Conclusion: for different parameters.

physicochemical properties. Cyt-LS exhibit specific activity, similar to non-liposomal Cyt-C solution.

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$_2$O$_2$ from mitochondria, acting as an antioxidant enzyme [1-3] (fig. 1)

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 preparation and characterization of the liposomal delivery system of natural heme protein.

METHODS AND MATERIALS

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 hydrogen peroxide H$_2$O$_2$ and superoxide O$_2^-$.
Preparation of cytochrome C containing liposomes

DPGG-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 CyC solution, on an IKA werke orbital shaker (Germany) until a homogenous 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) polyether sulfone 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). Particle size (DLS) and zeta potential were determined on a Malvern polyethersulfone filters with 0.22 μm pore size. Then the obtained LS-Cyt were subjected to sterilizing filtration in a clean room. Filtration possibility was evaluated on PALL (USA) polyether sulfone filters with 0.22 μm pore size.

Scanning electron microscopy

LS-Cyt imaging was performed by scanning electron microscopy [24] on a Shimadzu chromatograph (Japan), under the following conditions: Perfect Chrom 100 Diol column of 0.125 x 5 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: 66.35 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.

| 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 (7:4:23:3). 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 CyC (CyC total) was quantified with a spectrophotometric method on a Shimadzu UV1800 spectrophotometer (Japan) using the UV absorption spectrum of diluted CyC-LS in the range of 400-560 nm. The determination of non-liposomal CyC (CyC 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 "supersil 12" sorbent. Mobile phase 4.515 g/l KH2PO4, pH up to 6.0 2M NaOH; flow rate 0.5 ml/min; detection 409 nm; column temperature of 25 °C. Solutions of CyC substance and CyC-LS were chromatographed separately.

The percentage of encapsulation efficiency was determined by using the ratio amount of entrapped CyC/CyC total - CyC free to the amount of total CyC, which may be expressed by the following formula:

\[
\text{Encapsulation efficiency} = \left( \frac{\text{Amount of entrapped CyC}}{\text{Amount of total CyC}} \right) \times 100
\]

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 CyC 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 CyC can form complexes with anionic phospholipids in biological membranes [27-29], which is determinant in CyC protein-lipid interaction. Based on these data, anionic phospholipid-dipalmitoyl phosphatidylglycerol (DPGG) 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 CyC 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 prod-uct 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)

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). Lyso phosphatidylcholine 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
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).

**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 lipid 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      | 15.6±0.02                                            | 157.3±0.02                                          |
| Zeta potential, mV          | -57±0.10                                             | -57±0.10                                            |
| pH                          | 6.95±0.05                                            | 6.9±0.05                                            |
| LPC, %                      | 0.6±0.05                                             | 0.5±0.05                                            |
| Free fatty acids, %         | 0.4±0.05                                             | 0.3±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                                                 | Pass                                                |

Fig. 6: UV-spectrum of LS-Cyt
We also evaluated the reactivity of Cyt-C which is primarily regarded as its activity in providing electron transport function in mitochondrial respiratory chain. The specific activity study was performed in vitro. The reaction with sodium dithionite (Na$_2$S$_2$O$_4$) 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.

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 authors have contributed equally

CONFLICT OF INTERESTS
The authors declared no conflict of interest

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