a burner to an outer diameter of approximately 200 μm, and inserted into
122 the injection capillary. Finally, dispensing needles were placed at the junctions between
123 capillaries or their ends, and fix them to the slide with 5-minute Epoxy®.

124 **Generation of W/O/W double emulsion templates**

125 The W/O/W double emulsion templates were obtained using an innermost
126 aqueous phase containing 1% (w/v) PVA and 9% (w/v) dextran. The phospholipid
127 middle phase consisted of a mixture of 0.5% (w/v) soybean lecithin and 0.125% (w/v)

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128 β-carotene dissolved in the following organic solvent mixtures (1:1.8 v/v):
129 chloroform/hexane; ethyl acetate/hexane or ethyl acetate/pentane. Besides, the
130 continuous phase used in this study was an aqueous solution 10% (w/v) PVA. The
131 innermost, middle lipid, and continuous phases flowed into the microfluidic device
132 through connection of glass micro-syringe needles to the dispensing needles of the
133 device with polyethylene tubing using three syringe pumps (model PHD 2000, Harvard
134 Apparatus, Inc.; South Natick, MA, USA). The innermost (q_1) and middle oil (q_2)
135 phases were injected in stretched tube and cylindrical tube with smaller inner diameter,
136 respectively, at a flow rate 1000 μl/h, according to Figure 1. At the same time, the
137 continuous phase (q_3) flowed through the interstices between the cylindrical tapered
138 capillary and the square capillary, at a flow rate ranging between 3000 and 12000 μl/h.
139 The droplets were collected in a 50 mM sucrose solution, in order to adjust the
140 osmolarity between the innermost phase, continuous phase and collection solution to 50
141 mOsm/l evaluated by a micro-osmometer (model 3300, Advanced Instruments, Inc.;
142 Norwood, MA, USA). All experiments were performed at room temperature and the
143 process was operated in the discontinuous dripping regime, in which the formation of
144 W/O/W double and O/W single emulsions were monitored within the microfluidic
145 device using an 5× objective on an inverted microscope (model DM IRB, Leica
146 Microsystem; Mannheim, Germany) equipped with a high speed camera (model
147 Phantom v9.0, Vision Research; Wayne, NJ, USA).

148 **Characterization of giant liposomes**

149 Bright field and fluorescence images were obtained with a 10× objective on an
150 inverted fluorescence confocal microscope (model DM IRBE, Leica Microsystem;
151 Mannheim, Germany) at room temperature. For this, Argon (458 nm) laser was used as
152 excitation source and, the fluorescence emission was collected by the PMT detectors

153 through band pass filters between 488 and 543 nm for β-carotene. Besides, the contrast
154 provided by the presence of dextran and PVA in the inner core of the GUVs allowed to
155 visualize them in the bright field. Approximately 20 bright field micrographs were used
156 to determine the particle size distribution based on diameter measurements of 200
157 droplets using the open-source software ImageJ (version Java 1.6.0_24, National
158 Institutes of Health, Bethesda, MD, USA). The particle size was expressed in terms of
159 mean diameter, while the polydispersity of the system was expressed in terms of
160 coefficient of variation (CV), which relates standard deviation (sd) to mean diameter.
161 Besides, the bright field images were also used to estimate relative kinetic stability by
162 counting the GUVs number as a function of time.

163 **3. RESULTS AND DISCUSSION**

164 **3.1 Formation of W/O/W emulsion templates**

165 The W/O/W emulsion templates were successfully prepared using the glass-
166 capillary device and soybean lecithin by single-step process. This process configuration
167 forces the water droplets to become re-emulsified leading to formation of monodisperse
168 W/O/W emulsion droplets with an ultrathin middle oil phase at the orifice of the
169 capillary collection tube, as shown in Figure 1 (a,b) and Video 1 of the Supplementary
170 Material. The process was operated in the discontinuous dripping regime, producing
171 intermittently O/W single and W/O/W double emulsion. The W/O/W double and O/W
172 single droplets were separated by density difference between them. The O/W single
173 droplets coalesced and floated to the top of collection flask, which facilitated the oil
174 separation. Meanwhile, the W/O/W double emulsion droplets rapidly sank because they
175 are heavier than the collection solution. The micrograph in the Figure 1 (d) obtained
176 from inverted microscope shows the GUVs formed in the bottom of glass flask
177 collection. In dripping regime, the breakup of droplets is governed by the balance

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178 between the interfacial tension that constrains the droplet to the tip of the tapered tube
179 and the viscous forces exerted by the continuous phase that pulls the droplet
180 downstream. Therefore, droplets detachment is proportional to the viscosity of the
181 continuous phase, but mainly to the velocity difference between the continuous and oil
182 phase. Thus, an accurate control of W/O/W droplet diameter generation was observed
183 by finely tuning the flow rate of continuous phase, as shown in Figure 1 (b) and Figure
184 2 (a-c). The diameter of W/O/W emulsion droplets decreased with increasing flow rate
185 of continuous phase, which ranged between approximately 100 and 180 μm for all
186 solvent mixtures. Besides, the W/O/W emulsion exhibited high uniformity with
187 coefficients of variation in the range of ~3.0-6.0%.

188 Production of W/O/W double emulsion templates with an ultrathin middle oil
189 phase is directly associated to the design of glass-capillary devices and mainly to
190 chemical functionalization of the glass-capillary surfaces. Such ability is not specific to
191 a specific choice of organic phase composition or flow rates, provided that the inner and
192 outer phases viscosity shows an adequate ratio and the device is operated in the
193 discontinuous dripping regime [9]. The GUVs formation was only possible using
194 W/O/W double emulsion templates from utilization of a mixture of good and bad
195 volatile solvents of lipids with different solubility in water. Good volatile solvents, such
196 as, chloroform and ethyl acetate, allows phospholipids to remain fully dissolved during
197 double emulsion formation. At the same time, chloroform and ethyl acetate are more
198 soluble in water than the bad volatile solvents, such as hexane and pentane, allowing a
199 faster dissolution of the good solvents and a reduction of lipid solubility, triggering the
200 oil phase to dewet from soybean lecithin more easily. This dewetting process facilitates
201 the attractive interaction between the two layers covered by soybean lecithin at the
202 interface of the ultrathin shell, according to shown Figure 1 (c). The phospholipids can

203 adsorb to the interfaces of the inner core, ultrathin shell, and the outer aqueous phase,
204 reducing the overall interfacial energy [9]. The thickness of ultrathin shell shows
205 generally a few micrometers, as shown in Figures 1 (b,d) and 3, much smaller than in
206 typical double emulsion obtained by conventional methods; this enables the fabrication
207 of giant liposomes containing minimal residual solvent within their structure.

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

223 Thus, it was possible to confirm that single-step microfluidic production of
224 W/O/W emulsion droplet templates aiming giant liposomes formation was efficient
225 using solvents with lower toxicity potential to human health, such as ethyl acetate and
226 pentane, replacing solvents as chloroform and hexane, commonly used in these
227 processes. The choice of solvent for the industrial processing must take into account

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228 international regulations regarding the safety of consumers as well as the minimization
229 of production costs. The organic solvents ethyl acetate and pentane are classified as
230 Generally Recognized as Safe (GRAS) according to the US Food and Drug
231 Administration (toxicological class III) and could be used in food and pharmaceutical
232 applications [19-21]. Besides, the technical feasibility of using low-cost and non-
233 purified food-grade phospholipids was successfully demonstrated for GUVs production.

234 **3.2 β-carotene-incorporated giant liposomes formation**

235 Figure 3 shows that β-carotene was successfully incorporated inside
236 phospholipid ultrathin shell for all solvent mixtures, which was observed due to intrinsic
237 fluorescence of β-carotene. The β-carotene location was restricted only to the lipid
238 membrane and regulated by van der Waals interactions with the fatty acid chains,
239 because of the absence of polar groups in its structure [15]. The confocal micrographs
240 and particle size distributions indicated that giant liposomes were highly monodisperse.
241 It is possible to observe that presence of β-carotene inside oil shell did not affect
242 significantly the mean diameter and coefficients of variation (Figure 3 a-c) when
243 compared to liposomes without β-carotene (Figure 2 a-c). Within approximately 10
244 days, all giant liposomes were disrupted as shown in the Figure 4 (a). Breaking kinetic
245 profile of giant liposome rupture obtained in the presence and absence of β-carotene
246 using ethyl acetate and pentane mixture showed similar behavior, as shown in Figure 4
247 (b). Relative stability can be observed in micrographs for the incorporated β-carotene-
248 loaded GUVs (Figure 4 c). Our results indicate that solvent-type and β-carotene
249 presence did not exert significant influence on the stability of giant liposomes obtained
250 from soybean lecithin. Probably, the stability was achieved due to the same osmolarity
251 of the inner, continuous and collection aqueous solutions. Such good stability can be
252 also related to the significantly increased shear stress on the innermost droplet because

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253 of the lubrication effect associated to a very thin width of the middle phase [11]. The
254 lubricant effect between aqueous phases, continuous and innermost is only possible due
255 to the ultrathin oily intermediate phase of the W/O/W double emulsion, since the drag
256 force between aqueous phases is minimized which reduces the coalescence
257 phenomenon. Thus, the ultrathin middle layer provides stability to the W/O/W double
258 emulsion droplets, preventing coalescence between them. The same stability is
259 otherwise difficult to achieve using W/O/W double emulsion templates with oil phase
260 of wider thickness.

261 **4. CONCLUSION**

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

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279 **FIGURE CAPTIONS**

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

288 **Figure 2.** Influence of the continuous flow rate (q_3) on GUVs diameter distribution
289 (where green, red, blue and black bars represent 3000 µl/h, 6000 µl/h, 9000 µl/h and
290 12000 µl/h, respectively, at flow rate of innermost (q_1) and middle lipid (q_2) phases
291 equal 1000 µl/h) using different organic solvent mixtures (1:1.8 v/v): **(a)**
292 chloroform/hexane; **(b)** ethyl acetate/hexane and **(c)** ethyl acetate/pentane.

293 **Figure 3.** Confocal micrographs of β-carotene-loaded giant unilamellar liposome at
294 flow rate of innermost (q_1), middle lipid (q_2) and continuous (q_3) phases equal 1000
295 µl/h, 1000 µl/h and 12000 µl/h, respectively, using different organic solvent mixtures
296 (1:1.8 v/v): **(a)** chloroform/hexane; **(b)** ethyl acetate/hexane and **(c)** ethyl
297 acetate/pentane.

298 **Figure 4.** Stability of GUVs obtained at flow rate of innermost (q_1), middle lipid (q_2)
299 and continuous (q_3) phases equal 1000 µl/h, 1000 µl/h and 12000 µl/h, respectively. **(a)**

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300 Stability time of GUVs obtained using chloroform/hexane mixture (empty bars) and
301 ethyl acetate/pentane mixture (filled bars); **(b)** Fraction of unruptured GUVs as a
302 function of time using ethyl acetate/pentane in presence of β-carotene (circle) and
303 absence of β-carotene (square); **(c)** Optical microscopy as a function of time of GUVs
304 obtained using ethyl acetate/pentane in presence of β-carotene, where the scale bar
305 denotes 100 μm.

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Figure 1.

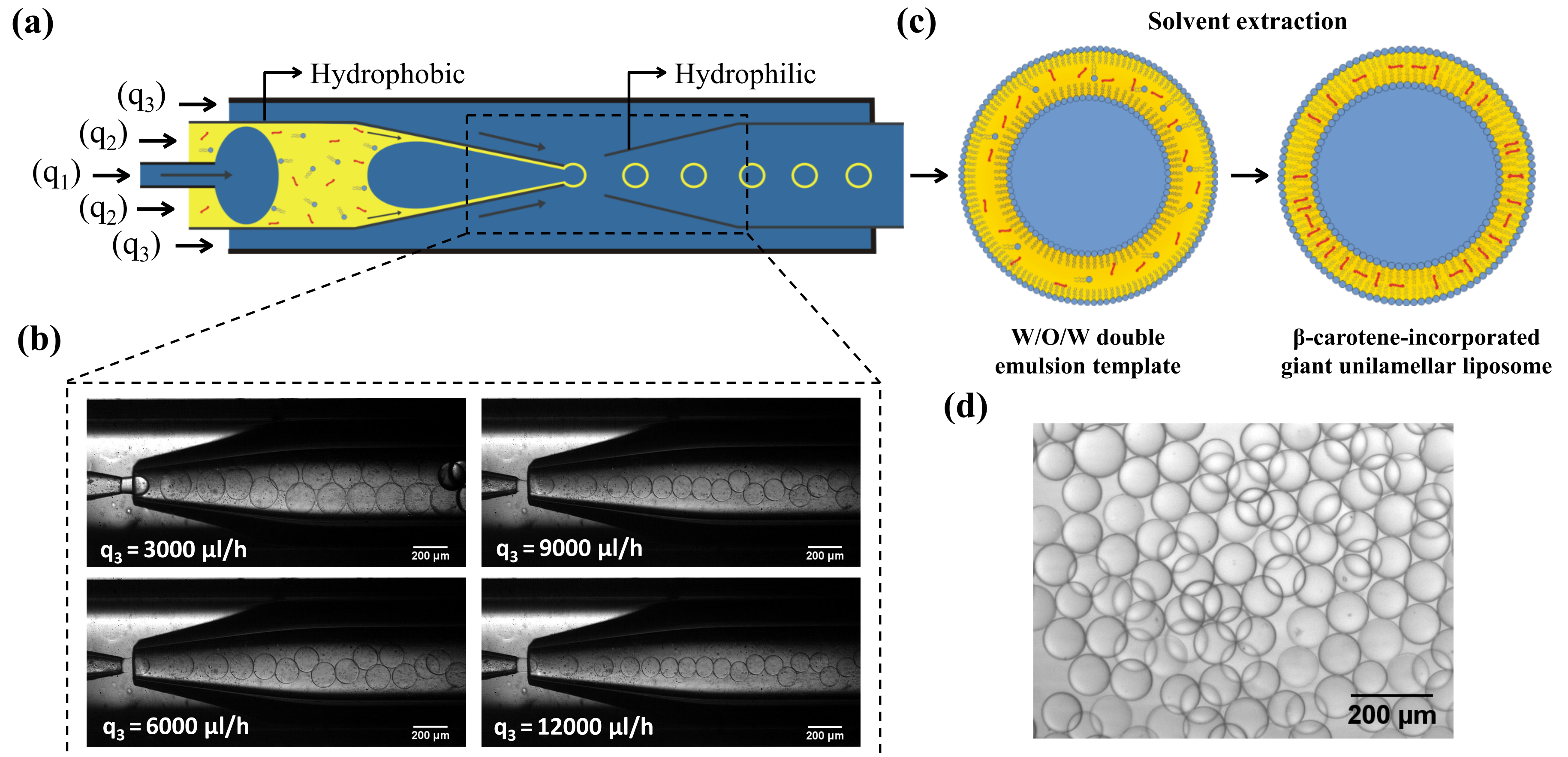

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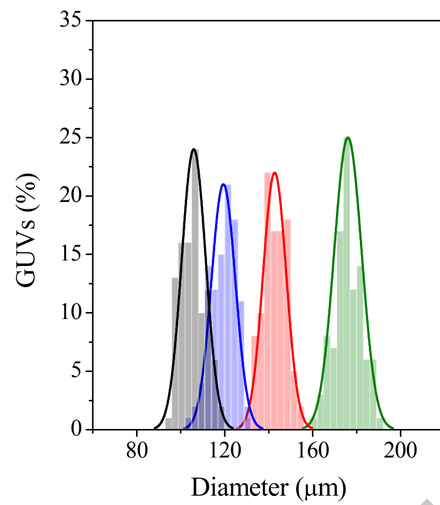
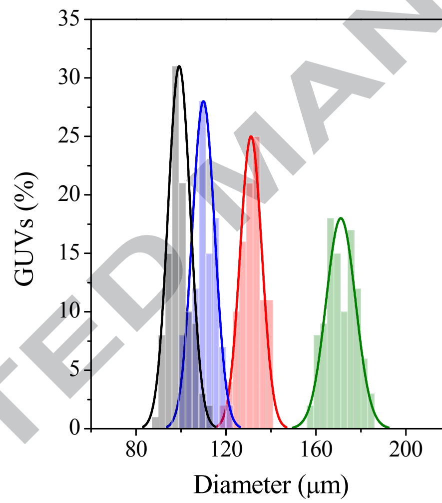
Figure 2.



(a)



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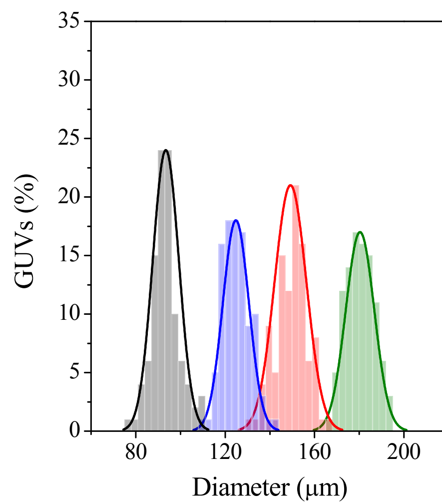

(c)

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Figure 3.

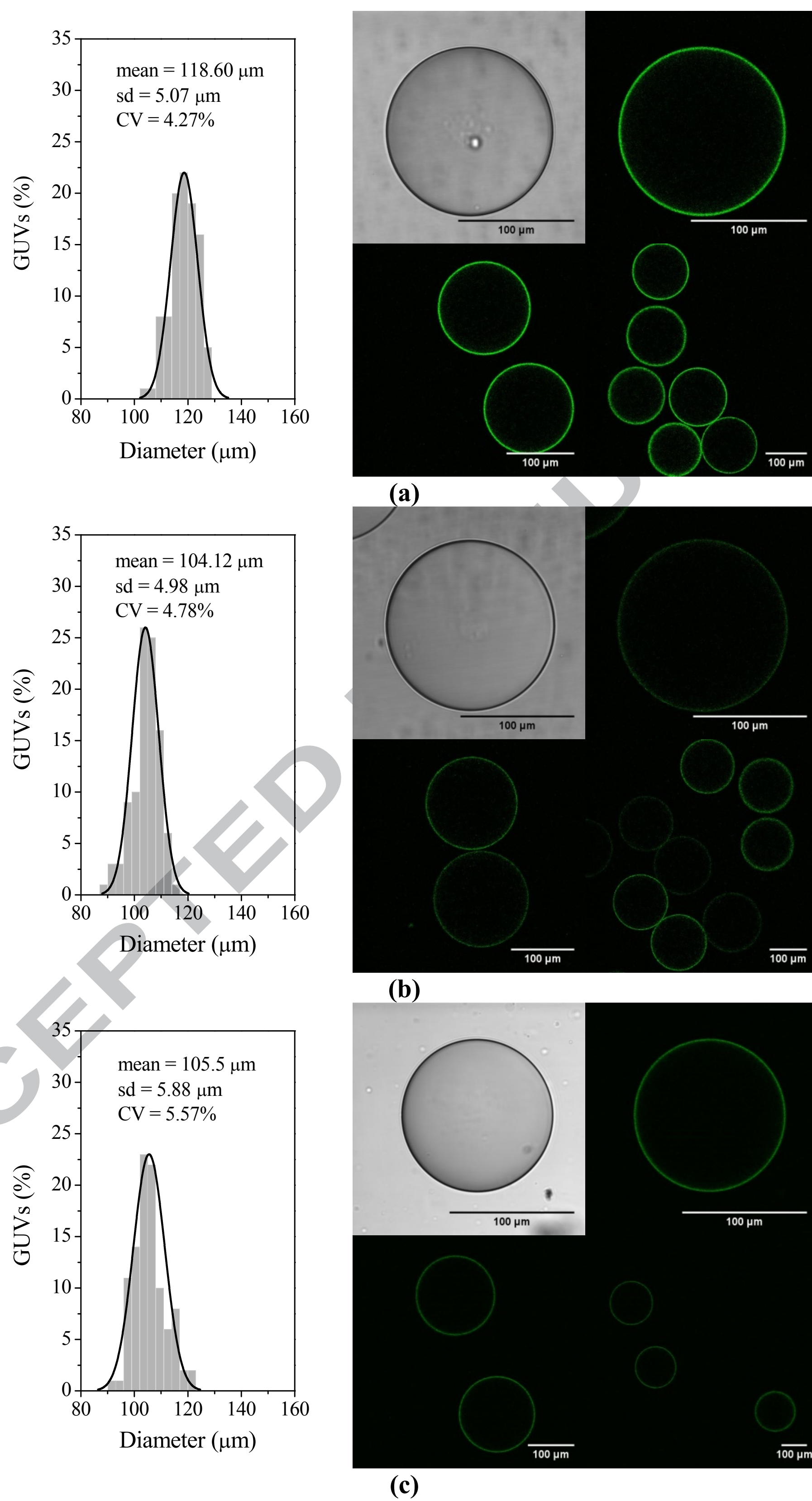

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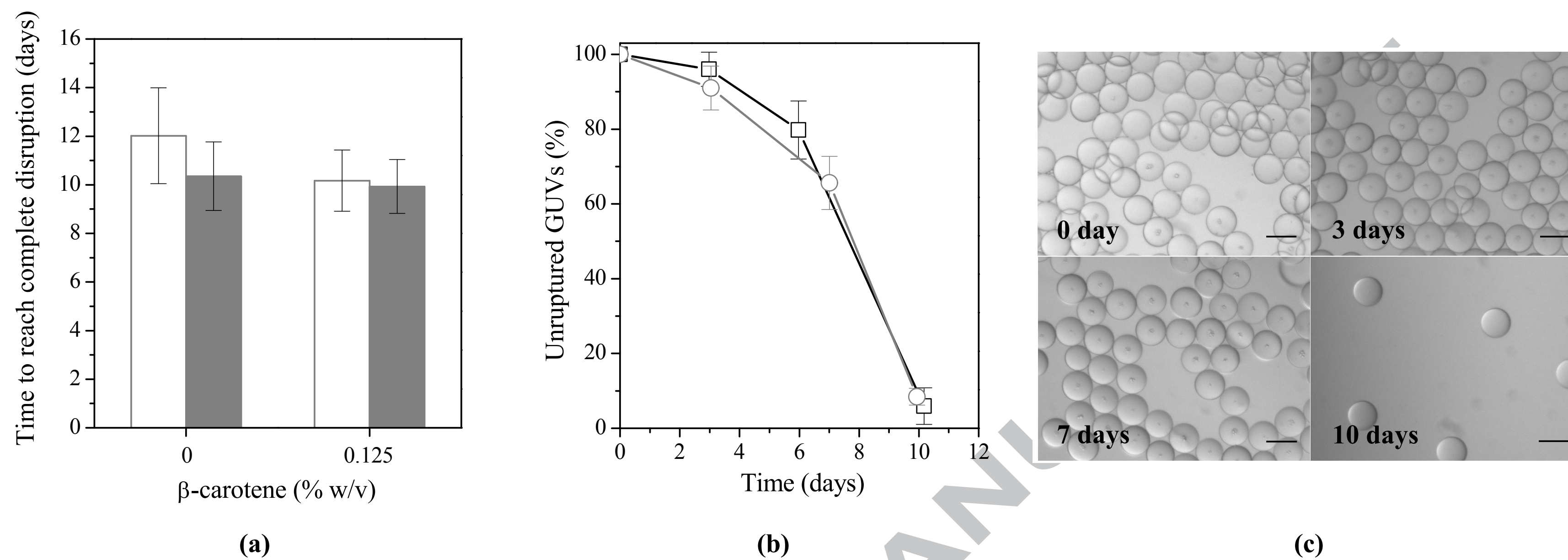
Figure 4.



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;

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- β-carotene was incorporated inside phospholipid shell forming giant liposome;
- β-carotene-incorporated giant liposomes can to applied in aqueous formulations.

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