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Abstract

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

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

1 Introduction

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

64 2017). Moreover, cystamine decreased ROS levels evoked by H_2O_2 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_2O_2 in B16 cells.
95 Finally, the skin penetration of free- and cyst HCl-loaded liposomes was compared using the
96 Franz diffusion cell.

2 Materials and Methods

2.1 Materials

Cyst HCl, cystamine dihydrochloride, metformin hydrochloride, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), DMSO, L-DOPA and NaOH were purchased from Sigma-Aldrich (Buchs, Switzerland). Phospholipon 90H (90% soybean PC, 4% lysoPC, 2% triglycerides, 2% water, and 0.5% ethanol) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was obtained from the Fisher chemical (Loughborough, UK). HP-β-CD-oral grade (MS=0.85) was purchased from Roquette (Lestrem, France). Sodium dodecyl phosphate and phosphoric acid were obtained from Sigma-Aldrich (China). (trimethylsilyl)-2,2,3,3-tetradeutepropropionic acid sodium salt, 2,7-dichlorodihydrofluorescein diacetate and H_2O_2 were purchased form Sigma-Aldrich (USA). Deuterium oxide (D_2O) was purchased from Acros organics (USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Dominique Dutscher (France). All other chemicals were of analytical grade.

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

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

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

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

2.3 Cyst HCl-loaded liposomes characterization

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

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

The encapsulation efficiency (EE %) of cyst HCl was calculated using the formula:

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

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

2.4 Cyst HCl and cystamine quantification

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

2.5 Ethanol quantification in the blank liposomal suspension

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

were centrifuged for 10 min at 11200g. The residual ethanol is supposed to be present in the upper phase. The internal standard used was (trimethylsilyl)-2,2,3,3-tetradeutepropropionic acid sodium salt (TSP) $(CH_3)_3SiCD_2CD_2CO_2Na$. 10 mg of TSP was dissolved in 400 µL D_2O and 300 µL of the supernatant of the liposomal suspension. The number of moles of ethanol is calculated from the NMR spectrum using the following equation:

$$n_{EtOH} = \frac{H_{IS}}{H_{CH2}} \times n_{IS} \times \frac{I_{CH2}}{I_{IS}}$$

Where H_{IS} and H_{CH2} are the numbers of hydrogen atoms present in the internal standard structure (equals to 9) and in CH_2 group of ethanol (equals to 2), n_{IS} is the number of moles of the internal standard (equals to 5.78×10^{-5} mole) and I_{CH2} and I_{IS} are the integration of the CH_2 group of ethanol and the internal standard peaks.

To simplify, we replaced H_{IS}, H_{CH2} and n_{IS} by their values to obtain the following equation:

$$n_{EtOH\ (mole)} = 0.00026 \times \frac{I_{CH2}}{I_{IS}}$$

2.6 Stability of free cyst HCl in solution, suspended cyst HCl-loaded liposomes (original and freeze-dried)

The stability of cyst HCl-loaded liposome was compared to that of free cyst HCl. The liposomal suspension (5 mL) was centrifuged, the supernatant was eliminated, and the pellet was re-suspended in water. The concentration of the encapsulated cyst HCl was determined after the destruction of liposomes by sonication. A solution of cyst HCl with the same concentration of the encapsulated cyst HCl was also prepared in water and stored at 4°C. After 6 months of storage at 4 °C, the concentration of the remaining cyst HCl in free cyst HCl solution and the suspensions of liposomes loading cyst HCl was determined. Also, after three months of storage at 4 °C, freeze-dried liposomes were re-suspended in 5 mL water and the remaining cyst HCl concentration was measured using ion-pair chromatography.

2.7 Cell culture

B16 murine melanoma cells (Elabscience, CliniSciences, Nanterre, France) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in an incubator at 37 °C in a humidified 5% CO_2 atmosphere and passaged weekly using trypsin 0.05%/EDTA 0.02% in PBS to maintain the optimum conditions for exponential growth.

2.8 Preparation of the formulations

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

2.9 Cytotoxicity analysis

B16 melanoma cell viability was determined by the (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay. Cells were seeded in 96-well microtiter plates at a density of $6x10^3$ cells/well. After 24 h of incubation at 37°C in 5% CO_2, cells were treated with 100 µL of each formulation described in section 2.8. After 24 and 48 h of incubation with the different formulations, the medium was removed, cells were washed twice and 100 µL of MTT solution (0.5 mg/mL in medium) were added to each well. Cells were incubated for 4 h at 37°C

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

2.10 Melanin measurement

B16 cells were seeded at a density of $10x10^4$ cells/well in 6-well plates. Plates were incubated for 24 h at 37°C in 5% CO_2 before being treated with cyst HCl (60 µM), cystamine (60 µM), kojic acid (700 µM), blank liposomes (original suspension and re-suspended freeze-dried form), total cyst HCl-loaded liposome suspension, encapsulated cyst HCl-loaded liposome suspension and suspended freeze-dried cyst HCl-loaded liposome. After 24 and 48 h, cells were collected by scraping into ice-cold PBS and pelleted. Intracellular melanin was extracted by solubilizing cell pellets in 1N NaOH containing 10% DMSO for 2 h at 80°C. The melanin content of cell lysate supernatants was measured spectrophotometrically at an absorbance of 405 nm against a standard curve of synthetic melanin. Intracellular melanin content was adjusted by the amount of protein.

2.11 Tyrosinase activity assay

Intracellular tyrosinase activity was determined by measuring the rate of oxidation of L-DOPA to DOPAchrome. B16 cells were seeded at a density of $10x10^4$ cells/well in 6-well plates. After 24 h, 2 mL of cyst HCl (60 µM), cystamine (60 µM), kojic acid (700 µM), blank liposomes (original suspension and re-suspended after freeze-drying), whole suspension of cyst HCl-loaded liposome, suspended cyst HCl-loaded liposome (encapsulated) and suspended freeze-dried cyst HCl-loaded liposome were added to cells. 48 and 72h post-treatment, cells were harvested by scraping into ice-cold PBS and lysed at room temperature for 15 min with 0.5% Triton X-100 in PBS. Lysed cell supernatants (100 µL) were mixed with 100 µL of freshly prepared L-DOPA solution (0.1% in PBS). Following incubation at 37°C for 1 h, absorbance was measured at 450 nm and compared with a standard curve using mushroom tyrosinase.

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

2.12 Reactive oxygen species content

Intracellular ROS production was detected using the oxidation-sensitive fluorescence dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were seeded at a density of $6x10^3$ cells/well in 96-well plates. After 24 h, they were firstly treated 2 times over a period of 72 h with 100 µL of tested substances, and secondly treated with H_2O_2 (100 µM in culture medium) for 6 h. Then DCFH-DA solution (1 µM in culture medium) was added to cells for 30 min at 37°C. Immediately after PBS washing, fluorescence was quantified using a spectrofluorophotometer (BioTek, Synergy H1) with 495 nm excitation and 527 nm emission filters. ROS were observed under an inverted fluorescence microscope (Olympus, DP50).

2.13 *Ex vivo* skin penetration study with Franz cell

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

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

2.14 Statistical analysis

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

3 Results and discussion

3.1 Characterization of cyst HCl-loaded liposomes

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

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

3.2 Stability of cyst HCl-loaded liposomes

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

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

3.3 Cytotoxicity of blank liposomes (original suspension and suspended freeze-dried form)

The cytotoxicity of the blank liposomes in suspension and the suspended freeze-dried form was evaluated by MTT assay on B16 murine melanoma cells after 24 and 48 h of incubation. Following the ISO 10993-5 guideline, percentages of cell viability above 80% are considered as non-cytotoxic. Blank liposomes in suspension form did not show any cytotoxic effect at the various volume concentrations (0.1; 1; 2; 2.8 and 5% v/v) (**Table S2)**. 5% of H_2O/ethanol solution decreased the cell viability to 83 ± 0.1%. Suspended freeze-dried blank liposomes showed a higher cytotoxicity than the liposomal suspension after 24 h of incubation. For 5% (v/v), the cytotoxicity of blank liposomal suspension was 90 ± 0.1% while it was 75 ± 0.1% for the suspended freeze-dried form **(Table S2)**. The cytotoxicity of the liposomes decreased after 48 h. The cytotoxicity observed is probably due to the presence of the HP-β-CD (used as a cryoprotectant) in the freeze-dried vesicles (Hammoud et al., 2019).

3.4 Cytotoxicity of free cyst HCl and cystamine

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

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

3.5 Cytotoxicity of cyst HCl-loaded liposomes

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

3.6 Inhibition of melanin synthesis

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

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

3.7 Inhibition of tyrosinase activity

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

To the best of our knowledge, one study investigated the depigmenting effect of cyst *in vitro* using pigmented melanoma MM418c5 cells. It showed that 100 µM of cyst inhibit 21.0% of melanin and 87.0% of tyrosinase while 50 µM of cystamine inhibit 5.0% of melanin and 89.0% of tyrosinase (Qiu et al., 2000).

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

3.8 Reactive oxygen species content

Intracellular oxidative stress was measured by the DCFH-DA assay. The cleavage of two acetate groups of DCFH-DA by esterase will lead to the formation of DCFH that can be oxidized to a highly fluorescent compound DCF by intracellular ROS. Therefore, the 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 μM 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±10.3% and 38.6±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±10.1% and 33.6±11.6% for the encapsulated cyst HCl in suspension and resuspended freeze dried forms **(Figure 6)**. After, the addition of 60 μM 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 resupended 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

quantity of retained cyst HCl in the epidermis was found for the cyst HCl-loaded liposome in the resuspended freeze-dried form (150 μg/cm^2). The presence of HP-β-CD could be the reason behind the increase of cyst penetration in the resuspended freeze-dried form. The cyclodextrin is known to be a penetration enhancer of the drug through the membranes (Loftsson & Sigurdardóttir, 1996; Yan et al., 2014). α-Cyclodextrin demonstrated its ability to promote the trans corneal diffusion of cyst (Pescina et al., 2016). A slight decrease of the retention of the molecule in the epidermis was observed for cyst HCl encapsulated in liposome (120.0 μg/cm^2) in comparison with the free drug (140.0 μg/cm^2) while a higher decrease was obtained for the total cyst HCl loaded liposome (85.0 μg/cm^2). No trace of cystamine was found in the epidermis.

4 Discussion

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

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

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

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

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-β-CD.

451 In this study, phopsholipon 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 Tm around 50°C (Dörfler & Brezesinski, 1983). A study successfully confirmed
464 that hydrogenated phospholipids penetrate into the strateum 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.

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

5 Conclusion

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

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal 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_2O_2 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_2O_2 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.

690

691 Figure 1

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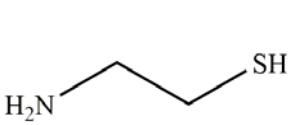
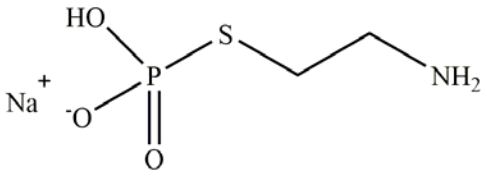
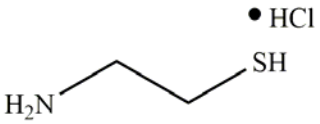


Cysteamine



Phosphocysteamine



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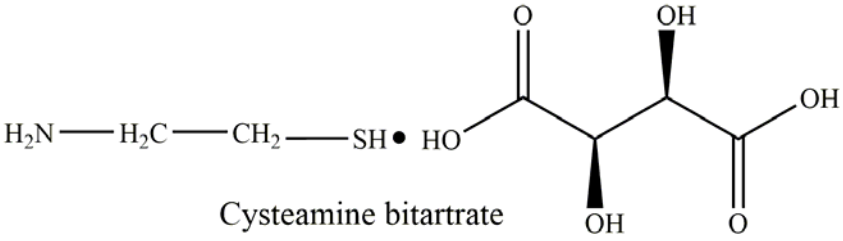

Cysteamine bitartrate

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697 Figure 2

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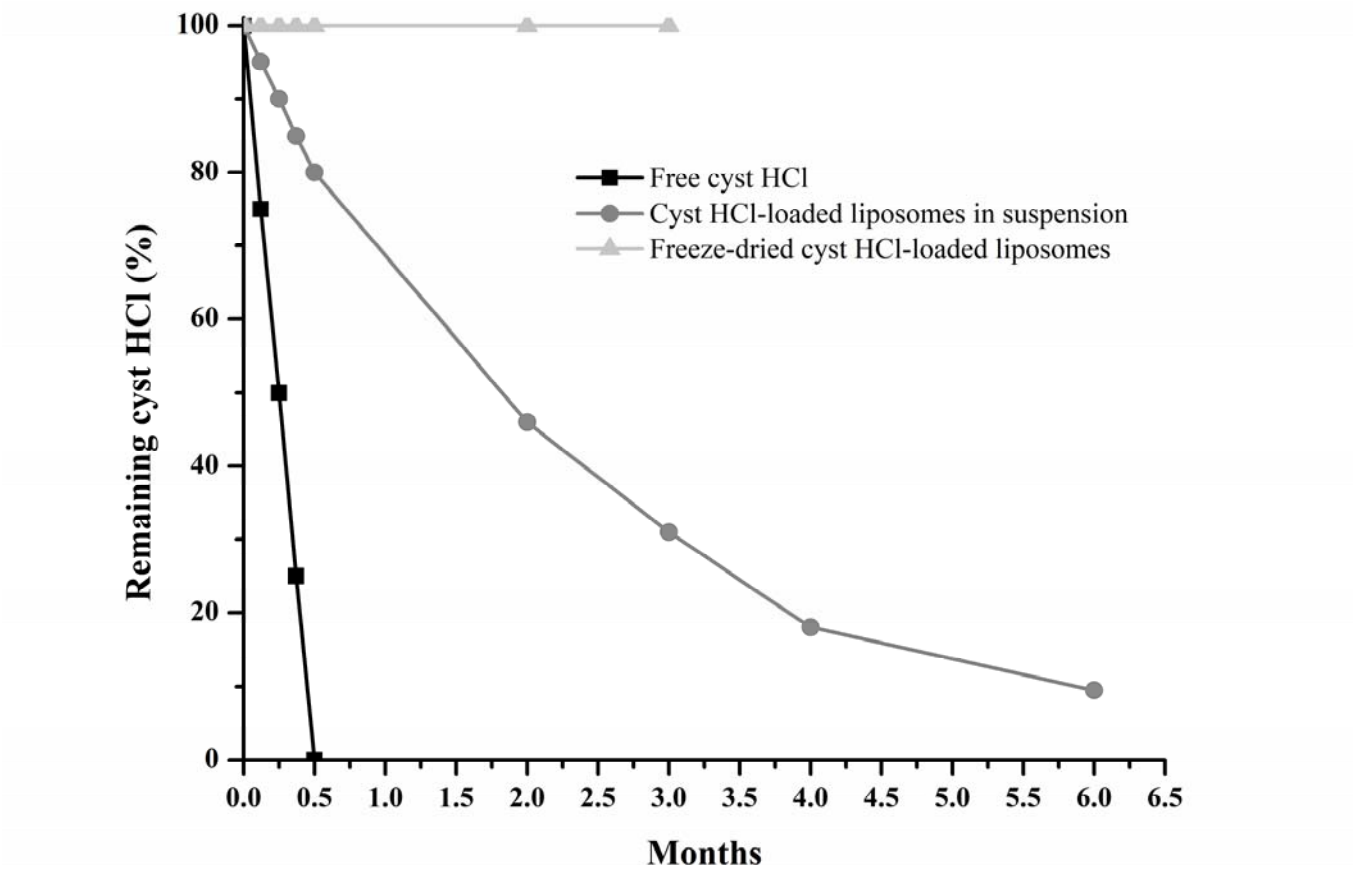

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703 Figure 3

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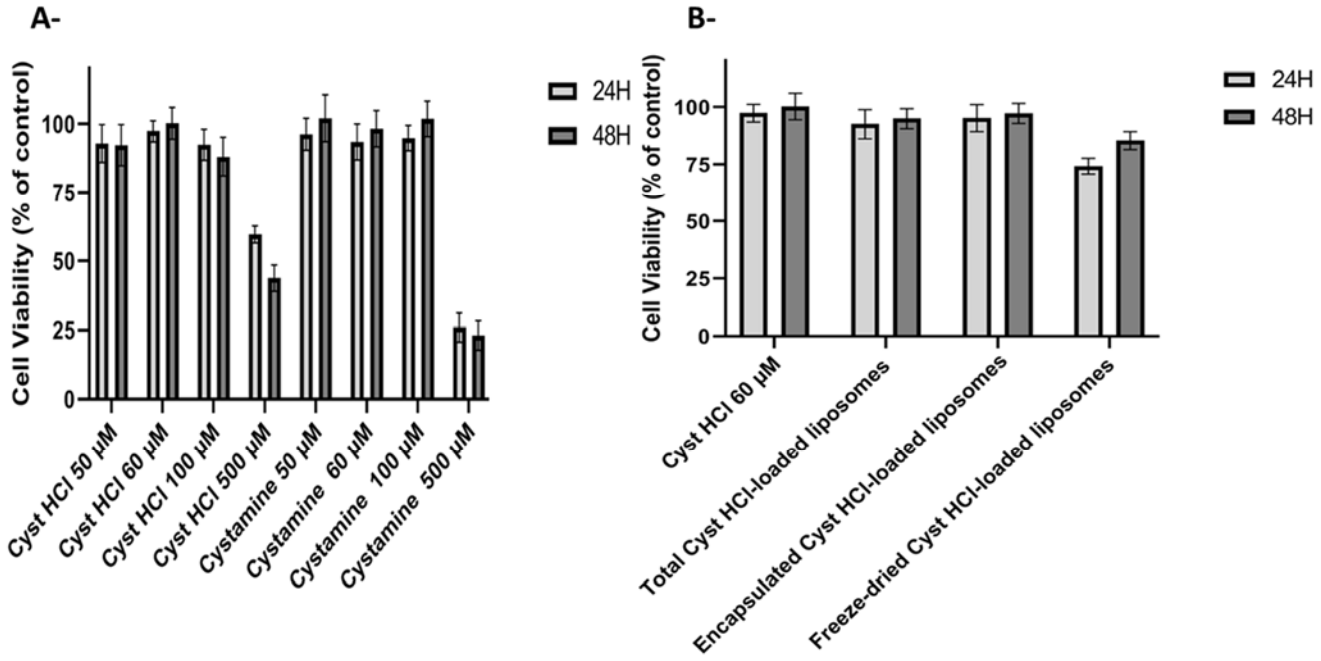

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709 Figure 4

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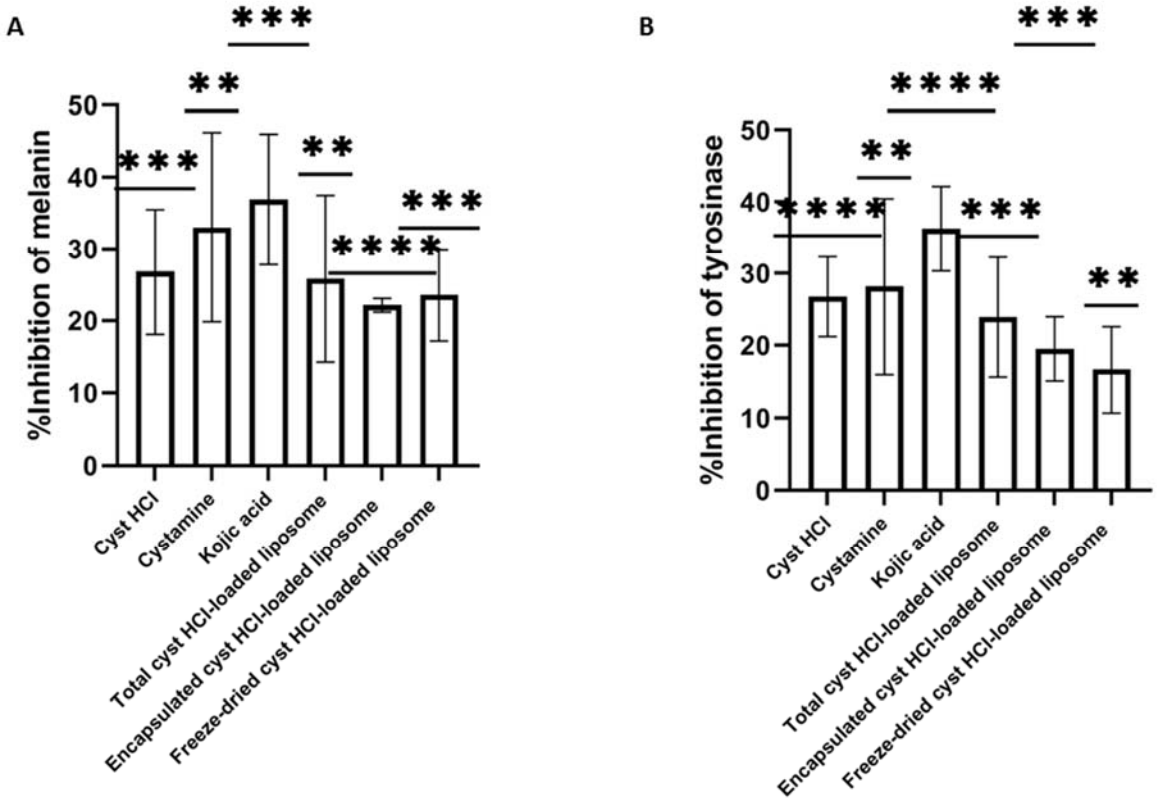

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714 Figure 5

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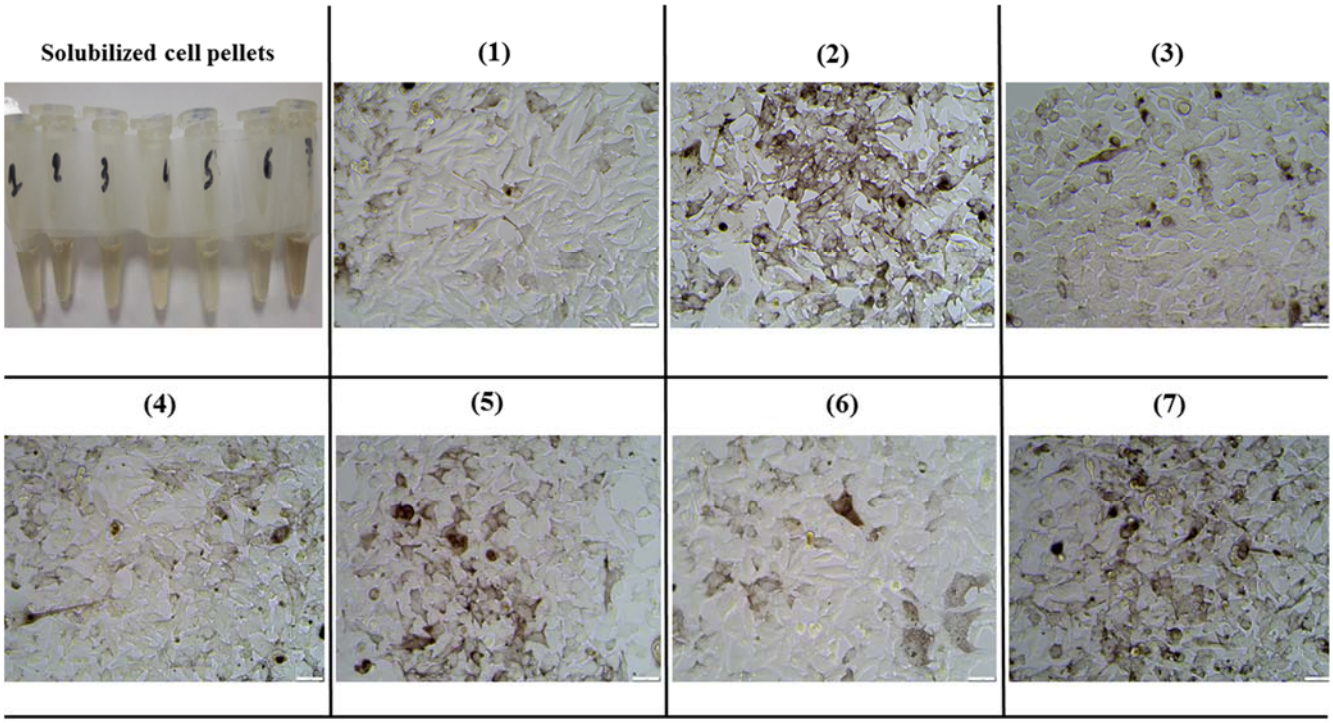

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721 Figure 6

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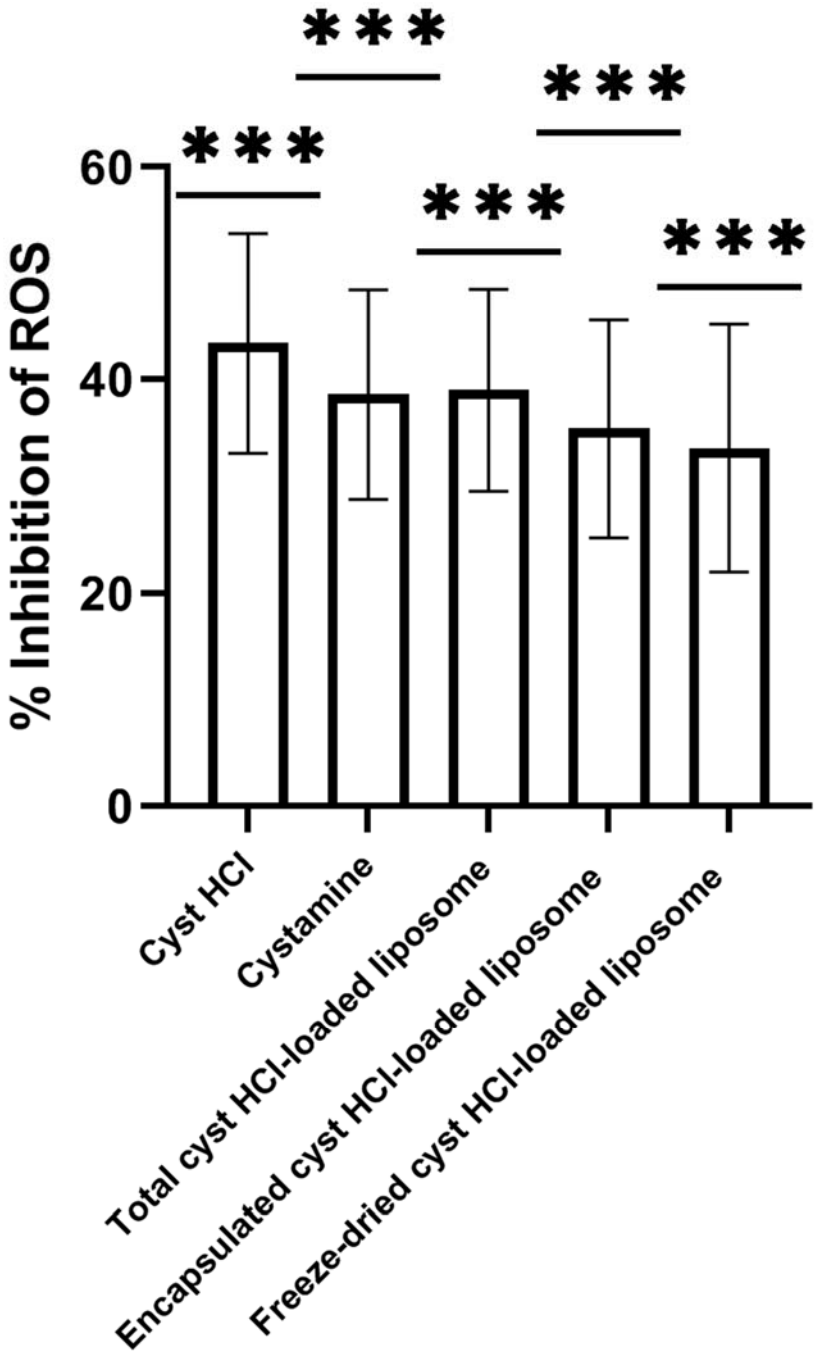

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726 Figure 7

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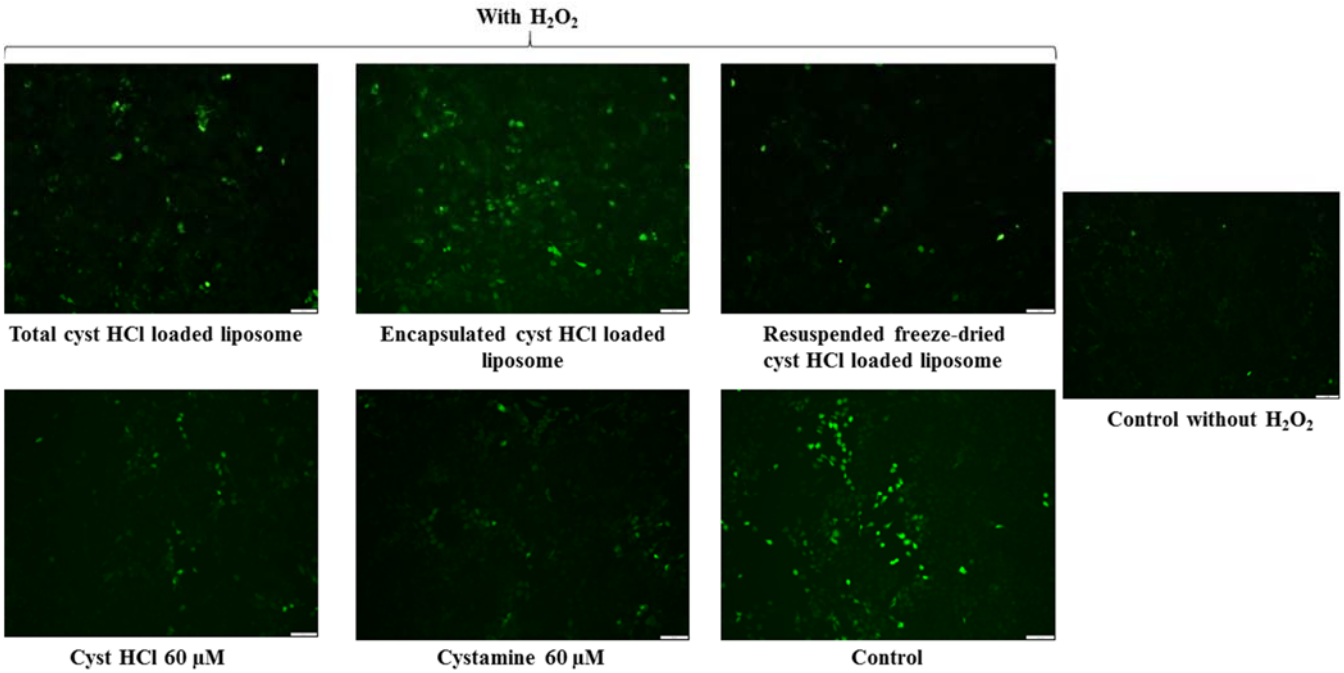

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731 Figure 8

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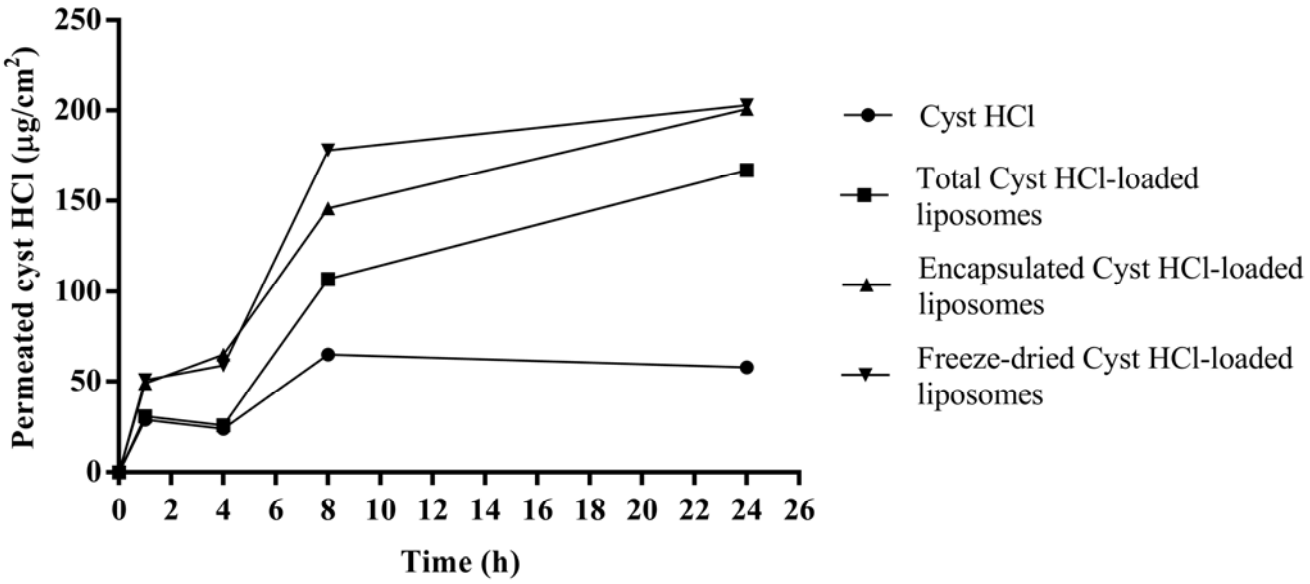

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737 Figure 9

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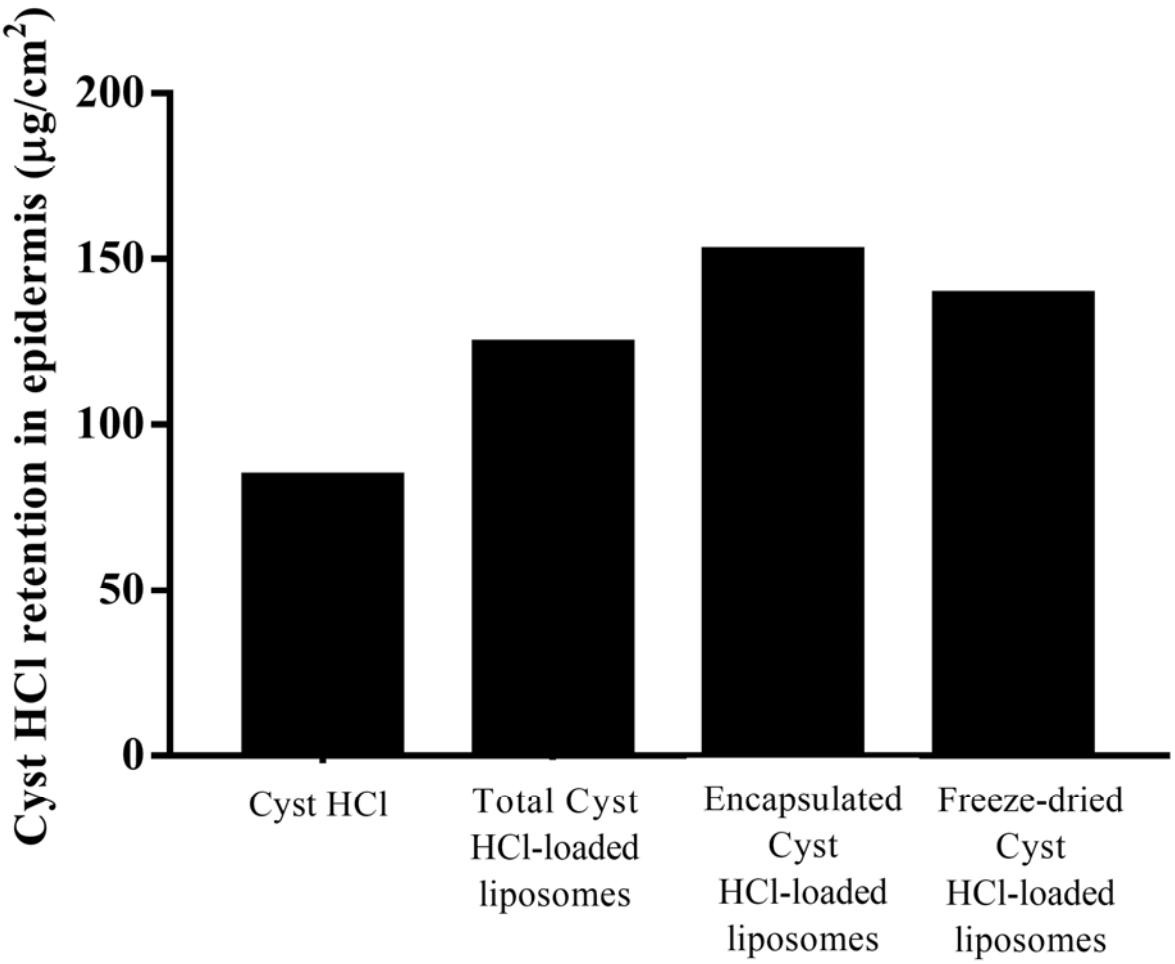

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