

1 **Title:** Microfluidic-controlled manufacture of liposomes for the solubilisation of
2 a poorly water soluble drug.

3

4 **Authors:** Elisabeth Kastner, Varun Verma, Deborah Lowry and Yvonne Perrie*

5

6

7 Aston Pharmacy School, School of Life and Health Sciences, Aston University,
8 Birmingham, UK, B4 7ET.

9

10

11

12

13 *Correspondence: Professor Yvonne Perrie
14 Aston Pharmacy School
15 School of Life and Health Sciences
16 Aston University, Birmingham, UK. B4 7ET.
17 Tel: +44 (0) 121 204 3991
18 Fax: +44 (0) 121 359 0733
19 E-mail: y.perrie@aston.ac.uk

20

21

22 **Keywords:** Liposomes, microfluidics, poorly soluble drugs, bilayer loading, high
23 throughput

24

25

26

27

28

29

30

31

32

33

34 **Abstract**

35 Besides their well-described use as delivery systems for water-soluble drugs,
36 liposomes have the ability to act as a solubilizing agent for drugs with low
37 aqueous solubility. However, a key limitation in exploiting liposome technology
38 is the availability of scalable, low-cost production methods for the preparation of
39 liposomes. Here we describe a new method, using microfluidics, to prepare
40 liposomal solubilising systems which can incorporate low solubility drugs (in
41 this case propofol). The setup, based on a chaotic advection micromixer, showed
42 high drug loading (41 mol%) of propofol as well as the ability to manufacture
43 vesicles with at prescribed sizes (between 50 to 450 nm) in a high-throughput
44 setting. Our results demonstrate the ability of merging liposome manufacturing
45 and drug encapsulation in a single process step, leading to an overall reduced
46 process time. These studies emphasise the flexibility and ease of applying lab-on-
47 a-chip microfluidics for the solubilisation of poorly water-soluble drugs.

48

49 **1 Introduction**

50 The delivery of drugs by liposomes was first described in the 1970s by
51 Gregoriadis (Gregoriadis and Ryman, 1971) and there is now a range of clinically
52 approved liposome-based products that improve the therapeutic outcome for
53 patients. Whilst liposomes are commonly considered for the delivery of aqueous
54 soluble drugs, they are also well placed to act as solubilisation agents for drugs
55 with low aqueous solubility. This is of considerable interest given that more than
56 40% of all new chemical entities in discovery have low solubility and subsequent
57 issues in bioavailability (Savjani et al., 2012; Williams et al., 2012). The
58 encapsulation of low solubility drugs into the bilayer of liposomes allows not
59 only for their solubilisation in an aqueous media, but furthermore can offer
60 protection from degradation and control over the pharmacokinetic drug
61 distribution profile and improved therapeutic efficacy.

62

63 When solubilising drug within the liposomal bilayer, drug incorporation and
64 release rates has been shown to depend on the properties of the drug, the
65 composition of the liposomes, the lipid choice and concentration (Ali et al., 2010;
66 Ali et al., 2013; Mohammed et al., 2004). For example, the log P and molecular
67 weight are often considered to impact on bilayer loading, and studies have
68 shown that molecular weight may play a dominant role (Ali et al., 2013). When
69 considering the design of liposomes, there are a range of parameters that impact
70 on bilayer loading efficacy. For example, we have previously shown that
71 increasing the bilayer lipophilic volume (by adopting longer alkyl chain lipids
72 within the liposomes) increases the loading ability of liposomal systems
73 (Mohammed et al., 2004; Ali et al., 2013). Similarly, incorporation of charged
74 lipids within the liposomal system may also impact on bilayer loading through
75 electrostatic repulsion of drugs with like-charged liposomal bilayers
76 (Mohammed et al., 2004). Incorporation of cholesterol, whilst stabilising the
77 liposomes was also shown to inhibit bilayer drug loading (Ali et al., 2010) due to
78 the space-filling action of cholesterol in the liposomal bilayer. By increasing the
79 orientation order of the phospholipid hydrocarbon chains, cholesterol decreases
80 bilayer permeability. Indeed, the presence of cholesterol in liposomes
81 solubilising propofol was shown to shift the drug release profile from zero-order

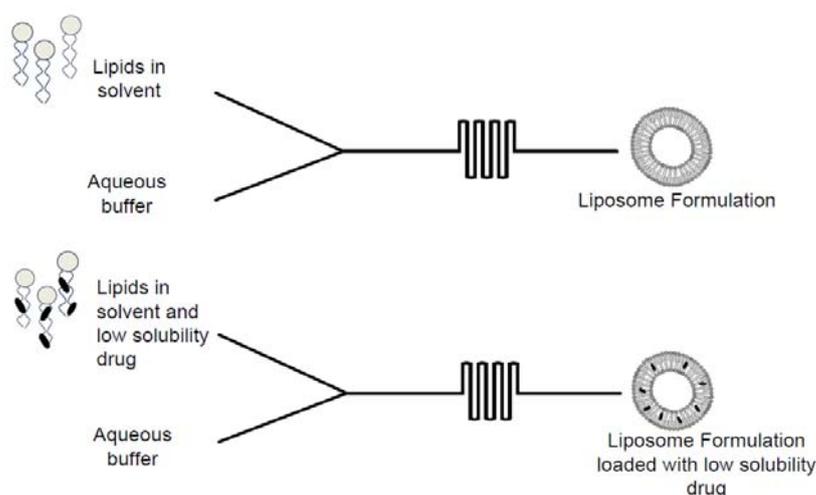
82 (when no cholesterol was present) to first order (when 11 to 33 mol% of
83 cholesterol was incorporated). This maps to the idea that without cholesterol
84 the bilayer can be thought of as more 'porous' in nature compared with the more
85 condensed and less permeable cholesterol-containing liposome bilayers (Ali et
86 al., 2010).

87

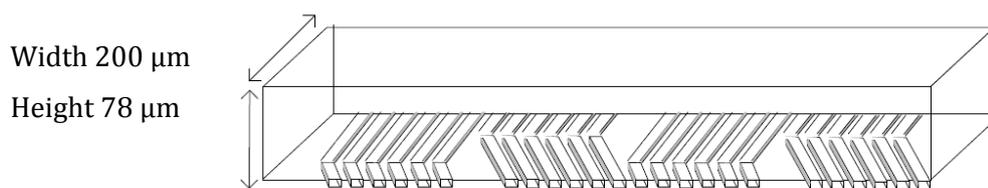
88 However, whilst a wide range of studies have looked at the effect of formulation
89 parameters on the application of liposomes as solubilising agents, more focus is
90 required into making liposomes a cost-effective solubilising agent. Recent
91 advances in lab-on-a-chip based tools for process development has already lead
92 to microfluidic-based methodologies in drug development (Dittrich and Manz,
93 2006; Weigl et al., 2003; Whitesides, 2006). Indeed, microfluidics-based methods
94 (which exploit controlled mixing of streams in micro-sized channels) have been
95 described for the manufacture of liposomes and lipid nanoparticles (van Swaay,
96 2013). Liposome formation by microfluidics primarily depends on the process of
97 controlled alterations in polarities throughout the mixer chamber, which is
98 followed by a nanoprecipitation reaction and the self-assembly of the lipid
99 molecules into liposomes. Generally, two or more inlet streams (lipids in solvent
100 and an aqueous phase) are rapidly mixed together and flow profiles in the
101 chamber itself are of low Reynolds numbers and categorized as laminar. Using
102 microfluidic systems a tight control of the mixing rates and ratio between
103 aqueous and solvent streams is achieved, with lower liquid volumes required,
104 which facilitates process development by reducing time and development costs.
105 The systems are designed with the option of high-throughput manufacturing and
106 are generally considered as less harsh compared to conventional methods of
107 liposome manufacturing that are based on mechanical disruption of large
108 vesicles into small and unilamellar ones (Wagner and Vorauer-Uhl, 2011).
109 Within the range of microfluidic mixing devices, we use a chaotic advection
110 micromixer, a Staggered Herringbone Micromixer (SHM). The fluid streams are
111 passed through the series of herringbone structures that allow for the
112 introduction of a chaotic flow profile, which enhances advection and diffusion. A
113 chaotic advection micromixer, as well as flow focusing methods, were shown to
114 allow for scalability, associated with defined vesicle sizes (Belliveau et al., 2012;

115 Jahn et al., 2007). The method based on chaotic advection was shown to
 116 reproducibly generate small unilamellar liposomes (SUV) with tight control of
 117 the resulting liposome sizes at flow rates as high as 70 mL/min in a parallelized
 118 mixer-setup. We have previously shown that microfluidics can be used to
 119 produce cationic liposomal transfection agents (Kastner et al., 2014), where
 120 design of experiments and multivariate analysis revealed the ratio between
 121 aqueous and solvent phase having a strong relevance for the formation of size-
 122 controlled liposomes. Within this study, we have exploited microfluidics to
 123 develop a high-throughput manufacturing process to prepare liposomes
 124 solubilising drug within their bilayer (Figure 1).

125



126



127

128 Figure 1: Schematic depiction of the liposome formation process based on the
 129 SHM design, a chaotic advection micromixer for (A) empty liposomes, (B) drug
 130 loaded liposomes and (C) chamber layout.

131

132

133

134

135

136

137 **2 Materials and Methods**

138 **2.1 Materials**

139 Egg Phosphatidylcholine (PC) and Cholesterol were obtained from Sigma-Aldrich
140 Company Ltd., Poole, UK. Ethanol and methanol were obtained from Fisher
141 Scientific UK, Loughborough, UK. TRIS Ultra Pure was obtained from ICN
142 Biomedicals, Inc., Aurora, Ohio. Propofol (2,6-Bis(isopropyl)phenol) and 5(6)-
143 Carboxyfluorescein (CF) was obtained from Sigma-Aldrich Company Ltd., Poole,
144 UK.

145

146 **2.2 Micromixer design and fabrication**

147 The micromixer was obtained from Precision NanoSystems Inc., Vancouver,
148 Canada. The mixer contained moulded channels which were 200 μm x 79 μm
149 (width x height) with herringbone features of 50 x 31 μm . 1 mL disposable
150 syringes were used for the inlet streams, with respective fluid connectors to the
151 chip inlets. Formulations using the micromixer were performed on a
152 NanoAssemblr™ (Precision NanoSystems Inc., Vancouver, Canada) that allowed
153 for control of the flow rates (0.5 to 6 mL/min) and the flow ratios (1:1 to 1:5,
154 ratio between solvent:aqueous) between the respective streams.

155 **2.3 Formulation of small unilamellar vesicles using microfluidics**

156 Lipids (16:4 molar ratio of PC and Cholesterol, 8:1 w/w) were dissolved in
157 ethanol. SUV were manufactured by injecting the lipids and aqueous buffer (TRIS
158 10mM, pH 7.2) into separate chamber inlets of the micromixer. The flow rate
159 ratio (FRR) (ratio between solvent and aqueous stream) as well as the total flow
160 rate (TFR) of both streams were controlled by syringe pumps, calibrated to the
161 syringe inner diameter. FRR varied from 1:1 to 1:5 and TFR varied from 0.5 to 6
162 mL/min, extrapolated from previous reported methods applying a SHM design
163 with a channel diameter of 200 μm (Kastner et al., 2014). The SUV formulation
164 was collected from the chamber outlet and dialysed at room temperature against
165 TRIS buffer (10mM, pH 7.2) for removal of residual solvent. The model drug of
166 low aqueous solubility was propofol (2,6-Bis(isopropyl)phenol), previously
167 shown to correspond to high encapsulation values in liposomal systems due to

168 its low molecular weight (Ali et al., 2013). To encapsulate propofol, the low
169 solubility drug was dissolved with the lipids in ethanol (0.5 to 3mg/mL) and
170 thereby liposome formation and encapsulation of the drug was performed
171 simultaneously using the micromixer method.

172

173 **2.4 Lipid film hydration and sonication**

174 Multilamellar vesicles (MLV) were prepared using the lipid film hydration
175 method (Bangham et al., 1965). Basically, lipids were dissolved in
176 chloroform/methanol (9:1 v/v) and the organic solvent was subsequently
177 removed by rotary evaporation under vacuum to form a dry lipid film which was
178 flushed with N₂ to ensure removal of solvent residues. The lipid film was
179 hydrated with TRIS buffer (10 mM, pH7.2) to form MLV. SUV were then formed
180 via probe sonication (Sonirep150plus, MSE; 5 min at an amplitude of 5).

181

182 **2.5 Measurement of particle characteristics**

183 Characterisation of the liposomes included size measurements using dynamic
184 light scattering (DLS) (Malvern NanoZS), reported as the z-average (intensity
185 based mean particle diameter) for monomodal size distributions and the zeta
186 potential using particle electrophoresis (Malvern NanoZS). Polydispersity (PDI)
187 measurements (Malvern NanoZS) were used to assess particle distribution.

188

189 **2.6 Quantification of drug concentrations**

190 Quantification of propofol was performed by reverse phase HPLC (Luna 5 μ C18,
191 Phenomenex, pore size of 100A, particle size of 5 μ m). Detector was UV/Vis, at
192 268 nm. The flow rate was constant at 1.0 mL/min throughout with a gradient
193 elution from 5% B (Methanol), 95% A (0.1% Trifluoroacetic Acid (TFA) in water)
194 to 100% B over 10 minutes. HPLC-grade liquids were used, sonicated and
195 filtered. The column temperature was controlled at 35°C. All analysis was made
196 in Clarity, DataApex version 4.0.3.876. Quantification was achieved by reference
197 to a calibration curve produced from standards (six replicates in ethanol) at
198 concentrations from 0.01 to 1 mg/mL. The calibration curve had a linearity $R^2 \geq$

199 0.997, and all measurements were within the level of detection and level of
200 quantification.

201

202 **2.7 Determination of drug loading into liposomes**

203 The amount of drug loaded into the bilayer was measured by determination of
204 the residual amount of drug in the liposome bilayer after removal of non-
205 entrapped drug by dialysis (sink conditions) against 1 L of TRIS buffer, 10mM pH
206 7.2 (3500 Da, Medicell Membranes Ltd., London, UK). The drug content was
207 measured by HPLC as described in section 2.6. This protocol was validated by
208 assessing the rate of propofol removal by dialysis.

209

210 **2.8 Stability study**

211 For the stability study, formulations of propofol-loaded SUV were stored at 4°C,
212 25°C and 40°C in pharmaceutical grade stability cabinets over 60 days (time
213 point measurements at day 0, 7, 14, 21, 28 and 60). Samples were taken at these
214 specific time points for measurement of particle characteristics (section 2.5) and
215 drug loading (section 2.6). Samples were dialysed against 500 mL TRIS buffer
216 (10 mM, pH7.2, sink conditions) at each time point to remove non-entrapped
217 propofol. Propofol content remaining in the liposome formulation was assessed
218 by HPLC as described in section 2.6.

219

220 **2.9 Recovery of lipids and propofol**

221 To assess the overall lipid and propofol recovery in the microfluidics method, the
222 amount of lipid and propofol was measured by HPLC and expressed as %
223 recovery compared to the initial amount of lipids or propofol available in the
224 stock. The HPLC method was the same as described section 2.6, and lipids were
225 quantified by an evaporative light scattering (ELS) detector (Sedere, Sedex 90),
226 set at 52°C and coupled to the HPLC.

227

228 **2.10 Freeze Fracturing Imaging**

229 Two microlitres of liposome suspension were placed in a ridged gold specimen
230 support and frozen rapidly by plunging into a briskly stirred mixture of

231 propane:isopentane (4:1) cooled in a liquid nitrogen bath. Fracturing, with a cold
232 knife, and replication were performed in a Balzers BAF 400D apparatus under
233 conditions similar to those described previously for freeze-fracture of liposomes
234 (Forge et al., 1978; Forge et al., 1989). The replicas generated were floated off on
235 water, cleaned in domestic bleach diluted 1:1 in distilled water, and then washed
236 several times in distilled water before mounting on grids for electron
237 microscopy. The replicas were viewed in a JEOL 1200EXII transmission electron
238 microscope operating at 80kv and digital images collected with a Gatan camera.
239 Images of the freeze-fractured samples are presented in reverse contrast so that
240 shadows appear black. Fracturing imaging was performed by Prof. Andrew Forge
241 at UCL Ear Institute, London, UK.

242

243 **2.11 Drug release study**

244 The in-vitro release rate of the drug was determined by incubating the drug-
245 loaded liposomes in 1 L TRIS buffer (10mM, pH 7.2) after removal of the non-
246 incorporated drug, at 37°C in a shaking water bath (150 shakes/min). Three
247 independent formulations of drug-loaded liposomes made by the microfluidics
248 method (TFR 2 mL/min, FRR 1:3) and standard lipid film hydration followed by
249 sonication were incubated (3 mL per formulation) and samples of 200 µL were
250 withdrawn at time intervals of 0.5 h, 1 h, 2 h, 4 h, 8 h and 16 h. Drug
251 quantification was performed as described in section 2.6 and expressed as %
252 cumulative release relative to the initial amount of drug encapsulated.

253

254 **2.12 Incorporation of an aqueous marker within liposomes**

255 To validate the formulation of liposomes, the presence of an aqueous core within
256 the nanoparticles manufactured was verified by including and imaging of an
257 aqueous fluorescent dye. Liposomes were manufactured as described in section
258 2.3 and 2.4 with 1 mM Carboxyfluorescein (CF) included in the aqueous buffer
259 (TRIS, 10 mM, pH 7.2). Liposomes with entrapped CF were separated from un-
260 entrapped dye by dialysis over night against 1 L fresh TRIS buffer, pH 7.2.
261 Liposomes were imaged under a confocal microscope SP5 TCS II MP, Leica

262 Microsystems, Leica TCSSP5 II, 63x objective (HCX PLAPO 63x/1.4-0.6 oil CS).
263 Images were taken by Charlotte Bland, Aston University, ARCHA facility.

264

265 **2.13 Statistical tools**

266 If not stated otherwise, results were reported as mean \pm standard deviation (SD).
267 One- or two-way analysis of variance (ANOVA) was used to assess statistical
268 significance, followed by Tukeys multiple comparing test and t-test was
269 performed for paired comparisons. Significance was acknowledged for p values
270 less than 0.05 (marked with *). All calculations were made in GraphPad Prism
271 version 6.0 (GraphPad Software Inc., La Jolla, CA).

272

273 **3 Results and discussion**

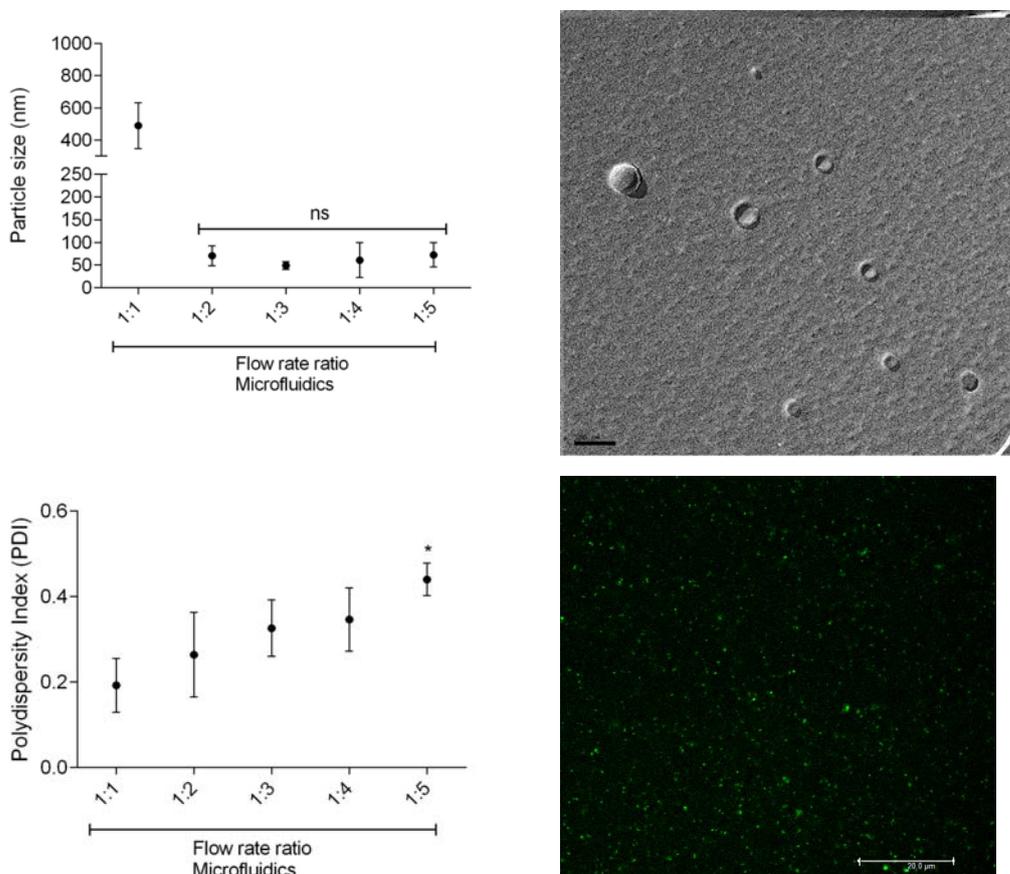
274 **3.1 Influence of the flow rate ratio of aqueous and solvent stream on** 275 **liposome size**

276 The increase in polarity throughout the chamber drives the formation of small
277 unilamellar liposomes (SUV) in milliseconds of mixing. For their formation, the
278 rate of mixing as well as the ratio of aqueous to solvent stream has been
279 anticipated as crucial factors. The formation of the liposomes is based on a
280 nanoprecipitation reaction, where supersaturation occurs and the liposomes are
281 formed by self-assembly after aggregation of the lipid molecules. The initial aim
282 of this work was to assess the formation of liposomes by microfluidic mixing and
283 assess the efficacy of this system to act as a solubilising agent. Therefore,
284 liposomes were prepared from PC and Cholesterol (16:4 molar ratio, 8:1 w/w) at
285 different total flow rates (TFR) and flow rate ratios (FRR) and the size,
286 polydispersity and zeta potential were measured.

287

288 Liposomes formed at low flow rate ratio (1:1) showed the largest size of around
289 450 nm; increasing the flow rate ratio resulted in smaller liposomes (around 40 -
290 50 nm) at constant flow rates of 2 mL/min (TRIS, 10 mM, pH7.2) (Figure 2A).
291 However, increasing the flow rate ratio increased polydispersity (to a maximum
292 of 0.4; Figure 2B). Liposomes prepared at a flow rate ratio of 1:3 are shown in

293 Figure 2C, demonstrating their small nature, with average sizes of the vesicles in
 294 agreement with average vesicle diameters obtained by particle sizing via
 295 dynamic light scattering (~40 nm). In contrast, the smallest vesicle size of a
 296 comparable formulation achievable via probe sonication with this lipid
 297 formulation was 100 nm in size at PDIs of 0.3 (data not shown). To verify the
 298 formation of liposomes, rather than micelles, the liposomes made by the
 299 microfluidics method were prepared encapsulating an aqueous fluorescent dye,
 300 carboxyfluorescein (CF, 1 mM), which was included in the aqueous phase during
 301 liposome manufacturing by microfluidics and lipid film hydration. After removal
 302 of the free CF by dialysis overnight, the remaining dye entrapped in the particles
 303 was visualized by confocal microscopy. Bright green fluorescent cores visible in
 304 the particles manufactured by the microfluidics method (Figure 2D) were in line
 305 with images obtained from liposomes manufactured with the lipid film hydration
 306 method (images not shown); which confirms the presence of aqueous cores and
 307 the formation of liposomes in the novel microfluidics method.
 308



309 Figure 2: Liposome size (A) and polydispersity (B) of vesicles formulated with
310 microfluidics method at increasing flow ratios. ns = not significant ($p>0.05$), *
311 denotes statistical significance ($p<0.05$) in comparison to FRR 1:1 (C) Freeze
312 fracturing electron microscopy images for empty liposomes manufactured with
313 the microfluidics method. Bar represents 100 nm. (D) Fluorescent microscope
314 images of liposomes manufactured with the microfluidics method,
315 carboxyfluorescein was encapsulated within the aqueous core of the vesicles as a
316 control for the manufacturing of bilayer liposomes. Bar represents 20 μm .

317

318

319

320 These impact of flow rate ratio on vesicle size are in agreement with previous
321 work showing that the increase in FRR reduces the resulting size of the
322 liposomes (Jahn et al., 2010; Kastner et al., 2014; Zook and Vreeland, 2010). A
323 correlation between higher flow rate ratios and smaller liposome particles has
324 been reported using liposomes composed of 1-palmitoyl, 2-oleoyl
325 phosphatidylcholine (POPC), cholesterol and the triglyceride triolein, which
326 resulted in the production of vesicular structures with sizes ranging from 140
327 nm to 40 nm dependent on the FRR chosen and triglyceride emulsions between
328 20– 50 nm size with nonpolar cores (Zhigaltsev et al., 2012). The overall lower
329 amount of residual solvent present at higher FRR employed decreases the
330 particle fusion (Ostwald ripening), which leads to the formation of smaller
331 particles (Zhigaltsev et al., 2012). The increase in polydispersity may be a result
332 of increased dilution at higher FRR reducing the rate of diffusional mixing within
333 the micromixer as noted in previous studies applying a SHM mixer for liposome
334 manufacturing (Kastner et al., 2014). With diffusion being proportional to the
335 lipid concentration, increasing FRR is effectively reducing the lipid
336 concentration, thus reducing the rate of diffusion, leading to partly incomplete
337 nucleation and a lower rate of liposome formation inside the micromixer
338 (Balbino et al., 2013b). Overall, these findings demonstrate that a FRR of 1:2 to
339 1:4 result in liposomes of the smallest size and polydispersity. The dilution factor
340 (due to flow ratios chosen involved in the SHM method) is overall lower
341 compared to ratios employed in the flow-focusing method, which can reach up to

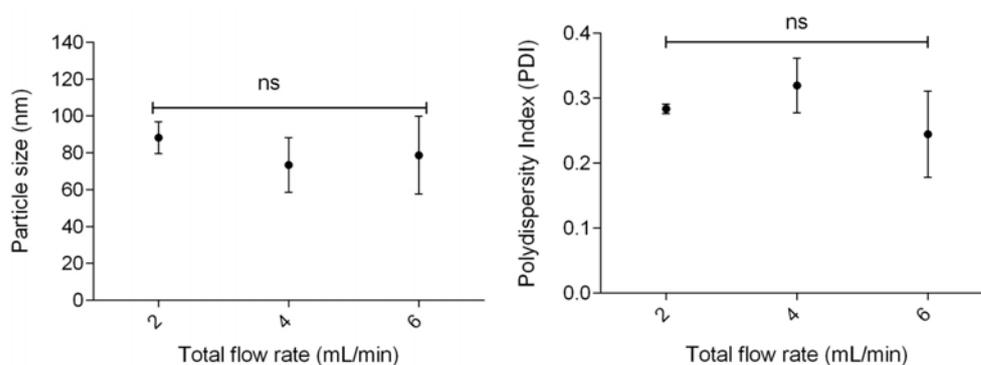
342 60 (Jahn et al., 2010; Jahn et al., 2007; Jahn et al., 2004). Furthermore, the SHM
343 method enhances the diffusional mixing due to the herringbone structures on
344 the channel wall (Stroock et al., 2002), which results in an enhanced mixing
345 profiles compared to the flow-focusing technique.

346

347 **3.2 Influence of flow rate on throughput and particle characteristics**

348 To assess the ability of the system as a potential high-throughput manufacturing
349 method for liposomal solubilisation systems, we increased the total flow rate 3-
350 fold whilst maintaining the ratio between aqueous and solvent stream constant.
351 Liposome size was shown to be independent of the applied flow rate, with no
352 significant change in vesicle size (Figure 3A), pdi (Figure 3B) and zeta potential
353 ($-3\pm 2\text{mV}$; data not shown). These results support the suitability of microfluidics
354 manufacturing as a high throughput method with liposome characteristics being
355 maintained constant whilst increasing the total flow rate in the system. Our
356 results also confirm that the flow rate ratio used in the system is the most crucial
357 variable on liposome size, which has previously been demonstrated with other
358 systems (Balbino et al., 2013a; Balbino et al., 2013b; Jahn et al., 2007; Jahn et al.,
359 2004; Kastner et al., 2014). The scalability of the microfluidics method has been
360 suggested by Belliveau et al. 2013, by parallelization of the mixer chamber.
361 Scalability and increase in throughput together demonstrate the industrial
362 applicability comparable with scale-up options available (Wagner and Vorauer-
363 Uhl, 2011).

364



365

366 Figure 3: Liposome size (A) and polydispersity (B) of vesicles formulated with
367 microfluidics at increasing flow rates and constant flow ratio of 1:3, n = 3, ns =
368 not significant ($p>0.05$).

369

370

371

372 As shown, the increase in FRR is the main contributing factor governing
373 liposome size (Figure 2A). Nevertheless, an increase in FRR will inevitably lead
374 to dilution and lower liposome concentrations in the final liposome suspension
375 produced. A subsequent concentration process based on filtration (Pattnaik and
376 Ray, 2009), chromatography (Ruysschaert et al., 2005) or centrifugation adds
377 additional processing time. Therefore, to circumvent this additional process step,
378 we counteracted the dilution of the lipids at higher FRR by increasing initial lipid
379 concentrations introduced to the micromixer at the desired FRR. Through this
380 method, liposomes were manufactured at up to 6 fold higher concentrations.
381 Increased lipid concentrations at FRR of 1:3 and 1:5 did not significantly
382 ($p>0.05$) influence size and polydispersity compared to the standard lipid
383 concentration (Figure 4A and B), whereas at a FRR of 1:1 a significant ($p<0.05$)
384 decrease in vesicle size was observed (Figure 4A). At this lower FRR, the higher
385 lipid concentrations may again decreasing particle fusion leading to the
386 formation of smaller particles (Zhigaltsev et al., 2012). Nevertheless, this setup
387 allows to increase the final liposome concentration according to the FRR chosen
388 without adversely changing resulting vesicle size or polydispersity for the
389 smallest vesicle sizes obtained at higher FRR (Figure 4A and B respectively), due
390 to the diffusional mixing process in the SHM design.

391

392

393

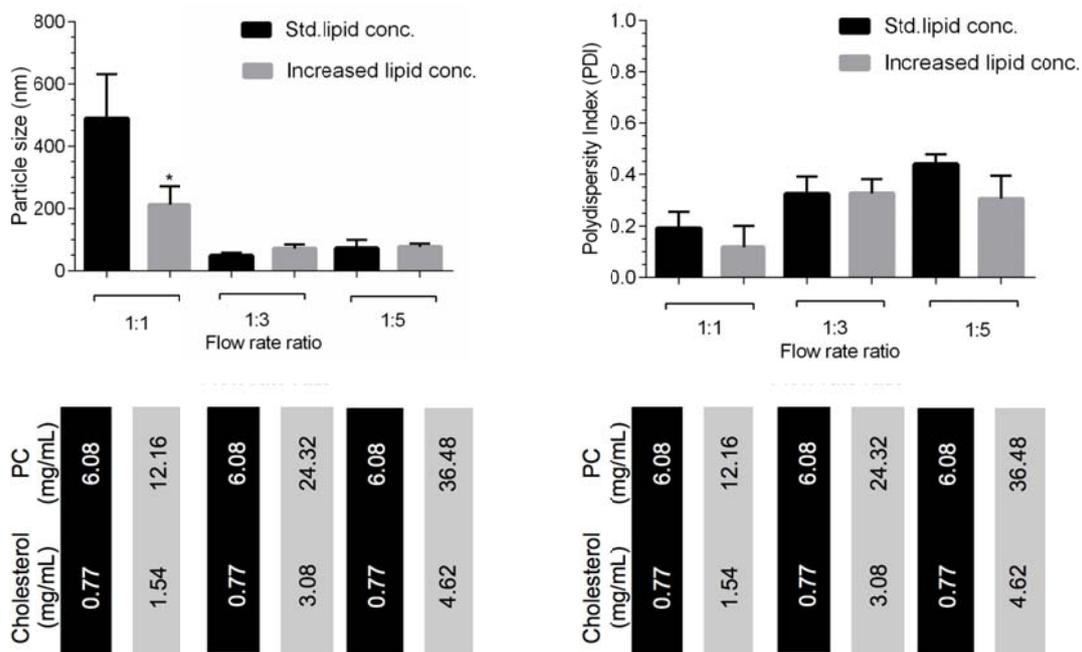
394

395

396

397

398



404 Figure 4: Increase in the lipid concentration in the ethanol stock to circumvent
 405 the dilution effect at flow ratios of 1:1, 1:3 and 1:5 for (A) liposome size, *
 406 denotes statistical significance ($p < 0.05$) in comparison to FRR 1:1 for the
 407 standard lipid concentration and (B) polydispersity with respective
 408 concentration of PC and Cholesterol in the inlet stream, $n = 3$.

405

406

408 3.3 Drug loading studies: The effect of drug encapsulation by the liposome 409 manufacturing method

416 So far, we have shown that the microfluidics method allows for size-controlled
 417 and rapid synthesis of liposomes. To consider the applicability of this method to
 418 be used for a high-throughput production of liposomes as solubilising agents the
 419 loading capacity of the formulation was considered. Based on the optimisation
 420 studies shown in Figure 2, propofol was solubilised within liposomes prepared
 421 at a FRR of 1:3 and a TFR of 2 mL/min. The particle characteristics and drug
 422 loading efficiency (mol%; Figure 5A) was determined at propofol concentrations
 423 ranging from 0.5 to 3 mg/mL (effective concentration in the solvent stream).

417

419 Using a propofol concentration of 1 mg/mL in the solvent stream showed high
 420 drug loading (~50 mol%), combined with particle size of ~50 nm and a low

419 polydispersity (Figure 5A). Particle size and polydispersity increased notably
420 (ca. 600 nm and 0.8 respectively) at the highest propofol concentration (3
421 mg/mL in the solvent stream, giving a loading of ~25mol%, Figure 5A),
422 suggesting the liposome system may have become saturated or destabilised at
423 high propofol concentrations (drug-to-lipid ratio 1.72 mol/mol). Based on this,
424 subsequent studies adopted a propofol concentration at 1 mg/mL in the solvent
425 stream for all performed encapsulation studies.

426

427 The drug encapsulation was further investigated as a function of FRR in the
428 microfluidics method. Propofol encapsulation (mol%) in liposomes prepared at
429 FRR 1:1, 1:3 and 1:5 remained at approximately 50 mol% with no statistical
430 difference. However this was significantly higher ($p < 0.0001$) than drug loading
431 in liposomes prepared via sonication (15 mol%; Figure 5B). The drug loading
432 efficiency of liposomes prepared by sonication is in line with previous reported
433 propofol encapsulation (Ali et al., 2013). Furthermore, drug encapsulation did
434 not alter vesicle size or polydispersity (Figure 5A) and vesicle sizes obtained by
435 dynamic light scattering were verified by freeze fracturing images (Figure 5D).
436 This higher drug loading may be a result of the highly efficient mixing processes
437 occurring during microfluidics that favours incorporation of propofol within the
438 bilayers in the same process as the vesicles form. Indeed, the here presented
439 method allows to achieve a propofol encapsulation of ~50 mol%, which
440 represents a total propofol amount of ~300 mg/mL in the final liposome
441 formulation, representing a 2000-fold increase to the reported aqueous
442 solubility of propofol, 150 $\mu\text{g/mL}$ (Altomare et al., 2003).

443

444

445

446

447

448

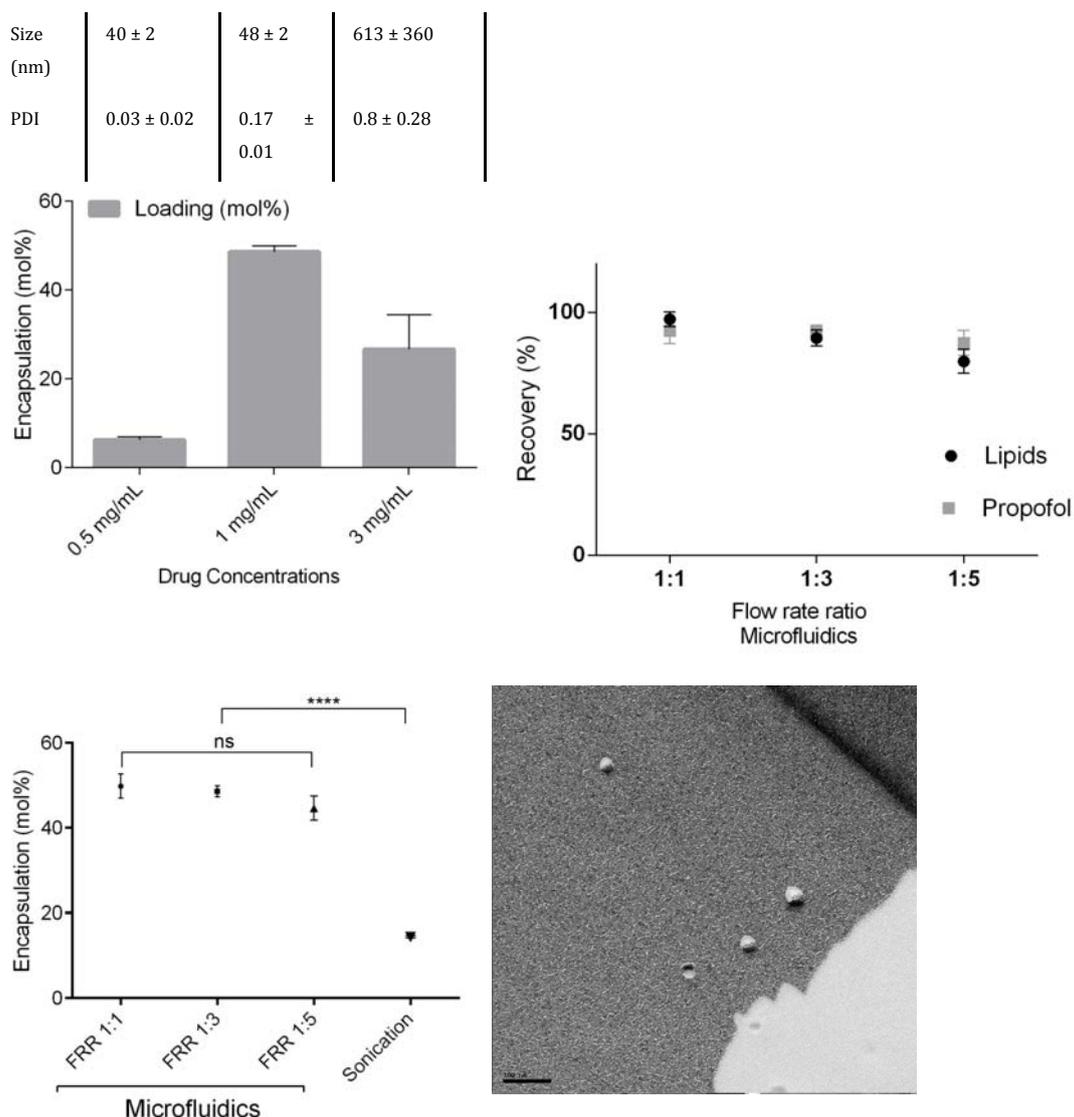
449

450

451

452

453



454 Figure 5: (A) Effect of drug concentrations in the ethanol inlet stream (0.5, 1 and
 455 3 mg/mL) on encapsulation efficiency (mol%), particle size and polydispersities
 456 at a flow ratio of 1:3.. (B) Encapsulation efficiency (mol%) of liposomes formed
 457 with the microfluidics method at flow ratios of 1:1, 1:3 and 1:5 compared to the
 458 encapsulation efficiency using the sonication method. Results are average out of
 459 triplicate formulations and measurements. ns = not significant ($p > 0.05$), *
 460 denotes statistical significance ($p < 0.00001$) in comparison to microfluidics-
 461 based samples. (C) Recovery of lipids and propofol in the microfluidics method
 462 at different flow ratios. Results are expressed as % compared to the initial lipid
 463 and propofol amount present ($n = 3$). (D) Freeze fracturing electron microscopy

464 images for liposomes loaded with the low solubility model drug (propofol)
465 manufactured with the microfluidics method. Bar represents 100 nm

466

467

468

469 To consider, drug release profiles, the *in-vitro* release of propofol encapsulated in
470 liposomes by microfluidics was monitored at 37°C over 16 h. Liposomes formed
471 with the microfluidics method had a significant higher drug encapsulated at the
472 start of the release study (~55 mol%) compared to those vesicles formed by
473 sonication (20 mol% drug encapsulation). However, relative to initial loading, an
474 initial release of ca 40% was observed at 1 h for both formulations, followed by a
475 continuous release of 90% of the encapsulated drug was observed over 8 h
476 (Figure 6). Whereas the fatty alcohol alkyl chain length was shown to affect the
477 release profile of encapsulated propofol (Ali et al., 2013), here the method of
478 liposome manufacturing was shown to mainly affect the amount of drug
479 incorporated into the liposomes, without altering the release profile of the
480 encapsulated drug against sink conditions. Previous we have shown that
481 solubilisation of propofol in phosphatidylcholine liposomes followed a zero-
482 order release kinetics, where the incorporation of a higher amount of cholesterol
483 shifted the release rates towards a first-order release model (Ali et al., 2010),
484 implying that the release kinetics itself are mainly dominated by the lipid
485 composition and physicochemical characteristics rather than the method of
486 liposome manufacturing. This may prove advantageous in the development of an
487 IV formulation; the pharmacokinetic release profile of propofol has been studied
488 previously in a colloidal dispersion between 20-100 nm (Cai et al., 2012), where
489 rapid distribution of propofol compared to the commercial product Diprivan®
490 highlighted the need on the development of new techniques for the
491 encapsulation of low solubility drugs.

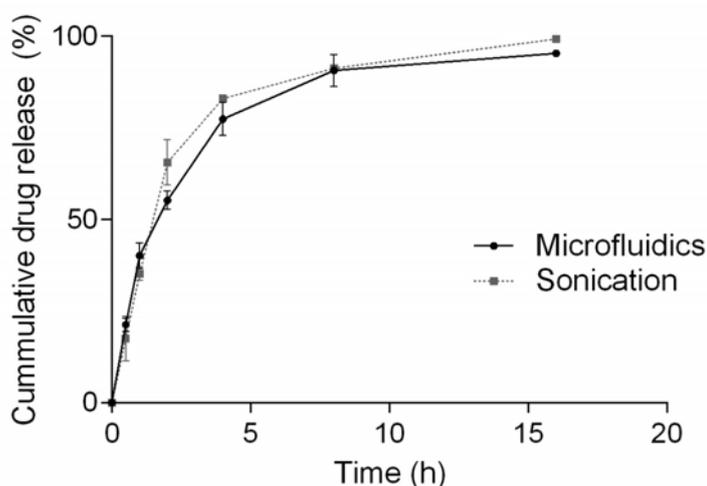
492

493

494

495

496



497

498 Figure 6: Effect of manufacturing method to the drug release of propofol from
 499 liposomes. Results show the cumulative drug release profile from formulations
 500 manufactured with the standard lipid film hydration / sonication method and
 501 microfluidics and represent percentage cumulative release of initially entrapped
 502 propofol, expressed as the means of three experiments \pm SD.

503

504 It is important to verify both lipid and drug recovery when using the
 505 microfluidics method, to ensure cost-effectiveness and that lipid and drug
 506 concentrations remain locked at the ratio initially designed prior to formulation.
 507 To date, the quantification of lipids is mainly dominated by time intensive assays
 508 like mass spectrometry (Moore et al., 2007). Here, we introduce a simple and
 509 robust method of lipid quantification based on evaporative light scattering (ELS)
 510 detection and HPLC separation. We coupled an ELS detector downstream a HPLC
 511 separation method, which allowed for quantification of any solids in the eluate
 512 with a lower volatility than the mobile phase. Microfluidics based liposomal-drug
 513 formulations showed good recovery of the drug (88 - 92%; Figure 5C),
 514 independent of the FRR. Similarly, lipid recovery was high at FRR of 1:1 and 1:3
 515 (97% and 89%; for FRR 1:1 and 1:3 respectively; Figure 5C). A significant drop
 516 (79%; $p < 0.01$) in lipid recovery was noted at a flow ratio of 1:5, suggesting that
 517 higher FRR employed in the microfluidics method may impede lipid recovery
 518 due to enhanced dilution in the chamber. Nevertheless, the smallest vesicle size
 519 (~ 50 nm) can be obtained at a FRR of 1:3 (Figure 2A) and any further increase in

520 FRR will not benefit the formulation (size, pdi and drug encapsulation). Based on
521 this, we chose the FRR 1:3 for a long-term stability study.

522

523 **3.4 The effect of manufacturing methods on liposome stability and drug** 524 **encapsulation over 8 weeks**

525 The SHM method was previously investigated for the encapsulation of a highly
526 soluble drug, with approximately 100% loading efficiencies being reported using
527 doxorubicin as a model drug (Zhigaltsev et al., 2012); the authors demonstrated
528 high drug retention of encapsulated drug with liposomes stored at 4°C over the
529 course of eight weeks (Zhigaltsev et al., 2012). Following the assessment that
530 liposomes manufactured by the microfluidics method yields significant higher
531 encapsulation of propofol, similarly we performed an eight-week stability study
532 to verify the integrity of the vesicles at different storage temperatures. Vesicles
533 were prepared using microfluidics as described above, and the initial amount of
534 propofol encapsulated was determined after removal of free drug by dialysis.
535 Vesicles were stored at 4°C, 25°C/60%RH and 40°C/75%RH (standard ICH
536 temperatures) in pharmaceutical grade stability cabinets and the formulations
537 made by the sonication method were stored at 25°C/60%RH (Figure 7, Table 1),
538 acting as the control method. The control liposomes formed by sonication
539 showed good stability in terms of size retention over the course of the study.
540 Similarly, for liposomes prepared using microfluidics, vesicle size remained
541 unaffected after storage over 8 weeks at 4°C and 25°C. In contrast, liposomes
542 stored at 40°C significantly increase in size from initially 55 nm to 120 nm
543 (Figure 7A), with no notable affect to polydispersity, suggesting the liposome
544 population as a whole has changed in size rather than a sub-set of the vesicles
545 (Table 1).

546

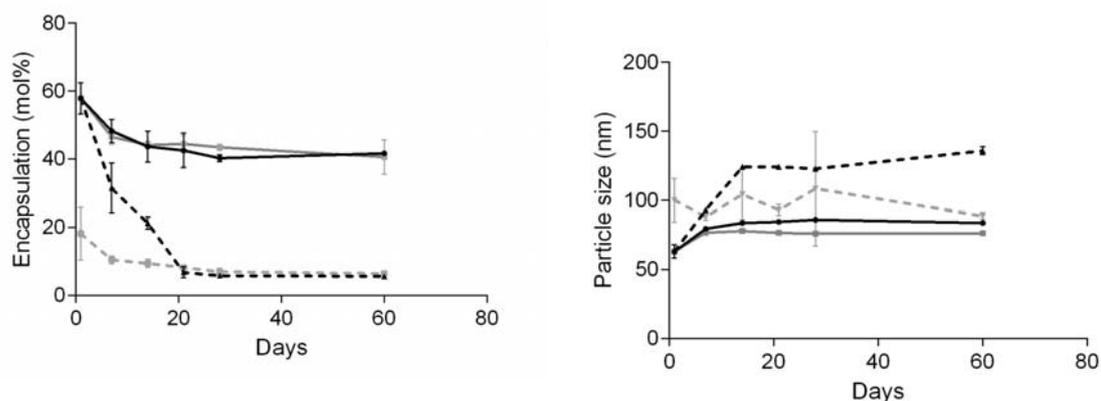
547

548 Table 1: Polydispersity at different storage conditions for 8 weeks. Results are
549 mean out of triplicate formulations and measurements.

Day	0	7	14	21	28	60
<u>Microfluidics</u>						
4°C	0.403 ± 0.02	0.286 ± 0.01	0.282 ± 0.01	0.295 ± 0.01	0.261 ± 0.01	0.305 ± 0.01

25°C	0.403 ± 0.02	0.295 ± 0.01	0.279 ± 0.01	0.301 ± 0.04	0.302 ± 0.03	0.266 ± 0.03
40°C	0.403 ± 0.02	0.254 ± 0.001	0.121 ± 0.02	0.119 ± 0.001	0.129 ± 0.01	0.221 ± 0.01
<u>Sonication</u>						
25°C	0.656 ± 0.02	0.652 ± 0.02	0.522 ± 0.15	0.658 ± 0.049	0.552 ± 0.04	0.505 ± 0.06

550



551 Figure 7: Size (A) and drug encapsulation (mol%) (B) at different storage
 552 conditions over 8 weeks. Results are mean of triplicate formulations and
 553 measurements.

554

555 Minor (but not significant) drug loss from the liposomes was detected for the
 556 formulations at 4°C and 25°C after the first 7 days of storage (Figure 7B), after
 557 which the formulations remained stable with final drug encapsulation values of
 558 41±1 mol% and 41±4 mol% at 4°C and 25°C storage conditions respectively
 559 (Figure 7B). Similarly, with liposomes formulated using sonication showed and
 560 initial drug loss when stored at 25°C/60%RH which then plateaued out (Figure
 561 7B). Notable drug loss from the microfluidic systems was only seen when they
 562 were stored at elevated temperatures with the formulation stored at 40°C
 563 showing almost complete drug loss over the course of the stability study, with
 564 only 5±1 mol% drug remaining encapsulated after 8 weeks, similar to the final
 565 drug encapsulated in the sonicated liposomes which were stored at
 566 25°C/60%RH (Figure 7B). Overall, vesicles produced with the microfluidics
 567 method were smaller with a lower polydispersity than those obtained by lipid
 568 film hydration / sonication. The vesicles manufactured by sonication maintained
 569 their size around 100±20 nm throughout the stability study (stored at 25°C) as
 570 well as their polydispersity (Table 1). Results suggest that the method of

571 manufacturing mainly impacts the drug encapsulation rather than the physical
572 properties (size, pdi, zeta potential). Stability of the formulations is crucial and
573 these results demonstrate that liposomes formed by the microfluidics method
574 remain over two months at conditions of 4 and 25°C.

575

576 **3.5 Conclusion**

577 Here, for the first time, we have demonstrated a high-throughput, robust method
578 of preparing size-controlled liposomes as solubilising agents using microfluidics.
579 These liposomes have well defined, scalable, process controlled, physico-
580 chemical attributes demonstrating this method is suitable for pre-clinical and
581 clinical production of liposomes. Drug loading was shown to be in an applicable
582 range for clinical application (Biebuyck et al., 1994). Furthermore, using this
583 novel method, liposome manufacturing and drug encapsulation are processed in
584 a single process step, circumventing an additional drug loading step
585 downstream, which notably reduces the time for production of stable drug-
586 loaded vesicles of specified physico-chemical characteristics.

587

588 **3.6 Acknowledgements**

589 Prof. Andrew Forge (UCL Ear Institute, London, UK) is acknowledged for the
590 imaging of the liposomes by freeze fracturing. Charlotte Bland (Aston University,
591 ARCHA facility) is acknowledged for the imaging of liposomes by fluorescent
592 microscopy. This work was part funded by the EPSRC Centre for Innovative
593 Manufacturing in Emergent Macromolecular Therapies and Aston University.

594

595 **References**

- 596 Ali, M.H., Kirby, D.J., Mohammed, A.R., Perrie, Y., 2010. Solubilisation of
597 drugs within liposomal bilayers: alternatives to cholesterol as a membrane
598 stabilising agent. *Journal of pharmacy and pharmacology* 62, 1646-1655.
- 599 Ali, M.H., Moghaddam, B., Kirby, D.J., Mohammed, A.R., Perrie, Y., 2013. The
600 role of lipid geometry in designing liposomes for the solubilisation of poorly
601 water soluble drugs. *International journal of pharmaceutics* 453, 225-232.
- 602 Altomare, C., Trapani, G., Latrofa, A., Serra, M., Sanna, E., Biggio, G., Liso,
603 G., 2003. Highly water-soluble derivatives of the anesthetic agent propofol: in
604 vitro and in vivo evaluation of cyclic amino acid esters. *European journal of*
605 *pharmaceutical sciences* 20, 17-26.
- 606 Balbino, T.A., Aoki, N.T., Gasperini, A.A., Oliveira, C.L., Azzoni, A.R.,
607 Cavalcanti, L.P., de la Torre, L.G., 2013a. Continuous flow production of
608 cationic liposomes at high lipid concentration in microfluidic devices for gene
609 delivery applications. *Chemical Engineering Journal* 226, 423-433.
- 610 Balbino, T.A., Azzoni, A.R., de La Torre, L.G., 2013b. Microfluidic devices for
611 continuous production of pDNA/cationic liposome complexes for gene delivery
612 and vaccine therapy. *Colloids and Surfaces B: Biointerfaces* 111, 203-210.
- 613 Bangham, A., Standish, M.M., Watkins, J., 1965. Diffusion of univalent ions
614 across the lamellae of swollen phospholipids. *Journal of molecular biology* 13,
615 238-IN227.
- 616 Belliveau, N.M., Huft, J., Lin, P.J., Chen, S., Leung, A.K., Leaver, T.J., Wild,
617 A.W., Lee, J.B., Taylor, R.J., Tam, Y.K., 2012. Microfluidic synthesis of highly
618 potent limit-size lipid nanoparticles for in vivo delivery of siRNA. *Molecular*
619 *Therapy—Nucleic Acids* 1, e37.
- 620 Biebuyck, J.F., Smith, I., White, P.F., Nathanson, M., Gouldson, R., 1994.
621 Propofol: an update on its clinical use. *Anesthesiology* 81, 1005-1043.
- 622 Cai, W., Deng, W., Yang, H., Chen, X., Jin, F., 2012. A propofol
623 microemulsion with low free propofol in the aqueous phase: Formulation,
624 physicochemical characterization, stability and pharmacokinetics. *International*
625 *journal of pharmaceutics* 436, 536-544.
- 626 Dittrich, P.S., Manz, A., 2006. Lab-on-a-chip: microfluidics in drug discovery.
627 *Nature Reviews Drug Discovery* 5, 210-218.
- 628 Forge, A., Knowles, P., Marsh, D., 1978. Morphology of egg
629 phosphatidylcholine-cholesterol single-bilayer vesicles, studied by freeze-etch
630 electron microscopy. *The Journal of membrane biology* 41, 249-263.
- 631 Forge, A., Zajic, G., Davies, S., Weiner, N., Schacht, J., 1989. Gentamicin
632 alters membrane structure as shown by freeze-fracture of liposomes. *Hearing*
633 *research* 37, 129-139.
- 634 Gregoriadis, G., Ryman, B., 1971. Liposomes as carriers of enzymes or drugs:
635 a new approach to the treatment of storage diseases. *Biochem. J* 124.
- 636 Jahn, A., Stavis, S.M., Hong, J.S., Vreeland, W.N., DeVoe, D.L., Gaitan, M.,
637 2010. Microfluidic mixing and the formation of nanoscale lipid vesicles. *Acs*
638 *Nano* 4, 2077-2087.
- 639 Jahn, A., Vreeland, W.N., DeVoe, D.L., Locascio, L.E., Gaitan, M., 2007.
640 Microfluidic directed formation of liposomes of controlled size. *Langmuir* 23,
641 6289-6293.

642 Jahn, A., Vreeland, W.N., Gaitan, M., Locascio, L.E., 2004. Controlled vesicle
643 self-assembly in microfluidic channels with hydrodynamic focusing. *Journal of*
644 *the American Chemical Society* 126, 2674-2675.

645 Kastner, E., Kaur, R., Lowry, D., Moghaddam, B., Wilkinson, A., Perrie, Y.,
646 2014. High-throughput manufacturing of size-tuned liposomes by a new
647 microfluidics method using enhanced statistical tools for characterization.
648 *International Journal of Pharmaceutics*.

649 Mohammed, A., Weston, N., Coombes, A., Fitzgerald, M., Perrie, Y., 2004.
650 Liposome formulation of poorly water soluble drugs: optimisation of drug
651 loading and ESEM analysis of stability. *International Journal of Pharmaceutics*
652 285, 23-34.

653 Moore, J.D., Caufield, W.V., Shaw, W.A., 2007. Quantitation and
654 standardization of lipid internal standards for mass spectroscopy. *Methods in*
655 *enzymology* 432, 351-367.

656 Pattnaik, P., Ray, T., 2009. Improving liposome integrity and easing
657 bottlenecks to production. *Pharmaceutical Technology Europe* 22.

658 Ruyschaert, T., Marque, A., Duteyrat, J.-L., Lesieur, S., Winterhalter, M.,
659 Fournier, D., 2005. Liposome retention in size exclusion chromatography.
660 *BMC biotechnology* 5, 11.

661 Savjani, K.T., Gajjar, A.K., Savjani, J.K., 2012. Drug solubility: importance and
662 enhancement techniques. *ISRN pharmaceutics* 2012.

663 Stroock, A.D., Dertinger, S.K., Ajdari, A., Mezić, I., Stone, H.A., Whitesides,
664 G.M., 2002. Chaotic mixer for microchannels. *Science* 295, 647-651.

665 van Swaay, D., 2013. Microfluidic methods for forming liposomes. *Lab on a*
666 *Chip* 13, 752-767.

667 Wagner, A., Vorauer-Uhl, K., 2011. Liposome technology for industrial
668 purposes. *J Drug Deliv* 2011, 591325.

669 Weigl, B.H., Bardell, R.L., Cabrera, C.R., 2003. Lab-on-a-chip for drug
670 development. *Advanced drug delivery reviews* 55, 349-377.

671 Whitesides, G.M., 2006. The origins and the future of microfluidics. *Nature*
672 442, 368-373.

673 Williams, R.O., Watts, A.B., Miller, D.A., 2012. Formulating poorly water
674 soluble drugs. Springer.

675 Zhigaltsev, I.V., Belliveau, N., Hafez, I., Leung, A.K., Huft, J., Hansen, C.,
676 Cullis, P.R., 2012. Bottom-up design and synthesis of limit size lipid
677 nanoparticle systems with aqueous and triglyceride cores using millisecond
678 microfluidic mixing. *Langmuir* 28, 3633-3640.

679 Zook, J.M., Vreeland, W.N., 2010. Effects of temperature, acyl chain length,
680 and flow-rate ratio on liposome formation and size in a microfluidic
681 hydrodynamic focusing device. *Soft Matter* 6, 1352-1360.

682

683

684

685

686

687

