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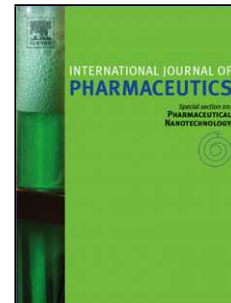


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Evaluation of Lipid-Stabilised Tripropionin Nanodroplets as a Delivery Route for Combretastatin A4

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Abstract

Lipid-based nanoemulsions are a cheap and elegant route for improving the delivery of hydrophobic drugs. Easy and quick to prepare, nanoemulsions have promise for the delivery of different therapeutic agents. Although multiple studies have investigated the effects of the oil and preparation conditions on the size of the nanoemulsion nanodroplets for food applications, analogous studies for nanoemulsions for therapeutic applications are limited. Here we present a study on the production of lipid-stabilised oil nanodroplets (LONDS) towards medical applications. A number of biocompatible oils were used to form LONDS with phospholipid coatings, and among these, squalane and tripropionin were chosen as model oils for subsequent studies. LONDS were formed by high pressure homogenisation, and their size was found to decrease with increasing production pressure. When produced at 175 MPa, all LONDS samples exhibited sizes between 100–300 nm, with polydispersity index PI between 0.1–0.3. The LONDS were stable for over six weeks, at 4 °C, and also under physiological conditions, showing modest changes in size (< 10%). The hydrophobic drug combretastatin A4 (CA4) was encapsulated

in tripropionin LONDS with an efficiency of approximately 76%, achieving drug concentration of approximately 1.3 mg/ml. SVR mouse endothelial cells treated with CA4 tripropionin LONDS showed the microtubule disruption, characteristic of drug uptake for all tested doses, which suggests successful release of the CA4 from the LONDS.

Keywords: Drug Delivery, Nanoemulsion, Combretastatin

1 **1. Introduction**

2 The pharmaceutical industry produces many new potential therapeutic
3 agents that have poor water solubility. Many of these hydrophobic agents
4 show promising results during *in vitro* studies, when administered dissolved
5 in solvents like DMSO. However, when undergoing pre-clinical testing, the
6 hydrophobicity of drugs often makes them difficult to deliver, resulting in re-
7 duced bioavailability and efficacy.[1]. A multitude of different routes for hy-
8 drophobic drug encapsulation have been proposed,[2] from micelles[3, 4, 5] to
9 porous nanoparticles.[2, 6] Among these methods, sub-micron emulsions, or
10 *nanoemulsions*,[7] are a versatile option for the encapsulation and delivery of
11 poor water soluble compounds.[8] Nanoemulsions consist of nanometer-sized
12 oil droplets dispersed in water; the oil is generally assumed to be insoluble in
13 the water phase. The adsorption of a surfactant in the oil-water interface al-
14 lows for the existence of the nanodroplets, and helps preventing nanodroplet
15 coalescence.[9] The use of nanoemulsions for hydrophobic drug delivery re-
16 lies on the encapsulation of the therapeutic agent within the oil core of the
17 nanodroplets. High-energy methods for the production of nanoemulsions in-
18 clude ultrasonication [10] and high pressure homogenisation[11, 12, 13, 14],
19 resulting in nanodroplets below 300 nm in diameter. Such nanodroplets are
20 particularly relevant for drug delivery as they are able to achieve good pene-
21 tration through small capillaries as well as uptake across tissue.[15] There are
22 several factors that influence the size of the nanodroplets within an emulsion.
23 For the specific case of high-pressure homogenisation, the production pressure
24 plays a crucial role as it determines the efficiency in dispersing the oil into the
25 water phase. The literature about this topic is extensive, but it has mostly
26 focused in nanoemulsions aimed at the food industry.[16, 17] The nature of
27 the surfactant used to stabilise the nanoemulsions is also known to affect the
28 size of the nanodroplets.[18] For example, increasing concentrations of PEG
29 in the surface of lipid-stabilised nanoemulsions has been reported to reduce

30 the size of the droplets.[19] Therapeutic nanoemulsions have been success-
31 fully prepared using a range of different biocompatible surfactants, including
32 Tween,[20] polyethylene glycol[21] and lipids.[22, 23, 24, 14, 25] Nevertheless,
33 lipid shells are of particular relevance for drug delivery systems, as phospho-
34 lipids are cheap, easy to handle and are versatile, i.e. to include different
35 ligands for nanodroplet functionalisation to enhance biocompatibility and
36 permit attachment of targeting agents such as antibodies.[26, 27, 19] In ad-
37 dition to the production pressure and the chosen surfactant, the viscosity
38 of the oil has been observed to influence the size of the nanoemulsion nan-
39 odroplets prepared using different methods.[28, 29] Lower viscosity oils have
40 been observed to produce smaller nanodroplets than higher viscosity oils such
41 as long chain triglycerides. For example, Sanguansri and co-workers observed
42 a size difference of around 33% when comparing nanodroplets prepared with
43 peanut oil (~ 57 mPa·s at 25 °C) and hexadecane (2.66 mPa·s at 25 °C).[30]
44 This effect is attributed to the enhancement of the droplet deformability
45 when using lower viscosity oils, which results in a more efficient dispersion
46 of the oil in the water phase.

47 To date, nanoemulsions have been used in wide range of treatments, such
48 as antiviral,[31] and antimalarial[22, 32]. Furthermore, a number of anti-
49 cancer drugs have been delivered to tumours using nanoemulsions.[33, 21, 14]
50 For example, Dacarbazine encapsulated in soybean oil nanodroplets was
51 found to have an increased efficacy when administered *in vivo*.[34] Com-
52 bretastatin A4 (CA4) belongs to a group of vascular disrupting agents which
53 selectively cause rapid shut down of established tumour vasculature.[35, 36].
54 CA4 has a high binding affinity to tubulin, which leads to morphological
55 changes of the endothelial cells cytoskeleton. *In vivo* treatments with CA4
56 are impaired by the toxicity and poor water solubility of the drug. Efforts
57 to improve treatments with CA4 have led to the development of the more
58 soluble prodrug combretastatin A4 phosphate (CA4P). CA4P has been evalu-
59 ated as a monotherapy in a number of Phase I clinical trials, with a reported
60 number of adverse side effects including nausea, vomiting, tumour pain and
61 more severe cases of acute coronary syndrome.[37, 38] There are ongoing
62 Phase II/III clinical trials assessing CA4P in combination with other drugs
63 such as Bevacizumab.[39]. Although converting CA4 to the water soluble pro-
64 drug CA4P permits delivery its toxicity still leads to a number of undesired
65 side effects. Therefore there is an unmet clinical need for the development
66 of a vehicle that is able to deliver compounds like CA4 in more targeted
67 strategies.

68 Various developments have been made towards enhancing the delivery of
69 CA4 through its incorporation in liposomes. Liposomal formulations rely on
70 the incorporation of CA4 in the lipid bilayer and therefore they are greatly
71 restricted in the amount of CA4 that can be loaded.[40, 41] Bibby and co-
72 workers studied the effect of CA4 delivered in a 10% DMSO/oil emulsion *in*
73 *vivo*. They found severe vascular shut-down and tumour growth delay as a
74 result of the treatment with 150 mg/kg of CA4 administered in this way.[42]
75 However DMSO is not an ideal solvent for the injection of poor water soluble
76 drugs due to its adverse side effects.

77 Here we report the encapsulation and *in vitro* delivery of CA4 in lipid-
78 stabilised oil nanodroplets (LONDS) (Figure 1). LONDS are formed in a
79 two-step homogenisation process that progressively refines the size distribu-
80 tion of the colloidal solution. This process includes a blending step, and
81 homogenisation under high pressure. To optimise the formation of LONDS,
82 and to determine the best oil to deliver CA4, a number of biocompatible oils
83 were screened. LONDS were prepared using seven different oils, and their
84 size, stability and concentration determined. Of these, squalane and tripropionin
85 were found to successfully form LONDS and were chosen as model oils
86 for further studies. Size differences were observed in LOND samples prepared
87 under different pressures, and also between the different oils. Squalane and
88 tripropionin LONDS were stable for at least six weeks when kept at 4 °C,
89 and for > 2 h at 37 °C. CA4 was successfully encapsulated in tripropionin
90 LONDS. *In vitro* evaluation of CA4 in SVR mouse endothelial cells at es-
91 calating doses revealed collapsed microtubule structures characteristic of the
92 activity of the CA4. This suggests successful drug release and intracellular
93 uptake of drug from LONDS, which is a promising step towards effective and
94 safe treatments with CA4 *in vivo* .

95 2. Methods

96 2.1. Materials

97 All lipids were purchased from Avanti Lipids (USA), including 1palmitoyl-
98 2-oleoyl-sn- glycerol- 3-phosphocholine (POPC), 1,2- distearoyl-sn- glycerol- 3-
99 phosphocholine (DSPC), 1,2-distearoyl-sn-glycerol- 3-phosphoethanolamine-
100 N-[methoxy (polyethylene glycol)- 2000] (PEG2000-DSPE) and 1,2-distearoyl-
101 sn-glycerol- 3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)- 2000]
102 (biotin-PEG2000-DSPE). Fluorescently tagged 1,2-dioleoyl-sn- glycerol- 3-
103 phosphoethanolamine (DOPE), Atto590 DOPE and Atto488 DOPE, were

104 obtained from Atto-TEC (Germany). All oils were purchased from Sigma-
105 Aldrich (UK), including squalane, squalene, triacetin, olive oil, isoamyl ac-
106 etate (IA), tripropionin and eicosapentaenoic acid (EPA). Combretastatin
107 A4 (CA4) was obtained from Sigma (UK). The primary mouse monoclonal
108 anti- β -tubulin antibody was also obtained from Sigma (UK). Secondary anti-
109 bodies biotin-labelled polyclonal rabbit anti-mouse was obtained from DAKO
110 (UK) and fluorescein-isothiocyanate-labelled Avidin D was obtained from
111 Vector Laboratories (UK). Antibody diluent ready to use reagent and Pro-
112 long Gold with DAPI were obtained from Thermo Fisher Scientific (UK).

113 *2.2. LONDS formation*

114 LONDS are emulsion droplets stabilised with phospholipids. LONDS were
115 formed in a two-step homogenisation process of oil-in-water mixtures contain-
116 ing lipid. All lipid mixtures were initially dissolved in a chloroform-methanol
117 mixture (1 : 1) and then dried under nitrogen for at least 30 min. The
118 lipid shell was tuned for the different oils, informed by preliminary opti-
119 misation on the production (data not shown). Hence, and unless otherwise
120 stated, POPC + 5% biotin-PEG2000-DSPE was used for stabilising squalane
121 LONDS, whereas DSPC + 20% Cholesterol + 5% biotin-PEG2000-DSPE was
122 used for the preparation of tripropionin LONDS; these lipid shell combina-
123 tions enhanced the stability of the LONDS and reduced premature coales-
124 cence. When required, fluorescence tracking of LONDS was performed by
125 incorporating 0.1 mol% Atto590-DOPE or Atto488-DOPE to the lipid mix.
126 The lipids were re-suspended in 0.7 ml ($\phi = 0.07$) of oil by vortexing. The oil
127 fraction was kept constant during the experiments. Occasionally the dried
128 lipid took longer to disperse in the oil, and an additional bath-sonication step
129 was required. For samples encapsulating drugs or drug mimics, the com-
130 pound was solubilised in the oil to the desired concentration before adding it
131 to the lipids. The homogenisation process began with a blending step using
132 the rotor-stator system Polytron PT1300 D (Kinematica AG, Switzerland).
133 Polytron blending of the sample was carried out at 12500 rpm for 10 min,
134 at 40 ° C and atmospheric pressure. LONDS formation was completed in
135 a high-pressure homogeniser EmulsiFlex-C5 (Avestin Europe GmbH, Ger-
136 many) for 20 min. The pressure was varied between 35 – 175 MPa to study
137 the dependence of LONDS size with the production pressure. Other than the
138 LOND samples used to study the effect of the production pressure, LONDS
139 were prepared under 175 MPa unless otherwise stated. Excess lipid and non-
140 encapsulated oil were removed from the LONDS solution by filtration using

141 a KrosFlow Research Iii Tangential Flow Filtration System (SpectrumLabs
142 Europe, The Netherlands) and 60 kDa Mini-Discover12 pore size column
143 (WaterSep, USA) for at least 2 h.

144 *2.3. LONDS size and concentration*

145 Filtered LONDS were sized by Dynamic Light Scattering (DLS) using
146 a Zetasizer Nano ZSP (Malvern Instruments, UK). For the measurements,
147 10 μl of the LOND sample were dissolved in 1 ml of PBS in an ordinary
148 disposable cuvette. Three measurements of the sample distribution were
149 performed, each of which consisted of 10 – 17 runs as determined by the
150 Malvern ZetaSizer Software. DLS was also used to study the stability of
151 LONDS size over time, with storage at 4 °C between measurements. The
152 stability of LONDS at 37 °C was also investigated using DLS. In this case,
153 the sample was kept at 37 °C for 2 h and measurements of its size distribution
154 were taken every 15 min. The sample was gently shaken between measure-
155 ments to avoid creaming (or sedimentation). The size of LOND samples
156 was alternatively determined by nanoparticle tracking analysis (NTA) using
157 a NanoSight instrument (Malvern Instruments, UK). This device was also
158 used to determine the concentration of LOND samples. NTA relies on the
159 detection of light scattered by sub-micron sized particles, which is used to
160 calculate individual diffusion coefficients and estimate their size.[43] Samples
161 containing around 10^6 LONDS/ml were used for the measurements with the
162 NanoSight instrument (normally involving a 1 : 10^6 dilution of the origi-
163 nal sample). All data regarding LOND size and concentration was analysed
164 using Origin 9 (OriginLab Corporation, USA).

165 *2.4. Transmission electron microscopy*

166 Transmission electron microscopy (TEM) images of LONDS were taken
167 using a JEM1400, 120 kV instrument (JEOL, USA). Samples for TEM
168 imaging were prepared by depositing 15 μl of the LONDS samples (10^{11}
169 LONDS/ml, in PBS) on a carbon coated grid. The solution was left on the
170 grid for 30 s, and the excess liquid dried out using blotting paper. Samples
171 were negatively stained using uranyl acetate by dropping 15 μl of uranyl ac-
172 etate (1%) on the grid and incubating for 15 s, after which the excess liquid
173 was removed with blotting paper. Images were analysed using ImageJ.

174 *2.5. Encapsulation efficiency of CA4 in LONDS*

175 The concentration of CA4 in LOND preparations was determined by com-
176 paring their UV-VIS absorption to a calibration curve for CA4 dissolved in
177 tripropionin. A Lambda 35 UV-VIS Spectrometer (Perkin Elmer, USA) was
178 used to record absorption spectra of the samples. All absorption spectra were
179 recorded between 200 – 700 nm. The peak absorption for CA4 diluted in the
180 oil (not encapsulated) lies around 309 nm. To measure the amount of CA4
181 encapsulated in the LONDS, 10 μ l of LONDS solution was dispersed in 990
182 μ l of DMSO. In this way, the LONDS are broken and their contents released,
183 eliminating light scattering from intact LONDS in the solution, which can
184 cause detection issues.

185 *2.6. In vitro cell culture*

186 SVR mouse pancreatic islet endothelial cells transformed with *Ras* onco-
187 gene, were cultured in high glucose Dulbecco's modified Eagle's medium
188 (DMEM) supplemented with 5% (v/v) foetal calf serum (FCS) at 37 °C and
189 5% CO₂. SVR cells were grown to 80 – 90% confluence, trypsinised and
190 seeded in μ -slide VI (ibidi, Germany) at a density of 3×10^5 overnight after
191 which they were treated with CA4 tripropionin LONDS. All treatments and
192 sequential staining were carried out directly in the μ -slide. Escalating doses
193 of CA4 tripropionin LONDS (4 mM stock concentration) were added to SVR
194 cells in fresh media for 2 h at 37 °C. Following the 2 h treatment the cells
195 were washed with PBS and fixed with 4%(v/v) paraformaldehyde in PBS
196 for 10 min and permeabilised with 0.1% Triton X-100 in PBS for 5 min. To
197 reduce non specific binding the cells were blocked with antibody diluent for
198 5 min at room temperature. Immunofluorescence staining was performed for
199 β -tubulin 1:500 in antibody diluent for 2 h at room temperature. The cells
200 were then washed with PBS and incubated with biotin-labelled polyclonal
201 rabbit anti-mouse 1:200 in antibody diluent for 1h at room temperature
202 followed by fluorescein isothiocyanate-labelled Avidin D 1:250 in antibody
203 diluent for 1h at room temperature. The μ -slide was mounted with Prolong
204 Gold and DAPI (4,6-diamidino-2-phenylindole) to visualise nuclei. Fluores-
205 cent images were acquired using a ZEISS Axioimager Z1 microscope (Carl
206 Zeiss Microscopy, USA) with AxioVision software at x40.

207 *2.7. Quantification of CA4 effect*

208 To quantitate the extent of disruption to microtubules caused by CA4
209 tripropionin LONDS, fluorescence images of control untreated cells and cells

210 treated with CA4 tripropionin LONDS were taken ($n = 5$). The length of
211 10 microtubules from five randomly selected cells were measured in each of
212 the five images (total of 250 microtubules). Image J and GraphPAD were
213 used to measure the microtubules and data plotting, respectively. Statistical
214 analysis was carried using a Mann-Whitney test.

215 3. Results and discussion

216 3.1. Preparation of LONDS with candidate oils

217 LONDS were prepared in a two-step homogenisation process of an oil-in-
218 water and phospholipids mixture. The crude emulsions was initially blent in
219 a rotor-stator system for around 10 min to create a rough dispersion of the oil
220 into the water phase, and to dissolve big lipid agglomerates. This mixing step
221 was followed by high pressure homogenisation for 20 min in an Emulsiflex.
222 In this device, the crude emulsion was forced through a *homogenising nozzle*,
223 where it experienced great mechanical stress for a short time. Figure 2 shows
224 the size distribution of squalane LONDS after processing under high pressure,
225 as measured with DLS, as an example. The high pressure homogenisation
226 step was performed at 175 MPa, and reduced the size distribution of the
227 sample giving a polydispersity index (PI) lower than 0.3 (figure 2b). The
228 average size of the LONDS was (165 ± 5) nm. After filtration, the average size
229 of the sample increased slightly to (188 ± 11) nm (figure 2). This apparent
230 size change of about 12% was accompanied by a slight increase in distribu-
231 tion width, as indicated by the increase of the full width at half maximum
232 (FWHM). This perhaps indicates that the filtration process gave rise to mild
233 sample coalescence.

234 A number of biocompatible oils were screened to determine their ability to
235 form LONDS stabilised with POPC + 5% biotin-PEG2000-DSPE and at 175
236 MPa. Table 1 summarises the results obtained for the different oils, including
237 the size and concentration of the LONDS, where applicable. Isoamyl acetate
238 (IA) and eicosapentaenoic acid (EPA) did not produce stable LONDS, thus
239 no further experiments were performed with these. The concentration of
240 triacetin LONDS was found to be lower than that of LONDS prepared with
241 any other oil. This was attributed to the higher solubility of triacetin in wa-
242 ter. Among all of the oils tested, tripropionin LONDS exhibited the smaller
243 size, and also the highest concentration. LONDS prepared with squalane,
244 squalene or olive oil produced LONDS of similar size, PI and concentra-
245 tion. Pure oils were chosen over blends such as olive oil, whose composition

246 strongly depends on its production environment and manufacturer. The size
247 of the LONDS prepared with different candidate oils remained stable for a
248 period of more than six weeks, at 4 °C. Details for the case of tripropionin
249 and squalane LONDS are provided below. Squalane and tripropionin were
250 used as oils for the optimisation of LONDS production, in order to compare
251 LOND production with oils of different density and viscosity.

252

253 3.2. Dependence of LONDS size and concentration on production pressure

254 Control over the size of the LONDS is important for reproducible pharma-
255 cokinetic/pharmacodynamic studies. Furthermore, the size of nanocarriers
256 greatly influences their biodistribution and bioavailability.[15] The produc-
257 tion of LONDS was monitored as a function of the homogenisation pressure
258 using two different oil/shell combinations: i) squalane LONDS stabilised with
259 POPC, and ii) tripropionin LONDS stabilised with DSPC + 20mol% Choles-
260 terol + 5mol% biotin-PEG2000-DSPE. Size measurements of both LOND
261 samples prepared under 35, 70, 105, 140 and 175 MPa were performed using
262 DLS and NanoSight. Figure 3 shows the changes in the size distribution of
263 the two LOND samples prepared under different pressures, as determined by
264 DLS. In the case of squalane, the average size of the droplets d was fitted
265 to the relationship $d = C \cdot P^{-b}$, where P is the pressure applied at the ho-
266 mogenising nozzle in the Emulsiflex. The parameter b is determined by the
267 turbulent regime existent in the homogenising device, which depends mostly
268 on the size of the appliance,[44] whereas C depends on efficiency of the droplet
269 disruption.[45] Through the fitting of the sizing data for squalane LONDS,
270 b was found to be $b = (0.70 \pm 0.06)$. This value is in agreement with previ-
271 ous observations, which reported values of b between 0.6 – 0.75 for regimes
272 dominated by inertial or shear forces, respectively.[46, 45] In the case of
273 tripropionin LONDS, their size was found to reduce with increasing produc-
274 tion pressure. It is also interesting to note that the size change undergone by
275 tripropionin LONDS between 35 and 175 MPa was of around 25%, whereas
276 the size change for squalane LONDS was of about 70%. This is thought
277 to relate primarily to the differences in viscosity between tripropionin and
278 squalane, as viscosity is known to influence the size of the nanodroplets in a
279 nanoemulsion.[30]

280 Upon preparation of a nanoemulsion, the number of nanodroplets formed
281 is generally assumed to be determined by the oil volume fraction ϕ . Although
282 this assumption is true in general, non-negligible solubility of the oil in water

283 could result in a reduced number of nanodroplets, since the disperse phase
284 could partially dissolve in the continuous prior to surfactant stabilisation.
285 The concentration and size of LONDS prepared under different pressures
286 (35, 70, 105, 140 and 175 MPa) were determined with the NanoSight instru-
287 ment. Figure 4 shows concentration change in LOND samples as a function
288 of the production pressure, for squalane (a) and tripropionin LONDS (b).
289 Squalane LONDS show a rapid decrease in their size distribution with in-
290 creasing pressure. The size reduction coincides with an increase in the num-
291 ber of LONDS in the solution. The concentration reached a steady value at
292 around $(2.2 \pm 0.4) \times 10^{13} \text{ ml}^{-1}$. This is in agreement with the predicted in-
293 crease in the efficiency of the oil breakage upon increase of the shear forces at
294 the homogenising nozzle (i.e. increasing production pressure). These values
295 are, within experimental error, consistent with the theoretical value for the
296 concentration of LONDS calculated through size considerations for $\phi = 0.07$
297 ($\sim 2 \times 10^{13}$ LONDS/ml). In the case of tripropionin LONDS (figure 4b),
298 increasing production pressures (35 – 145 MPa) resulted in distribution sizes
299 changes of around 4%. Sample processing under 175 MPa for 10 min re-
300 sulted in a significant size reduction ($\sim 16\%$), accompanied by an increase
301 of the LOND concentration ($\sim 53\%$). As expected, the concentration of the
302 the tripropionin LOND sample prepared under 175 MPa ($(1.4 \pm 0.1) \times 10^{14}$
303 LONDS/ml) is in agreement with the theoretical value for the concentration
304 of LONDS, based on LONDS dimension and oil volume fraction ($\phi = 0.07$),
305 1.3×10^{14} LONDS/ml.

306 3.3. Stability of LONDS

307 The stability of LONDS as a function of time was followed by monitoring
308 the size distribution of LOND samples using DLS. Sample agglomeration or
309 degradation was expected to translate into changes in the size distribution,
310 such as broadening of the original size distribution and increases in the *PI*.
311 Two different studies were performed: i) to determine the stability of LONDS
312 during six weeks, and ii) to determine the stability of LONDS at 37 °C in
313 *in vitro* conditions for 2 h. There was an interest in monitoring the stability
314 of LONDS for a time period that would resemble the time required for the
315 LONDS to initially reach the target site and accumulate over time in an *in*
316 *vivo* setting. Hence the choice of a 2 h period for this experiment. The
317 temperature conditions in this experiment aimed to better resemble those to
318 which the LONDS would be exposed to *in vivo*.

319 As reflected in table 1, LONDS prepared from all the candidate oils showed
320 good stability over a six weeks period. During this time, the PIs were < 0.3
321 in all cases. As an example of the type of data recorded, figure 5a shows the
322 size variation of squalane and tripropionin LONDS over six weeks. Each ex-
323 perimental point was produced averaging three DLS runs. This graph shows
324 a maximum size variation of around 16% for squalane LONDS, and around
325 15% for tripropionin LONDS. This small variation of the LONDS diameter
326 over time suggests good stability of the LONDS against agglomeration and
327 coalescence. Figure 5b shows the size variation for squalane and tripropionin
328 LONDS during 2 h, and incubated $37\text{ }^{\circ}C$ more consistent with conditions *in*
329 *vitro*. The sizes of both LOND types did not change, showing good stabil-
330 ity under these conditions. This suggests that LONDS are suitable vehicles
331 for the delivery of hydrophobic compounds *in vivo* and have an excellent
332 potential to enhance the delivery of poor water soluble drugs.

333 3.4. TEM imaging of LONDS

334 TEM was used to image LOND samples. Figure 6 shows the TEM images
335 of squalane and tripropionin LONDS. Squalane LONDS appeared as globular
336 structures ranging 100 – 300 nm, with an average size of 111 nm ($n = 187$,
337 with a standard deviation of the sample $\sigma = 85$ nm). In contrast, tripropionin
338 LONDS appeared to lose their structure under vacuum conditions in TEM,
339 and exhibited a shell appearance. These LONDS were found to have an
340 average size 89 nm ($n = 71$, with a standard deviation of the sample $\sigma = 23$
341 nm). A small discrepancy was observed between the TEM and DLS sizing
342 of both squalane and tripropionin LONDS. This was expected and it is
343 attributed to the different conditions under which the samples are studied
344 in one and another techniques (i.e. the sample is dried for TEM imaging,
345 whereas the hydrodynamic radius of the colloids are measured by DLS).

346 3.5. Encapsulation of CA4 in LONDS

347 Drug delivery using nanoemulsions, and LONDS in particular, rely on
348 drug encapsulation within their structure. It is generally accepted that the
349 drug is encapsulated within the oil core of the nanoemulsions, and that its
350 lipophilicity directly affects its release from the nanodroplets and therefore
351 its biodistribution.[47] It is also known that the polarity of a therapeutic com-
352 pounds defines its solubility in oils.[32, 48] CA4 is a largely polar molecule
353 with poor water solubility. However, its polarity made it difficult to dissolve
354 in squalane, as it is a saturated, non-polar oil. Triacetin and tripropionin

355 were profiled as more suitable oils for the preparation of LONDS encapsulat-
356 ing CA4, as the polarity of the oil enhanced the solubility of CA4. Tripropi-
357 onin was chosen over triacetin, in light of its reduced water solubility. The
358 concentration of the drug encapsulated in LONDS was determined by mea-
359 suring its absorption in the UV-VIS. The calibration curve produced for the
360 CA4 in tripropionin is presented in the supplementary. The encapsulation
361 efficiency of CA4 in tripropionin LONDS was approximately 76%, with 1270
362 $\mu\text{g}/\text{ml}$ in the final LOND sample.

363 3.6. CA4 tripropionin LONDS cause microtubule disruption

364 To qualitatively and quantitatively assess LOND-based drug delivery,
365 CA4 tripropionin LONDS were used to treat SVR endothelial cells. The cells
366 were treated with escalating concentrations of CA4 tripropionin LONDS from
367 2 – 100 nM for 2 h at 37 °C. Figure 7 is a representative panel of images
368 showing the effect caused by CA4 tripropionin LOND exposure. CA4 tripro-
369 pionin LONDS caused complete microtubule disruption evident in all SVR
370 cells treated with 40 – 100 nM, with microtubules appearing as a uniform flu-
371 orescence surrounding the cells nuclei. At concentrations lower than 2 – 20
372 nM, some short microtubules could be seen. Control untreated SVR cells
373 showed normal microtubule structures.

374 To quantitate the effect on SVR cells and to evaluate the effect caused
375 to microtubules by CA4 tripropionin LOND treatment, the lengths of the
376 cellular microtubules were measured. Figure 8 shows the average microtubule
377 length for the different treatment concentrations. Compared to control, cells
378 treated with doses of CA4 tripropionin LONDS between 4 – 100 nM are
379 significantly different $***P < 0.001$. A significant difference $*P < 0.024$
380 was observed in the smallest concentration of 2 nM. It should be noted that
381 the exact mechanism of drug uptake is unclear however the fact that CA4
382 tripropionin LONDS at 37°C are stable suggests that the lipid membrane of
383 the LONDS fuses with cell membranes leading to drug release or the LONDS
384 are endocytosed leading to intracellular release of CA4.

385 4. Conclusion

386 We have carried out a systematic study on the formation of lipid-stabilised
387 oil nanodroplets (LONDS) with a range of biocompatible oils, towards med-
388 ical applications. LONDS are formed in a two-step process that includes

389 blending using a rotor-stator system and high pressure homogenisation. Pro-
390 duction pressure has been found to be a factor affecting the size of the
391 LONDS, as it also affects LONDS concentration in the final sample. LOND
392 samples exhibit excellent stability over time and under different conditions.
393 The hydrophobic therapeutic drug Combretastatin A4 (CA4) was encapsu-
394 lated in tripropionin LONDS with an efficiency of around 76%. Preliminary
395 studies on LOND-based delivery of this compound to SVR cells have shown
396 successful drug release from the LONDS, attending at the effect on cellular
397 microtubules by the encapsulated CA4. Tripropionin LONDS show promis-
398 ing results for the delivery of CA4 *in vitro*, and they hold exciting potential
399 for facilitating the delivery of the compound *in vivo*. We anticipate that
400 studies concerned with the use of CA4 in anticancer combination therapies
401 will benefit from the enhancement of the delivery routes for CA4.

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408 <http://doi.org/10.5518/98>

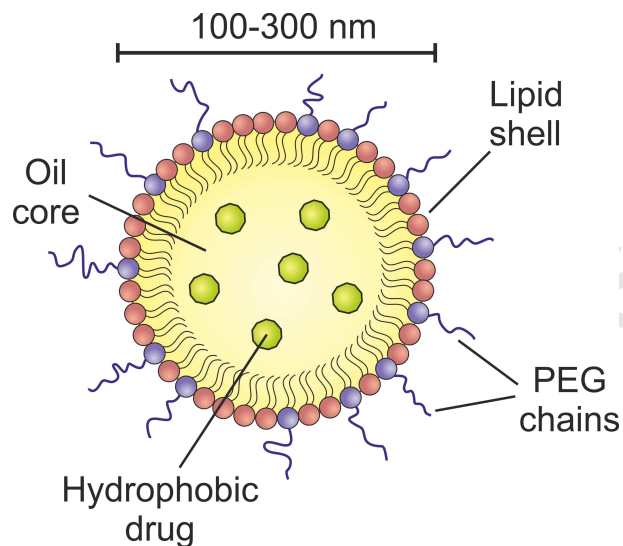


Figure 1: Schematic of a lipid-stabilised oil nanodroplet (LONDs). Hydrophobic drugs, such as CA4, are contained in the oil core of the LONDs, which is stabilised by a lipid monolayer.

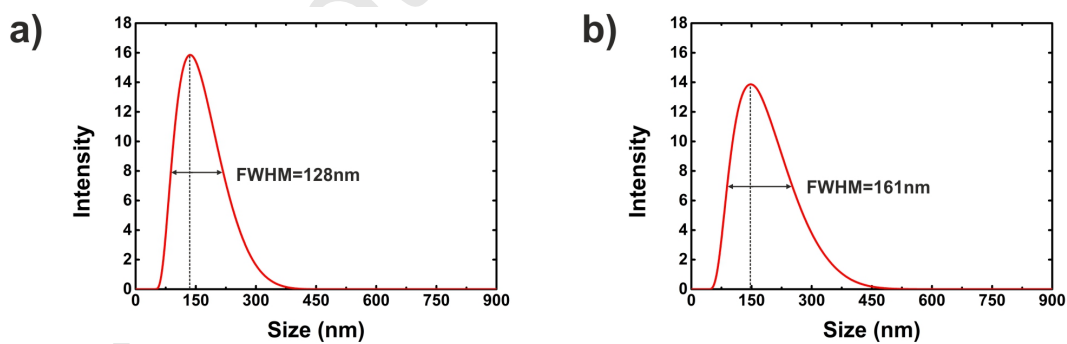


Figure 2: Changes in the size distribution of a squalane LONDs sample: a) after high pressure homogenisation (at 175 MPa), with average size (165 ± 5) nm and b) after sample cross-filtration, mode size (188 ± 11) nm. The width of the distribution (full width at half maximum, FWHM) is indicated in the figures.

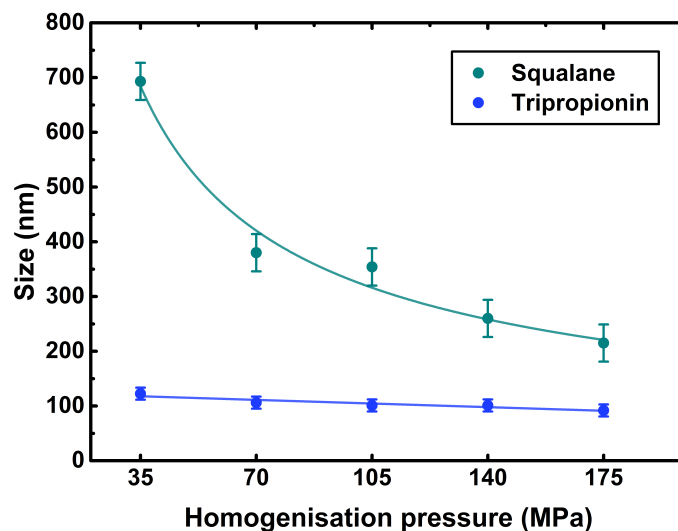


Figure 3: DLS sizing of squalane and tripropionin LOND samples prepared at different pressures. Squalane LONDS were stabilised with POPC, and the shell of the tripropionin LONDS was DSPC + 20% Cholesterol + 5% biotin-PEG2000-DSPE. The lines show fits to the data.

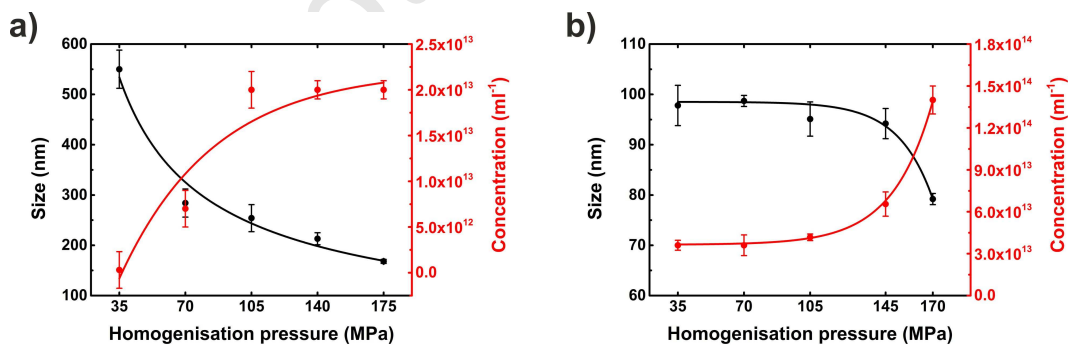


Figure 4: Nanoparticle Tracking Analysis (NTA) measurements on a) squalane LONDS stabilised with POPC and b) tripropionin LONDS stabilised with DSPC + 20% cholesterol + 5% biotin-PEG2000-DSPE. The graph shows the changes in size and concentration of the LONDS in the solution, and the data points were fitted as a guide to the eye.

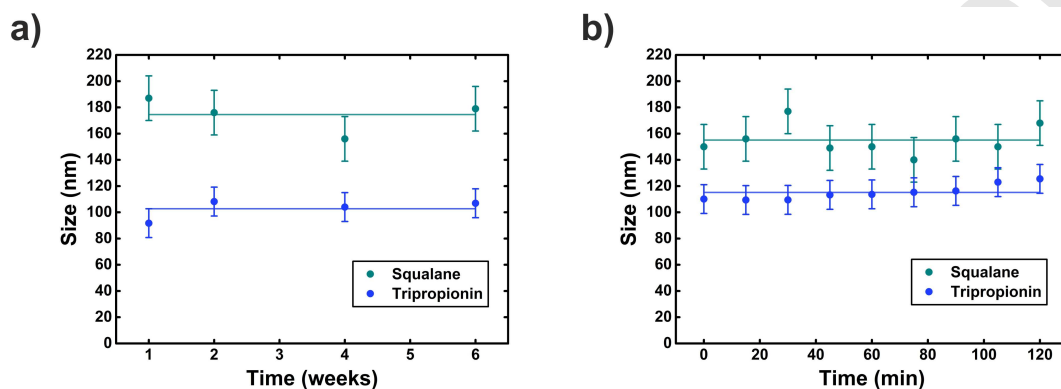


Figure 5: a) Size change of squalane and triacetin LONDS over a six week period, stored at 4 °C, and measured using DLS. b) Size change of squalane and triacetin LONDS at 37 ° over 2 h, as measured by DLS. Experimental points in both graphs were produced averaging three size measurements on the same sample.

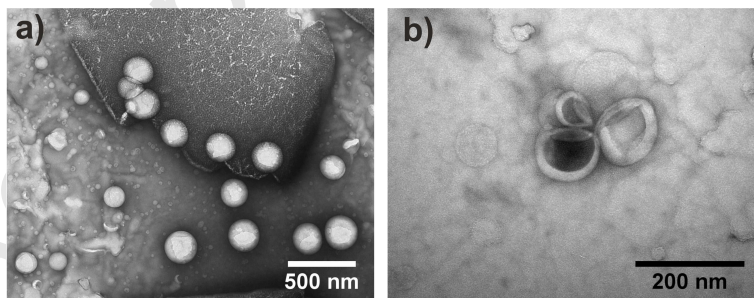
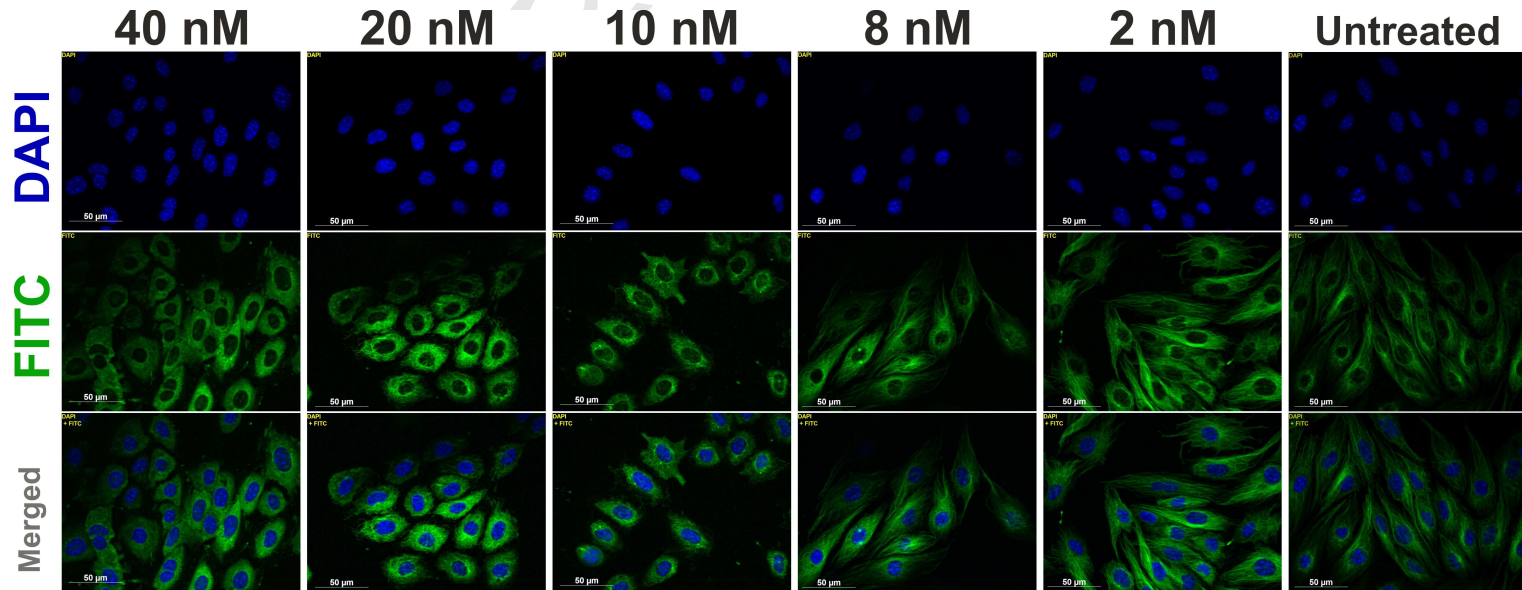


Figure 6: TEM images of LONDS. a) Squalane LONDS stabilised with POPC + 5% biotin-PEG2000-DSPE. b) Tripropionin LONDS stabilised with DSPC + 20% Cholesterol + 5% biotin-PEG2000-DSPE. Both samples were prepared under at 175 kPa.

Figure 7: CA4 tripropionin LONDS disrupt microtubules *in vitro*. SVR mouse endothelial cells were treated with CA4 tripropionin LONDS at a dose escalation range between 2 nM to 100 nM for 2h at 37 °C followed by washing and fixing with 4% paraformaldehyde. Immunofluorescence staining of β -tubulin was carried out. Prolong Gold with DAPI was used to mount and visualise the nuclei. The results show at higher doses of 100 nM to 40 nM CA4 released from the LONDS causes complete microtubule disruption leading to a uniform fluorescence around the nuclei. Lower doses of 20 nM to 2 nM show a varying lengths of microtubule filaments. Untreated cells were used as control where normal microtubules can be seen. Scale bar 50 μ m.



17

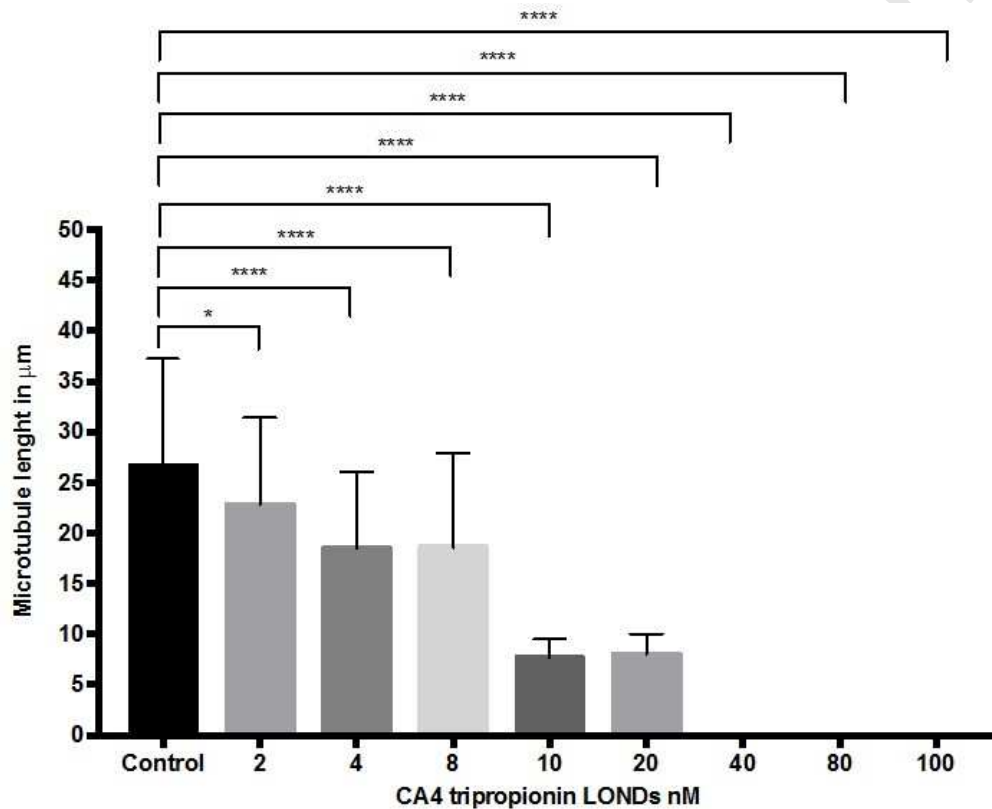


Figure 8: Quantification of microtubule lengths following CA4 tripropionin LOND exposure of 2 h at 37 °C. A total of 250 microtubule filaments were measured with *Image J* from five images taken from different positions on the ibidi μ -Slide. Error bars were calculated by Mann-Whitney test. * $P < 0.0240$, *** $P < 0.0001$. Results shown as media with interquartile range.

Table 1: LOND preparation and stability for different oil types and a POPC + 5% biotin-PEG200-DSPE* shell.

| Oil | Solubility in H_2O (g/L) | Viscosity at 20 °C (cP) | Density (g/cm ³) | Lipid stabilised | Diameter ^a (nm) | Concentration (ml ⁻¹) | Stability (weeks) |
|--------------|----------------------------|-------------------------|------------------------------|------------------|----------------------------|-----------------------------------|-------------------|
| Squalane | - | 12 | 0.805 | Yes | 165 | 10 ¹³ | > 6 |
| Squalene | 0.124×10 ⁻³ | 11 | 0.858 | Yes | 133 | 10 ¹³ | - |
| Olive oil | - | 85 | 0.918 | Yes | 191 | - | > 6 |
| Triacetin | 61 | 23 | 1.160 | Yes | 250 | 10 ¹¹ | > 6 |
| Tripropionin | 0.003 | 7 | 1.082 | Yes | 100 | 10 ¹⁴ | > 6 |
| IA | 2 | 7.2 | 0.876 | No | - | - | - |
| EPA | 0.284×10 ⁻³ | 35 | 0.923 | No | - | - | - |

* Tripropionin LONDS shell was 75mol% DSPC + 5mol% Cholesterol + 5mol% PEG2000-DSPE. Olive oil, IA and EPA had POPC shells.

^a Two-step preparation process and cross filtration. Emulsiflex pressure was 175 kPa in all cases. Sizing data on this table was measured with DLS. Concentration data for squalane and squalene LONDS was obtained with NTA, and Qnano was used to obtain the concentration of triacetin LONDS.

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