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1 **Development of cysteamine loaded liposomes in liquid and dried forms for**
2 **improvement of cysteamine stability**

3
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18 **Abstract**

19 Despite the high aqueous solubility of cysteamine, its unpleasant organoleptic properties,
20 hygroscopicity, instability in solutions, and poor pharmacokinetic profile are the main
21 drawbacks that limit its use for medical and cosmetic purposes. In this study, cysteamine-
22 loaded liposomes were prepared using the ethanol injection method. Liposomes were
23 characterized for their size, homogeneity, surface charge, and morphology. The incorporation
24 ratios of cholesterol and phospholipids, the encapsulation efficiency and the loading ratio of
25 cysteamine in liposomes were determined. Moreover, the stability of free and encapsulated
26 cysteamine was assessed at different temperatures (4, 25, and 37°C) in the presence and
27 absence of light. Cysteamine-loaded liposomes were freeze-dried and reconstituted liposomes
28 were characterized. Finally, the storage stability of the freeze-dried cysteamine-loaded
29 liposomes was studied. Liposomes were nanometric, oligolamellar, and spherical. The
30 encapsulation efficiency and the loading ratio of cysteamine varied between 12 and 40% in
31 the different formulations. The encapsulation improved the stability of cysteamine in the
32 various storage conditions. The dried form of cysteamine-loaded liposomes conserved the size
33 of the vesicles and retained 33% of cysteamine present in the liposomal suspension before
34 lyophilization. The freeze-dried liposomes formulations were stable after four months of
35 storage at 4°C.

36 **Keywords:** Cysteamine; Encapsulation; Freeze-drying; Liposomes; Stability.

37 **1. Introduction**

38 Cysteamine (Cyst) is an aminothiols compound synthesized by human body cells and derives
39 from coenzyme A degradation (Besouw et al., 2013; Gallego-Villar et al., 2017) (**Fig. 1**). Cyst
40 is described to have a radioprotective (Bacq et al., 1953), an anti-cancer (Apffel et al., 1975),
41 and anti-malarial (Min-Oo et al., 2010) effects. It can be used for hyperprolactinemia
42 treatment by reducing the size of pituitary glands and depleting plasma prolactin (Sagar et al.,
43 1985). This molecule represents the only treatment of cystinosis disease, which is
44 characterized by cystine accumulation in many tissues (Elmonem et al., 2016). In lysosomes,
45 cysteamine reacts with cystine to form a mixed disulfide of half-cystine and Cyst, which then
46 leaves the cells via a lysine transporter (Bozdağ et al., 2008). It can also be used as a
47 depigmenting agent (Chavin and Schlesinger, 1966; Farshi et al., 2018; Frenk et al., 1968;
48 Mansouri et al., 2015). Cyst is freely soluble in water (23.5 mg/mL), has a foul odor and a
49 bitter taste, and presents a weak pharmacokinetic profile (Lahiani-Skiba et al., 2007). Besides,
50 Cyst is unstable in aqueous solutions due to the rapid oxidation of the sulfhydryl group,
51 leading to the formation of cystamine. This reaction is catalyzed by metal ions such as Cu^{2+} ,
52 Fe^{3+} , and Zn^{2+} and is stimulated in alkaline pH (Atallah et al., 2020). To increase its stability
53 and improve its biological effects, Cyst has been encapsulated in liposomes (Butler et al.,
54 1978; Jaskierowicz et al., 1985; Jeitner and Oliver, 1990), cyclodextrins (Lahiani-Skiba et al.,
55 2007), and emulsions (Gresham et al., 1971).

56 Liposomes are spherical vesicles that comprise one or more lipid bilayer structures enclosing
57 an aqueous core. They are used to encapsulate active compounds for various applications
58 (Laouini et al., 2012). Their biocompatibility, biodegradability, and low toxicity make them
59 very appropriate carriers for many drugs (Zylberberg and Matosevic, 2016). Cyst has been
60 encapsulated in liposomes to enhance its absorption through the intestinal wall (Butler et al.,
61 1978), its effectiveness in cystinosis (Jaskierowicz et al., 1985), and the period of prolactin
62 depletion action (Jeitner and Oliver, 1990). To the best of our knowledge, the liposomes

63 encapsulating Cyst were not characterized in previous studies, and the effect of encapsulation
64 on Cyst stability was not evaluated.

65 The freeze-drying is a promising approach to increase the shelf life of liposomes
66 incorporating active agents. Among several cryoprotectants (sucrose, trehalose, glucidex 6D
67 and 19D), *Sebaaly et al.* demonstrated that hydroxypropyl beta-cyclodextrin (HP- β -CD) was
68 the most effective cryoprotectant of liposomes during the freeze-drying of eugenol-loaded
69 Phospholipon 90H (PH 90H) liposomes (Sebaaly et al., 2016). Besides, *Gharib et al.* proved
70 that HP- β -CD protects hydrogenated but not unsaturated liposomes during freeze-drying
71 (Gharib et al., 2018).

72 In this study, blank and Cyst-loaded liposomes composed of PH 90H and Lipoid S100 were
73 prepared by the ethanol injection method. The liposomal suspensions were characterized for
74 their size, polydispersity index (PdI), and zeta potential by diffusion light scattering (DLS) as
75 well as for their morphology using transmission electron microscopy (TEM). The
76 concentrations of Cyst, phospholipid, and cholesterol (Chol) in liposomes were determined
77 spectrophotometrically by the Ellman method with some modifications, Bartlett method and
78 Chol quantitation kit, respectively. The encapsulation efficiency (EE%) and the loading ratio
79 (LR %) of Cyst in liposomes were also determined. Freeze-drying of PH 90H liposomes was
80 realized according to the optimized protocol previously described (Gharib et al., 2018), and
81 the characteristics of the reconstituted vesicles were determined. Moreover, the stability of
82 free and encapsulated Cyst was assessed at different temperatures (4, 25, and 37°C) and in the
83 presence and absence of room light. Finally, the storage stability of the freeze-dried form of
84 encapsulated Cyst was evaluated after four months of storage at 4°C.

85

86 **2. Materials and methods**

87 *2.1 Materials*

88 Cyst, monobasic phosphate, cystamine dihydrochloride, DL-dithiothreitol, and metformin
89 hydrochloride were purchased from Sigma-Aldrich (Buchs, Switzerland). 5,5'- Dithiobis(2-
90 nitrobenzoic acid) (DTNB), ammonium molybdate were purchased from Sigma-Aldrich (St
91 Louis, Missouri, USA). Ethylenediaminetetraacetic acid (EDTA), dibasic phosphate, sulfuric
92 acid, and hydrogen peroxide were purchased from Sigma-Aldrich (Steinheim, Germany). PH
93 90 H (90% soybean PC, 4% lysoPC, 2% triglycerides, 2% water, and 0.5% ethanol) and
94 Lipoid S100 (94% soybean PC, 3% lysoPC, 0.5% N-acyl-PE, 0.1% PE, 0.1%
95 phosphatidylinositol, 2% water, 0.2% ethanol) were obtained from Lipoid GmbH
96 (Ludwigshafen, Germany). Chol was obtained from the Fisher chemical (Loughborough,
97 UK), and sodium bisulfite was purchased from Combi Blocks (San Diego, California, USA).
98 4-Amino-3-hydroxy-1-naphthalene sulfonic acid was purchased from Sigma-Aldrich
99 (Bangalore, India). Chol quantitation kit was purchased from SPINREACT (Barcelona,
100 Spain). HP- β -CD-oral grade (MS=0.85) was purchased from Roquette (Lestrem, France).
101 Sodium dodecyl phosphate and phosphoric acid were obtained from Sigma-Aldrich (China).
102 All other chemicals were of analytical grade.

103 *2.2 Cyst quantification*

104 The quantification of Cyst was done using two methods. For the quantification of Cyst in
105 liposomal suspension, a modified Ellman method was used. First, a stock solution of Cyst was
106 prepared in water (1 mg/mL) and then diluted to obtain final concentrations of Cyst ranging
107 between 2.5 and 100 μ g/mL. Ellman's reagent was prepared by dissolving 4 mg DTNB in 1
108 mL of reaction buffer (pH 8.0). The latter is composed of 0.1 M sodium phosphate and 1 mM
109 EDTA. 250 μ L of water (blank) or Cyst solution was added to 1.25 mL reaction buffer, 1.25
110 mL of methanol, and 50 μ L Ellman's reagent solution. This reaction mixture was then

111 vortexed and incubated at room temperature for 15 min. The absorbance was measured at 412
112 nm using the Spectrophotometer UV5 Mettler Toledo (Columbus Ohio, USA). The
113 calibration curve was constructed by plotting the absorbance against the concentration of Cyst
114 ranging from 2.5 to 100 $\mu\text{g/mL}$.

115 For the stability studies, the simultaneous quantification of Cyst and its degradation product
116 cystamine was needed. An ion-pair chromatography with an isocratic mode consisting of
117 acetonitrile: water containing 0.1% phosphoric acid and SDS (4 mM) (v/v, 45:55) was used.
118 20 μL of the samples were injected into a reverse-phase Kinetex C18 column (4.6×100 mm,
119 2.6 μm). The flow rate was set at 1.0 mL/min, the column temperature was maintained at
120 25°C, and the detection wavelength was 215 nm. Metformin was used as an internal standard
121 with a concentration of 1 $\mu\text{g/mL}$. Linearity was between 2.5 and 100 $\mu\text{g/mL}$ for Cyst and
122 between 5 and 100 $\mu\text{g/mL}$ for cystamine.

123 2.3 *Preparation of liposomes*

124 Liposomes were prepared by the ethanol injection method, as described by Sebaaly et al.
125 (2016). Saturated (PH 90H) or unsaturated lipids (Lipoid S100) (10 mg/mL) and Chol (5
126 mg/mL) were dissolved in absolute ethanol to obtain the organic phase (10 mL). Then, the
127 organic phase was injected into the aqueous phase (20 mL) containing different
128 concentrations of Cyst (0; 0.5; 0.75; 1; 2.5; 5 mg/mL), using a syringe pump (Fortuna optima,
129 GmbH-Germany), at a temperature above the transition temperature of the phospholipid
130 (55°C for PH 90H and -20°C for Lipoid S100) and under magnetic stirring at 400 rpm. The
131 phospholipid:Chol:Cyst molar ratios ranged between 1:0.98:1 and 1:0.98:10; for PH 90H and
132 between 1:1.17:1.17 and 1:1.17:11.7 for Lipoid S100. The contact between the organic
133 solution and the aqueous phase leads to a spontaneous liposome formation. The liposomal
134 suspensions were then left for 15 min at 25 °C under stirring (400 rpm). The ethanol was
135 removed by rotary evaporation (Heidolph GmbH, Germany) under reduced pressure at 40 °C

136 and the obtained liposomal solutions were stored at 4 °C for further characterization. Each
137 preparation was performed in triplicate.

138 2.4 *Characterization of the liposomal suspensions*

139 2.4.1 *Determination of size, pDI and zeta potential by dynamic light scattering*

140 Malvern Zetasizer Nanoseries (Zetasizer Nano ZS; Malvern Instruments Ltd, France) was
141 used to determine the mean size of the vesicles. All batches were diluted with ultrapure water.
142 The particle-size distribution data were collected using the DTS (nano) software (version
143 5.10) provided with the instrument. The polydispersity index, which indicates the particle size
144 distribution, ranges from 0 (monodispersed) to 1 (very broad distribution). Zeta potential was
145 calculated using Smoluchowski's equation from the electrophoretic mobility of liposomes.
146 All measurements were carried out at 25°C after 2 min of equilibration and were performed in
147 triplicate. Data were expressed as the mean values \pm SD.

148 2.4.2 *Morphological characterization by transmission electron microscopy*

149 The morphology of the liposomal suspensions was imaged by transmission electron
150 microscope (TEM) (CM 120; Philips, Eindhoven, Netherlands) operating at an accelerating
151 voltage of 120 kV. A drop of a solution of the liposomal suspension with or without dilution
152 was placed onto a carbon-coated copper grid for 2 min, and the remaining liquid was removed
153 using a filter paper. The grid was placed on negative staining using a 1% sodium silico-
154 tungstate solution during 30 s. The excess amount of sodium silicotungstate solution was
155 removed using a filter paper, and dried samples were observed.

156 2.4.3 *Determination of phospholipid:Chol:Cyst molar ratios in the final liposome structures*

157 The concentrations of incorporated compounds (phospholipids, Chol, and Cyst) in lipid
158 vesicles were obtained by subtracting the concentrations of free compounds from their total
159 concentrations determined in the suspensions. The suspensions underwent centrifugation at

160 21382xg during 1 h at 4°C using Vivaspin 500 centrifugal concentrator (Sartorius Stedim
161 Biotech, Germany, MWCO = 10 kDa). The filtrates contained the free compounds.

162 2.4.4 *Phospholipid quantification assay*

163 Bartlett's method was used to determine free and total concentrations of phospholipids in the
164 various liposomal suspensions. This method is based on several steps; first, the digestion of
165 the organic phosphate (0.5 mL from total liposomal suspensions, filtrates, and standard
166 solutions of phosphorus) using diluted sulfuric acid (5 mM) at 200°C for 1h. Then, the
167 oxidation to the inorganic phosphates was done in the presence of 10% hydrogen peroxide
168 (0.1 mL) for 30 min at 200°C. A phosphomolybdic complex was formed after the addition of
169 ammonium molybdate (4.6 mL) and the reduction of this complex by the 4-amino-3-
170 hydroxyl-1-naphthalene sulfonic acid (0.2 mL) at 100°C for 15 min. A blue solution appeared
171 and was measurable at 815 nm spectrophotometrically.

172 The incorporation ratio (IR) of phospholipids was calculated as follows:

$$\text{IR \%} = \frac{\text{Incorporated mass of phospholipids}}{\text{Initial added mass of phospholipids}} \times 100 \text{ Eq. (1)}$$

173 where the incorporated mass of phospholipids stands for the phospholipid mass determined in
174 the liposome structure; the initial mass of phospholipids represents the mass initially used to
175 prepare liposomes.

176 2.4.5 *Chol quantification assay*

177 The quantification of Chol (free form or total amount present in the suspension) was
178 performed after its enzymatic hydrolysis and oxidation using the Chol CHOD-POD kit
179 (SPINREACT, Spain). Chol in the samples was oxidized by Chol oxidase into 4-cholestenona
180 and hydrogen peroxide. The latter reacts with 4-aminophenazone in the presence of
181 peroxidase to form the colorimetric indicator quinonimine, having an absorbance proportional
182 to Chol concentration in the sample. Standard solutions (0 to 2 mg/mL) were prepared from

183 Chol stock solution (2 mg/mL); the latter was prepared in 10% Triton X-100. Samples from
184 the liposome suspensions were diluted in 10% Triton X-100 and sonicated for 20 min at room
185 temperature to ensure the dissolution of vesicles and the release of incorporated Chol. 1 mL of
186 the working reagent containing buffer and enzyme was then added to 10 μ L of each sample
187 (liposome suspension, filtrate, and Chol standard solution) and incubated for 10 min at room
188 temperature. The absorbance was monitored at a wavelength of 505 nm.

189 The incorporation ratio (IR) of Chol was calculated as follows:

$$\text{IR \%} = \frac{\text{Incorporated mass of Chol}}{\text{Initial added mass of Chol}} \times 100 \text{ Eq. (2)}$$

190 Where the incorporated mass of Chol stands for the Chol mass determined in the liposome
191 structure, the initial mass of Chol represents the mass initially used to prepare liposomes.

192 2.4.6 *Encapsulation efficiency and loading ratio of Cyst*

193 The EE and LR% of Cyst in liposomes were determined using the optimized Ellman method.
194 250 μ L of the Cyst standard solution, the filtrate containing free Cyst, or the diluted solution
195 of the liposomal suspension were added to 1.25 mL phosphate buffer, 1.25 mL of methanol,
196 and 50 μ L of Ellman's reagent. This method allows the determination of the free ($[\text{Cyst}]_{\text{free}}$)
197 and the total ($[\text{Cyst}]_{\text{tot}}$) Cyst concentrations. EE% was calculated as the following equation:

$$\text{EE}_{\text{cyst}} \% = \frac{[\text{Cyst}]_{\text{tot}} - [\text{Cyst}]_{\text{free}}}{[\text{Cyst}]_{\text{tot}}} \times 100 \text{ Eq. (3)}$$

198 where $[\text{Cyst}]_{\text{tot}}$ and $[\text{Cyst}]_{\text{free}}$ correspond to the concentration of total and free Cyst in the
199 liposomal suspension, respectively.

200 LR% of Cyst was calculated as follows:

$$\text{LR\%} = \frac{m_{\text{total}} - m_{\text{free}}}{m_{\text{initial}}} \times 100 \text{ Eq. (4)}$$

201 where m_{total} and m_{free} are the mass of total and free Cyst in the liposomal suspension and m_{initial}
202 is the mass of Cyst initially added to the aqueous phase during the preparation of the
203 liposomes.

204 2.5 Stability study

205 The formulation PH 90H: Chol: Cyst (1:0.98:10) was selected to compare the stability of Cyst
206 loaded in liposome to that of free Cyst. The liposomal suspension (5 mL) was centrifuged, the
207 supernatant was eliminated, and the pellet was re-suspended in water. The concentration of
208 the encapsulated Cyst was determined after the destruction of liposomes by sonication; Cyst
209 solutions with a similar concentration were prepared in water and placed at 4, 25, and 37°C in
210 the dark. Also, the same concentration of free Cyst was placed in light at 25°C. At different
211 times, aliquots were removed, and the concentration of Cyst remaining in solution and that of
212 formed cystamine were determined by ion-pair chromatography.

213 The percentage of remaining Cyst was calculated as follows:

$$\text{Remaining Cyst (\%)} = \frac{[\text{Cyst}]_t}{[\text{Cyst}]_{t_0}} \times 100 \text{ Eq. (5)}$$

214 where $[\text{Cyst}]_t$ is the concentration of remaining Cyst determined at time t and $[\text{Cyst}]_{t_0}$ is the
215 initial Cyst concentration.

216 The percentage of formed cystamine was calculated as follows:

$$\text{Formed cystamine (\%)} = \frac{[\text{Cystamine}]_t}{[\text{Cystamine}]_f} \times 100 \text{ Eq. (6)}$$

217 where $[\text{Cystamine}]_t$ is the concentration of formed cystamine determined at time t and
218 $[\text{Cystamine}]_f$ is the final cystamine concentration obtained after the total conversion of
219 cysteamine to cystamine.

220 2.6 Freeze-drying of liposomes

221 The freeze-drying of blank and Cyst loaded liposomes composed of PH 90H and Chol was
222 performed according to *Gharib et al.* (Gharib et al., 2018). Blank liposomes and Cyst loaded

223 liposomes (PH 90H: Chol: Cyst molar ratios of 1:0.98:1 and 1:0.98:10) were freshly prepared;
224 5 mL were then ultracentrifuged at 170000 g for 1 h at 4°C. The supernatant was removed,
225 and the pellet was reconstituted in an aqueous solution of 50 mM HP-β-CD (2 mL). The
226 samples were kept at -20°C overnight then placed into the drying chamber of Cryonext 23020
227 freeze-dryer (Trappes, France), pre-cooled to -20°C, then lowered to -40°C with a slow
228 cooling profile of 0.5°C/min. The product was stabilized for 30 min at -38°C before the
229 vacuum was applied. Primary drying was executed at a pressure of 150 μbar for 3 h at -10°C,
230 then the temperature of the drying chamber was progressively increased to 5°C for 6 h at 250
231 μbar, to reach finally 10°C at 350 μbar for 9 h. The temperature was adjusted at a product
232 temperature higher than that of the sublimation temperature of the water. During primary
233 drying, the ice crystals are sublimated, resulting in the porous cake of the freeze-concentrated
234 matrix. A secondary drying step for 10 h at 20°C and 100 μbar pressure was applied. This
235 step is crucial to decrease the residual water content of the amorphous matrix. Finally, the
236 vials were removed from the freeze-dryer, closed with rubber caps, and stored at 4°C. The
237 lyophilized liposomes were then reconstructed with ultra-pure water to its original volume (5
238 mL) before characterization and further analysis.

239 2.7 Storage stability of freeze-dried forms

240 After four months of storage at 4°C, freeze-dried liposomes were resuspended in 5 mL water;
241 the particle size, PDI, and zeta potential values of the obtained suspensions were determined.
242 The remaining Cyst concentrations were measured using ion-pair chromatography. The
243 following equation was used to determine the percentage of remaining Cyst:

$$\text{Percentage of remaining Cyst} = \frac{[\text{Cyst}]_t}{[\text{Cyst}]_{t_0}} \times 100 \text{ Eq. (7)}$$

244 where $[\text{Cyst}]_t$ and $[\text{Cyst}]_{t_0}$ are the total concentrations of Cyst in the liposomal suspension
245 before and after storage of dried liposomes at 4°C for 4 months. The experiment was carried
246 out in triplicate.

248 **3. Results and Discussion**

249 *3.1 Liposomes characterization*

250 *3.1.1 Size, polydispersity index, and zeta potential*

251 The characterization of liposomes composed of saturated (PH 90H) and unsaturated (Lipoid
252 S100) phospholipids and Chol was realized by DLS. The mean particle size, polydispersity
253 index (PdI), and zeta potential values were determined (**Table 1**).

254 Two populations were observed in blank and Cyst-loaded liposomes. The first population was
255 nanometric and represented more than 94% of the suspensions. The second population was of
256 a micrometric size and represented a negligible percentage varying between 1 and 6%.

257 The size of blank PH 90H liposomes was 211 ± 8 nm, while that of blank Lipoid S100
258 liposomes was lower (151 ± 11 nm). Similar results were obtained by *Gharib et al.* (Gharib et
259 al., 2017).

260 The PdI values were close to 0.2 for all the formulations, signifying that the liposomes had
261 homogeneous size. The zeta potential indicates the vesicle's surface charge; this parameter
262 reflected the liposome stability since it shows repulsive forces between particles (Domingues
263 et al., 2008). All liposomal formulations had a negative charge due to the presence of the
264 phosphate group at the surface of the vesicles (Ascenso et al., 2013). Liposomes incorporating
265 Cyst presented zeta potential values lower than blank ones. A variation of the pH between the
266 supernatants of blank liposomal formulation (pH 7) and Cyst loaded liposomes (pH 9.5) was
267 noticed. Also, the positively charged amino group of Cyst can interact with the negative
268 charges of phosphate groups of lipid membranes. The decrease of zeta potential values could
269 be due to the negative sulfur group of cysteamine attached to the surface. Indeed, after the
270 incubation of Cyst with the blank liposomes, the centrifugation of the mixture, and the
271 reconstitution of the pellet with water, the vesicles presented lower zeta potential values than
272 blank liposomes indicating that electrostatic interaction between Cyst and liposomes surface
273 may occur.

274 *Berleur et al.* studied the interaction of Cyst with dipalmitoylphosphatidylcholine (DPPC)
275 using differential scanning calorimetry, electron spin resonance, and turbidimetry. They
276 demonstrated that Cyst interacts with the DPPC membranes in their polar head region. At a
277 low concentration (10^{-4} M), Cyst behaved like a divalent cation (amine and SH groups),
278 creating electrostatic bridges with the negatively charged phosphate groups of the polar heads
279 of lipids leading to an increase in membrane stability. These bridges are disrupted when Cyst
280 is present at high concentration (10^{-2} and 10^{-1} M) inducing a decrease in the lipid membrane
281 rigidity; Cyst acted like a monovalent cation and displacement of the slightly charged SH
282 extremity by the amine of Cyst is occurred (Berleur et al., 1985).

283 3.1.2 *Incorporation ratios of phospholipid and Chol, encapsulation efficiency and loading* 284 *ratio of Cyst*

285 The quantification of phospholipids showed a high incorporation ratio of phospholipids in all
286 the formulations since the IR values were above $94 \pm 1.41\%$ (**Table 2**). Our results are in
287 accordance with those of *Sebaaly et al.* for blank PH 90 liposomes (Sebaaly et al., 2016). Cyst
288 did not affect the incorporation ratio of phospholipids since it is a hydrophilic molecule, and
289 therefore, it is expected to be encapsulated in the aqueous core of the liposomes. Concerning
290 Chol incorporation, the IR of Chol was higher for Lipoid S100 blank liposome ($95 \pm 2.0\%$)
291 than for PH 90H blank liposome ($80 \pm 0.0\%$). Similar results were reported by *Azzi et al.*,
292 where the IR values of Chol were higher for Lipoid S100 ($85.2 \pm 2.4\%$) when compared to PH
293 90H liposomes ($84.2 \pm 3.8\%$) (Azzi et al., 2018). Regarding Chol incorporation into Cyst-
294 loaded liposomes, the IR% values were slightly decreased for Cyst-loaded PH 90H liposomes
295 compared to blank liposomes. While for Lipoid S100-liposomes, a noticeable decrease of the
296 IR of Chol was observed at high Cyst to lipid molar ratios (phospholipid:Chol:Cyst of
297 1:1.17:5.89 and 1:1.17:11.78). The mechanism underlying this decrease is unclear.

298 On the other hand, EE and LR% of Cyst decreased while increasing the concentration of Cyst
299 in both types of liposomes. The higher EE and LR% values were obtained for the liposomal

300 suspensions containing the lowest concentration of Cyst (**Table 2**). At this same Cyst
301 concentration, the EE and the LR% of Cyst were higher for unsaturated liposomes when
302 compared to saturated ones. The preparation of PH 90H liposomes required heating above the
303 main transition temperature of the phospholipid (55°C), which could accelerate the oxidation
304 of the product. The EE% values of Cyst were generally lower (less than 40%) than those
305 determined for hydrophobic bioactive molecules (Azzi et al., 2018; Sebaaly et al., 2015) using
306 the same liposome formulations. A low encapsulation efficiency usually results when
307 encapsulating a hydrophilic drug (Eloy et al., 2014) because of its diffusion in and out of the
308 lipid membrane. Thus, the drug is difficultly retained inside the liposomes (Çağdaş et al.,
309 2014). The EE% values of caffeine were 7, 18, and 30% in Lipoid S100, Phospholipon 90G
310 (phosphatidylcholine 95%) liposomes (Tuncay Tanriverdi, 2018), and egg or soy lecithin
311 (Budai, 2013). Also, the EE% value of ciprofloxacin was 9% in liposomes composed of 1,2-
312 distearoyl-sn-glycero-3-phosphoglycerol-distearoylphosphatidylcholine-Chol (5:5:5 molar
313 ratio) (Oh et al., 1995); that of mitomycin C was 31% in Phospholipon 80:Chol (9:6 molar
314 ratio) (Chetoni et al., 2007) and the EE% value of ascorbic acid was 40% in
315 phosphatidylcholine liposomes (Serrano et al., 2015).

316 *3.1.3 Liposomes morphology*

317 TEM images of blank liposomes and Cyst-loaded liposomes are shown in **Fig. 2**. These
318 images correlated with the DLS results and showed the formation of nanometer-sized
319 vesicles. Oligolamellar spherical-shaped vesicles were obtained for blank and Cyst-loaded
320 liposomes.

321 *3.2 Stability study*

322 The stability of free and encapsulated Cyst was assessed at different temperatures (4, 25, and
323 37°C) and in dark and light. Aliquots were withdrawn at different time intervals, and the
324 concentration of Cyst was determined by ion-pair chromatography. The results were

325 expressed as the percentage of Cyst remaining in the solution as a function of time. The plots
326 showed a straight line indicating that the oxidation of Cyst followed a zero-order reaction, and
327 the slope corresponds to $-k$.

328 The stability of Cyst decreased when the temperature increased (**Fig. 3**). Cyst was oxidized
329 after 15 h at 4°C in the dark with a degradation rate of $5.25 \pm 0.09 \mu\text{g}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$ (**Table 3**).
330 Whereas at 37°C in the dark, the total oxidation of Cyst was achieved after 8h of incubation
331 with a degradation rate of $11.52 \pm 0.16 \mu\text{g}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$. *Pescina et al.* confirmed the temperature
332 dependence of Cyst oxidation after measuring the stability of the molecule at -20, 4, and 25°C
333 (*Pescina et al.*, 2016).

334 The effect of light on Cyst stability was also evaluated, and the results showed that there
335 wasn't any significant difference between the stability of incubated Cyst in light or dark (**Fig.**
336 **4**). The degradation rate of Cyst was slightly higher at 25°C in light ($10.68 \pm 0.57 \mu\text{g}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$)
337 ¹) than in dark ($10.06 \pm 0.07 \mu\text{g}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$).

338 The encapsulation of Cyst slowed down the oxidation of Cyst in all the studied conditions.
339 For example, at 4°C in the dark, the total oxidation was delayed for 43 h for the encapsulated
340 Cyst compared to the free Cyst. The protection factor, defined as the degradation rate of the
341 free Cyst divided by the degradation rate of the encapsulated Cyst, was also calculated.
342 Following the encapsulation of Cyst in liposomes, protection factors from its oxidation were
343 of 2.96, 4.7, 5, and 4.14 after storage at 4°C, 25°C at dark, 25°C in light, and 37°C,
344 respectively (**Table 3**). Whereas, the use of cyclodextrin didn't affect the stability of Cyst
345 (*Pescina et al.*, 2016). Besides, *Dixon et al.* found that the addition of antioxidants except the
346 enzyme catalase had an insignificant effect on the prevention of Cyst oxidation (*Dixon et al.*,
347 2018).

348 To our knowledge, the quantification of Cyst and its degradation product cystamine is done
349 for the first time in liposomal suspension. Cyst was totally converted to cystamine. The latter

350 was the only product present in the liposomal suspension after the total oxidation of Cyst. The
351 encapsulation delayed this conversion in all the studied conditions (**Fig. 5**).

352 3.3 Freeze-drying

353 *Gharib et al.* succeeded to freeze-dry PH 90H liposomes formulations using HP- β -CD (50
354 mM) as a cryoprotectant (Gharib et al., 2018). Two formulations of Cyst loaded liposomes
355 (PH 90H:Chol:Cyst 1:0.98:1 and 1:0.98:10 molar ratios) were selected for freeze-drying. The
356 liposomes were then reconstituted in water and characterized for their morphology,
357 homogeneity, size, surface charge, and LR% (**Table 4**).

358 The morphology of the liposomes was preserved after lyophilization (**Fig. 6**); the vesicles
359 appeared to be oligolamellar and spherical. The presence of HP- β -CD did not affect liposome
360 lamellarity or morphology.

361 The size of blank and Cyst PH 90H loaded liposomes was maintained after lyophilization
362 regardless of the concentration of Cyst. A slight increase of the pDI values was observed after
363 lyophilization and reconstitution for blank and Cyst-loaded liposomes. The zeta potential
364 value was conserved for the blank liposomes; nevertheless, it significantly decreased for Cyst-
365 loaded liposomes from -42 to -19 mV for the formulation PH 90H:Chol:Cyst molar ratio of
366 1:0.98:1 and from -48 to -33 mV for the formulation PH 90H:Chol:Cyst molar ratio of
367 1:0.98:10. The variation of the zeta potential was due to the loss of Cyst bound to the outer
368 side of the membranes of the vesicles during lyophilization. Furthermore, the loading ratio of
369 Cyst after the freeze-drying decreased from 24 to 8% in PH 90H:Chol:Cyst (1:0.98:1) and
370 from 16 to 5% in PH 90H:Chol:Cyst (1:0.98:10). After lyophilization, liposomal suspension
371 retained 33% of Cyst present in the PH 90H liposomal suspension. A significant decrease in
372 the loading rate of anethole after freeze-drying in anethole loaded liposomes was also
373 observed previously (Gharib et al., 2018).

374 *3.4 Storage stability of freeze-dried forms*

375 The mean particle size, PDI, and zeta potential values of the reconstituted liposomes after
376 freeze-drying and after four months of storage at 4°C were evaluated (**Table 4**). The two
377 formulations used in this study were stable with adequate size, PDI, and zeta potential values.
378 Besides, the total concentrations of Cyst in the reconstituted liposomes after freeze-drying
379 were determined after four months and compared to those obtained at t_0 . The percentage of
380 remaining Cyst was 16% for the formulation PH 90H:Chol:Cyst (1:0.98:10). This result was
381 satisfactory, especially when considering the high instability of Cyst; Cyst was oxidized
382 entirely to Cystamine after 15 h in aqueous solution and 60 h in liposomal suspensions after
383 storage at 4°C (**Fig. 3A**). The storage conditions are critical in the case of Cyst. Taking into
384 account its instability, the eye-drop CystaranTM (Cyst ophthalmic solution 0.44%) has to be
385 stored at -20°C, and administered hourly during the daytime. After its opening, it should be
386 used within one week (Huynh et al., 2013). Cyst hydrochloride (0.5%) formulated ophthalmic
387 preparation is easily oxidized within the first week after storage at +4°C, rendering the
388 preparation less effective (Reda et al., 2017). In comparison with the aqueous liposomal
389 suspension, the freeze-dried form conserved Cyst for a more extended period. The elimination
390 of the water from the liposomal suspension is the factor that induced this improvement of
391 Cyst stability. The presence of water will lead to the degradation of Cyst to cystamine (Gana
392 et al., 2015).

393 **4. Conclusion**

394 In this study, we proved that the encapsulation of Cyst in conventional liposomes improved
395 its stability. Cyst-loaded liposomes were nanometric and presented a negative surface charge.
396 In addition to its incorporation in vesicles, Cyst binds to the surface of the vesicle. The
397 loading ratio of Cyst in liposomes varied between 12 and 24% depending on Cyst to lipid
398 molar ratio. Besides, in this work, we succeeded in preparing freeze-dried liposomes that
399 retain a noticeable amount of Cyst after four months of storage at 4°C. These results could
400 help to develop various pharmaceutical forms of Cyst and enlarge its applications.

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402
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407 University of Lyon.

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409

410 **Declaration of Competing Interest**

411 The authors declare that they have no known competing financial interests or personal
412 relationships that could have appeared to influence the work reported in this paper.

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560 **Figures Legends**

561 **Figure 1:** Structure of Cyst.

562 **Figure 2:** TEM images for blank PH 90H liposomes (A), Cyst-loaded PH 90H liposomes (PH
563 90H: Chol: Cyst 1:0.98:1) (B) blank Lipoid S100 liposomes (C), Cyst-loaded Lipoid S100
564 liposomes (Lipoid S100: Chol: Cyst 1: 1.17: 1.77) (D).

565 **Figure 3:** Stability study of free and encapsulated Cyst in the dark at 4°C (A), 25°C (B), and
566 37°C (C).

567 **Figure 4:** Stability study of free and encapsulated Cyst at 25°C in the dark (A) and in light
568 (B).

569 **Figure 5:** Percentage of formed cystamine in free and Cyst-loaded liposomes in the dark at
570 4°C (A), 25°C (dark (B), and light (C)), and 37°C (D).

571 **Figure 6:** TEM images for blank (A) and Cyst-loaded-PH 90H liposomes (B) after freeze-
572 drying and reconstitution with water.

573

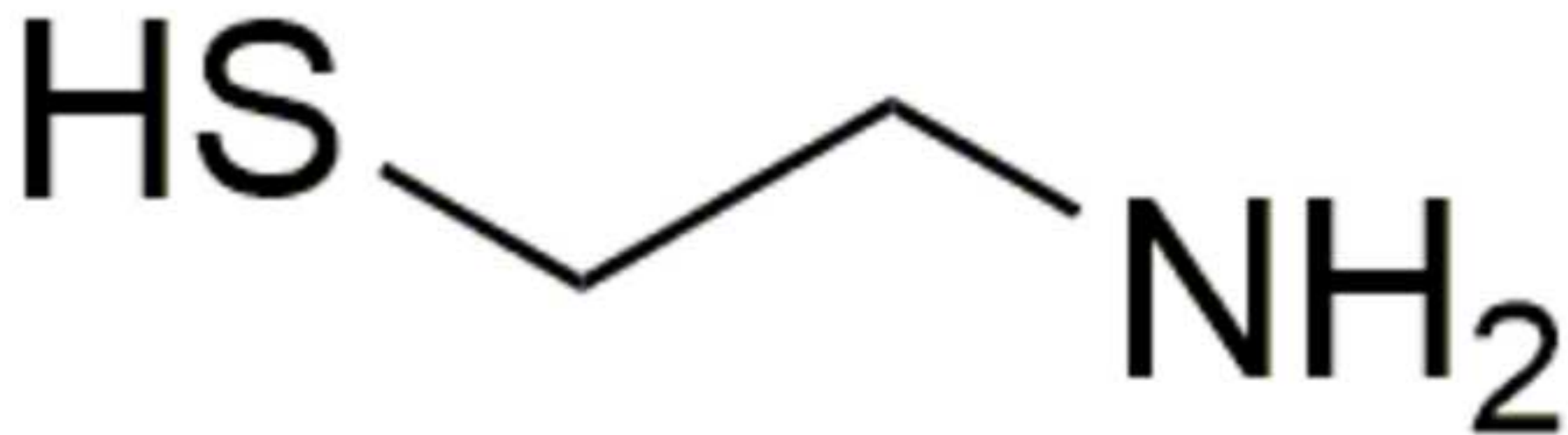


Figure 2

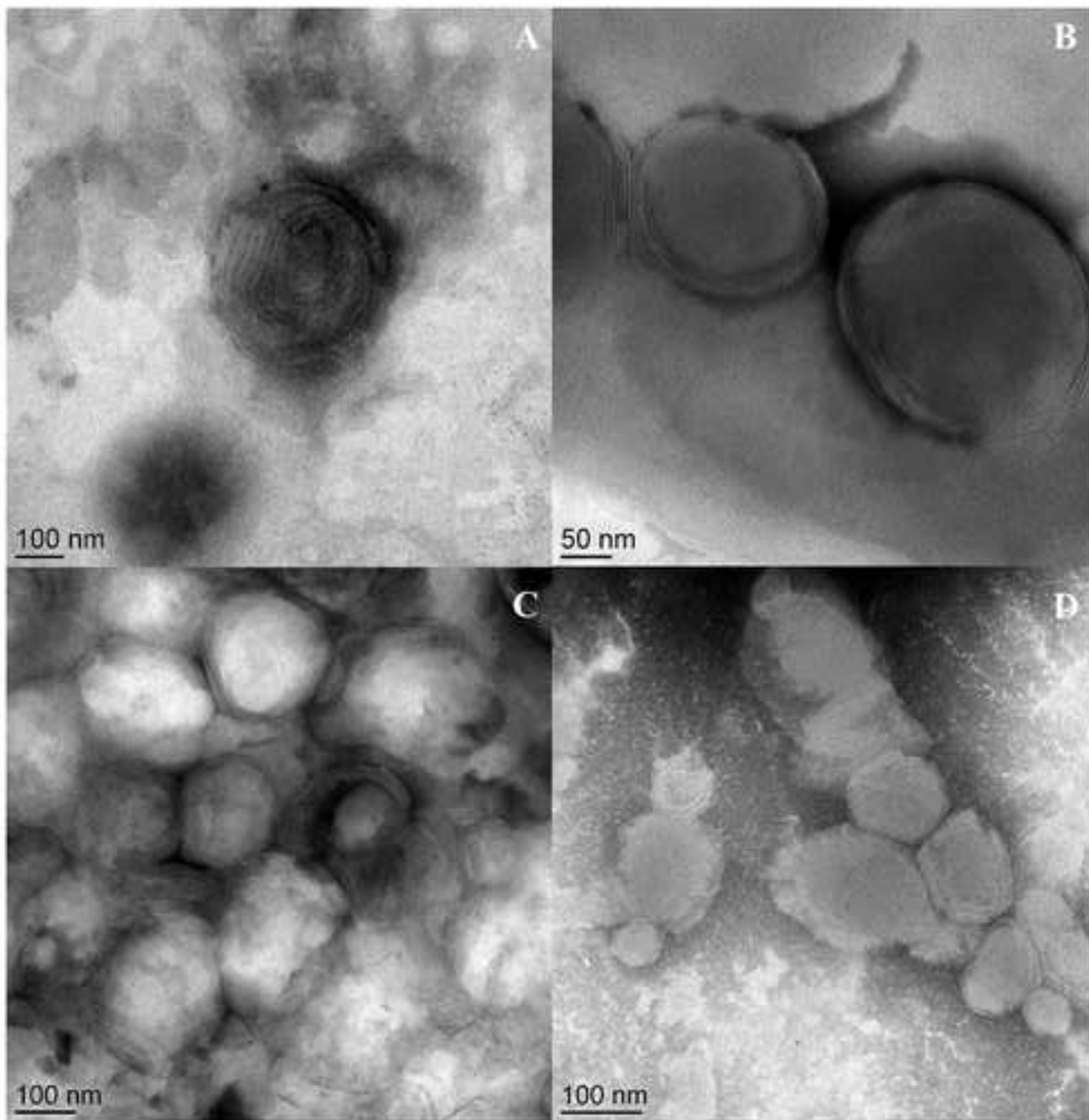


Figure 3

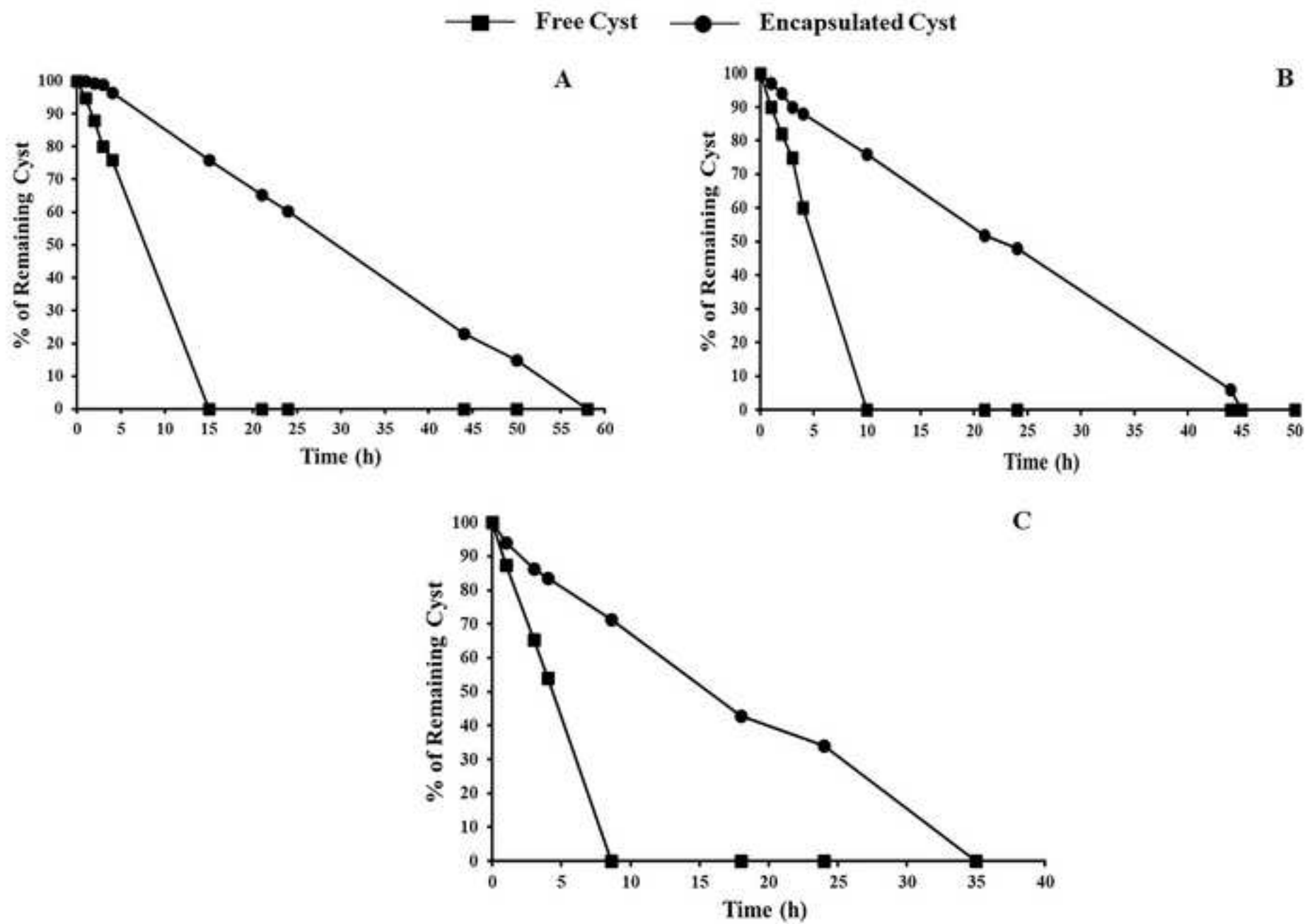


Figure 4

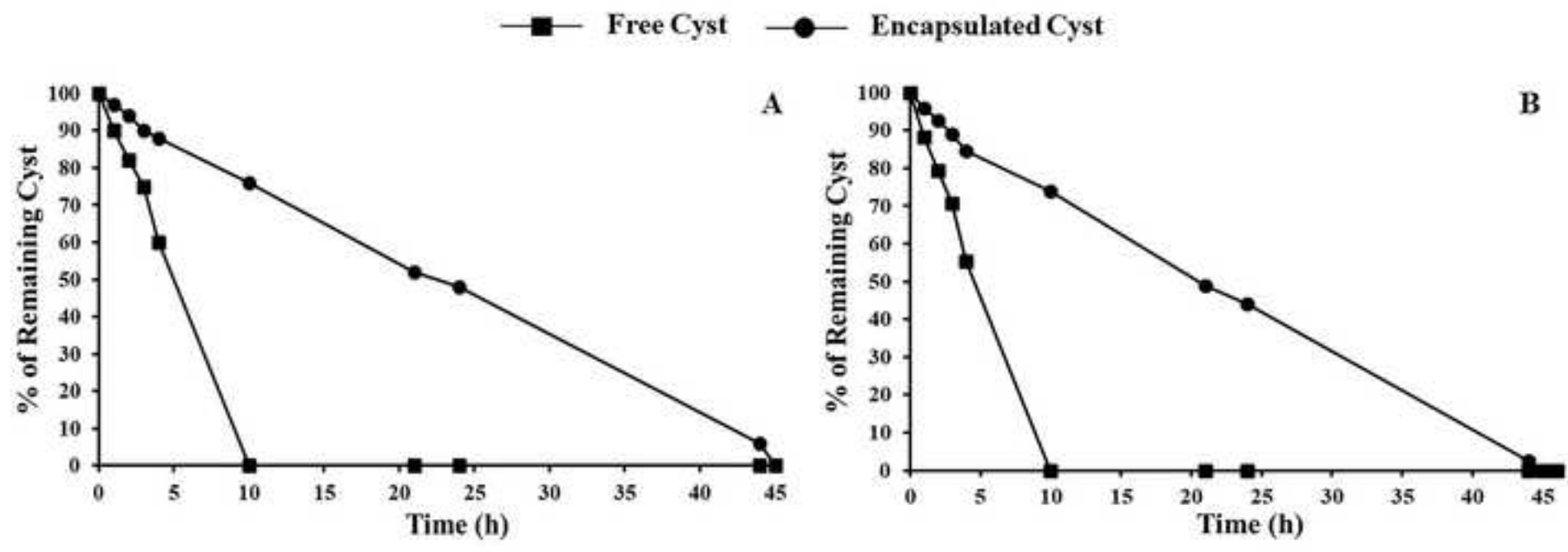


Figure 5

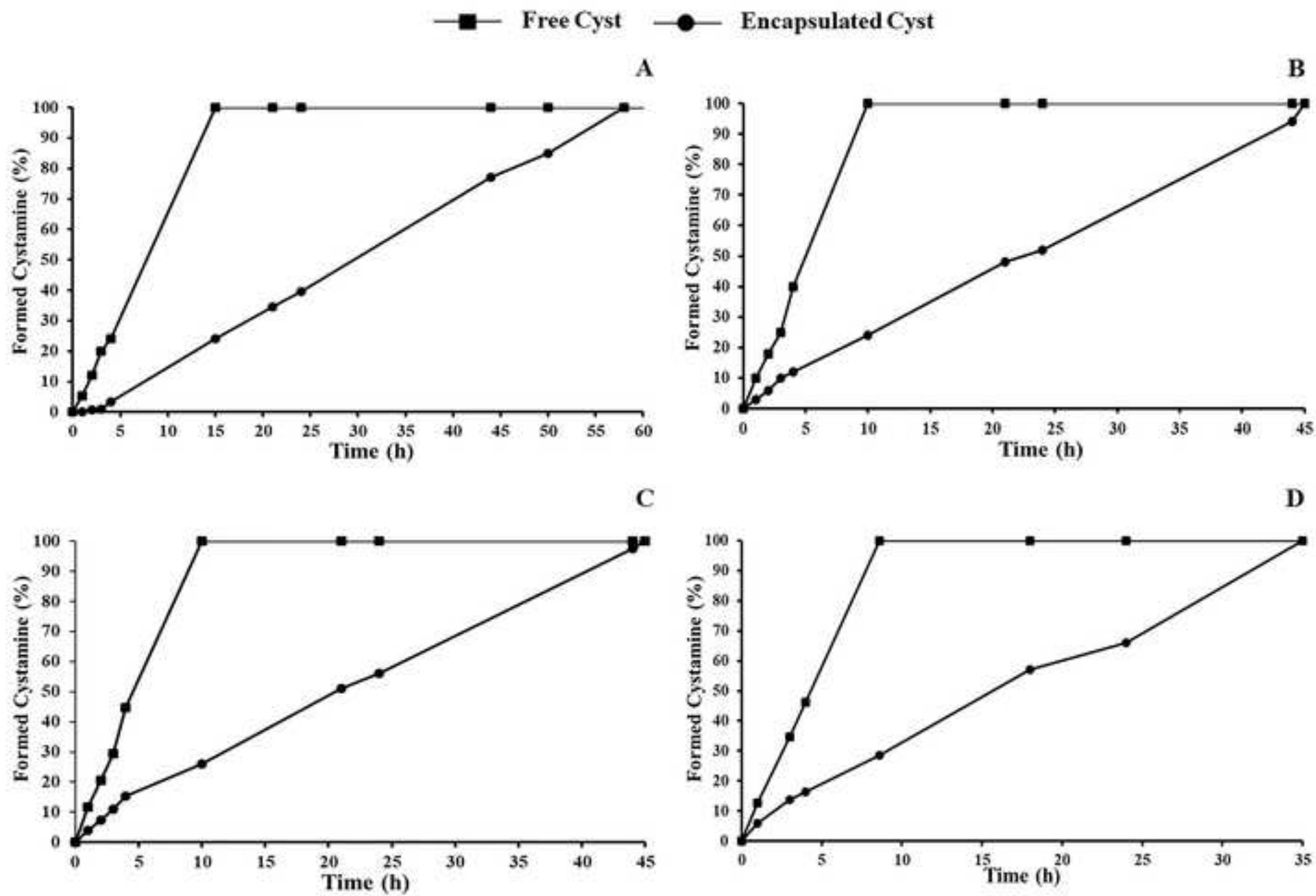


Figure 6

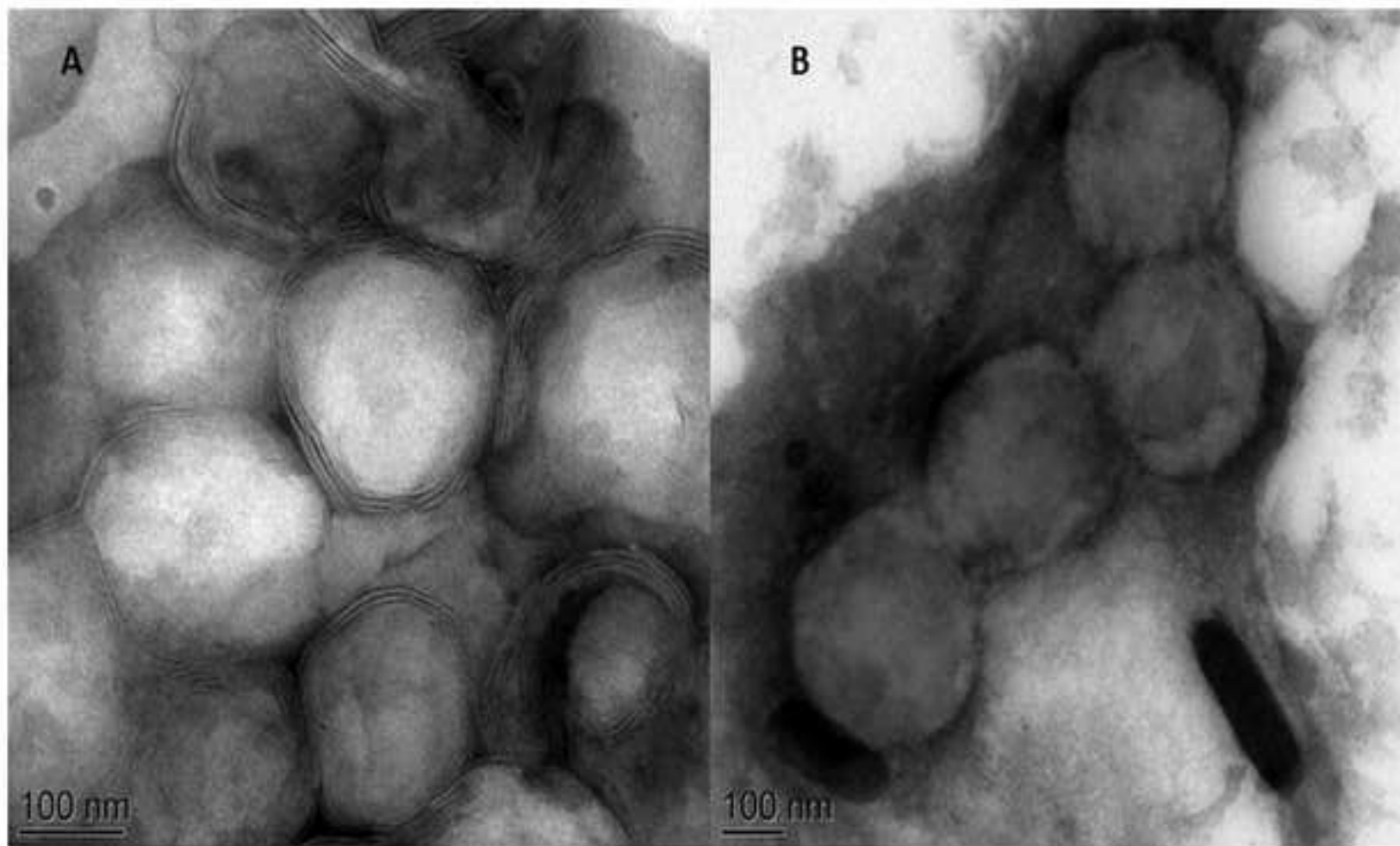


Table 1: Size, pDI, and zeta potential of blank and Cyst loaded PH 90H and Lipoid S100

liposomes.

Formulation	Lipid:Chol: Cyst molar ratio	Size				Zeta potential (mV)	PdI
		Population 1		Population 2			
		%	Mean size (nm)	%	Mean size (μ m)		
Blank liposomes (PH 90H:Chol)	1:0.98	99 \pm 0.4	211 \pm 8	0.9 \pm 0.4	5 \pm 0.1	-17.9 \pm 4.0	0.2 \pm 0.0
	1:0.98:1	98 \pm 1.3	301 \pm 56	2 \pm 1.3	5 \pm 0.3	-35.8 \pm 0.8	0.2 \pm 0.0
Cyst-loaded liposomes (PH 90H:Chol:Cyst)	1:0.98:1.5	98 \pm 1.1	318 \pm 47	2 \pm 1.1	5 \pm 0.3	-37.1 \pm 0.7	0.2 \pm 0.1
	1:0.98:2	98 \pm 1.7	248 \pm 86	2 \pm 1.7	5 \pm 3.0	-37.0 \pm 1.2	0.2 \pm 0.1
	1:0.98:5	97 \pm 2.7	278 \pm 55	3 \pm 2.7	5 \pm 2.9	-45.0 \pm 2.8	0.2 \pm 0.1
	1:0.98:10	95 \pm 0.7	262 \pm 33	5 \pm 0.7	5 \pm 0.1	-45.0 \pm 1.0	0.2 \pm 0.1
Blank liposomes (Lipoid S100:Chol)	1:1.17	98 \pm 0.7	151 \pm 11	2 \pm 0.0	5 \pm 0.0	-24.0 \pm 0.1	0.2 \pm 0.0
	1:1.17:1.17	94 \pm 2.0	156 \pm 13	6 \pm 2.0	5 \pm 0.2	-32.0 \pm 0.41	0.3 \pm 0.0
Cyst loaded liposomes (Lipoid S100:Chol:Cyst)	1:1.17:1.77	97 \pm 1.0	150 \pm 3	3 \pm 1.0	5 \pm 0.3	-34.0 \pm 3.70	0.3 \pm 0.0
	1:1.17:2.36	97 \pm 1.0	149 \pm 1	3 \pm 1.0	5 \pm 1.0	-34.3 \pm 1.45	0.3 \pm 0.0
	1:1.17:5.89	97 \pm 1.0	152 \pm 11	3 \pm 1.0	5 \pm 0.2	-42.5 \pm 1.17	0.3 \pm 0.1
	1:1.17:11.78	97 \pm 1.6	160 \pm 8	3 \pm 1.8	4 \pm 0.2	-39.2 \pm 1.30	0.2 \pm 0.0

Table 2: Incorporation ratios of phospholipids (%) and Chol (%), EE%, and LR% values of Cyst in liposomes suspensions.

Formulations	Lipid:Chol:Cyst molar ratios	IR % of phospholipid	IR % of Chol	EE%	LR%	Final liposome composition (Lipid:Chol:Cyst molar ratio)
Blank liposomes (PH 90H:Chol)	1:0.98	94 ± 1.4	80 ± 0.0			0.94:0.81
	1:0.98:1	97 ± 0.8	74 ± 0.5	29 ± 3.0	24 ± 2	0.97:0.72:0.24
Cyst-loaded liposomes (PH 90H:Chol:Cyst)	1:0.98:1.5	100 ± 0.0	74 ± 1.5	26 ± 6.0	22 ± 6	1:0.72:0.33
	1:0.98:2	98 ± 0.5	72 ± 3.7	23 ± 4.0	20 ± 4	0.98:0.70:0.40
	1:0.98:5	94 ± 3.6	72 ± 2.0	24 ± 2.0	18 ± 3	0.94:0.70:0.90
	1:0.98:10	94 ± 2.5	74 ± 2.5	21 ± 5.0	16 ± 4	0.94:0.73:0.16
Blank liposomes (Lipoid S100:Chol)	1:1.17	99 ± 1.0	95 ± 2.0			0.99:1.11
	1:1.17:1.17	100 ± 1.0	99 ± 2.0	40 ± 4.0	30 ± 2	0.99:1.16:0.35
Cyst loaded liposomes (Lipoid S100:Chol:Cyst)	1:1.17:1.77	100 ± 2.0	91 ± 3.8	29 ± 4.0	24 ± 4	1:1.06:0.42
	1:1.17:2.36	99 ± 0.7	97 ± 5.0	22 ± 0.4	20 ± 1	0.99:1.13:0.47
	1:1.17:5.89	99 ± 1.0	35 ± 2.7	13 ± 2.0	12 ± 2	0.94:0.34:0.71
	1:1.17:11.78	99 ± 1.3	12 ± 0.0	13 ± 2.0	12 ± 2	0.94:0.12:1.41

Table 3: Degradation rate constants of free and encapsulated Cyst at 4°C (dark), 25°C (dark and light), and 37°C (dark).

Formulations	Temperature (°C)	Degradation rate constant k (µg. mL⁻¹.h⁻¹)	Protection factor
<i>Free Cyst</i>	4 (dark)	5.25 ± 0.09	-
<i>Cyst loaded liposome</i>		1.77 ± 0.18	2.96
<i>Free Cyst</i>	25 (dark)	10.06 ± 0.07	-
<i>Cyst loaded liposome</i>		2.14 ± 0.12	4.7
<i>Free Cyst</i>	25 (light)	10.68 ± 0.57	-
<i>Cyst loaded liposome</i>		2.12 ± 0.07	5
<i>Free Cyst</i>	37 (dark)	11.52 ± 0.16	-
<i>Cyst loaded liposome</i>		2.78 ± 0.05	4.14

Table 4: Size, polydispersity index, zeta potential values for blank and Cyst-loaded liposomes and loading rate of Cyst in liposomes made from PH 90H before and after freeze-drying at t_0 and after four months of storage in powder form (**bold**). The values obtained after lyophilization were compared to those before lyophilization.

	Before freeze-drying				After freeze-drying			
	Size (nm)	pdI	Zeta potential (mV)	LR%	Size (nm)	pdI	Zeta potential (mV)	LR%
Blank PH 90H liposome	187 ± 42	0.15 ± 0.08	-17 ± 2.8		172 ± 14	0.23 ± 0.03	-18 ± 2.5	
PH 90H:Chol:Cyst 1:0.98:1	181 ± 19	0.12 ± 0.01	-37 ± 0.2	24 ± 2	166 ± 2 231 ± 36	0.15 ± 0.05 0.29 ± 0.05	-19 ± 0.8 -18 ± 3.5	8 ± 1.2 0 ± 0
PH 90H:Chol:Cyst 1:0.98:10	187 ± 18	0.18 ± 0.06	-48 ± 4	16 ± 4	193 ± 27 226 ± 15	0.32 ± 0.02 0.23 ± 0.02	-33 ± 1.4 -34 ± 0.5	5 ± 0.5 0.8 ± 0.11