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### ► To cite this version:

Carla Atallah, Celine Viennet, Sophie Robin, Sami Ibazizen, H el ene Greige-Gerges, et al.. Effect of cysteamine hydrochloride-loaded liposomes on skin depigmenting and penetration. *European Journal of Pharmaceutical Sciences*, 2022, 168, pp.106082. 10.1016/j.ejps.2021.106082 . hal-03759788

**HAL Id: hal-03759788**

**<https://hal.science/hal-03759788>**

Submitted on 24 Aug 2022

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1 **Effect of cysteamine hydrochloride-loaded liposomes on skin depigmenting**  
2 **and penetration**

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13

14 **Abstract**

15 Skin hyperpigmentation is caused by an excessive production of melanin. Cysteamine, an  
16 aminothioliol compound physiologically synthesized in human body cells, is known as  
17 depigmenting agent. The aim of this study was to evaluate the depigmenting activity and skin  
18 penetration of liposome formulations encapsulating cysteamine hydrochloride. First,  
19 cysteamine hydrochloride-loaded liposomes were prepared and characterized for their size,  
20 polydispersity index, zeta potential and the encapsulation efficiency of the active molecule. The  
21 stability of cysteamine hydrochloride in the prepared liposome formulations in suspension and  
22 freeze-dried forms was then assessed. The *in vitro* cytotoxicity of cysteamine and cysteamine-  
23 loaded liposome suspensions (either original or freeze-dried) was evaluated in B16 murine  
24 melanoma cells. The measurement of melanin and tyrosinase activities was assessed after cells  
25 treatment with free and encapsulated cysteamine. The antioxidant activity of the free and  
26 encapsulated cysteamine was evaluated by the measurement of ROS formation in treated cells.  
27 The *ex vivo* human skin penetration study was also performed using Franz diffusion cell. The  
28 stability of cysteamine hydrochloride was improved after encapsulation in liposomal  
29 suspension. In addition, for the liposome re-suspended after freeze-drying, a significant increase  
30 of vesicle stability was observed. The free and the encapsulated cysteamine in suspension  
31 (either original or freeze-dried) did not show any cytotoxic effect, inhibited the melanin  
32 synthesis as well as the tyrosinase activity. An antioxidant activity was observed for the free  
33 and the encapsulated cysteamine hydrochloride. The encapsulation enhanced the skin  
34 penetration of cysteamine hydrochloride. The penetration of this molecule was better for the re-  
35 suspended freeze-dried form than the original liposomal suspension where the drug was found  
36 retained in the epidermis layer of the skin.

37 **Keywords:** Cysteamine hydrochloride, cystamine, cytotoxicity, liposome, melanin, skin  
38 penetration, tyrosinase.

## 39 **1 Introduction**

40 Many compounds such as hydroquinone, kojic acid, and arbutin are used to treat  
41 hyperpigmentation; however, their use was associated with cytotoxic effects (Ephrem et al.,  
42 2017; Rendon & Gaviria, 2005; Solano et al., 2006). Cysteamine (cyst), an aminiothiol  
43 synthesized by human body cells; and its disulfide form, cystamine, have shown to possess a  
44 depigmenting effect on black gold fish (Chavin & Schlesinger, 1966; Frenk et al., 1968).  
45 Besides, the application of cysteamine Cream® on the ear of black female guinea pigs  
46 demonstrated a high depigmenting effect (Hsu et al., 2013). A randomized, double-blind  
47 vehicle-controlled clinical trial was also conducted to evaluate the efficacy and safety of this  
48 cream to treat epidermal melasma; the treatment with cysteamine cream® decreased the content  
49 of melanin (Farshi et al., 2017; Mansouri et al., 2015). It was reported that the depigmenting  
50 action of cyst and cystamine is due to the inhibition of tyrosinase activity which is the enzyme  
51 that plays a crucial role in the melanogenesis. Unlike hydroquinone, cyst and cystamine  
52 mechanisms of action are a melanogenesis inhibition and not a melanocytotoxicity (Qiu et al.,  
53 2000). Qiu et al. reported that 100  $\mu\text{M}$  of cyst inhibit 21% of melanin and 87% of tyrosinase  
54 while 50  $\mu\text{M}$  of cystamine inhibit 5% of melanin and 89% of tyrosinase (Qiu et al., 2000).  
55 These molecules could also reduce the reactive oxygen species (ROS) generation and enhance  
56 the intracellular glutathione (Lee et al., 2017; Okamura et al., 2014; Ribeiro et al., 2013; Shin  
57 et al., 2011; Sun et al., 2010). The production of (ROS) was significantly suppressed by low  
58 dose of cyst on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic  
59 neurodegeneration mice (Sun et al., 2010). Cyst also inhibited tert-butyl hydroperoxide-induced  
60 ROS production in human corneal endothelial cells (Shin et al., 2011). It reduced ROS  
61 production by 33% in phorbol 12-myristate-13-acetate stimulated macrophages (Okamura et  
62 al., 2014). In addition, cyst inhibited vascular endothelial growth factor-induced ROS  
63 generation in a concentration-dependent manner, with maximum effect at 50  $\mu\text{M}$  (Lee et al.,

64 2017). Moreover, cystamine decreased ROS levels evoked by H<sub>2</sub>O<sub>2</sub> or staurosporine in mutant  
65 cells (Ribeiro et al., 2013).

66 However, cyst presents an offensive odor and is unstable in solutions where it undergoes  
67 oxidation to cystamine (Atallah, Charcosset, et al., 2020). The encapsulation of many skin  
68 whitening agents in delivery systems improved their stability (Ephrem et al., 2017). Moreover,  
69 it increased their concentration at the targeted site by improving skin permeation, penetration  
70 or distribution (Ephrem et al., 2017). Liposomes are nanometric spherical vesicles able to  
71 encapsulate hydrophilic, hydrophobic and amphiphilic molecules. They are biodegradable,  
72 biocompatible and present a low toxicity (Zylberberg & Matosevic, 2016).

73 Cyst can be used in different forms such as cyst (base form), cyst hydrochloride (cyst HCl),  
74 phosphocyst, and cyst bitartrate (**Figure 1**). An increase of the stability of the base form of cyst  
75 was previously demonstrated after encapsulation in liposomes suspension (original and from  
76 freeze-dried form) compared to free cyst (base form) during storage at 4°C (Atallah, Greige-  
77 Gerges, et al., 2020). However, after 60 hours, the remaining cyst in liposome suspension was  
78 very low. Therefore, cyst HCl was used in this study because the salt form of this molecule is  
79 more stable than its base form (Wiedmann & Naqwi, 2016).

80 The aim of this study was to investigate the effect of cyst HCl-loaded liposomes on  
81 melanogenesis and oxidative stress in cultured B16 cells. Skin penetration assays were also  
82 conducted on *ex-vivo* human skin. Blank and cyst HCl-loaded liposomes were prepared by the  
83 ethanol injection method and characterized for their size, polydispersity index and zeta  
84 potential. The encapsulation efficiency of cyst HCl (EE%) in liposomes was also calculated.  
85 The amount of residual ethanol in the blank liposomes was determined by NMR technique.  
86 Blank and cyst HCl-loaded liposomes were freeze-dried according to the optimized protocol  
87 previously described (Gharib et al., 2018). The lyophilized liposomes were then re-suspended  
88 with ultra-pure water. The stability of free cysteamine dissolved in water and that of the cyst

89 HCl-loaded liposome suspensions (original or freeze-dried form) was evaluated at 4°C during  
90 6 months. The cytotoxicity of free cyst HCl, cystamine and the suspensions of blank- and cyst  
91 HCl-loaded liposomes (original or freeze-dried) were evaluated by MTT assay. In addition, the  
92 effects of the same formulations on the *in vitro* melanin content and tyrosinase activity were  
93 measured. Moreover, the antioxidant activity of the free and the encapsulated cyst HCl was  
94 assessed by the measurement of ROS formation induced by the presence of H<sub>2</sub>O<sub>2</sub> in B16 cells.  
95 Finally, the skin penetration of free- and cyst HCl-loaded liposomes was compared using the  
96 Franz diffusion cell.

## 97 **2 Materials and Methods**

### 98 **2.1 Materials**

99 Cyst HCl, cystamine dihydrochloride, metformin hydrochloride, (3-(4, 5-dimethylthiazol-2-  
100 yl)-2, 5-diphenyltetrazolium bromide), DMSO, L-DOPA and NaOH were purchased from  
101 Sigma-Aldrich (Buchs, Switzerland). Phospholipon 90H (90% soybean PC, 4% lysoPC, 2%  
102 triglycerides, 2% water, and 0.5% ethanol) was obtained from Lipoid GmbH (Ludwigshafen,  
103 Germany). Cholesterol was obtained from the Fisher chemical (Loughborough, UK). HP- $\beta$ -  
104 CD-oral grade (MS=0.85) was purchased from Roquette (Lestrem, France). Sodium dodecyl  
105 phosphate and phosphoric acid were obtained from Sigma-Aldrich (China). (trimethylsilyl)-  
106 2,2,3,3-tetradecylpropanoic acid sodium salt, 2,7-dichlorodihydrofluorescein diacetate and  
107 H<sub>2</sub>O<sub>2</sub> were purchased from Sigma-Aldrich (USA). Deuterium oxide (D<sub>2</sub>O) was purchased from  
108 Acros organics (USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum  
109 (FBS), penicillin, and streptomycin were purchased from Dominique Dutscher (France). All  
110 other chemicals were of analytical grade.

### 111 **2.2 Cyst HCl-loaded liposomes suspensions preparation (original and re-suspended** 112 **after freeze-drying)**

113 Liposomes were prepared by ethanol injection method, as described by Sebaaly et al. (2016).  
114 The organic phase was prepared by dissolving phospholipon 90H (saturated lipid) (100 mg)  
115 and cholesterol (50 mg) in absolute ethanol (10 mL). Then, the organic phase was injected into  
116 the aqueous phase (20 mL) containing 5 mg/mL of cyst HCl, using a syringe pump (Fortuna  
117 optima, GmbH-Germany), at a temperature above the transition temperature of the  
118 phospholipid (55°C) and under magnetic stirring at 400 rpm. The contact between the organic  
119 solution and the aqueous phase leads to a spontaneous liposome formation. The liposomal  
120 suspensions were then left for 15 min at 25°C under stirring (400 rpm). The ethanol was  
121 removed by rotary evaporation (Heidolph GmbH, Germany) under reduced pressure at 40°C

122 and the obtained liposomal solutions were stored at 4°C. Each preparation was performed in  
123 triplicate.

124 The freeze drying of blank- and cyst HCl-loaded liposomes was done as previously reported  
125 (Atallah, Greige-Gerges, et al., 2020). In brief, blank liposomes and cyst HCl-loaded liposomes  
126 (PH 90H: Chol: cyst molar ratios 1:0.98:10) were freshly prepared; 5 mL were then  
127 ultracentrifuged at 170,000g for 1 h at 4 °C. The supernatant was removed, and the pellet was  
128 re-dissolved in an aqueous solution of 50 mM HP-β-CD (2 mL). The samples were kept at -20  
129 °C overnight then placed into the drying chamber of Cryonext 23,020 freeze-dryer (Trappes,  
130 France), pre-cooled to -20°C, then lowered to -40°C with a slow cooling profile of 0.5°C/min.  
131 The product was stabilized for 30 min at -38 °C before the vacuum was applied. Primary drying  
132 was executed at a pressure of 150 µbar for 3 h at -10°C, then the temperature of the drying  
133 chamber was progressively increased to 5 °C for 6 h at 250 µbar, to reach finally 10°C at 350  
134 µbar for 9 h. A secondary drying step for 10 h at 20°C and 100 µbar pressure was applied.  
135 Finally, the vials were removed from the freeze-dryer, closed with rubber caps, and stored at  
136 4°C. The lyophilized liposomes were then re-suspended with ultra-pure water to the original  
137 volume (5 mL).

### 138 **2.3 Cyst HCl-loaded liposomes characterization**

139 The size, polydispersity index and zeta potential were determined using Malvern Zetasizer  
140 Nanoseries (Zetasizer Nano ZS; Malvern Instruments Ltd, France). All batches were diluted  
141 with ultrapure water. The particle-size distribution data were collected using the DTS (nano)  
142 software (version 5.10) provided with the instrument. Zeta potential was calculated using  
143 Smoluchowski's equation from the electrophoretic mobility of liposomes. All measurements  
144 were carried out at 25°C after 2 min of equilibration and were performed in triplicate. Data  
145 were expressed as the mean values ± SD.



146 For the calculation of EE%, liposomal suspensions (5 mL) were ultra-centrifuged (Optima™  
147 Ultracentrifuge, Beckman Coulter, USA) at 170,000g for 1 h at 4°C to separate the free cyst  
148 HCl (supernatant) from the liposomes loading cyst HCl (pellet). The pellet was re-suspended  
149 in water (5 mL). The total liposomal suspension as well as the re-suspended pellet were  
150 sonicated and then analyzed by ion pair chromatography method as described below. This  
151 allows to determine the total and encapsulated cyst HCl in the liposomal suspensions.  
152 The encapsulation efficiency (EE %) of cyst HCl was calculated using the formula:

$$153 \quad EE_{\text{Cyst HCl}} \% = \frac{[\text{Cyst HCl}]_{\text{encapsulated}}}{[\text{Cyst HCl}]_{\text{total}}} \times 100$$

154 Where  $[\text{Cyst HCl}]_{\text{encapsulated}}$  and  $[\text{Cyst HCl}]_{\text{total}}$  represent the concentration of encapsulated cyst  
155 HCl (pellet) and the concentration of the total cyst HCl present in the liposomal suspension,  
156 respectively.

#### 157 **2.4 Cyst HCl and cystamine quantification**

158 To quantify cyst HCl and cystamine in the different formulations, an ion-pair chromatography  
159 with an isocratic mode consisting of acetonitrile:water containing 0.1% phosphoric acid and  
160 sodium dodecyl phosphate (4 mM) (v/v, 45:55) was used. The column was a reverse-phase  
161 Kinetex C18 column (4.6 × 100 mm, 2.6 μm). 20 μL were injected in the column maintained at  
162 25°C with a flow rate of 1.0 mL/min, and the detection wavelength was 215 nm. Metformin (1  
163 μg/mL) was used as an internal standard. Linearity was between 2.5 and 100 μg/mL for cyst  
164 HCl and between 5 and 100 μg/mL for cystamine. This method was previously used for the  
165 quantification of cyst in its base form; the same chromatographic results were obtained as for  
166 cyst HCl (retention time, limits of detection and quantification).

#### 167 **2.5 Ethanol quantification in the blank liposomal suspension**

168 The quantity of ethanol present in the blank liposomal suspension was also determined by NMR  
169 technique according to Zuriarrain et al. (Zuriarrain et al., 2015). Blank liposomal suspensions

170 were centrifuged for 10 min at 11200g. The residual ethanol is supposed to be present in the  
171 upper phase. The internal standard used was (trimethylsilyl)-2,2,3,3-tetra-deutero-propionic  
172 acid sodium salt (TSP)  $(\text{CH}_3)_3\text{SiCD}_2\text{CD}_2\text{CO}_2\text{Na}$ . 10 mg of TSP was dissolved in 400  $\mu\text{L}$   $\text{D}_2\text{O}$   
173 and 300  $\mu\text{L}$  of the supernatant of the liposomal suspension. The number of moles of ethanol is  
174 calculated from the NMR spectrum using the following equation:

$$175 \quad n_{\text{EtOH}} = \frac{H_{\text{IS}}}{H_{\text{CH}_2}} \times n_{\text{IS}} \times \frac{I_{\text{CH}_2}}{I_{\text{IS}}}$$

176 Where  $H_{\text{IS}}$  and  $H_{\text{CH}_2}$  are the numbers of hydrogen atoms present in the internal standard  
177 structure (equals to 9) and in  $\text{CH}_2$  group of ethanol (equals to 2),  $n_{\text{IS}}$  is the number of moles of  
178 the internal standard (equals to  $5.78 \times 10^{-5}$  mole) and  $I_{\text{CH}_2}$  and  $I_{\text{IS}}$  are the integration of the  $\text{CH}_2$   
179 group of ethanol and the internal standard peaks.

180 To simplify, we replaced  $H_{\text{IS}}$ ,  $H_{\text{CH}_2}$  and  $n_{\text{IS}}$  by their values to obtain the following equation:

$$181 \quad n_{\text{EtOH (mole)}} = 0.00026 \times \frac{I_{\text{CH}_2}}{I_{\text{IS}}}$$

## 182 **2.6 Stability of free cyst HCl in solution, suspended cyst HCl-loaded liposomes**

### 183 **(original and freeze-dried)**

184 The stability of cyst HCl-loaded liposome was compared to that of free cyst HCl. The liposomal  
185 suspension (5 mL) was centrifuged, the supernatant was eliminated, and the pellet was re-  
186 suspended in water. The concentration of the encapsulated cyst HCl was determined after the  
187 destruction of liposomes by sonication. A solution of cyst HCl with the same concentration of  
188 the encapsulated cyst HCl was also prepared in water and stored at  $4^\circ\text{C}$ . After 6 months of  
189 storage at  $4^\circ\text{C}$ , the concentration of the remaining cyst HCl in free cyst HCl solution and the  
190 suspensions of liposomes loading cyst HCl was determined. Also, after three months of storage  
191 at  $4^\circ\text{C}$ , freeze-dried liposomes were re-suspended in 5 mL water and the remaining cyst HCl  
192 concentration was measured using ion-pair chromatography.

## 193 **2.7 Cell culture**

194 B16 murine melanoma cells (Elabscience, CliniSciences, Nanterre, France) were grown in  
195 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal  
196 bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained  
197 in an incubator at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere and passaged weekly using trypsin  
198 0.05%/EDTA 0.02% in PBS to maintain the optimum conditions for exponential growth.

## 199 **2.8 Preparation of the formulations**

200 Different concentrations of cyst HCl and cystamine (50; 60; 100 and 500 µM) were prepared  
201 in culture medium. Blank liposomes (original and re-suspended after freeze-drying) were  
202 prepared between 0.1 to 5.7% v/v in culture medium to evaluate their cytotoxic effect. The  
203 suspensions containing total cyst HCl-loaded liposome (encapsulated and non-encapsulated)  
204 were centrifuged and the pellet was re-suspended to obtain the encapsulated cyst HCl-loaded  
205 liposome. The total (present in the whole suspension) and the encapsulated cyst HCl-loaded  
206 liposome were diluted in culture medium to obtain 0.1 and 2.8 v/v%, respectively. The freeze  
207 dried cyst HCl-loaded liposomes were re-suspended in water and then diluted in culture  
208 medium to obtain 5.7 v/v%. These dilutions were made to obtain the same concentration of cyst  
209 HCl (60 µM) in all formulations. Kojic acid (700 µM) was prepared in water and used as  
210 positive control.

## 211 **2.9 Cytotoxicity analysis**

212 B16 melanoma cell viability was determined by the (3-(4, 5-dimethylthiazol-2-yl)-2, 5-  
213 diphenyltetrazolium bromide) MTT assay. Cells were seeded in 96-well microtiter plates at a  
214 density of  $6 \times 10^3$  cells/well. After 24 h of incubation at 37°C in 5% CO<sub>2</sub>, cells were treated with  
215 100 µL of each formulation described in section 2.8. After 24 and 48 h of incubation with the  
216 different formulations, the medium was removed, cells were washed twice and 100 µL of MTT  
217 solution (0.5 mg/mL in medium) were added to each well. Cells were incubated for 4 h at 37°C

218 and the produced formazan salts were then dissolved in DMSO. Absorbance was measured at  
219 571 nm by spectroscopy on a microplate reader (Thermo Scientific, Multiskan FC).

## 220 **2.10 Melanin measurement**

221 B16 cells were seeded at a density of  $10 \times 10^4$  cells/well in 6-well plates. Plates were incubated  
222 for 24 h at 37°C in 5% CO<sub>2</sub> before being treated with cyst HCl (60 μM), cystamine (60 μM),  
223 kojic acid (700 μM), blank liposomes (original suspension and re-suspended freeze-dried form),  
224 total cyst HCl-loaded liposome suspension, encapsulated cyst HCl-loaded liposome suspension  
225 and suspended freeze-dried cyst HCl-loaded liposome. After 24 and 48 h, cells were collected  
226 by scraping into ice-cold PBS and pelleted. Intracellular melanin was extracted by solubilizing  
227 cell pellets in 1N NaOH containing 10% DMSO for 2 h at 80°C. The melanin content of cell  
228 lysate supernatants was measured spectrophotometrically at an absorbance of 405 nm against a  
229 standard curve of synthetic melanin. Intracellular melanin content was adjusted by the amount  
230 of protein.

## 231 **2.11 Tyrosinase activity assay**

232 Intracellular tyrosinase activity was determined by measuring the rate of oxidation of L-DOPA  
233 to DOPACHrome. B16 cells were seeded at a density of  $10 \times 10^4$  cells/well in 6-well plates. After  
234 24 h, 2 mL of cyst HCl (60 μM), cystamine (60 μM), kojic acid (700 μM), blank liposomes  
235 (original suspension and re-suspended after freeze-drying), whole suspension of cyst HCl-  
236 loaded liposome, suspended cyst HCl-loaded liposome (encapsulated) and suspended freeze-  
237 dried cyst HCl-loaded liposome were added to cells. 48 and 72h post-treatment, cells were  
238 harvested by scraping into ice-cold PBS and lysed at room temperature for 15 min with 0.5%  
239 Triton X-100 in PBS. Lysed cell supernatants (100 μL) were mixed with 100 μL of freshly  
240 prepared L-DOPA solution (0.1% in PBS). Following incubation at 37°C for 1 h, absorbance  
241 was measured at 450 nm and compared with a standard curve using mushroom tyrosinase.

242 Tyrosinase activity was normalized to the amount of protein (determined using the Bradford  
243 assay).

#### 244 **2.12 Reactive oxygen species content**

245 Intracellular ROS production was detected using the oxidation-sensitive fluorescence dye 2,7-  
246 dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were seeded at a density of  $6 \times 10^3$   
247 cells/well in 96-well plates. After 24 h, they were firstly treated 2 times over a period of 72 h  
248 with 100  $\mu$ L of tested substances, and secondly treated with  $H_2O_2$  (100  $\mu$ M in culture medium)  
249 for 6 h. Then DCFH-DA solution (1  $\mu$ M in culture medium) was added to cells for 30 min at  
250 37°C. Immediately after PBS washing, fluorescence was quantified using a  
251 spectrofluorophotometer (BioTek, Synergy H1) with 495 nm excitation and 527 nm emission  
252 filters. ROS were observed under an inverted fluorescence microscope (Olympus, DP50).

#### 253 **2.13 *Ex vivo* skin penetration study with Franz cell**

254 *Ex vivo* percutaneous permeation of cyst HCl was performed on thawed skin from a female  
255 caucasian donor. Skin was defatted using a scalpel and then dermatomed with a dermatome  
256 (Zimmer Biomet Electric® Dermatome). Skin discs were realized using a circled cutter and the  
257 thickness range was between 400 and 600 microns. Skin discs were mounted on a Franz cells  
258 system (PermeGear). Franz cells were previously filled with receptor fluid (RF) consisting of  
259 PBS-EDTA 0.1% at pH 7.4. Skin discs were equilibrated during 1 h before measurement of  
260 TEWL (Trans Epidermal Water Loss) using a tewameter TM300 (MDD 4, Courage &  
261 Khazaka). The tested formulations were cyst HCl solution, total and encapsulated cyst HCl-  
262 loaded liposomes and resuspended freeze-dried cyst HCl-loaded liposomes. 1 mL of each  
263 formulation diluted to obtain 1200 $\mu$ M of cyst HCl as a final concentration has been deposited  
264 on the skin. RF was sampled at different time points 0, 1, 4, 18 and 24h. At 24 h, formulations  
265 were removed and skin discs were washed with RF (1 mL). Skin compartments were separated  
266 using forceps in order to obtain epidermis and dermis. Samples (Washing, Epidermis and

267 Dermis) were weighed and then the active ingredient was extracted in methanol:water  
268 extraction solvent (50:50, v/v) after stirring during 24 h. Finally, extracts were quantified by  
269 ion pair chromatography.

#### 270 **2.14 Statistical analysis**

271 The differences between the treated and the control cells were calculated using ANOVA  
272 (GraphPad Prism 9; GraphPad Software, San Diego, CA) with  $p < 0.05$  defining statistical  
273 significance.

## 274 **3 Results and discussion**

### 275 **3.1 Characterization of cyst HCl-loaded liposomes**

276 The sizes of the blank and cyst HCl-loaded liposomes were similar with  $211 \pm 8$  nm and  $217 \pm$   
277  $22$  nm for blank and loaded liposomes, respectively. The polydispersity index was  $0.2 \pm 0.1$  for  
278 the blank and cyst HCl-loaded liposomes indicating that the liposomal population was  
279 homogeneous. The zeta potential of the blank liposomes ( $-17.95 \pm 4.0$  mV) was increased after  
280 the addition of cyst HCl ( $-2.37 \pm 0.7$  mV). This result indicates the presence of an interaction  
281 between cyst HCl and the liposomal suspension. The EE% of the studied formulation was  $3.74$   
282  $\pm 0.46$  % which is lower than the EE% of cyst-loaded liposome ( $21 \pm 5.0\%$ ) found in our  
283 previous work using the base form of cyst (Atallah, Greige-Gerges, et al., 2020). Cyst (base  
284 form) and cyst HCl differ by their aqueous solubility, pH of their aqueous solution, and their  
285 stability. The base form may interact with negatively charged phosphate of phospholipids  
286 leading to a greater encapsulation when compared with cyst HCl form.

287 The remaining percentage of ethanol in the liposomal suspension depended on the time of  
288 evaporation and the remaining volume of liposomal suspension. In fact, the percentage of the  
289 ethanol in the liposomal suspension increased with the increase of the final volume of liposomal  
290 suspension. The percentage of ethanol in all the studied conditions was less than 2.6% (**Table**  
291 **S1**).

### 292 **3.2 Stability of cyst HCl-loaded liposomes**

293 The stability of cyst HCl-loaded liposomes and free cyst HCl prepared in water was assessed  
294 after storage at 4°C. The total degradation of free cyst HCl was obtained after 16 days, whereas  
295 a remaining cyst HCl percentage of 9.5% was obtained in the cyst HCl-loaded liposomes in  
296 suspension form after 6 months of storage at 4°C. The total concentration of cyst HCl in the  
297 resuspended freeze dried liposomes was determined after 3 months of storage at 4°C showing  
298 that the concentration of cyst HCl determined at time 0 was stable after 3 months of storage

299 **(Figure 2)**. The stability of cysteamine in its hydrochloride form was much better than in its  
300 base form. For the same concentration of the free drug (200 µg/mL), cysteamine in its base  
301 form was totally converted to cystamine after 15h of storage at 4°C (Atallah, Greige-Gerges, et  
302 al., 2020) while cyst HCl was totally converted after 16 days of storage at 4°C. The  
303 encapsulation of cyst HCl increased its stability in suspension form (total degradation after 6  
304 months of storage) and for a longer time when the formulation was freeze-dried (no degradation  
305 after 3 months of storage). The base form of cyst (200 µg/mL) was totally oxidized after 60 h  
306 in the liposomal suspension and 4 months in the freeze-dried form (Atallah, Greige-Gerges, et  
307 al., 2020).

### 308 **3.3 Cytotoxicity of blank liposomes (original suspension and suspended freeze-dried** 309 **form)**

310 The cytotoxicity of the blank liposomes in suspension and the suspended freeze-dried form was  
311 evaluated by MTT assay on B16 murine melanoma cells after 24 and 48 h of incubation.  
312 Following the ISO 10993-5 guideline, percentages of cell viability above 80% are considered  
313 as non-cytotoxic. Blank liposomes in suspension form did not show any cytotoxic effect at the  
314 various volume concentrations (0.1; 1; 2; 2.8 and 5% v/v) (**Table S2**). 5% of H<sub>2</sub>O/ethanol  
315 solution decreased the cell viability to 83 ± 0.1%. Suspended freeze-dried blank liposomes  
316 showed a higher cytotoxicity than the liposomal suspension after 24 h of incubation. For 5%  
317 (v/v), the cytotoxicity of blank liposomal suspension was 90 ± 0.1% while it was 75 ± 0.1% for  
318 the suspended freeze-dried form (**Table S2**). The cytotoxicity of the liposomes decreased after  
319 48 h. The cytotoxicity observed is probably due to the presence of the HP-β-CD (used as a  
320 cryoprotectant) in the freeze-dried vesicles (Hammoud et al., 2019).

### 321 **3.4 Cytotoxicity of free cyst HCl and cystamine**

322 The cytotoxicity of free cyst HCl and cystamine was for the first time evaluated on B16  
323 melanoma cell line. Free cyst HCl and cystamine did not show any cytotoxicity between 50 and



324 100  $\mu\text{M}$  (cell viability > 92%). However, cystamine showed a higher cytotoxicity than cyst HCl  
325 at 500  $\mu\text{M}$ ; cell viability was decreased to  $59 \pm 0.1\%$  and  $26 \pm 0.1\%$  after 24h of 500  $\mu\text{M}$  cyst  
326 HCl and cystamine treatments, respectively (**Figure 3A**). Similarly, Qiu et al. showed that  
327 cystamine (50 to 100  $\mu\text{M}$ ) did not present any cytotoxic effect on MM418c5, MM96L and HeLa  
328 cells after 6 days of treatment (Qiu et al., 2000). According to these results, cyst HCl at a  
329 concentration of 60  $\mu\text{M}$  was chosen for the next analysis.

### 330 **3.5 Cytotoxicity of cyst HCl-loaded liposomes**

331 The cytotoxicity of the total and encapsulated cyst HCl loaded liposomes was also evaluated  
332 on B16 melanoma cells. Cell viability of the total and encapsulated cyst HCl-loaded liposomes  
333 was  $92 \pm 0.1\%$  and  $95 \pm 0.1\%$ , respectively, after 24 h of incubation. However, the suspended  
334 freeze-dried form was more cytotoxic ( $74 \pm 0.1\%$ ) than the liposomal suspension (**Figure 3B**).

### 335 **3.6 Inhibition of melanin synthesis**

336 To study the effect of cyst HCl loaded liposomes on melanogenesis, we examined the level of  
337 melanin produced in B16 cells after 2 treatments during 72 h of incubation with free cyst HCl  
338 and cystamine (culture media as control), whole liposome suspension encapsulating cyst HCl  
339 and cyst HCl-loaded liposome (original liposome suspension as control), and resuspended  
340 freeze-dried cyst HCl-loaded liposome (resuspended freeze-dried liposome as control). Kojic  
341 acid was used as a positive control.

342 For the same concentration of cyst HCl and cystamine (60  $\mu\text{M}$ ), cystamine was able to reduce  
343 the melanin content in the cells ( $33.0 \pm 13.0\%$ ) more than cyst HCl ( $26.0 \pm 8.6\%$ ). Kojic acid  
344 used as a reference presented a melanin inhibition of  $36.0 \pm 9.1\%$ . Suspended freeze-dried blank  
345 liposome did not show any inhibition of melanin (data not shown). The inhibition of melanin  
346 was lower for the encapsulated ( $22.0 \pm 0.9\%$ ) form compared to the free form ( $26.0 \pm 8.6\%$ ).  
347 The suspended freeze-dried cyst HCl-loaded liposome ( $23.5 \pm 6.3\%$ ) showed a better inhibition  
348 of melanin than the original suspension (**Figure 4**).

### 349 **3.7 Inhibition of tyrosinase activity**

350 The tyrosinase enzyme plays a crucial role in melanogenesis. Kojic acid (used as positive  
351 control) showed a strong inhibition of the enzyme activity ( $36.2 \pm 5.8\%$ ). Cystamine ( $28.2 \pm$   
352  $12.2\%$ ) and cyst HCl ( $26.8 \pm 5.5\%$ ) inhibited the tyrosinase activity. Therefore, melanogenesis  
353 inhibition by cyst HCl and cystamine in B16 cells occurs via direct inhibition of tyrosinase  
354 activity, as previously described by Qiu et al. The effect of the cyst HCl encapsulated in  
355 liposomes on the inhibition of the tyrosinase activity was investigated. The encapsulation of  
356 cyst HCl decreased its inhibition of tyrosinase activity effect ( $26.8 \pm 5.5\%$  was inhibited in  
357 presence of free cyst HCl while  $19.6 \pm 4.4\%$  and  $18.3 \pm 2.9\%$  was inhibited for the encapsulated  
358 cyst HCl loaded liposome and resuspended freeze-dried cyst HCl-loaded, respectively) (**Figure**  
359 **4**).

360 To the best of our knowledge, one study investigated the depigmenting effect of cyst *in vitro*  
361 using pigmented melanoma MM418c5 cells. It showed that  $100 \mu\text{M}$  of cyst inhibit  $21.0\%$  of  
362 melanin and  $87.0\%$  of tyrosinase while  $50 \mu\text{M}$  of cystamine inhibit  $5.0\%$  of melanin and  $89.0\%$   
363 of tyrosinase (Qiu et al., 2000).

364 **Figure 5** shows the microscopic observation of the cells after 2 treatments during 72 h of  
365 incubation with free, total and encapsulated cyst HCl-loaded liposome in suspension and  
366 resuspended freeze-dried forms. It also shows the solubilized cell pellets of the different treated  
367 cells. A whitening effect was observed for all the formulations in comparison with the control.

### 368 **3.8 Reactive oxygen species content**

369 Intracellular oxidative stress was measured by the DCFH-DA assay. The cleavage of two  
370 acetate groups of DCFH-DA by esterase will lead to the formation of DCFH that can be  
371 oxidized to a highly fluorescent compound DCF by intracellular ROS. Therefore, the  
372 fluorescence intensity of DCF can directly reflect the level of ROS in the cell (Rhee et al.,

2010). Hydrogen peroxide ( $H_2O_2$ ) is a well-known inducer of oxidative stress. To estimate the effect of cyst HCl and its encapsulation on the intracellular production of ROS in cultured B16 cells, cells were pretreated 2 times over a period of 72 h with 60  $\mu$ M of cyst HCl and cystamine; total and encapsulated cyst HCl-loaded liposome in suspension and resuspended freeze-dried forms, and then treated for 6 h with  $H_2O_2$ . The inhibited percentage of ROS formation was 43.4 $\pm$ 10.3% and 38.6 $\pm$ 9.8% in the presence of free cyst HCl and cystamine, respectively. This percentage was slightly decreased after the encapsulation of cyst HCl where it was 35.4 $\pm$ 10.1% and 33.6 $\pm$ 11.6% for the encapsulated cyst HCl in suspension and resuspended freeze dried forms (**Figure 6**). After, the addition of 60  $\mu$ M cyst or cystamine to the B16 cells, a decrease in ROS levels by a factor of 1.8 and 1.6, respectively, in comparison with the control was observed. The addition of the encapsulated cyst HCl in suspension and resuspended freeze dried forms also decreased the relative fluorescence by 1.4 and 1.5 times in comparison with the control. **Figure 7** shows fluorescence microscopy images of B16 cells. A decrease of the DCFH-DA fluorescence in all cultures treated with the formulations containing cyst HCl was observed in comparison to the control.

### 3.9 Skin penetration study

The cumulative amounts of cyst HCl penetrating the skin and found in the receptor fluid from each of the liposomal formulation and controls over 24 h are shown in **Figure 8**. The encapsulation of cyst HCl in liposomes increased its penetration through the human skin. The quantity of cyst HCl found in the fluid receptor after 24 h of deposition was increased by 2.9-; 3.5- and 3.5-fold for the total, encapsulated and lyophilized cyst HCl loaded liposomes, respectively in comparison to the free drug in solution (**Figure 8**). Cystamine did not penetrate the skin since it was not found in the receptor fluid nor in the epidermis layer.

**Figure 9** shows the retention of cyst HCl in the epidermis when the drug was free or encapsulated in liposomes in suspension or in resuspended freeze-dried forms. The highest

398 quantity of retained cyst HCl in the epidermis was found for the cyst HCl-loaded liposome in  
399 the resuspended freeze-dried form ( $150 \mu\text{g}/\text{cm}^2$ ). The presence of HP- $\beta$ -CD could be the reason  
400 behind the increase of cyst penetration in the resuspended freeze-dried form. The cyclodextrin  
401 is known to be a penetration enhancer of the drug through the membranes (Loftsson &  
402 Sigurdardóttir, 1996; Yan et al., 2014).  $\alpha$ -Cyclodextrin demonstrated its ability to promote the  
403 trans corneal diffusion of cyst (Pescina et al., 2016). A slight decrease of the retention of the  
404 molecule in the epidermis was observed for cyst HCl encapsulated in liposome ( $120.0 \mu\text{g}/\text{cm}^2$ )  
405 in comparison with the free drug ( $140.0 \mu\text{g}/\text{cm}^2$ ) while a higher decrease was obtained for the  
406 total cyst HCl loaded liposome ( $85.0 \mu\text{g}/\text{cm}^2$ ). No trace of cystamine was found in the  
407 epidermis.

#### 408 **4 Discussion**

409 Liposomes represent the most studied system for drug delivery through the skin. These delivery  
410 systems are used to enhance stability, skin penetration, depigmenting effect and antioxidant  
411 activity of several compounds. For example, vitamin A demonstrated better stability when  
412 loaded into liposomes compared to the free form during exposure to UV radiation (Arsić &  
413 Vuleta, 1999). The encapsulation of curcumin in liposomes provided substantially more  
414 protection than the encapsulation in CDs (Matloob et al., 2014). Amphotericin B liposome  
415 formulations showed a higher stability (shelf life of 1 year) compared to the free drug (4 and  
416 14 days) (Manosroi et al., 2004). The chemical stability of fasudil, an anti-vasospastic agent,  
417 was also enhanced after its encapsulation (Ishida et al., 2002). In this article, the encapsulation  
418 of cyst HCl in liposomes enhanced its stability; the total degradation of cyst HCl was obtained  
419 after 16 days in aqueous medium, and lasted for 6 months for the liposomal suspension.  
420 Moreover, cyst HCl loaded liposomes were stable during freeze-drying, and stability was  
421 maintained after 3 months of storage.

422 In addition, the cream containing liposome-encapsulated 4-n-butylresorcinol and resveratrol  
423 was shown to be effective and safe for the treatment of melasma. (Kwon et al., 2020). Aloe  
424 Vera loaded into liposomes was proposed for melasma treatment and as an effective skin care  
425 formulation especially for anti-aging and skin regeneration (Ghafarzadeh & Eatemadi, 2017).  
426 In this study, the encapsulation of cyst HCl in liposomes showed a decrease of the depigmenting  
427 effect that could be due to the entrapment of the drug in the liposomes aqueous compartment.

428 Hydroxytyrosol liposomes presented better DPPH radical scavenging activity than free  
429 hydroxytyrosol, which could be due to the fact that hydroxytyrosol was encapsulated inside the  
430 liposomes with a high encapsulation rate (Yuan et al., 2017). Stronger free radical scavenging  
431 effect was obtained in *Orthosiphon stamineus* liposomes than in nonformulated extract (Yücel  
432 & Şeker Karatoprak, 2017). No variation of the antioxidant activity was observed for the  
433 hydrophilic *Crithmum maritimum* after its encapsulation in liposomes (Alemán et al., 2019).  
434 Also, our results are in accordance with Aleman et al. where the antioxidant activity was slightly  
435 decreased after encapsulation in liposomes.

436 Liposomes also showed an increase of skin penetration of entrapped and non-entrapped  
437 hydrophilic substances into human skin. The penetration study of the liposomal formulation  
438 containing encapsulated and non-encapsulated carboxyfluorescein exhibited maximum  
439 deposition of this compound in the stratum corneum, whereas the liposomes containing just the  
440 encapsulated carboxyfluorescein inside the liposomes showed a higher penetration into deeper  
441 skin layers, such as the viable epidermis, and through the skin to the receiver compartment of  
442 Franz diffusion cell (Verma et al., 2003). Another study showed that maximum idebenone  
443 penetration in dermis was achieved by liposomes compared to leciplex and invasomes (Shah et  
444 al., 2015). The skin penetration of itraconazole (ITZ) was studied using an aqueous solution of  
445 ITZ, CD/ITZ inclusion complex, ITZ loaded liposomes and the CD/ITZ inclusion complex  
446 loaded into liposomes. The complex improved slightly the drug penetration while ITZ-HP- $\beta$ -

447 CD-loaded liposomes exhibited significant increase in ITZ skin deposition compared to  
448 liposomes containing ITZ alone (Alomrani et al., 2014). Our results are in accordance with the  
449 literature as an increase of the skin penetration was observed with cyst HCl encapsulation  
450 especially the freeze dried form containing HP- $\beta$ -CD.

451 In this study, phospholipon 90H (PH90H) and cholesterol were chosen to prepare liposomes  
452 encapsulating cyst HCl. Previous studies used PH90H liposomes to enhance the skin  
453 penetration of drugs of various pharmacological classes, like methotrexate, idoxuridine and  
454 acyclovir. It was shown that their dermal penetration was improved (Ita et al., 2010). Tarnowska  
455 et al. also used PH90H liposomes to study the skin absorption of  $Mg^{2+}$  and  $Ca^{2+}$  from thermal  
456 spring water, and showed that the suspension improved the skin absorption of  $Ca^{2+}$  (Tarnowska  
457 et al., 2020). Beside, a mixture of PH90H and cholesterol was used for preparation of liposomes  
458 encapsulating terbinafine HCl by the ethanol injection method. The cutaneous application of  
459 this formulation prolonged the antifungal activity (Beeravelli et al., 2014). Similarly, a  
460 liposomal formulation based on PH90H and cholesterol encapsulating nadifloxacin showed a  
461 better penetration in deeper layers of skin, when compared with the free drug (Shiny et al.,  
462 2016). In addition, saturated PH90H are in a gel like state on the skin, due to their melting  
463 temperature  $T_m$  around  $50^\circ C$  (Dörfler & Brezesinski, 1983). A study successfully confirmed  
464 that hydrogenated phospholipids penetrate into the stratum corneum, a high amount of the  
465 lipid was found in the upper skin layers and a gradual decrease was noticed in the deeper layers  
466 showing an accumulation of saturated phosphatidylcholine in upper epidermal layers  
467 (Gutekunst et al., 2018).

468 Liposomes can be applied on the skin under different forms such as gel or cream. Topical gels  
469 are commonly prescribed for skin diseases. The use of topical gels is convenient and could  
470 provide a prolonged skin retention of the drug, which is beneficial for the treatment of skin  
471 disease. Several studies used the liposomal gel for skin applications. For example, Wang et al.

472 used liposomal gel dual-loaded with all-trans retinoic acid and betamethasone for enhanced  
473 therapeutic efficiency of psoriasis (Wang et al., 2020). The dermal delivery of nadifloxacin was  
474 enhanced using liposomal gel (Shiny et al., 2016). In addition, liposomal gels were developed  
475 for the topical delivery of anthralin (Fathalla et al., 2020).

## 476 **5 Conclusion**

477 The encapsulation of cyst HCl increased the stability of the molecule especially when the  
478 formulation was freeze-dried. The formulation was not cytotoxic with the chosen  
479 concentrations. Cyst HCl and cystamine showed an inhibition of melanin and tyrosinase activity  
480 but cystamine was not able to penetrate the skin layers. The encapsulation of cyst HCl decreased  
481 its inhibition of melanin and tyrosinase activity but increased its penetration in the skin and its  
482 retention in the epidermis. The encapsulation didn't affect the antioxidant activity observed for  
483 the free cyst HCl. These findings are important for further application of cyst HCl liposomal  
484 formulations as a depigmenting agent. In this study, the results of depigmenting and anti-  
485 oxidant activities are based on vitro assays, precisely 2D cultures of B16. The *B16 cell* line is a  
486 widely used *model* for skin whitening and pigmentation studies. But potentially 3D models  
487 where cells are embedded in complex environments offer a better model of the *in vivo* cell  
488 environment than traditional 2D culture.

## 489 **Acknowledgements**

490 Authors thank the Research Funding Program at the Lebanese University and the “Agence  
491 Universitaire de la Francophonie, projet PCSI” for supporting the project (2018-2020). Authors  
492 also thank the Lyon 1 University NMR facility for setting up the NMR experiments.

## 493 **Declaration of Competing Interest**

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



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661 **Figure legends**

662 **Figure 1:** The structure of the different forms of cyst.

663 **Figure 2:** Stability study at 4°C of free cyst HCl, suspended cyst HCl-loaded liposome, and  
664 freeze dried form of cyst HCl-loaded liposome suspended in water the day of HPLC analysis.

665 **Figure 3:** Cell viability of B16 melanoma cells in presence of different concentrations of cyst  
666 HCl and cystamine (A) and in presence of free, total and encapsulated cyst HCl-loaded  
667 liposome in suspension and resuspended freeze-dried form (B). Data are presented as means ±  
668 SD (n = 6).

669 **Figure 4:** Inhibition percentages of melanin (A) and tyrosinase (B) for cyst HCl, cystamine,  
670 kojic acid, total and encapsulated cyst HCl-loaded liposome in suspension and resuspended  
671 freeze-dried forms after 2 treatments in 72 h incubation time. Data are presented as means ±  
672 SD (n = 6). Statistical analysis was performed using ANOVA test; ns: not significant, \*p < 0.05,  
673 \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

674 **Figure 5:** Microscopic observations of cells after 72 h of incubation with the different  
675 formulations (2 treatments) and representative image of the different solubilized pellets  
676 obtained ((1)Total cyst HCl loaded liposome (2) Encapsulated cyst HCl loaded liposome (3)  
677 Resuspended freeze-dried cyst HCl loaded liposome (4) Cyst HCl (5) Cystamine (6) Kojic acid)

678 **Figure 6:** Intracellular ROS formation measured by relative DCFH-DA fluorescence in  
679 cultured B16 pretreated by H<sub>2</sub>O<sub>2</sub> and treated with 60 μM of cyst HCl and cystamine; total and  
680 encapsulated cyst HCl-loaded liposome in suspension and resuspended freeze-dried forms. The  
681 formation of ROS was assayed by measuring the fluorescence of DCFH-DA. Data are presented  
682 as means ± SD (n = 6). Statistical analysis was performed using ANOVA test; \*\*\*p < 0.001,  
683 \*\*\*\*p < 0.0001.

684 **Figure 7:** Fluorescence microscopy images of B16 cells pretreated with H<sub>2</sub>O<sub>2</sub> and treated for  
685 72 h with free and encapsulated cyst HCl and cystamine.

686 **Figure 8:** Skin permeation profile of free and cyst HCl loaded liposome in suspension and  
687 resuspended freeze-dried forms.

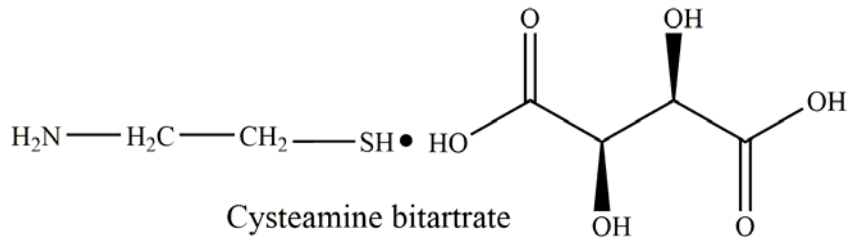
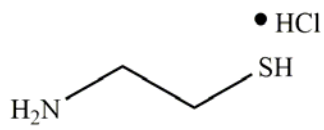
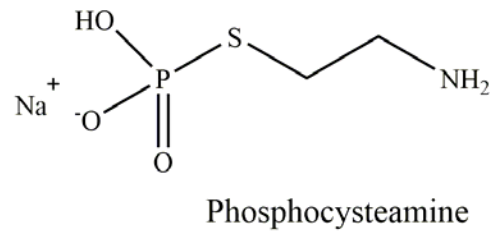
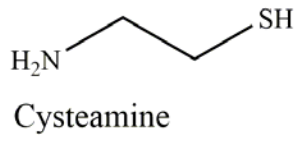
688 **Figure 9:** Cyst HCl retention in the epidermis for the free, total and encapsulated cyst HCl-  
689 loaded liposome in suspension and resuspended freeze-dried forms.

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691 Figure 1

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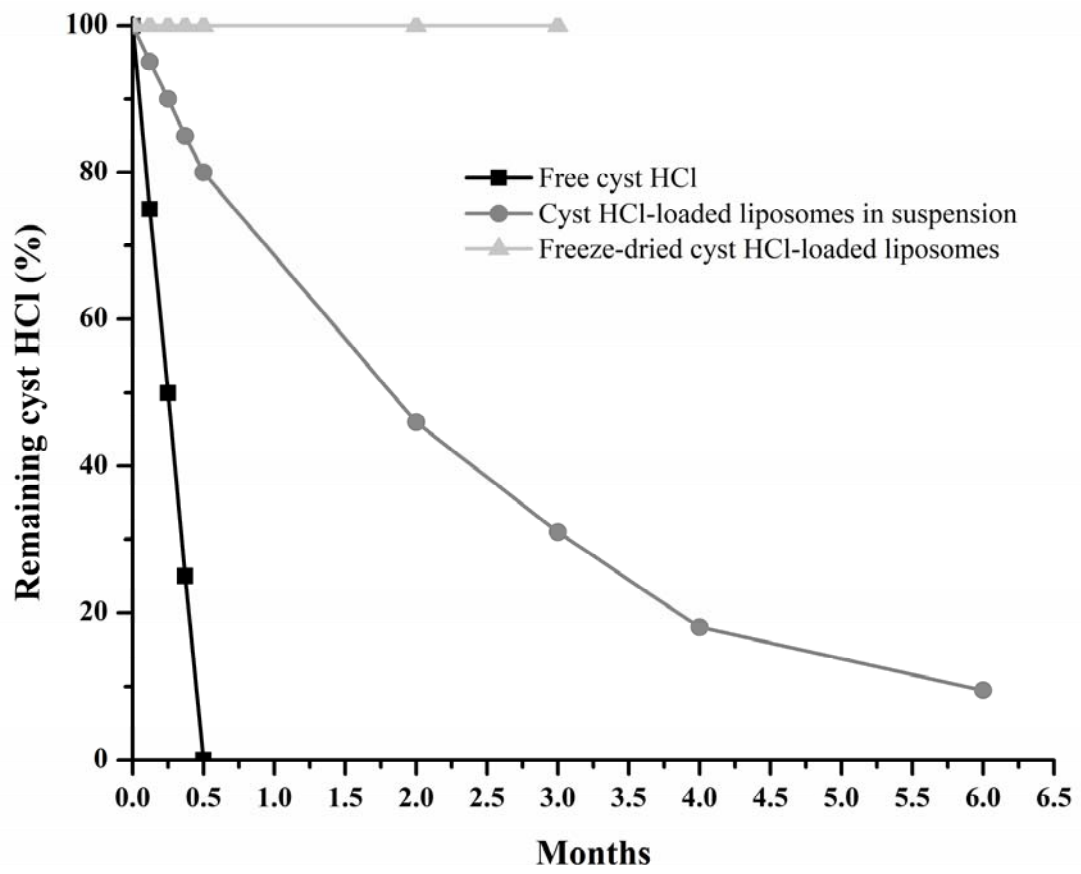
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697 Figure 2

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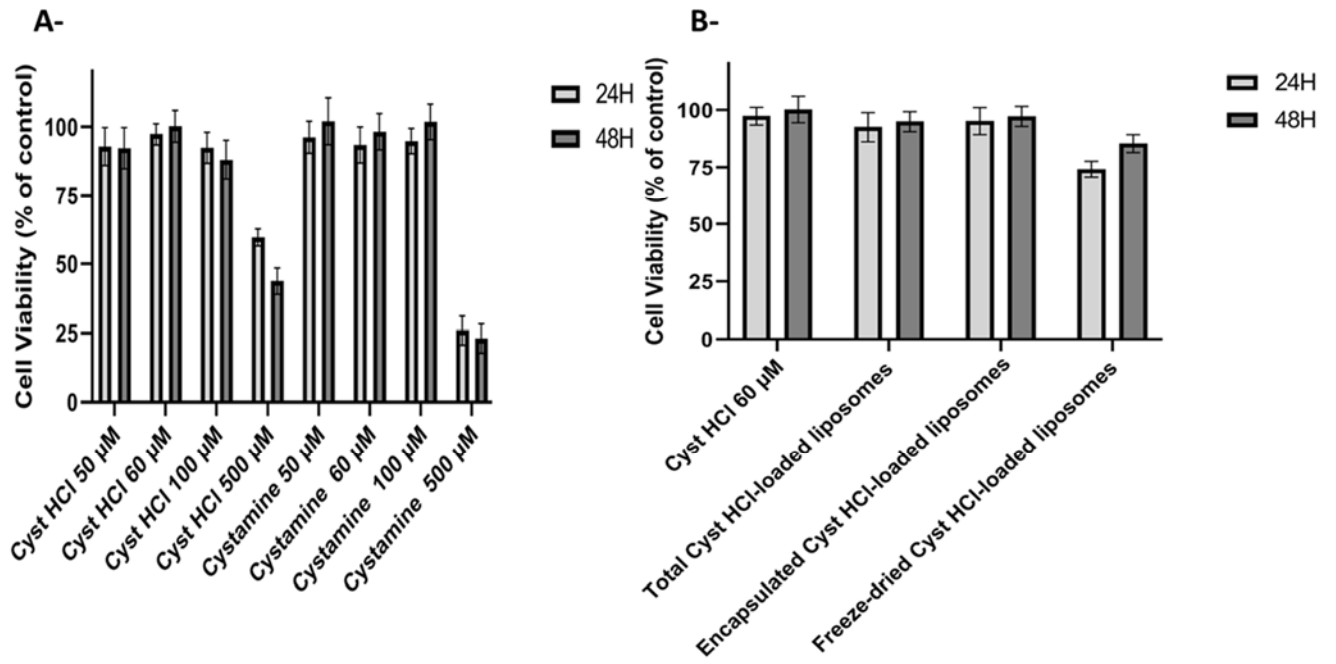
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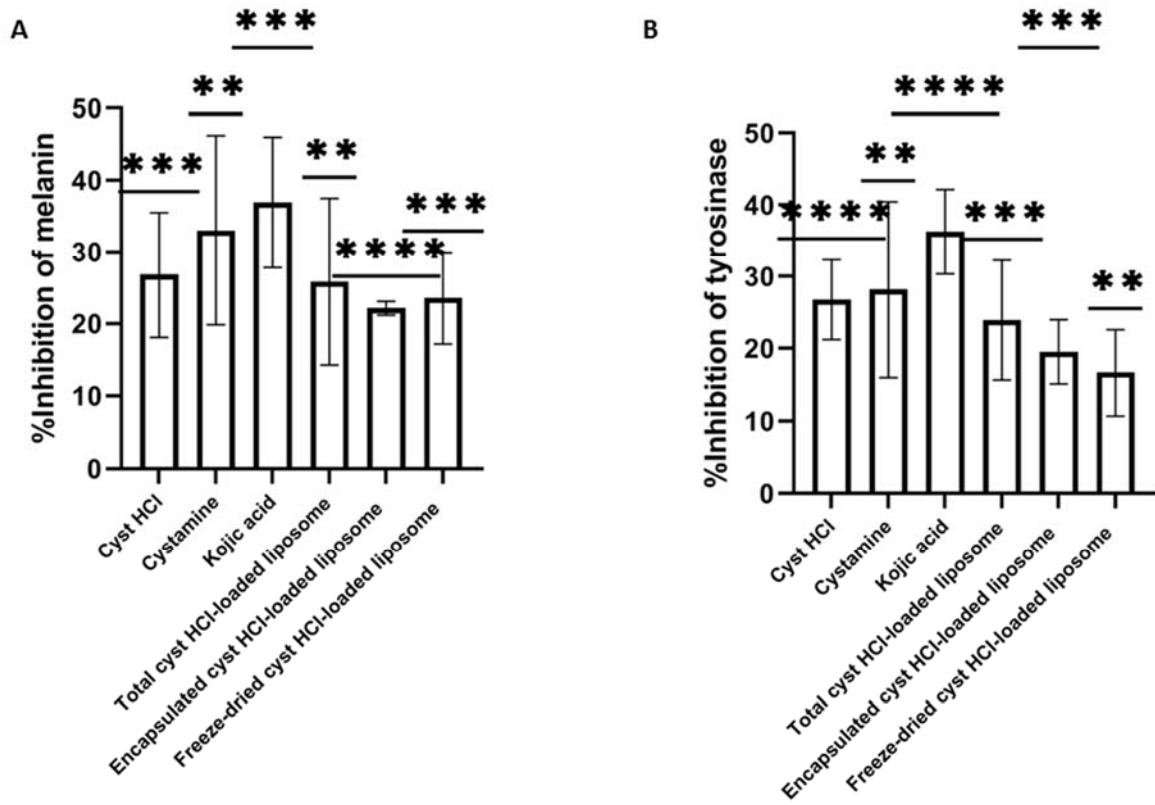
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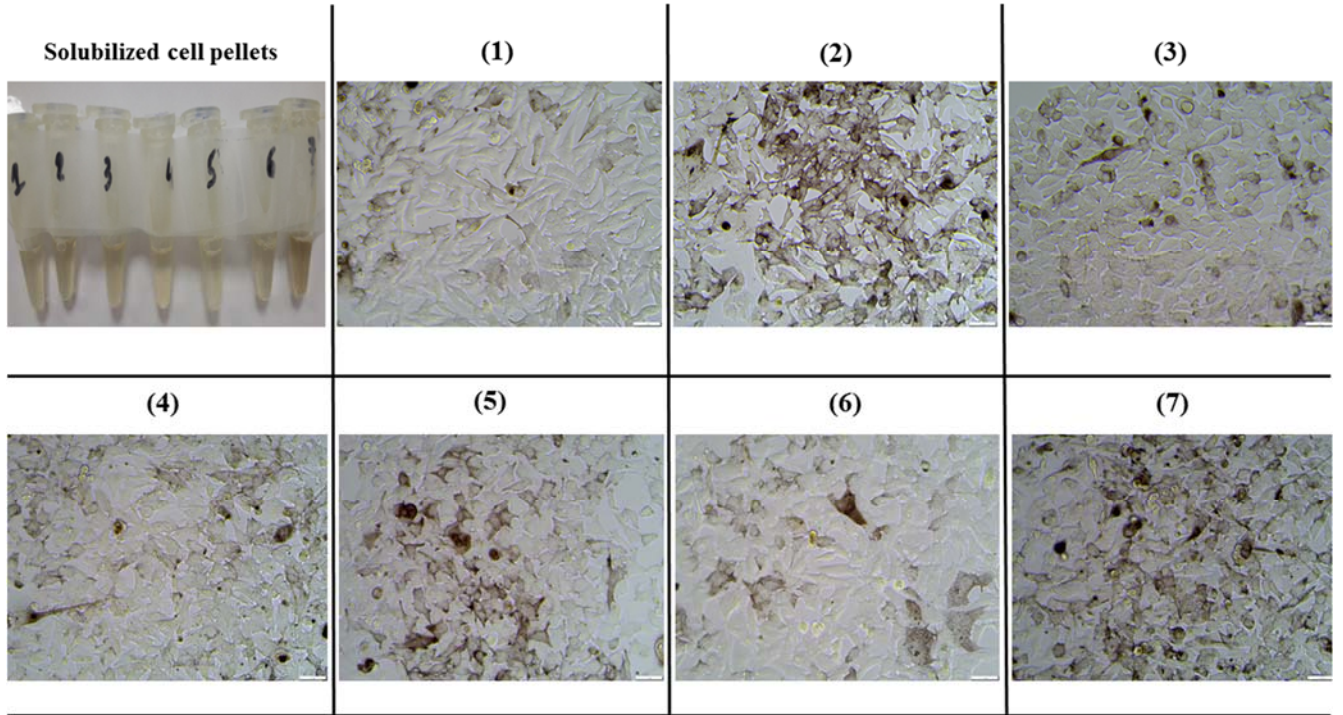
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714 Figure 5

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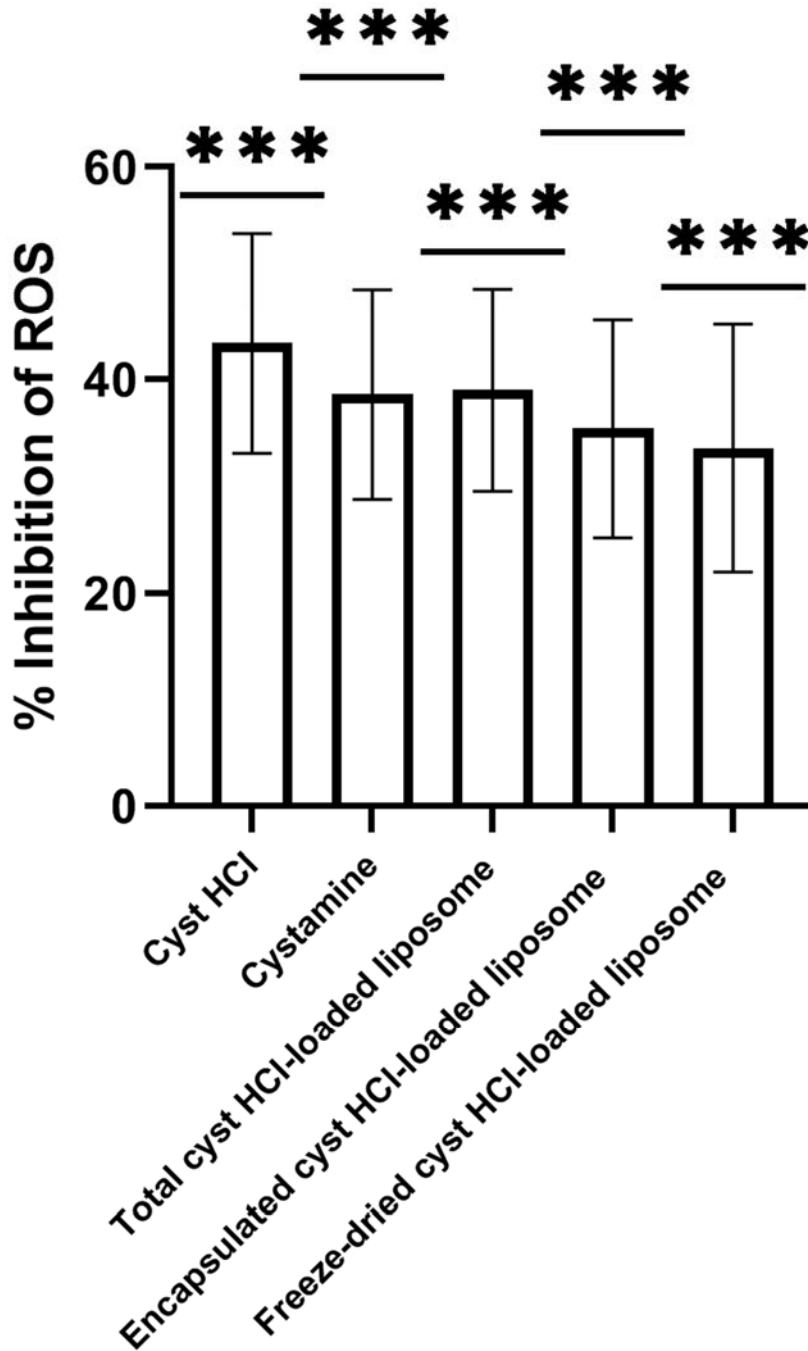
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721 Figure 6

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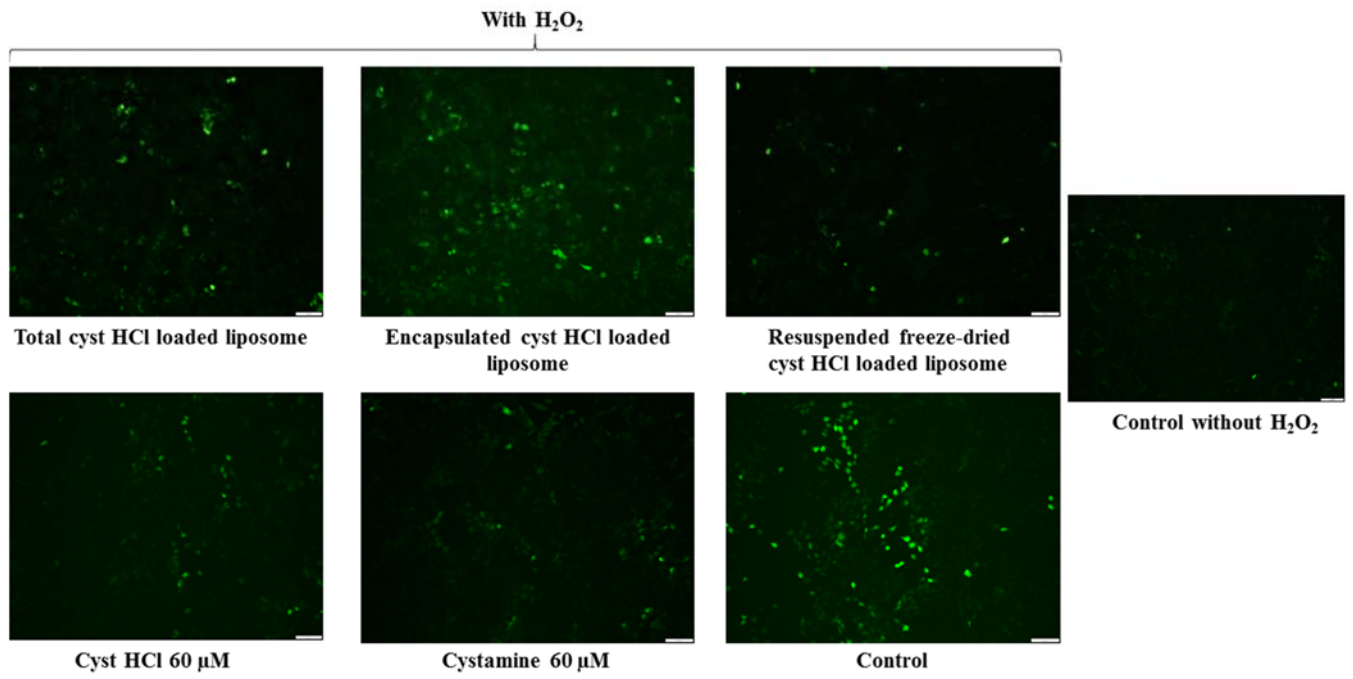
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726 Figure 7

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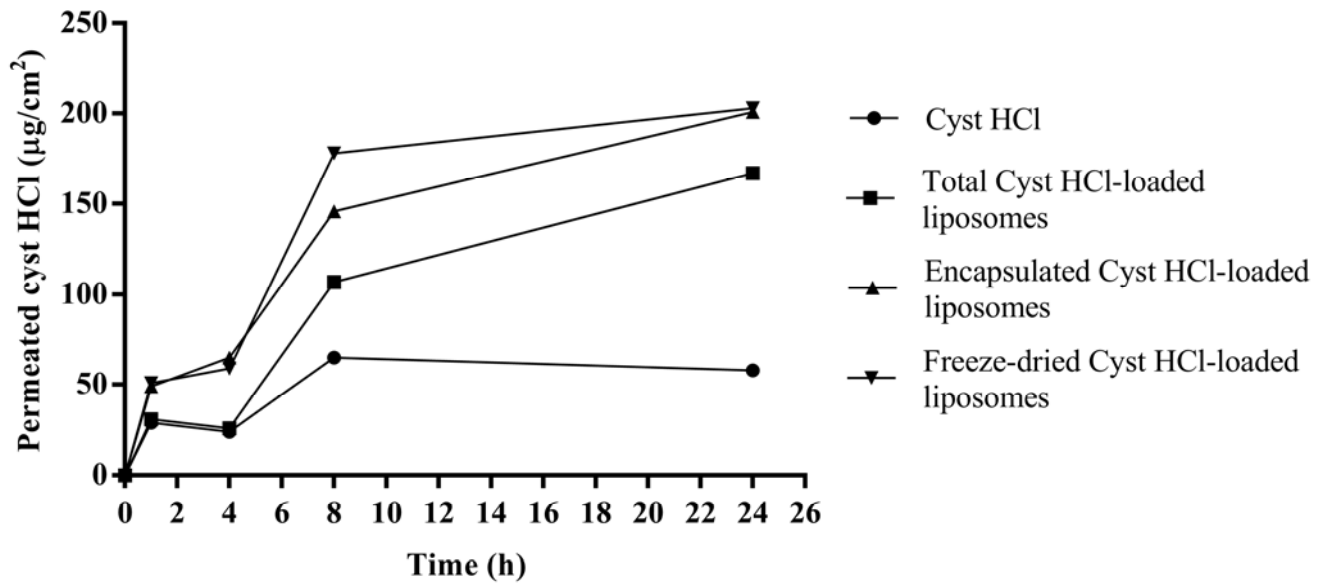
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731 Figure 8

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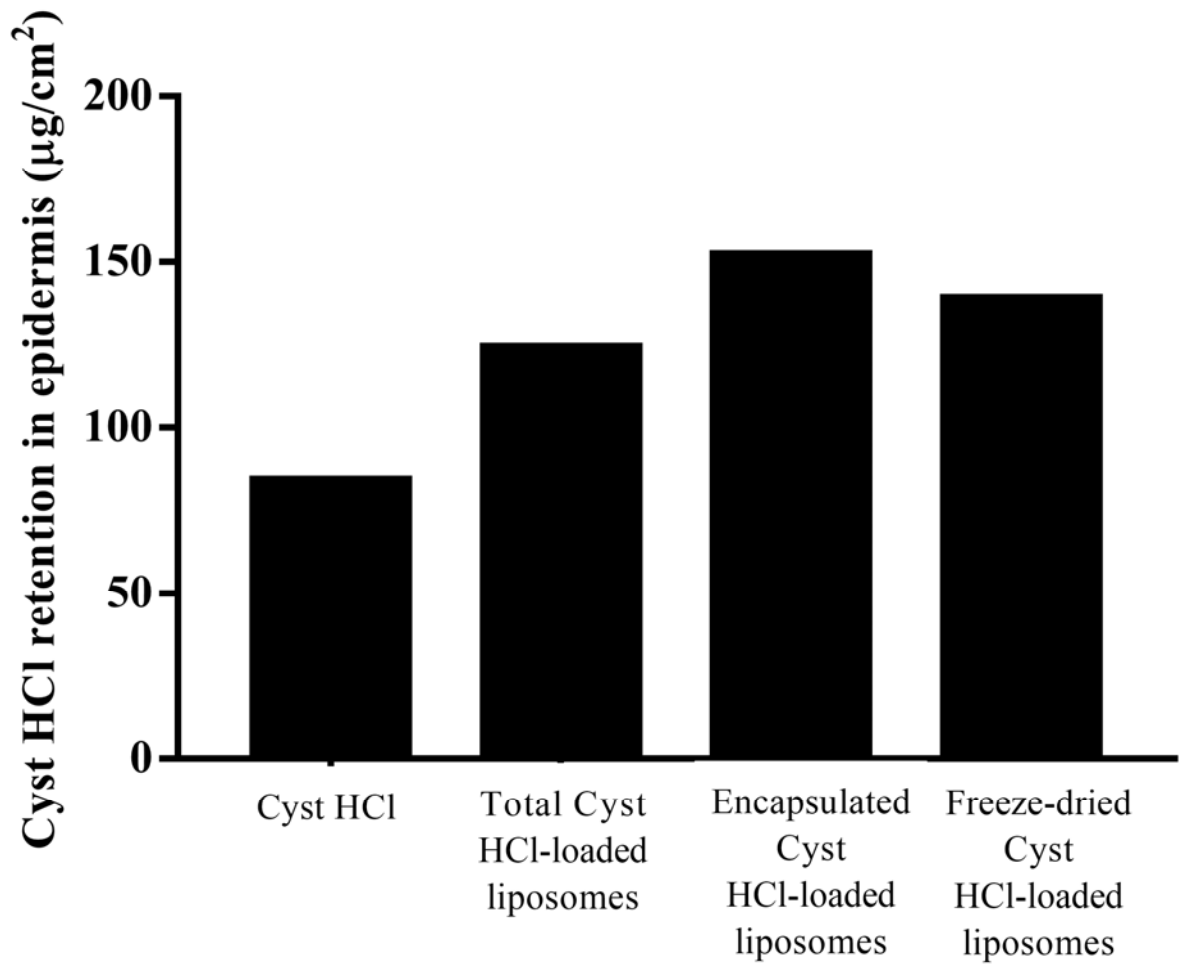
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737 Figure 9

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