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1 **Full title: Preparation and characterization of phospholipid stabilized**

2 **nanoemulsions in small-scale**

3 **Short title: Phospholipid stabilized Nanoemulsions in small-scale**

4 **Shila Gurung[a, b*], Martin Holzer[a], Sabine Barnert[a], Rolf Schubert[a]**

5 [a]Department of Pharmaceutical Technology and Biopharmacy, Albert Ludwig University of Freiburg,

6 Freiburg im Breisgau, Germany

7 [b]Current address: School of Health and Allied Sciences, Pokhara University, Kaski, Nepal

8 *gshila@gmail.com (SG)

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9 **Abstract**

10 Phospholipids have been used to prepare liposomes. The use of phospholipids to stabilize
11 nanoemulsions may cause spontaneous formation of liposomes. The main objective of this study is to
12 develop a method to prepare phospholipid stabilized nanoemulsions in small scale (< 1 mL) and to
13 minimize the formation of liposomes.

14 A combination of hand extrusion and detergent removal methods was used in this study. Extrusion
15 through polycarbonate membranes was performed in two steps, firstly using membranes of 400 nm
16 followed by 200 nm membranes as the second step. Sodium cholate was used as a detergent to
17 solubilize the formed liposomes which was later removed via dialysis. Nanoemulsions were characterized
18 by measuring their particle size, polydispersity index and zeta-potential using Photon Correlation
19 Spectroscopy and Cryo-TEM pictures. The stability of nanoemulsion stored under refrigeration was also
20 studied.

21 Fifty-one extrusion cycles through polycarbonate membrane of 400 nm pore size followed by one-
22 hundred fifty-three cycles through polycarbonate membrane of 200 nm produced nanoemulsions having
23 particle size below 200 nm (diameter). The nanoemulsions were found to be homogenous as depicted by
24 polydispersity index (PDI) value below 0.1. Similarly, the zeta-potential was measured to be above -30
25 mV which is sufficient to keep nanoemulsions stable for as long as 7 months when stored under
26 refrigeration. The Cryo-TEM pictures revealed 30 mM to be an optimum concentration of sodium cholate
27 to prepare homogenous nanoemulsions with negligible proportion of liposomes.

28 It was concluded that this method could be established as a small scale method of preparing
29 nanoemulsions which will not only reduce the cost of preparation but also the disposal cost of toxic
30 chemicals used for functionalizing nanoemulsions for scientific research.

31

32 Keywords: Small-scale nanoemulsions, Functionalized nanoemulsions, Phospholipids, Photon
33 Correlation Spectroscopy, Hand extrusion

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34 ## Introduction

35 Nanoemulsions are transparent or translucent systems generally covering a size range between 20-500
36 nm. Due to the small droplet size, the Brownian motion is sufficiently high to overcome the phenomena of
37 physical destabilization caused by gravitational separation, flocculation and/or coalescence [1-4]. When
38 the maximum droplet size of an emulsion is below 80 nm, it gains advanced properties compared to
39 conventional emulsions, such as optical transparency, high colloidal stability and large interfacial area to
40 volume ratio [5]. Nanoemulsions differ from microemulsions with respect to stability, preparation methods,
41 dilution behavior and temperature fluctuations. In addition, nanoemulsions are thermodynamically
42 unstable, but kinetically stable systems and require less surfactant than microemulsions. They are
43 sometimes referred to as ‘approaching thermodynamic stability [5, 6].

44 Nanoemulsions have been extensively investigated as a promising drug delivery system for poorly water
45 soluble substances. They have been used in intravenous, oral and ocular drug administrations to reduce
46 drug side effects and improve the pharmacological effects of the loaded drugs [3].

47 Parenteral emulsions provide a number of potential advantages as drug delivery vehicle, such as
48 reduction in pain, irritation and thrombophlebitis, reduced toxicity, improved stability and solubility, and the
49 option for a targeted drug delivery approach [7]. The droplets remain stable under the conditions of
50 temperature changes and/or dilutions and do not differ markedly from bulk values. This is especially
51 advantageous for nanoemulsions because when they are injected into the bloodstream, changes in
52 temperature, pH values, osmolarity etc. are likely to occur, affecting the physical properties of the loaded
53 drug [8, 9].

54 Emulsion formation is a non-spontaneous process and therefore requires high energy [5, 10]. The basic
55 composition of an emulsion system is oil, water and emulsifier. For the oil phase, long chain triglycerides
56 such as triolein, soybean oil, safflower oil, sesame oil, and castor oil are approved for clinical use.
57 Medium chain triglycerides are used alone or in combination and the approved ones include fractionated

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58 coconut oil, Miglyol® 810 and 812, Neobee®, and Captex® 300 [7]. Generally, the oil phase does not
59 exceed 30% (w/w), due to which the application of emulsions in drug delivery is limited [11].

60 Emulsifier plays major role in the formation of nanoemulsions by lowering the interfacial tension and
61 hence less stress is needed to break up a droplet [5, 12]. Depending upon the nature of the emulsifier
62 used, they form an interfacial film at the o/w interface, which provides a mechanical barrier and repulsive
63 force to stabilize the emulsion system. The repulsive forces provided by the emulsifiers can be
64 electrostatic (e.g. lecithin), steric (e.g. block copolymer like Poloxamer 188) or electrosteric (a
65 combination of both lecithin and block copolymers). Unfortunately, only a limited number of emulsifiers
66 are approved and recognized as safe by the regulatory authorities [7, 13].

67 Most commonly used methods for preparing emulsions are simple pipe flow, static mixers and general
68 stirrers, high speed mixers, colloid mills, high pressure homogenizers and ultrasonication. Other methods
69 include the low energy emulsification method at constant temperature [4, 14] and the phase inversion
70 temperature (PIT) [5, 15]. However, for preparing nanoemulsions, one is limited to the higher energy
71 sources like high pressure homogenizer and ultrasonication [5, 16]. These so-called high energy methods
72 supply enough energy to increase the interfacial area for generating submicronic droplets [8, 17].

73 Depending upon the preparation method, the size of nanoemulsions varies. Those prepared using PIT
74 are relatively polydisperse and generally give higher Ostwald ripening rates than those prepared by high
75 pressure homogenisation techniques [5]. By using ultrasonic devices having frequencies from 20 kHz to
76 1.0 MHz droplets typically between 100 and 1000 nm in diameter can be prepared. Therefore, they are
77 mostly milky in appearance. However, use of a higher frequency of 2.4 MHz, could produce clear and
78 transparent emulsions which are stable for 12 months even under surfactant-free conditions [18]. The
79 main disadvantages of these high energy methods are difficulties for small scale preparation and
80 therefore expensive.

81 In this study, we investigated a method to prepare stable and homogenous nanoemulsions having definite
82 size and narrow size distribution (PDI below 0.1) in a small scale. This method would be very useful and

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83 cost effective in scientific research for the following reasons: a) for in vitro studies, very less volume of
84 nanoemulsion (< 1 ml) is sufficient, b) to minimize the sample waste and disposal cost, c) to simplify the
85 working set up by avoiding the use of compressed air (like in high pressure homogenizers), and d) to
86 minimize the cost of preparation, especially when expensive chemicals like isotopes, fluorescence
87 markers, nanoparticles surface modifiers like anchors and ligands or antibodies are used.

88 Materials and Methods

89 Materials

90 Medium chain triglyceride (Miglyol® 812) was obtained from Sasol GmbH, Witten, Germany. Egg
91 phospholipid (Lipoid® E80) and Poloxamer 188 (Lutrol® F68) were generously gifted by Lipoid GmbH,
92 Ludwigshafen, Germany and BASF, Ludwigshafen, Germany, respectively. Glycerol (purity >99 %) and
93 sodium cholate hydrate were purchased from Sigma-Aldrich Life Science, Steinheim, Germany. Similarly
94 cholic acid (2, 4-^{3}H) was obtained from American Radiolabeled Chemicals, Inc. St. Louis, MO, USA and
95 phosphatidylcholine (DPPC, L-a-dipalmitoyl [1-palmitoyl-1-^{14}C] from PerkinElmer, Inc. Albany Street,
96 Boston, USA. Deionized water (18.2 MΩ.cm) was used for all dilutions. Slide-A-Lyzer® (10 kDa
97 membrane cut off) was commercially available from Thermo Scientific, Rockford, USA. All other
98 chemicals were of analytical grade.

99 Preparation of nanoemulsions

100 *Hand extruded nanoemulsions*

101 Crude emulsion was prepared by phase inversion temperature method. Briefly, the ingredients of
102 aqueous phase (Table 1) were mixed in a round bottomed flask at 70 °C for about 30 min and 700 rpm
103 using magnetic stirrer (MR 3001, Heidolph Instruments, Schwabach, Germany). Similarly, the oily phase
104 was also prepared by mixing the ingredients (Table 1) in similar conditions. The aqueous phase was then
105 added slowly to the oil phase upon constant stirring (700 rpm) but at an increased temperature of 80 °C.
106 The mixture appeared apparently transparent in the beginning and then turned into translucent upon
107 further addition of remaining aqueous phase. This translucent preparation was cooled for about 1 h in ice

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108 bath and was termed as crude emulsion. The theoretical concentration of phospholipid was maintained at
109 15.75 mM (1.2 %, w/w) throughout the study.

110 ***Table 1. Composition of nanoemulsion***

111	Oil Phase	Aqueous Phase
112	Miglyol® (10 %, w/w)	Poloxamer 188 (1.0 %, w/w)
113	Phospholipid E80® (1.2 %, w/w)	Glycerol (2.5 %, w/w)
114		Deionized water (~ 86 %, w/w)
115		Sodium cholate (20 – 50 mM)

116 Then the crude emulsion was extruded through polycarbonate membranes pre-equilibrated in deionized
117 water for 15 – 30 min using a LiposoFast Basic device (Avestin Inc., Ottawa, Canada). The extrusion was
118 done in two steps in a water bath at 65 °C. In the first step, the crude emulsion was extruded for 51 cycles
119 (400 nm pore size) which was followed by additional 153 extrusion cycles (200 nm pore size). The
120 membrane was changed after every 51 cycles to ensure that the membrane is not damaged. The odd
121 number of extrusion cycle was used to collect the nanoemulsion from the second syringe so that the
122 unextruded larger particles remained in the first syringe.

123 *Nanoemulsions with sodium cholate*

124 It is likely that nanoemulsions stabilized with phospholipids may contain liposomes. Therefore, detergents
125 are used to solubilize lipid membranes [19] because at sufficient concentration, detergents such as
126 sodium cholate solubilize the phospholipid to form mixed micelles (MMs). When the amount of detergent
127 in the MMs is reduced further, the MMs enlarge to form liposomes [20, 21]. Thus, different concentrations
128 (20 – 50 mM) of sodium cholate were used to solubilize the liposomes formed during the preparation of
129 nanoemulsions. The concentration of sodium cholate influences the formation and solubilization of
130 liposomes. The sodium cholate was added to the aqueous phase and the crude emulsion was prepared
131 as described previously.

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132 The detergent was removed by dialysis as in the preparation of unilamellar liposomes [22-24]. Sodium
133 cholate was used in this study because it is non-toxic at low concentration and is easy to remove by
134 dialysis [25].

135 *Radiolabelling of nanoemulsions*

136 Nanoemulsions were either single- or double-labeled with radioactive cholate and phospholipid. Single
137 labeling refers to the use of [^{3}H]-labeled cholic acid and double labeling refers to the use of both [^{3}H]-
138 labeled cholic acid and [^{14}C]-phosphatidylcholine. Single labeling was performed by an overnight
139 incubation of [^{3}H]-labeled cholic acid (55.5 kBq) with nanoemulsion. The resulting radiolabelled
140 nanoemulsion was called hot nanoemulsion. For double labeling, [^{3}H]-labeled cholic acid (55.5 kBq) was
141 used in the aqueous phase and L-α-dipalmitoyl-[^{14}C]-phosphatidylcholine (DPPC) (5.55 kBq) was used in
142 the oil phase. Thus obtained hot crude emulsion was extruded as usual. The initial concentrations of
143 sodium cholate and phospholipid were 30 mM and 15.75 mM, respectively.

144 **Removal of sodium cholate by dialysis**

145 After extrusion, the detergent was removed via dialysis through a very high permeability membrane (10
146 kDa cutoff). The nanoemulsion was dialyzed against a detergent free medium (glycerol, 2.5 % w/w) using
147 an in-house built dialyzer (Fig 1) and a commercial dialyzer (Slide-A-Lyzer®).

148 *Dialysis using in-house built dialyzer*

149 The dialyzer was developed at an institute of Albert Ludwig University of Freiburg, Germany. The
150 membrane (very high permeability 10 kDA cutoff membrane, Dianorm GmbH, Munich, Germany) was
151 equilibrated with the dialysis fluid (glycerol, 2.5 % w/w) for at least 30 min prior to use and fixed in
152 between the two compartments of a dialysis cell (4.9 cm^2 cavity area) and the dialysis was performed as
153 described elsewhere [26]. Briefly, a specified volume of nanoemulsion was placed in one compartment
154 and the dialysis fluid was allowed to flow continuously through the other compartment. Both the
155 compartments equipped with small magnetic stir bars were separated by the pre-equilibrated membrane.
156 Any air bubbles in both the compartments were excluded to ensure enough osmotic pressure for

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157 detergent removal. Failure to seal the compartments tightly might result into loss of sample due to
158 leakage. The flow rate (2.5 mL·min^{-1}) of dialysis fluid was regulated by using a pump (Ismatec SA, Zurich,
159 Switzerland). The dialysis was performed at continuous stirring (700 rpm) for 28 h at room temperature.

160 ***Fig 1. In-house built dialyzer***

161 *Dialysis using commercial dialyzer*

162 Dialysis was performed by using a commercial dialyzer (Slide-A-Lyzer®) available in different capacities
163 (0.5 mL – 3.0 mL) and membrane cutoffs. The dialyzer cassette (1 mL) having a membrane cutoff of 10
164 kDa was pre-equilibrated with the dialysis fluid (glycerol, 2.5 % w/w) for at least 30 min prior to dialysis.
165 Nanoemulsion (1 mL) was pipetted into the cassette and a small magnetic stir bar was inserted into the
166 cassette. Entrapped air was removed by lightly pressing the membrane and immediately closing the lid.
167 Nanoemulsion was dialyzed against the fixed volume of dialysis fluid (500 ml) at constant stirring
168 (300 rpm) for 28 h at room temperature.

169 *Dialysis of radiolabelled nanoemulsions*

170 Efficiency of dialysis was investigated for radiolabeled nanoemulsions dialyzed using two types of
171 dialyzers. The radioactivity [^{3}H and/or ^{14}C] in nanoemulsions was analyzed by using liquid scintillation
172 counter (LSC). As negative and positive controls, cold and hot nanoemulsions (before dialysis),
173 respectively, were used. The negative control represented the background value, whereas, the positive
174 control represented the reference value. For LSC measurement, the samples were withdrawn hourly,
175 diluted with Ultima Gold® at a ratio of 1:6 and analyzed under LSC to detect the radioactivity. All the
176 measurements were performed in triplicates.

177 **Characterization of nanoemulsion**

178 Nanoemulsions were characterized by measuring the particle size (Z-average), size distribution
179 (polydispersity index, PDI) and the surface charge (zeta-potential) using a photon correlation
180 spectroscopy (PCS, Malvern Nano ZS® series, Malvern, UK) which is based on Mie scattering theory [7].
181 A monochromatic laser 633 nm, fixed at a scattering angle of 173° is used to measure the Brownian

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182 motion of the particles which is correlated with the hydrodynamic diameter. Morphological
183 characterization of nanoemulsions was performed by the help of cryo-transmission electron microscopic
184 (cryo-TEM) pictures. The phospholipid content of the nanoemulsions was quantified by Bartlett assay
185 [27]. Similarly, other parameters such as stability were also investigated.

186 *Z-average and polydispersity index (PDI)*

187 The z-average (nm) and polydispersity index (PDI) which represent the hydrodynamic diameter and size
188 distribution of a particle, respectively was calculated as an average value of 3 consecutive measurements
189 each consisting of 15 sub-runs lasting 10 s per sub-run. For the measurement, samples were prepared in
190 a small volume disposable cuvette by diluting 5 µL nanoemulsion with 995 µL particle free deionized
191 water (1:200). Prior to dilution deionized water was filtered through a cellulose acetate filter (Minisart®,
192 Sartorius Stedim Biotech GmbH, Goettingen, Germany, pore size 0.2 µm) to avoid the effects of multiple
193 scattering from dust particles. During the measurement, an equilibration period of 80 s and temperature of
194 25 °C were set up. The intensity average diameter and PDI of each sample was calculated from the
195 Cumulant analysis (Zetasizer software 6.2) of each sample's correlation function. The PDI indicates the
196 homogeneity of the particle size distribution. A PDI value below 0.1 is an indication for a narrow size
197 distribution [28].

198 *Zeta potential (ζ)*

199 The zeta-potential (ζ) is a charge acquired by a particle or molecule in a given medium and is measured
200 by laser Doppler anemometry (Malvern Nano ZS® series, Malvern, UK). Samples were diluted (1:200)
201 similar to that for the particle size measurement and filled in a folded capillary cell. After an equilibration
202 period of 120 s at 25 °C, measurements were performed in triplicate in an automatic mode so that the
203 total sub-runs are between 10 and 100. Since the similar charges repel each other, the particles avoid
204 phenomena such as flocculation and aggregation making the samples stable for longer period. Therefore,
205 measurement of zeta-potential is an important parameter to study the stability of colloidal systems.
206 Absolute values larger than ± 30 mV are considered as an indicator for a stable emulsion system [7].

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207 **Cryo-transmission electron microscopy (Cryo-TEM)**

208 Cryo-TEM is a widely used method to morphologically characterize the colloidal particles such as
209 liposomes [26] and nanoemulsions [29]. This advanced microscopic method captures the two
210 dimensional image of the sample and gives accurate information about the size, lamellarity and size
211 distribution. The images of nanoemulsions were taken using a LEO 912 OMEGA electron microscope
212 (Zeiss, Oberkochen, Germany) operating at 120 kV and 'zero-loss' conditions. Approximately 5 µL of
213 sample (diluted if necessary) was placed on a copper grid (Quantifoil® S7/2 Cu 400 mesh, holey carbon
214 films, Quantifoil Micro Tools GmbH, Jena, Germany) and any excess liquid was absorbed by a filter
215 paper, so that only a thin (100 – 500 nm) liquid film remained on the copper grid [30]. The sample was
216 then immediately shock-frozen by plunging it into liquid ethane. The vitrified sample was stored at 90 K
217 (-183° C) in liquid nitrogen until it was loaded into a cryogenic sample holder (D626, Gatan Inc,
218 Pleasanton, USA). The specimens were examined at -174 °C. Digital images were recorded with a slow
219 scan CCD camera system (Proscan HSC 2 Oxford instruments, Abingdon, USA), and at a minimal
220 under-focus of the microscope objective lens to provide sufficient phase contrast [31]. All the pictures
221 were analysed using the software iTEM 5.0 Build 1054 (Soft Imaging System GmbH, Muenster,
222 Germany). Various scales (2 µm, 1 µm, 500 nm, 200 nm, 100 nm) could be used to estimate the size of
223 individual particle.

224 **Determination of phospholipid content**

225 Phospholipid used in the preparation was quantified by performing phosphorous assay. This assay
226 measures the phosphorous present in the head region of phospholipid as a phosphate molecule. The
227 assay was performed according to the previously established method with some modifications [27]. The
228 principle behind this assay is that one phosphorous atom corresponds to one phospholipid molecule.

229 The complete assay was conducted in phosphate free glass tubes. For a calibration curve, a standard
230 solution of KH_2PO_4 (1 mM) in HCl (0.05 N) was prepared and the volumes of 50 µL, 100 µL, 150 µL, 200
231 µL, 250 µL, 300 µL and 350 µL were weighed in glass tubes on the assumption of Lambert-Beer law that
232 absorbance is linear with concentration. Similarly, the sample volume was calculated from the theoretical

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233 concentration so that the phosphate content falls within the calibration curve. An empty glass tube
234 (without standard solution) was used as blank value and treated in a similar manner as calibration and
235 sample tubes. Then 500 µL of H_2SO_4 (10 N) were added to all the tubes including the blank tube and
236 mixed well by vortexing and incubated at 160 °C for 3 h. After 3 h, the samples appeared dark brown in
237 color due to oxidation. In order to completely oxidize the organic compounds, 200 µL of H_2O_2 (30 % w/w)
238 was added, vortexed and incubated further at 160 °C for 1.5 h. Upon complete incubation, the solution in
239 the tubes should turn clear. If this was not the case, incubation at 160 °C for 1.5 h was repeated with
240 additional 200 µL of H_2O_2 (30 % w/w). Clear solutions marked complete oxidation and were ready for
241 reduction process.

242 Then 4.75 mL of ammonium molybdate solution (0.22 % w/v) and 200 µL of freshly prepared Fiske
243 Subbarrow reducer solution (14.8 % w/v) were added. After each addition of reagents, the contents of the
244 glass tubes were mixed properly by vortexing. Then the tubes were covered with glass marbles and
245 incubated for another 10 min at 95 °C in a heating block (MTB 250, Development and Technology,
246 Ilmenau, Germany). After the incubation, the tubes were cooled down and vortexed. The solutions were
247 transferred to a 2 mL disposable plastic cuvette and absorbance was measured using a
248 spectrophotometer at a wavelength of 833 nm (Lambda XLS, Perkin Elmer, Hamburg, Germany). The
249 blank value was deducted from the standard solutions and a calibration curve was prepared by plotting
250 absorbance against the amount of phospholipid (micromoles). Using the slope of a straight line, the
251 phospholipid concentration of the sample was calculated. The acceptable regression value of calibration
252 curve was greater than 0.99.

253 **Stability studies**

254 Nanoemulsions were stored under refrigeration for as long as 7 months. The particle size, PDI and zeta-
255 potential were measured every month and compared with the initial value (day of preparation). Any
256 significant increase in the above mentioned parameters was considered to be an unstable preparation. In
257 addition, any phase separation if observed during the storage period was noted and concluded to fail the
258 stability study.

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259 # Results and discussion

260 ## Characterization of nanoemulsion

261 ### *Hand extruded nanoemulsions*

262 The z-average of nanoemulsions after four sets of extrusion (51x400 nm and 153x200 nm membranes)
263 was measured and the results are presented in Table 2. The z-average was found to be about 235 nm
264 with PDI of about 0.135 after the first set of extrusion. Further extrusion through 200 nm membrane
265 resulted in a sharp reduction in droplet size to about 185 nm with PDI below 0.09. Similarly, after the four
266 sets of extrusion, the particle size reduced to about 166 nm and the PDI value was much lower (about
267 0.05). The size of emulsion droplet is smaller than the pore size of membrane (200 nm) because the
268 droplets break down into droplet size closer to the pore size of the membrane [32]. Thus, it was observed
269 that the reduction in droplet size can be improved by increasing the number of extrusion cycles. In case of
270 liposomes, extrusion could reduce the vesicles to be in the size range between 50 and 100 nm when
271 extruded through 100 nm pore size membranes [33]. However, the use of 100 nm membrane was not
272 able to reduce the emulsion droplet below 100 nm probably due to the oily inner core (data not shown).
273 The PDI value between 0.04 and 0.08 is defined to be extremely highly mono-disperse [28]. Therefore,
274 the resulting nanoemulsion could also be considered as extremely mono-disperse nanoemulsions.

275 ***Table 2. Characteristics of nanoemulsions after subsequent sets of extrusion cycles***

Extrusion cycles	Z-average (nm)	Poly Dispersity Index (PDI)	Zeta-Potential (mV)
51 x 400 nm	234.7 ± 2.69	0.135 ± 0.014	-22.5 ± 0.351
51 x 200 nm	185.1 ± 0.60	0.091 ± 0.034	-24.1 ± 0.265
102 x 200 nm	174.4 ± 0.70	0.063 ± 0.003	-20.7 ± 0.361
153 x 200 nm	166.2 ± 1.01	0.048 ± 0.007	-21.4 ± 0.635

276 Results are represented as mean values ± SD (n=3).

277 The zeta-potential of the emulsions during the various extrusion steps was found to be between -20 and
278 -25 mV (Table 2). According to Hippalgaonkar and group, the obtained nanoemulsions are not
279 electrostatically stable as the zeta-potential is below ± 30 mV [7]. It was also observed that the zeta-
280 potential was not affected by increasing the extrusion cycle.

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281 *Nanoemulsions with sodium cholate*

282 Since the sodium cholate was used only to disorganize the liposomal membrane [19], it was removed
283 after extrusion by dialysis against detergent-free aqueous medium [25, 26]. Sodium cholate at various
284 concentrations (20 – 50 mM) was studied for optimizing the appropriate concentration of sodium cholate
285 to increase the number of nanoemulsions and reduce the number of liposomes. It was observed that the
286 particle size changed minimally with an increase in sodium cholate concentration (Table 3).

287 ***Table 3. Characteristics of nanoemulsions with different concentrations of sodium cholate***

Sodium Cholate (mM)	Z-average (nm)		Poly Dispersity Index (PDI)		Zeta-Potential (mV)	
	Before Dialysis	After Dialysis	Before Dialysis	After Dialysis	Before Dialysis	After Dialysis
20	161.6 ± 1.44	160.8 ± 2.69	0.079 ± 0.025	0.072 ± 0.018	-45.4 ± 6.11	-35.8 ± 16.7
30	168.6 ± 10.32	165.9 ± 8.90	0.050 ± 0.006	0.056 ± 0.004	-47.3 ± 2.57	-37.8 ± 6.7
40	165.9 ± 11.66	166.1 ± 9.12	0.023 ± 0.017	0.069 ± 0.005	-47.9 ± 7.95	-36.1 ± 4.6
50	168.9 ± 12.05	NA	0.043 ± 0.001	NA	-44.4 ± 3.03	NA

288 Extrusion cycles: 51x400 nm and 153x200 nm, Results are represented as mean values ± SD (n=3). NA: Not
289 available.

290 However, the PDI value was found to remain below 0.1 at all concentrations of sodium cholate. After
291 complete extrusion (51x400 nm and 153x200 nm), the size of nanoemulsions was found to be below 170
292 nm at all concentrations of sodium cholate which is comparable to the size of nanoemulsions without
293 sodium cholate (Table 2). Additionally, dialysis seemed to have negligible effect on particle size and PDI
294 of nanoemulsions. Thus, the findings suggest that all the studied concentration of sodium cholate (20 –
295 50 mM) can be used to prepare homogenous nanoemulsions without affecting the Z-average and PDI
296 values.

297 Similarly, an increase in sodium cholate concentration did not increase the zeta-potential of
298 nanoemulsions (Table 3).The zeta-potential was found to be in the range of -45 mV to -48 mV when
299 sodium cholate was used at different concentrations (Table 3) which is an increment of about -20 mV
300 when compared to that of nanoemulsions without sodium cholate (Table 2). Nevertheless, after dialysis
301 the zeta-potential was reduced to about -36 mV which accounts to a loss of about -10 mV. Since the

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302 values were above -30 mV, the nanoemulsions were stable electrostatically. It was observed that the use
303 of sodium cholate provided additional electrostatic stability to the nanoemulsion even after its removal via
304 dialysis. Therefore, the use of sodium cholate at higher concentration (50 mM) does not seem to be
305 beneficial.

306 **Removal of sodium cholate by dialysis**

307 Two types of dialyzers were used to compare their detergent removal performance and the results are
308 shown in Table 4.

309 ***Table 4. Comparison of dialyzers***

Dialyzers	Z-average (nm)		Poly Dispersity Index (PDI)		Zeta-Potential (mV)	
	Before Dialysis	After Dialysis	Before Dialysis	After Dialysis	Before Dialysis	After Dialysis
In-house dialyzer	164.5 ± 7.31	165.1 ± 9.11	0.039 ± 0.006	0.058 ± 0.022	-50.4 ± 4.15	-43.5 ± 1.48
Slide-A-Lyzer®	165.5 ± 7.59	164.3 ± 7.96	0.049 ± 0.013	0.062 ± 0.006	-49.47 ± 4.80	-40.37 ± 2.39

310 Extrusion cycles: 51x400 nm and 153x200 nm, Sodium cholate concentration: 30 mM. Results are represented as
311 mean values ± SD (n=3).

312 The concentration of sodium cholate was maintained at 30 mM. It was observed that the nanoemulsions
313 before and after the dialysis were similar in size and homogeneity in both dialyzers. But the zeta-potential
314 after dialysis using the Slide-A-Lyzer® was found to be slightly lower than the in-house built dialyzer.
315 However, the nanoemulsions were stable with zeta-potential above -30 mV in both the cases. These
316 findings showed that the two dialyzers showed similar performance and are easily replaceable as per the
317 convenience and availability.

318 *Sodium cholate removal efficiency*

319 Sodium cholate removal efficiency was investigated using the two types of dialyzers and the results are
320 shown in Fig 2.

321 ***Fig 2. Sodium cholate removal efficiency in radiolabelled nanoemulsions (single labeling)***

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322 Labeling: Incubation of cold nanoemulsion with [^{3}H]-labelled cholic acid (55.5 kBq). Sodium cholate: 30 mM. Results
323 are normalized with a dilution factor calculated after dialysis. Error bars represent SD (n=3).

324 The study illustrated that sodium cholate removal profile differed slightly in the early phase but overlapped
325 with each other in the later phase, showing similar pattern of detergent removal in both the dialyzers.
326 Within the first hour of dialysis only about 16 % of cholate was removed by the Slide-A-Lyzer® whereas
327 already 58 % was removed by the in-house dialyzer. However, after 7 hours of dialysis, both the dialyzers
328 exhibited similar efficiency (detergent removal of about 85 %). After the completion of dialysis period of
329 28 h, the residual cholate for both dialyzers was found to be between 3 and 5 % when the molar ratio of
330 phospholipid-to-detergent was 0.525 (15.75 mM phospholipid and 30 mM sodium cholate). In a previous
331 study, the residual cholate was measured to be less than 0.5% after 24 h of dialysis when the molar ratio
332 of phosphatidylcholine-to-cholate was maintained at 0.625 [28]. This explains that the amount of residual
333 detergent depends upon the phospholipid-to-detergent ratio used.

334 Similarly, in double labeling [^{3}H and ^{14}C] study, along with the sodium cholate depletion, phospholipid
335 content was also analyzed to monitor the loss of phospholipid during dialysis. Radiolabelled
336 nanoemulsion was dialyzed using Slide-A-Lyzer®. The samples were analyzed hourly for ^{3}H and ^{14}C
337 under LSC and the results are summarised in Fig 3. It was observed that about 65 % of sodium cholate
338 was removed within 2 h, whereas, after 28 h of dialysis, about 7 % of sodium cholate remained in
339 nanoemulsion (Fig 3A). This value was slightly higher than the amount obtained in the previous study
340 where labeling was performed by incubating cold nanoemulsion with [^{3}H]-labelled cholic acid (Fig 2, Slide-
341 A-Lyzer®). This study thus showed that the method of radiolabeling affects the sodium cholate removal
342 profile. The ratio ^{3}H/^{14}C was about one which means that almost an equal proportion of ^{3}H and ^{14}C
343 remained in the nanoemulsion after dialysis (Fig 3B).

344 ***Fig 3. Analysis profile of nanoemulsion dialyed using Slide-A-Lyzer® (double labeled)***
345 ***A) ^{3}H and ^{14}C (Radioactivity %); B) Ratio of ^{3}H and ^{14}C (^{3}H/^{14}C)***
346 Labeling: Extrusion of hot crude emulsion containing [^{3}H]-labelled cholic acid (55.5 kBq) and [^{14}C]-
347 Phosphatidylcholine (5.55 kBq). Sodium cholate: 30 mM. Results are normalized with a dilution factor calculated after
348 dialysis. Results are represented as mean values. Error bars represent SD (n=3).

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349 **Cryo-transmission electron microscopy (Cryo-TEM)**

350 The cryo-TEM pictures were not only essential to observe the presence of liposomes but also to find out
351 the appropriate concentration of sodium cholate required to prepare homogenous and stable
352 nanoemulsions. The Cryo-TEM pictures of nanoemulsions at different conditions are shown in Fig 4.

353 ***Fig 4. Cryo-TEM pictures of nanoemulsions at different conditions.***
354 ***A) Without sodium cholate; B) 20 mM sodium cholate after dialysis; C) 30 mM sodium cholate***
355 ***after dialysis; D) 40 mM sodium cholate after dialysis; E) 30 mM sodium cholate before dialysis; F)***
356 ***30 mM sodium cholate after dialysis and after 23 weeks of storage under refrigeration.***
357 Extrusion cycles: 51x400 nm and 153x200 nm. Dialyzer: in-house dialyzer.

358 It was surprisingly observed that very few nanoemulsion droplets (dark circular structures) and many
359 liposomes (transparent circular structures) were present when sodium cholate was not used in the
360 preparation of nanoemulsions (Fig 4A). In cryo-TEM images, liposomes appeared as transparent circular
361 structures due to their aqueous interior and the dark border represents the phospholipid bilayer [26],
362 whereas, nanoemulsions appeared as dark circular structures due to their oily inner core. According to
363 Torchilin and Weissig, liposomes are formed spontaneously upon rehydration of phospholipids [34, 35].
364 Therefore, the phospholipid used as emulsifying agent in the preparation of nanoemulsions could also
365 form liposomes. Previously, nanoemulsions and solid lipid nanoparticles were prepared by using
366 extrusion method but without the use of sodium cholate. Also, the simultaneous formation of liposomes
367 was not mentioned earlier [36]. Our findings suggest that cryo-TEM pictures are necessary in complete
368 characterization of nanoemulsions. If cryo-TEM pictures are not taken, the presence of liposomes along
369 with nanoemulsions could not be identified. From Table 3, it was difficult to find out the optimum
370 concentration of sodium cholate to prepare homogenous and stable nanoemulsions which are free from
371 liposomes because at all concentrations of sodium cholate, nanoemulsions were found to be
372 homogeneous and stable on the basis of PDI and zeta-potential values. Therefore, cryo-TEM pictures of
373 nanoemulsions were supportive to find out the optimum concentration of sodium cholate.

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374 Fig 4 revealed that after dialysis, liposomes of about 200 nm (marked with white arrows) were present at
375 sodium cholate concentration of 20 mM (Fig 4B), but at 30 mM, no such liposomes were detected (Fig
376 4C). As the concentration of sodium cholate was increased further to 40 mM, numerous but very small
377 liposomes (marked with white boundaries) were observed again (Fig 4D). At 20 mM, the sodium cholate
378 was perhaps adequate to solubilize the lipid membrane and reorganise them to form liposomes upon
379 dialysis. But at 30 and 40 mM, the ratio of detergent-to-phospholipid was perhaps inadequate to form
380 liposomes having defined size. In a previous study, the critical molar ratio of detergent-to-lipid for the
381 formation of liposomes by detergent removal via dialysis was found to be between 1.2 and 2 with lipid up
382 to 25 mM [20, 28]. With the help of cryo-TEM pictures, it was thus concluded that the appropriate
383 concentration of sodium cholate to prepare nanoemulsions without liposomes was 30 mM.

384 The cryo-TEM picture of nanoemulsion before (Fig 4E) and after dialysis (Fig 4C) seem to be similar
385 indicating no influence of dialysis on the size of nanoemulsion and the results were also supported by
386 Table 3 and Table 4. Additionally, Fig 4C and Fig 4F do not seem to differ much which means that the
387 nanoemulsion was stable for as long as 23 weeks when stored under refrigeration.

388 **Determination of phospholipid content**

389 Phospholipid content of nanoemulsions prepared using 30 mM sodium cholate was quantified before and
390 after dialysis by means of phosphorous assay. With 1.2 % (w/w) of phospholipid (E80®), the initial
391 concentration was theoretically calculated to be 15.75 mM. After phosphorous assay, the phospholipid
392 content was found to differ slightly (Table 5).

393 ***Table 5. Phosphorous assay of nanoemulsions***

Type of dialyzer	Phospholipid content (mM)	
	Before dialysis	After dialysis
In-house built dialyzer	14.9 ± 2.38	11.73 ± 1.38
Slide-A-Lyzer®	14.54 ± 0.92	14.11 ± 1.62

394 Composition: Table 1 (with 30 mM sodium cholate). Dialysis: against aqueous glycerol (2.5 %, w/w). Results are
395 normalized with a dilution factor calculated after dialysis and are represented as mean values ± SD (n=3).

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396 It was observed that phospholipid was lost during dialysis. Among the two types of dialyzers, the loss was
397 found to be higher in in-house dialyzer (21 %) than in Slide-A-Lyzer® (3 %). The variation in loss of
398 phospholipid in both dialyzers could be explained by the variation in dialysis conditions such as volume
399 and flow rate of dialysis fluid. The dialysis process in in-house dialyzer was an open-system where the
400 dialysis fluid (about 4.5 L) was allowed to flow continuously at a fixed flow rate (2.5 mL min^{-1}) for a fixed
401 period of time (28 h) whereas, dialysis using Slide-A-Lyzer® was a closed-system where a fixed volume of
402 dialysis fluid (500 mL) at constant stirring (300 rpm) was used and the dialysis for continued for 28 h at
403 room temperature. From this, it is clear that the volume of dialysis fluid affects the loss of phospholipid but
404 does not affect the characteristics of nanoemulsions.

405 In spite of the loss of phospholipid during dialysis (Table 5), the cryo-TEM images revealed that the
406 nanoemulsions were still stable even after storage under refrigeration for as long as 23 weeks (Fig 4 F)
407 when compared to the cryo-TEM images before dialysis (Fig 4 E) and after dialysis (Fig 4 C).

408 **Stability studies**

409 The stability of nanoemulsions dialyzed by using two different dialyzers was studied for a duration of 7
410 months. Since the measurement of particle size, PDI and zeta-potential and Cryo-TEM images are useful
411 techniques to confirm the stability of nanoemulsions [37], the samples were monitored every month for
412 particle size, PDI and zeta-potential. Three samples per dialysis method were stored under refrigeration
413 (4 – 8 °C) and studied for their stability. Fig 5 represents the summary of stability of nanoemulsions
414 dialyzed using in-house built dialyzer.

415 ***Fig 5. Stability of nanoemulsions dialyzed using in-house built dialyzer***

416 It was observed, that the changes in size during the storage period was negligible. The PDI values were
417 found to change with time but remained below 0.1 even after 7 months of storage. A slight fluctuation was
418 noted in zeta-potential for all samples. Nevertheless, zeta-potential was measured to be above -30 mV for
419 all samples even after 7 months. Therefore, the samples were concluded to be stable under refrigeration
420 for as long as 7 months.

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421 Similarly, the stability of nanoemulsions dialyzed using Slide-A-Lyzer® is summarized in Fig 6. As shown
422 in figure, the changes in both size and PDI were observed to be negligible like in the case of in-house
423 built dialyzer (Fig 5). The zeta-potential throughout the storage period was measured to be above -30 mV.
424 Due to all these reasons, the samples were found to be stable for about 7 months. Therefore, both
425 dialyzers were found to be suitable for preparing nanoemulsions without liposomes which are stable for
426 as long as 7 months under refrigeration. Thus, both the dialyzers are conveniently replaceable to each
427 other.

428 ***Fig 6. Stability of nanoemulsions dialyzed using Slide-A-Lyzer®***

429 Conclusions

430 Preparation of homogenous nanoemulsions in small-scale is a challenge, especially if the emulsion is
431 stabilized by phospholipids due to the unavoidable formation of liposomes along with the emulsion
432 droplets. Extensive extrusion of crude emulsion through polycarbonate membranes (51x 400 nm and
433 153x 200 nm) at 65 °C not only reduced the emulsion droplet to nanometer range and but also prepared
434 homogenous droplets as depicted by a PDI value below 0.1. Furthermore, the study showed that the use
435 of a physiological detergent, sodium cholate at 30 mM concentration and later removal via dialysis after
436 extrusion minimized the formation of liposomes resulting into nanoemulsions which are stable under
437 refrigeration for as long as seven months. The cryo-TEM pictures provided sufficient evidences that the
438 use of sodium cholate was indeed beneficial to prepare liposome-free nanoemulsions in small scale (less
439 than 1 mL). The easy availability of commercial dialyzers at variable capacities makes the preparation
440 process even easier.

441 Thus, this method could be regarded as an economic and yet promising technique especially for
442 preparing functionalized or modified nanoemulsions where expensive ligands or antibodies, fluorescence
443 or radioactive markers must be used to target such nanoemulsions to a specific cell or location.

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447 ## Author contributions

448 Conceived and designed the experiments: SG and RS. Performed the experiments: SG and SB.
449 Analyzed the data: SG, MZ and RS. Contributed reagents/materials/analysis tools: SG, MZ and SB.
450 Wrote the paper: SG, MZ, SB and RS.

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