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Original Article

PREPARATION AND CHARACTERIZATION OF LIPOSOMAL DELIVERY SYSTEM OF NATURAL HEME PROTEIN

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ABSTRACT

Objective: The objective of the present study was to develop and optimize the methods for preparation and characterization of the liposomal delivery system of natural heme protein.

Methods: Cytochrome C containing liposomes (Cyt-LS) were prepared by high-pressure homogenization technique using phosphatidylcholine (PC) and dipalmitoyl phosphatidylglycerol (DPPG). Nanoparticles were characterized by using: dynamic light scattering, zeta potential measurements, scanning electron microscopy and HPLC. The specific activity was studied *in vitro.*

Results: The study of homogenization regimes for obtaining unilamellar Cyt-LS was carried out. The selected temperature regime of homogenization was kept within 38–44 °C with optimal homogenization pressure of 800 bar. The obtained Cyt-LS were characterized by the main physicochemical parameters showed: Encapsulation efficiency 95.8±2.0%, Zeta potential-57±1.0 mV, pH-6.95±0.05. Phospholipid impurities had the following content: lysophosphatidylcholine-0.60±0.05% and free fatty acids-0.4±0.05%. The average particle diameter was 156±2 nm. Also, the size of Cyt-LS particles was confirmed by the ability of emulsion subjected to the sterilizing filtration with the preservation of its main physicochemical properties. Cyt-LS exhibit specific activity, similar to non-liposomal Cyt-C solution.

Conclusion: The formulation of the liposomal delivery system of heme protein was successfully prepared using natural components and evaluated for different parameters.

Keywords: Liposome, Heme protein, Cytochrome C, Phospholipids, Oxidative stress, Antioxidant, Homogenization

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INTRODUCTION

One of the known natural heme protein involved in the respiratory electron transport chain and cell apoptosis is cytochrome C (Cyt-C). Cyt-C is also involved in the removal of superoxide (O_2^-) and hydrogen peroxide H_2O_2 from mitochondria, acting as an antioxidant enzyme. [1-3] (fig. 1)

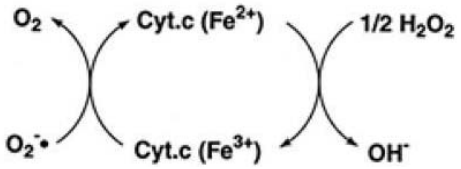


Fig. 1: Removal of O_2^- and H_2O_2 by cytochrome c

The ability to capture and neutralize reactive oxygen species (ROS) defines some properties of Cyt-C as a medical preparation for the prevention and treatment of the oxidant-induced injury caused by ROS formation under oxidative stress which often accompanies the processes of tissue hypoxia. There are the following Cyt-C containing drugs on the world pharmaceutical market used for the treatment of cardiological and ophthalmologic diseases, e. g. in preparations like "Oftan catachrom" (Santen), "Cytochrome C Eye Drops" (Samson-med), "Cytochrome C for injection" (Pharmstandard biolik) "Ractovit" (Ibn Sina),"Vitafol" (Popular Pharmaceuticals).

However, the water-soluble Cyt C form has a number of significant drawbacks: Cyt C insignificantly penetrates cells through biological membranes and is rapidly removed from parts of the body damaged by hypoxia, which causes its extremely low bioavailability after administration. At the same time, under hypoxia, the structural damage leads to the simultaneous loss of Cyt-C and phospholipids in the mitochondrial respiratory chain [4].

Liposomes (LS) are of special importance among modern drug delivery systems. Phospholipids derived from natural sources of plant or animal origin (e. g. soybeans or chicken eggs) are often used as the main component of the liposomal membrane. LS derived from phospholipids of natural origin have a number of undoubted advantages: they are nonimmunogenic, low-toxic and under certain conditions can connect with the phospholipids of cell membranes and deliver their contents inside the cells [5, 6].

LS can act as a delivery system of small molecules [7, 8] as well as various macromolecules with a molecular mass of more than 10 kDa including various heme proteins (hemoglobin, cytochromes) [9-12]. The earlier *in vivo* studies of Cyt-LS confirm the greater efficacy of using Cyt-LS in comparison with Cyt-C non-liposomal form after instillation of eye and parenteral administration [12-15]. Thus, the feasibility of developing a Cyt-C encapsulated in LS (Cyt-LS) dosage form consisting of natural phospholipids is obvious. However, the preparation and characterization of such liposomal systems as medicines in accordance with modern requirements for the pharmaceutical industry [16-19] is quite a challenge. High molecular mass, special structure and sensitivity to heat of all heme proteins will largely determine the approaches to obtain such liposomal systems as medicines.

The objective of the present study was to develop and optimize the methods for the preparation and characterization of the liposomal delivery system of natural heme protein.

MATERIALS AND METHODS

Materials

Cytochrome C (Farmasino pharmaceuticals Co., ltd, China), Egg Phosphatidylcholine, Lipoid E 100 (Lipoid GmbH, Germany), dipalmitoyl phosphatidylglycerol (DPPG-Na) (Lipoid GmbH, Germany), phosphatidylcholine standard Lipoid E PC RS (Lipoid GmbH, Germany), dipalmitoyl phosphatidylcholine standard DPPG-Na RS (Lipoid GmbH, Germany), lysophosphatidylcholine (Lipoid GmbH, Germany), chloroform (Sigma Aldrich), methanol (Sigma Aldrich), potassium dihydrogen phosphate (Fluka), sodium

dihydrogen phosphate (Sigma Aldrich), disodium hydrogen phosphate (Sigma Aldrich).

Preparation of cytochrome C containing liposomes

DPPG-Na and EPC were dissolved in chloroform: ethanol mixture (4:1). The mixture was evaporated on a BUCHI Rotavapor R215 rotary evaporator (Switzerland) until a lipid film was formed. The lipid film was hydrated with a Cyt C solution, on an IKA werke orbital shaker (Germany) until a homogeneous emulsion of multilamellar liposomes was formed. The resulting emulsion of multilamellar drugs was homogenized on a high-pressure homogenizer-microfluidizer M110P (USA) [20, 21].

Then the obtained LS-Cyt were subjected to sterilizing filtration in a clean room. Filtration possibility was evaluated on PALL (USA) polyethersulfone filters with 0.22 μm pore size.

Physicochemical characterization of liposomes

Particle size (DLS) and zeta potential were determined on a Malvern zetasizer nano ZS (UK). The pH of the liposomal emulsion with Cyt C was measured on a Schott instruments Lab860 pH meter (Germany) [22].

Scanning electron microscopy

LS-Cyt imaging was performed by scanning electron microscopy [23]. A sample of Cyt-LS was applied to a copper mesh covered with a thin carbon layer. The sample was contrasted with 2% uranyl acetate solution. Next, the sample was tested on a Tescan Mira 3 LMU (Czech Rep.) using a STEM detector which allows conducting studies in scan mode with detection of the signal that passed through the sample.

Phospholipids and their impurities assay (lysophosphatidylcholine, free fatty acids)

The content of phospholipids in liposomes was determined by HPLC [24] on a Shimadzu chromatograph (Japan), under the following conditions: PerfectChrom 100 Diol column of 0.125x4 mm, 5 μm, with column temperature of 55 °C, ELSD Sedere SEDEX 85 detector (France), sample volume of 20 μl, Mobile phase A: 1341.6 g of n-hexane, 334.1 g of 2-propanol, 39.4 g of acetic acid, and 1.45 g of triethylamine. Mobile phase B: 663.5 g of 2-propanol, 140.0 g of water, 15.8 g of acetic acid, and 0.58 g of triethylamine. The gradient elution program is shown in table 1.

Table 1: The gradient elution program

Time (min)	Flow rate (ml/min)	Mobile phase A: (% vol)	Mobile phase B (% vol)
0	1.0	95	5
5.0	1.0	80	20
8.5	1.0	60	40
15.0	1.0	0	100
17.5	1.0	0	100
17.6	1.0	95	5
21.0	1.0	95	5
22.0	2.0	95	5
27.0	2.0	95	5
29.0	1.0	95	5

Phospholipids included in liposomes and phospholipid standards were dissolved in chloroform: methanol: water (74:23:3), The content of phospholipids was calculated by PC, DPPG-Na, lysophosphatidylcholine (LPC) and Free fatty acids calibration graphs.

Encapsulation efficiency studies

The total amount of Cyt C (Cyt C total) was quantified with a spectrophotometric method on a Shimadzu UV1800 spectrophotometer (Japan) using the UV absorption spectrum of diluted Cyt-LS in the range of 400-560 nm. The determination of non-liposomal Cyt C (Cyt C free) was evaluated by gel chromatography method. Shimadzu "Nexera" chromatograph was used, a Tricorn chromatographic column of 5/200 size (Ge Healthcare) filled with a "superose 12" sorbent. Mobile phase: 4.515 g/l KH_2PO_4 pH up to 6.0 2M NaOH; flow rate 0.5 ml/min; detection 409 nm; column temperature of 25 °C. Solutions of Cyt-C substance and Cyt-LS were chromatographed separately

The percentage of encapsulation efficiency was determined by using the ratio amount of entrapped Cyt C "Cyt C total–Cyt C free" to the amount of total Cyt C, which may be expressed by the following formula:

$$\%\ \text{Encapsulation efficiency} = \frac{\text{Amount of entrapped Cyt C}}{\text{Amount of total Cyt C}} \times 100$$

Sterility evaluation

Sterility evaluation was performed by the method of membrane filtration [25]. LS-Cyt was filtered through two pre-moistened membrane filters. After the filtration was finished, the filters were washed with sterile water. Then one tank of the sterilization system was filled with thioglycolic, and the second with soy-casein nutrient media. Next, inoculations in the thioglycolic medium were incubated at a temperature of 30-35 °C, and in the soy-casein medium, at a temperature of 20-25 ° C., all the inoculations were incubated for 14 d.

***In vitro* activity study**

UV absorption spectra of the reduced and oxidized forms of the LS-Cyt solution were compared with the solution of cytochrome C substance in the range of 190-600 nm on Shimadzu UV1800 spectrophotometer (Japan).

The reduced form of cytochrome C was obtained by adding sodium dithionite to the aqueous solutions of LS-Cyt and Cyt substance. After that, cytochrome C was oxidized by adding potassium ferricyanide to the same solutions [26]

RESULTS AND DISCUSSION

Preparation of cytochrome C containing liposomes

It is well known that Cyt-C can form complexes with anionic phospholipids in biological membranes [27-29], which is determinant in Cyt-C protein-lipid interaction. Based on these data, anionic phospholipid-dipalmitoyl phosphatidylglycerol (DPPG) was used in the composition of nanoparticles. Phosphatidylcholine (PC), the main component of eukaryotic membranes, was used as a membrane-forming lipid. The technological scheme of LS-Cyt production comes down to the preparation of a lipid film and its emulsification in Cyt-C solution to obtain an emulsion of multilamellar vesicles, followed by high-pressure homogenization until unilamellar vesicles were obtained.

Critical quality attributes (CQAs) of the liposomal product are: encapsulation efficiency of the active substance, particle size, and liposomal nanoparticles morphology. One of the key factors of the technology influencing these indicators is the choice of the method of unilamellar vesicles production. Today, one of the main industrial methods for obtaining small unilamellar liposome vesicles with active pharmaceutical ingredients included is high-pressure homogenization. The advantage of this method, in comparison with the methods of ultrasound, freeze-thaw, injection, etc., is the standard approaches and potential scaling ability to large-scale production, high yield, minimal oxidation and hydrolysis of phospholipids, integrity of the drug, stability of the resulting

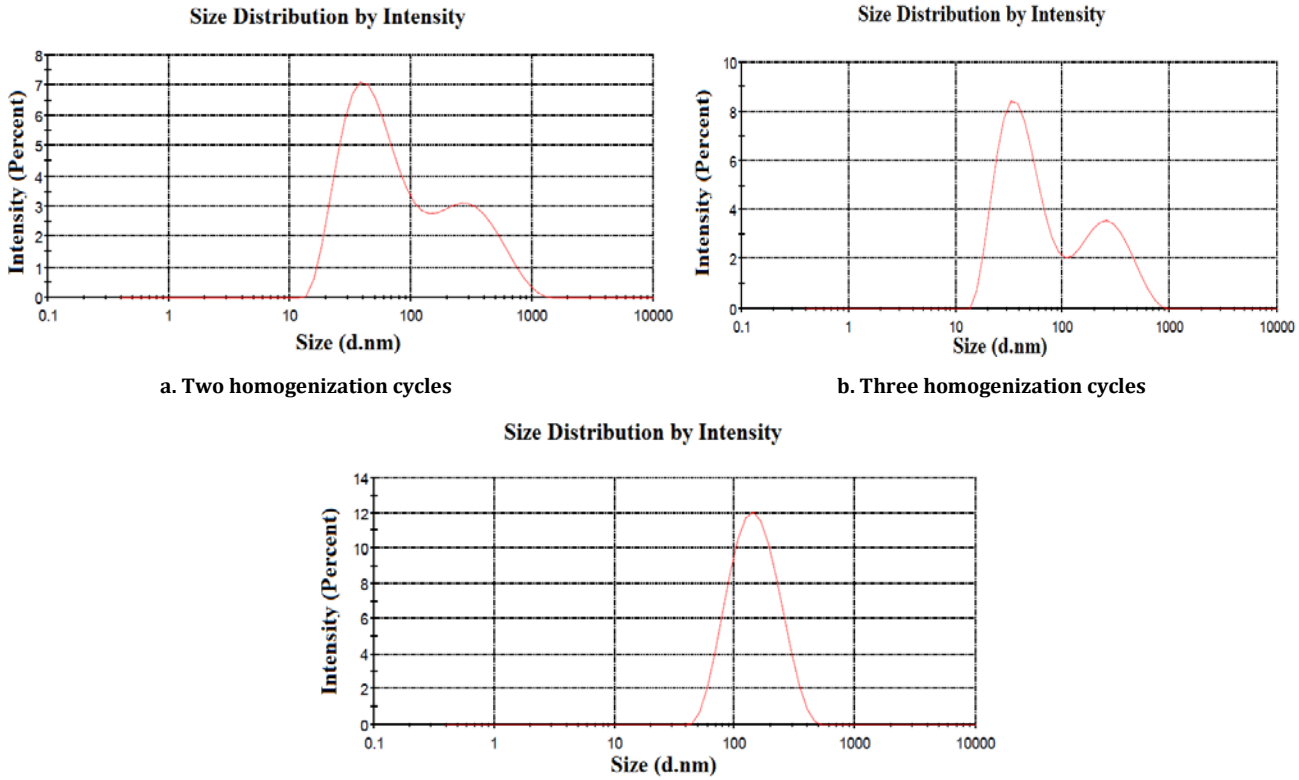
liposomes and control over key parameters in the technology process [30-32]. The selected homogenization mode may allow obtaining liposomes of standard composition, the bulk of which is represented by particles no larger than 200 nm. In addition to pharmacological properties, such liposome size will also determine the possibility of carrying out a sterilizing filtration process.

The optimal temperature regime of homogenization was kept within 38–44 °C. Firstly, such a temperature range is above the transition phase temperature of the phospholipids used, secondly, it is maximum close to temperature values at which Cyt-LS is in biological systems. In addition, natural phospholipids which are the main component of LS are also quite sensitive to the temperature increase at which the increase in degradation products (LPC and free fatty acids) can be observed. [33, 34]

The choice of optimal pressure, on one hand, was aimed to obtain the minimum number of homogenization cycles required to have the main LS mass up to 200 nm-which allows sterilizing filtration. On the other hand, the choice of the optimal pressure level was supposed to prevent the selected temperature regime from exceeding. Thus, experimentally, the pressure level was selected within 800 bar.

Physicochemical characterization of liposomes

Particle size control during homogenization was performed using the DLS method (fig. 2)



a. Two homogenization cycles

b. Three homogenization cycles

c. The final homogenization cycle

Fig. 2: Particle size control during homogenization a-Cyt-LS after two homogenization cycles, b-Cyt-LS after three homogenization cycles, c-Cyt-LS after the final homogenization cycle

Dynamic light scattering (DLS), is broadly used in liposome size distribution analysis. The strengths of the technique include the ability to make measurements in the original state of liposomes; its sensitivity; ease of commercially available operating instrument; minimal sample volume, concentration and preparation requirements; large size range of species spanning up 1 nm to several µm. However, the technique does not yield particle shape information; it can yield a bias towards reporting larger diameters when small quantities of high molecular weight or aggregates or impurities are present in the sample [35, 36].

After homogenization, Cyt-LS were characterized to confirm homogeneity by a number of parameters including encapsulation efficiency, lipid composition content, surface charge, and particle size assessment.

As a result, the mean particle of Cyt-LS were observed to be 156.6±0.02 (mean±SD, n = 3). Lysophosphatidylcholine content (from the amount of lipids): (LPC) of 0.60±0.05% (mean±SD, n = 3), free fatty acids — 0.40±0.05% (mean±SD, n = 3).

Encapsulation efficiency studies

The liposome preparations are a mix of encapsulated and non-encapsulated drug fractions. The first step for the determination of the encapsulation efficiency is the separation between the encapsulated drug (within the carrier) and the non-encapsulated. Gel chromatography methods can be applied for the determination of the encapsulation efficiency [37, 38]. In this case, the encapsulation percent can be expressed as the ratio of the un-encapsulated peak area to that of a reference standard at the same initial concentration.

To determine the encapsulation efficiency, methods for determining the total Cyt-C concentration and the concentration of non-encapsulated Cyt-C were proposed.

The total concentration of Cyt-C in Cyt-LS (Cyt total) was determined spectrophotometrically using the UV absorption spectrum of a diluted Cyt-LS emulsion in the range of 400–560 nm (fig. 3).

The determination of "non-encapsulated" Cyt-C (Cyt free) was performed by gel chromatography method.

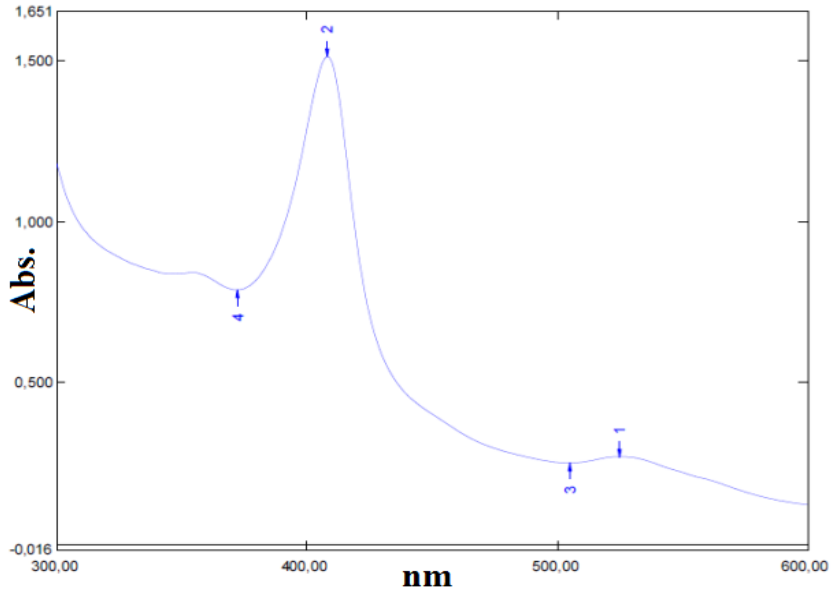
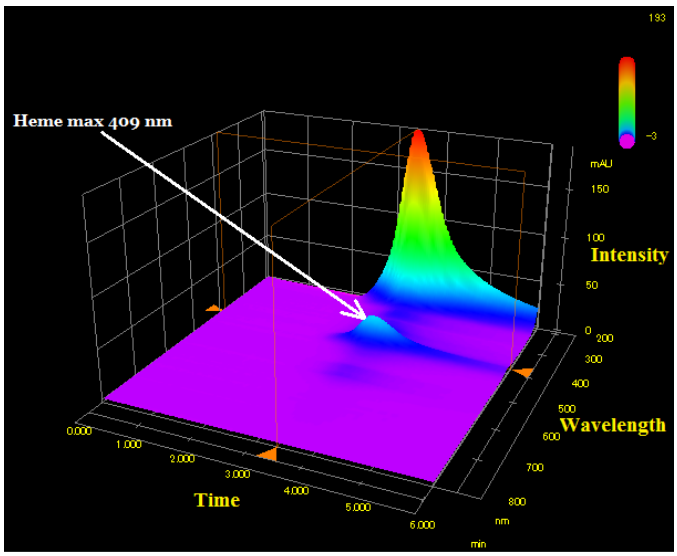
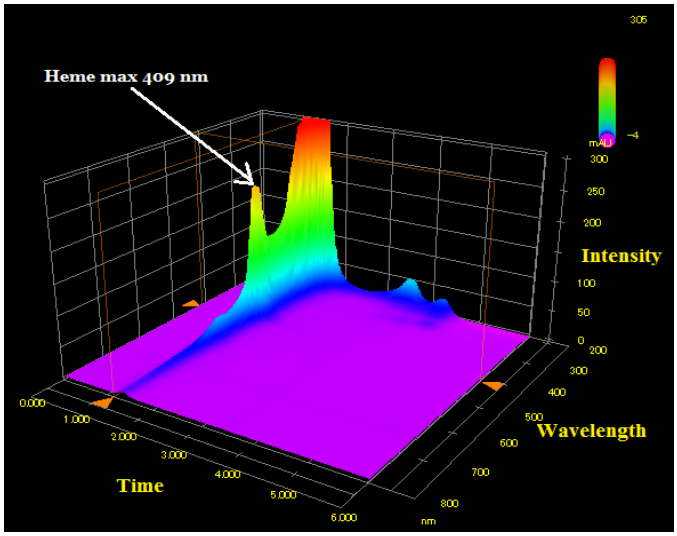


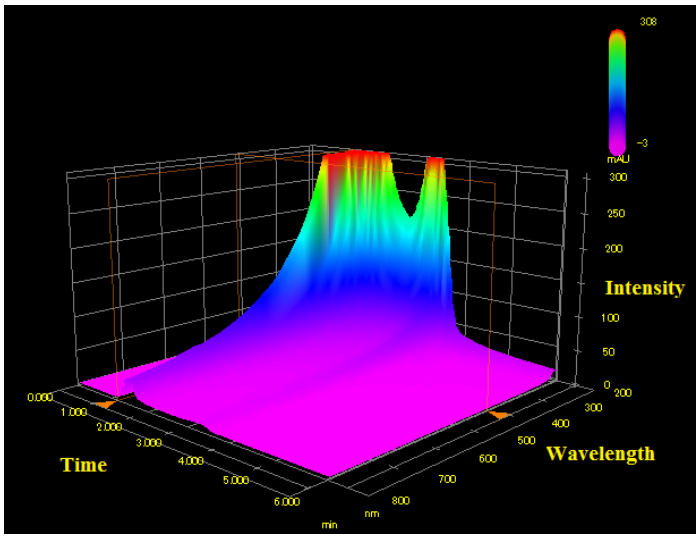
Fig. 3: UV-spectrum of Cyt-LS



a. Cyt-C (Non-liposomal)



***b.* Cyt-LS**



c. Placebo Cyt-LC (Without Cyt-C)

Fig. 4: 3D Chromatograms of Cyt-C standard, Cyt-LS and Cyt-LS placebo solutions: a-3D chromatogram of a Cyt-C standard solution (non-liposomal); b-3D Cyt-LS chromatogram; c-3D placebo Cyt-LS chromatogram (without Cyt-C)

As can be seen from Fig. 4 the main peaks in Cyt-LS and non-liposomal Cyt-C chromatograms have different retention times. The characteristic maximum of the UV spectrum in the 400–410 nm ranges is observed in the standard Cyt-C solution peaks spectra of (200–800 nm) and Cyt-LS (200–800 nm), which confirms the encapsulation of Cyt-C in liposomes. As a result, Cyt-LS were obtained with encapsulation efficiency 95.8±0.02% (mean±SD, n = 3)

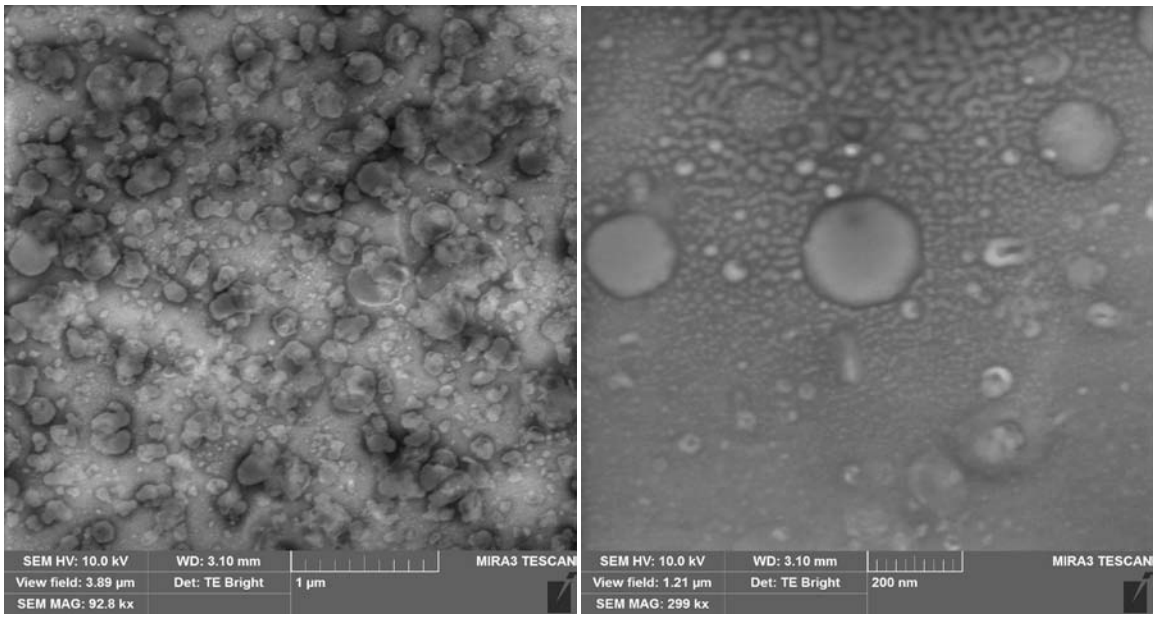


Fig. 5: SEM images of Cyt-LS

Scanning electron microscopy

Controlling and confirming the nanoparticles morphology is also key to the effective clinical use of LS drugs. Currently, there are a lot of visualization methods for evaluating LS and other nanoparticle morphology, each of which has its advantages and disadvantages that should be considered when interpreting the data. One of such visualization method is Scanning Electron Microscopy, SEM. We use SEM with a STEM detector, which allows conducting research in a scanning mode with the detection of a signal that passed through a sample. The results of the study are presented in fig. 5. On the obtained images LS have an average size of about 200 nm.

Sterility evaluation

The potential use of Cyt-LS in clinical practice as a medical substance and its long-term storage will be connected with obtaining sterile LS.

To ensure the production of sterile LS there is the possibility to obtain LS under aseptic conditions, carrying out thermal sterilization or sterilizing filtration [39]. In the first case, it will be necessary to ensure that all technological operations are performed in order to obtain LS under sterile conditions starting from the stage of obtaining a lipid film and its rehydration with sterile Cyt-C buffer solution, which in turn will entail quite considerable costs on implementation of this scheme for obtaining LS [40].

Thermal sterilization will involve LS sterilization at 121 °C for 10–30 min. At the same time, the use of thermal sterilization will be determined by a number of factors: the lipid LS composition and their oxidative stability; the heat resistance of substances included in LS. An additional problem unique to liposomes is the resultant leakage of encapsulated contents [41, 42]. Therefore it will obviously have a negative impact on the use of natural phospholipids and biologically active proteins, which are quite sensitive to heating at high temperatures.

The method of sterilizing filtration involves the filtering of liposome preparations through sterile filtration units (0.22 μm) under pressure, allowing liposomes smaller than 200 nm to pass through. This sterilization technique is suitable for thermolabile API (for example hemeproteins) since it does not involve any form of heating nor conditions that can result in the formation of degradation products or leakage of liposomal contents associated with the other terminal sterilization techniques. One drawback of this technique is size restriction limits of LS which are suitable for filtering. However, this limitation is insignificant in manufacturing liposomes for parenteral usage since a small vesicle size (of<500 nm) [43] is recommended to minimize complications such as retention and trapping of the vesicles in the narrower capillaries. Unfortunately, all the other conventional techniques result in the formation of degradation products via the aforementioned degradation pathways.

The physical and chemical complexity of LS drugs creates unique challenges for the sterilization filtration process [44, 45]. The LS components can, for example, interact with the filter matrix or, having an average particle size exceeding 0.22 μm, can block it up. At the same time, conducting sterilizing filtration allows standardization of LS preparations.

We have evaluated the sterilization filtration. Cyt-LS in the amount of 1 liter was subjected to sterilizing filtration through Pall (USA) 0.22 μm membrane filters. After sterilizing filtration, the main indicators of LS product quality were monitored. The comparison is shown in table 2. The sterility of the obtained product was also assessed by membrane filter assay. Thus, the obtained LS-Cyt can be subjected to sterilizing filtration with the preservation of the main physicochemical properties.

Table 2: Study of sterilizing filtration of Cyt-LS before and after sterilizing filtration

Test	Cyt-LS before sterilizing filtration (mean±SD, n = 3)	Cyt-LS after sterilizing filtration (mean±SD, n = 3)
Encapsulation efficiency,%	95.8±0.02	95,8±0.02
Mean particle size, nm	156.6±0.02	157.3±0.02
Zeta potential, mV	-57±0.10	-57±0.10
pH	6,95±0.05	6,91±0.05
LPC, %	0,60±0.05	0,59±0.05
Free fatty acids,%	0,4±0.05	0,39±0.05
Cyt C, %	100,1±0.05	100,3±0.05
PC, %	99,8±0.05	99,6±0.05
DPPG-Na, %	100,2±0.05	100,0±0.05
Sterility	-	Pass

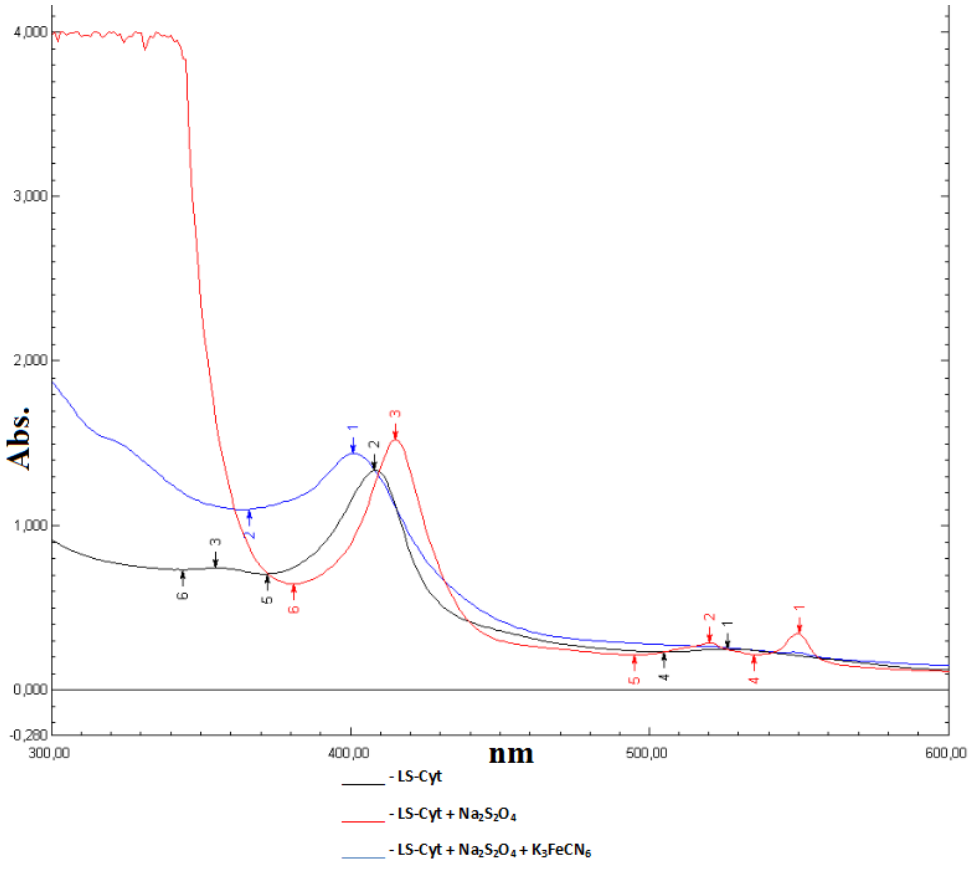


Fig. 6: UV-spectrum of LS-Cyt

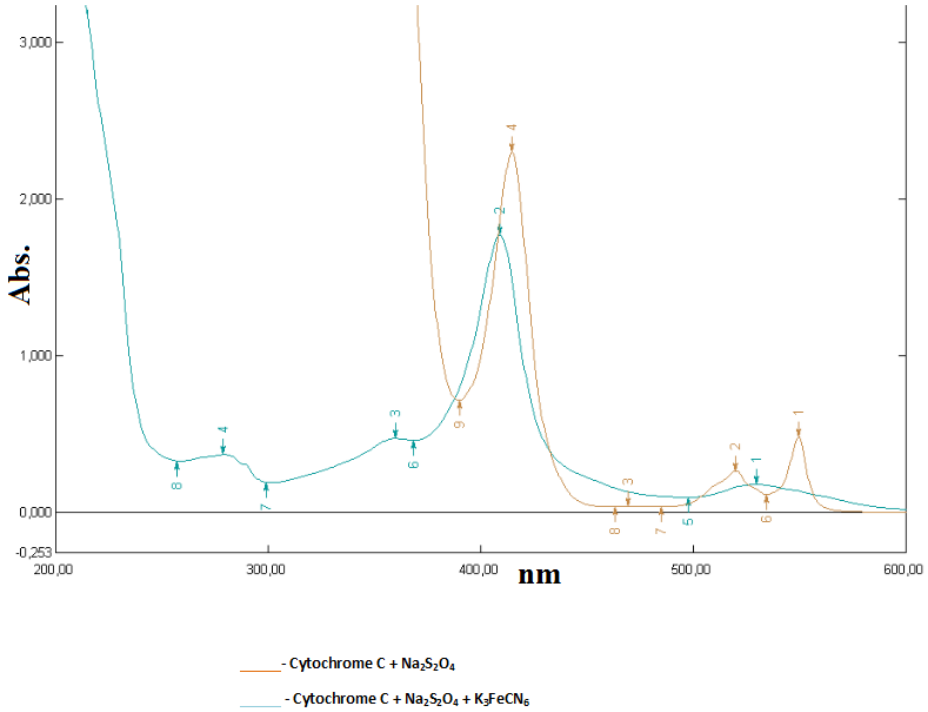


Fig. 7: UV-spectrum of Cyt solution

In vitro activity study

We also evaluated the reactivity of Cyt-C which is primarily regarded as its activity in providing electron transport function in mitochondria respiratory chain. The specific activity study was performed *in vitro*. The reaction with sodium dithionite ($Na_2S_2O_4$) is used to determine Cyt-C activity in Cyt-C solutions for injection [46]. The ability of Cyt to transfer from the reduced to oxidized form was evaluated by adding potassium ferrocyanide to the solution of Cyt-C in the reduced form [47]. To compare the specific activity of Cyt-LS, the spectra of the LS-Cyt solution that were reduced with sodium dithionite and oxidized ferrocyanide were compared with the spectra of a non-liposomal Cyt-C solution in reduced and oxidized forms respectively fig. 6, 7.

After adding sodium dithionite the absorption maxima at 520 and 550 nm identically appear in the UV spectra of LS-Cyt and Cyt solutions. That corresponds to the characteristic UV spectrum for all types of Cyt in a reduced state [48].

Thus, the obtained UV spectra demonstrate that Cyt-LS retains the ability to receive and release electrons, similarly to a non-liposomal Cyt solution.

CONCLUSION

Applications of heme proteins in medicine can be improved through liposomal delivery systems. However, the preparation and characterization of such systems as medicines in accordance with modern requirements for the pharmaceutical industry is quite a challenge. This project was conducted to study the methods for the preparation and characterization of heme protein containing a liposomal delivery system consisting of natural components, for its potential use in the prevention and treatment of oxidant-induced injuries. Thus, preclinical and a clinical trial is required for the future development of this formulation.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

The authors declared no conflict of interest

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