Two types of magnetite-containing liposomes for magneto-controlled drug release

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Abstract. The paper is dedicated to a comparative study of pharmaceutical properties of magnetite containing liposomes. We have investigated two types of liposomes: 1) magnetic shell liposomes containing magnetic nanoparticles in the coating lipid bilayer and 2) magnetic core liposomes containing the nanoparticles in the internal volume (water phase). Both types of liposomes were obtained by the thin film hydration method from phosphatidylcholine and cholesterol. Fluorescent dye 5,6-carboxyfluorescein was used as a marker substance to indicate release. The dimensional characteristics, the degree of dye release under the influence of an alternating magnetic field, the speed of spontaneous release and the stability of liposomes during storage were investigated. It has been shown that liposomes with nanoparticles in the internal space are more stable, have low rate of spontaneous release of the incorporated substance, but the rate of release under the influence of an alternating field is also low. Liposomes with magnetic nanoparticles inside the lipid bilayer have high release degree influenced by alternating magnetic field, but they are less stable during storage and more prone to spontaneous loss of the active substance.

1. Introduction

Currently, methods of targeted drug delivery using various remote control systems are actively developed. One of such systems is liposomes. Since its discovery, liposomes have been attracting undying interest due to their biocompatibility, the possibility of variation of the size and the composition of lipid membranes, the ability to encapsulate different substances in the internal volume or in the lipid bilayer [1-3]. The release of the encapsulated drug substance can be achieved by destabilizing the lipid bilayer caused by external influences, such as temperature, pH change, exposure to ultrasound or electromagnetic fields, mechanical compression, etc. [4-10]. Liposomes containing magnetic nanoparticles (MNPs) are of great interest in biomedicine. They can also be used as MRI markers [11], magnetic biosensors [12].

The presence of magnetic nanoparticles in liposomes makes it possible their selective accumulation in the area of pathological process using an external constant magnetic field and further release of the drug under the action of alternating magnetic field [13-15].
The effectiveness of the release of the encapsulated substance depends on various factors: the membrane lipids composition, chemical structure of drug, field parameters (amplitude, frequency and exposure time), localization of magnetic nanoparticles in liposomes, etc. [16, 17].

There are two possible ways of incorporating magnetic nanoparticles into liposomes: firstly, into the inner space of the vesicles and secondly, into the lipid bilayer [14-17]. As a rule, particles with a hydrophilic shell are used in the first case, and carriers stabilized by hydrophobic substances are taken in the second one. There are studies showing that liposomes with MNPs incorporated in the lipid bilayer release the incorporated agent more effectively in comparison with vesicles containing MNPs in the internal aqueous phase [18, 19]. However, for practical application, not only the effectiveness of the induced release is important, but also some pharmaceutical properties, such as storage stability and the rate of a spontaneous release. MNPs contained in liposomes can influence these processes.

The purpose of this work is the study of the physicochemical and pharmaceutical properties of liposomes with MNPs included in their shell and in their internal space.

2. Materials and methods

2.1. Materials

The following reagents were used in the work: iron chloride (III) 99.99%, iron chloride (II) 99.99%, citric acid 99.9%, L-α-phosphatidylcholine (PC), cholesterol, hexane, 5(6)-carboxyfluorescein (CF) >95% - Sigma-Aldrich, USA; ammonium hydroxide 25%, oleic acid 99.8%, triton X-100, acetone 99.5%, hydrochloric acid 99.8% - Vekton, Russia.

2.2. Preparation of hydrophobic magnetic nanoparticles coated with oleic acid (OA-MNPs)

The synthesis of magnetic nanoparticles was carried by the chemical coprecipitation method in a nitrogen atmosphere [20-22]. Iron chloride (II) and iron chloride (III) (0.86 g and 2.35 g respectively) were dissolved in 40 ml of deionized water. The solution was heated up to 100°C and then 5.4 ml of 25% ammonia solution was slowly poured. After that, 4 ml of oleic acid was quickly added and the pH of the reaction medium was adjusted to 6 with hydrochloric acid. The suspension of the obtained particles was mixed for 90 minutes at 100°C. The magnetic particles were separated by magnetic decantation, washed several times with deionized water and acetone. After collecting the precipitate with a magnet, the particles were dispersed in 25 ml of hexane. Next, the dispersion was centrifuged at 14000 RPM for 15 minutes to remove larger clusters of particles, and the supernatant containing stable nanoparticles was collected for further use.

2.3. Preparation of hydrophilic magnetic nanoparticles coated with citric acid (CA-MNPs)

The first stage of the synthesis was similar to the described above. The solution of iron (II) and (III) chlorides in deionized water was heated to 80°C and 5.4 ml of 25% aqueous ammonia solution was slowly poured. Then 2 ml of 50% citric acid solution was added to the suspension of Fe₃O₄ particles. The particle suspension was shacked for 90 minutes at 95°C. The resulting colloid was purified by dialysis through cellulose membrane with a pore size of 12-14 kDa (Orange Scientific, Belgium) for 72 hours, changing the dialysis medium every 12 hours until a negative qualitative reaction to the chloride ions. To get rid of large aggregates, the CA-MNPs colloid was centrifuged at 12000 RPM for 10 minutes.

2.4. Preparation of liposomes containing MNPs

For the synthesis of magnetic liposomes, the thin-film hydration method was used. L-α-phosphatidylcholine and cholesterol (300 and 3 mg respectively, mass ratio 100:1) were dissolved in 10 ml of chloroform, and then 5 ml of OA-MNPs suspension in hexane were added to the resulting lipid solution. Then the mixture was evaporated on a rotary vaporizer at 40°C until complete removal of organic solvents. The formed thin lipid film containing magnetic nanoparticles was hydrated with CF buffer solution. The suspension of multilamellar polydisperse liposomes was extruded through polycarbonate membrane with a pore size 400 and 200 nm. The resulting liposomes were purified from
unincorporated magnetic nanoparticles and CF using size exclusion chromatography with Sephadex G-25. Thus, magnetic shell liposomes (MSLs) containing magnetic nanoparticles in the coating lipid bilayer were obtained.

Magnetic core liposomes (MCLs) containing the nanoparticles in the internal volume (water phase) were obtained by the same method, but the lipid film of L-α-phosphatidylcholine and cholesterol was hydrated with hydrophilic CA-MNPs and a CF buffer solution.

2.5. Characterization of MNPs and magnetic liposomes
Size distribution of MNPs and liposomes was determined by dynamic light scattering (DLS) (analyzer NANO-flex, Microtrac Inc., Germany). The size and morphology of the obtained liposomes were studied using transmission electron microscopy (TEM) (microscope FEI Tecnai Osiris, USA). The phase composition of synthesized iron oxide particles was checked with an Empyrean PANalitical X-ray diffractometer (CuKα radiation). Phase was identified using HighScore Plus package and JSPDS PDF-2-1911 database. Sample magnetization was measured by the induction method with an EZ11 vibration magnetometer (Microsense Inc., Lowell, Massachusetts, USA). Quantitative determination of iron oxide in colloid and purified liposomal suspensions was performed using a photocolorimetry based on measuring the optical density of colored iron (III) ions complexes with sulfosalicylic acid in an alkaline medium (spectrophotometer Shimadzu-UV-2600).

2.6. Determination of carboxyfluorescein release
To estimate the total amount of included CF, liposomes were destroyed by adding Triton X-100 to liposomal suspension and the fluorescence of the resulting solution was measured.

To estimate the induced release, MSLs and MCLs were exposed to an alternating magnetic field (AMF) with a frequency of 100 kHz and an amplitude of 200 Oe for 30 minutes. After processing in a magnetic field, the suspension was centrifuged at 12000 RPM for 10 minutes and the fluorescence intensity of the supernatant was determined (spectrofluorometer Shimadzu RF-5301PC, Japan). The percentage of released CF was calculated as:

$$R = \frac{(I_t - I_0)}{(I_f - I_0)} \times 100\%,$$

where $I_0$, and $I_t$ are the fluorescence intensities of the supernatant at the beginning of the experiment and after exposure to AMF respectively and $I_f$ is the fluorescence intensity of the supernatant after destruction of the liposomes by Triton X-100.

The spontaneous release of CF from MSLs and MCLs was evaluated at 2, 4, 8, 12, 16, 20, 24 and 28 days of storage at 4°C. To evaluate the spontaneous release, the liposome suspension was centrifuged at 12000 RPM for 10 minutes and the fraction of released CF was determined by the above described method.

2.7. Liposomes stability study
The stability of MSLs and MCLs was assessed by the change in their average hydrodynamic diameter during storage at 4°C. The hydrodynamic diameter of liposomes after 2, 4, 8, 12, 16, 20, 24 and 28 days of storage was determined by the above described DLS method.

3. Results and discussion
The XRD pattern of the OA-MNPs is shown in figure 1 (the pattern for the CA-MNPs is similar). The XRD spectrum contains peaks typical for Fe₂O₃ and γ-Fe₂O₃.
The magnetization curve of OA-MNPs samples is shown in figure 2. Both OA-MNPs and CA-MNPs have similar form of the curves. The hysteresis loops have exhibited superparamagnetic behaviour. The saturation magnetization was about 75 emu/g for OA-MNPs and 72 emu/g for CA-MNPs.

The results of particles size distribution study for MSLs and MCLs samples are shown in figure 3. The average size of particles was 230 ± 55 nm and 202 ± 37 nm for MSL and MCL respectively. The iron oxide concentration in the purified liposomal emulsion was 2.97 mg/ml for MSLs and 3.32 mg/ml for MCLs.
Figure 4 shows the TEM images of the obtained samples. Both types of liposomes look like round-shaped structures about 200 nm in size with a high degree of contrast. The size of the vesicles, determined by the TEM method, correlates with the size obtained by the DLS method.

![TEM images of MSLs (a) and MCLs (b).](image)

The results of a study on the CF release from liposomes in the alternating magnetic field are shown in figure 5. One can see that the CF release from MSLs was higher than from MCLs (54% vs 35%, respectively). The possible reason for the higher permeability of the MSLs liposomal membrane is the heating of the ferromagnetic nanoparticles in the lipid shell. When particles are localized in the shell, heat is transferred directly to the lipids, whereas when they are localized in the core, the heat is transmitted to the lipids through the water. Apparently, in the first case, the destabilization of the liposomal membrane is more pronounced, despite the lower relative iron oxide concentration.

![CF release from MSLs and MCLs when exposed to AMF. “Control” is the background content of CF in the aqueous phase of the liposomal emulsion.](image)

The results of the evaluation of CF spontaneous release from liposomes are shown in figure 6. It can be seen that the release rate of the fluorescent dye from both types of liposomes was maximal in the first 4 days, and then gradually decreased. The total amount of lost dye was significantly larger for liposomes containing MNPs in the lipid bilayer compared to liposomes with particles in the inner space (16 ± 5 and 5 ± 2%, respectively).

The results of the stability assessment of MSLs and MCLs are presented in figure 7. The mean hydrodynamic diameter of the MCLs did not change during the entire observation period. The size of the MSLs was stable during the first 4 days, and then steadily increased, reaching 922 ± 130 nm by 28 days.

It is known that the type of supramolecular structures formed by amphiphilic molecules in the aqueous phase depends on the length of the alkyl chain and on its molecular volume. For PC molecules, the optimal form of their packing is a lipid bilayer, which forms a kind of film in the aqueous phase. The addition of nanoparticles with a hydrophobic surface between the lipid layers increases the molecular volume of the hydrophobic part of this film. On the other hand, it is known that for the substances with a predominance of the hydrophobic molecular volume over the volume of the
hydrophilic part, the more energetically favorable is forming not the lipid bilayer, but structures with a hydrophilic core and a hydrophobic shell \[23, 24\]. Thus, magnetic nanoparticles located between the layers of amphiphilic PC molecules destabilize the membrane of vesicles. This can cause both spontaneous loss of the dye from this type of liposomes and their greater tendency to aggregate.

4. Conclusion
Thus, the obtained results demonstrate the advantages and disadvantages of different types of liposomes with magnetic nanoparticles. Liposomes with nanoparticles in the internal space are more stable, have a lower rate of spontaneous release of the incorporated substance, but the rate of release under the influence of an alternating field is also lower. Liposomes with MNPs inside the lipid bilayer have a higher release degree under the influence of AMF, but they are less stable during storage and more prone to spontaneous loss of the active substance. The results can be used to optimize the method of synthesis of liposomal drug forms for magnetic controlled delivery.

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