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THERMODYNAMIC AND SPECTROSCOPIC STUDY OF COMPLEX CALCIUM-CONTAINING LIPOSOMES

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ABSTRACT

In the study, both uncomplexed and calcium-containing complex liposomes made of DPPA and DPPC phospholipids were studied calorimetrically and spectrophotometrically. Liposomes with a diameter of 200 nm were produced by new nanotechnology. As calcium molecules, $CaCO_3$ was used, which is used in medicine as a calcium supplement or antacid, which is used to relieve heartburn, stomach acidity. Complex liposomes were prepared in both water and 20 % glycerol solvent. As calorimetric and spectrophotometric experiments show, $CaCO_3$ molecules made by new technology are incorporated into the structure of liposomes, which allows them to be used for treatment. In addition, calcium is placed in large quantities in such complex liposomes, although their entry into the blood does not cause side effects. The structure and thermal stability of complex liposomes were determined in the paper.

In particular, according to the obtained experimental results, we believe that the structure of calcium-containing nanoparticles can be both unilaminar and multilaminar, in particular, the structure of uncomplexed and calcium-containing DPPC liposomes prepared in water and glycerol are multilaminar. The structure of uncomplexed DPPA liposomes prepared in water is unilaminar, while calcium-containing DPPA liposomes prepared in water form a multilayer structure. As for the structure of both pure and calcium-containing DPPA liposomes prepared in glycerolare multilamellar. With some preliminary considerations, calcium-containing liposomes can be tested in experimental animals, and after obtaining positive results, they can be recommended for the treatment of hypocalcemia in humans.

Introduction

Bone-related diseases are nowadays a very big social and economic problem, especially these problems are often related to human age. It is known that elderly people often suffer from osteoporosis and osteopenia [9]. If we take into account that the number of elderly people on earth is increasing, the number of such diseases is also increasing. Due to the lack of effective treatment for the diseases mentioned above, the treatment period is very long, which affects both the patient's quality of life and the costs of the healthcare system [2, 4, 18, 21, 22].

In addition, there are frequent cases of bone fractures, which are also associated with long-term treatment, so it is important to develop such drugs that will purposefully and quickly fill the calcium deficiency. At the same time, it should be noted that taking a large amount of calcium is not allowed for humans, because an increase in the concentration of calcium in the blood causes a number of diseases such as pancreatitis, high blood pressure, kidney stones. Severe hypercalcemia impairs neuromuscular and myocardial depolarization, leading to muscle weakness and ar-

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rhythmias. Also, the increased concentration of calcium in the blood leads to a decrease in the volume of erythrocytes, which causes anemia [7, 15, 20]. Another adverse event associated with hypercalcemia is the fact that red blood cells actively promote blood coagulation and clot formation, which is initiated by calcium [6, 21]. As a result, it is important to create a calcium drug, which on the one hand will fill the calcium deficiency in the body and on the other hand will not cause life-threatening secondary effects [3, 8]. This can be achieved by using liposomes, in which the amount of calcium is significantly increased, and its penetration into the body will not cause secondary effects that are dangerous to human life. Liposomes mainly consist of lipid bilayers, the composition of which is similar to the cell membrane, which is why they have good biocompatibility, and without increasing the concentration of calcium in the blood, they bring it to the tissue and increase the concentration of calcium [22, 23]. That is why our research is focused on the creation of such complex nanoparticles that can deliver the required concentration of calcium to the site of bone damage without causing side effects [14, 28–30]. The most common liposomes are made of phospholipids, the composition of which is represented by phosphatidylcholine amphiphilic molecules. Its structure is characterized by two pairs of hydrophobic acyl hydrocarbon chains connected to a polar, hydrophilic head by a glycerol bridge [12, 27, 31, 40, 41].

In our studies, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA) phospholipids were used as drug deliverysystems.It should be noted that in the case of hydrophobic drugs, by incorporating them into liposomes, the final product becomes soluble in water, which is also important.We have taken $CaCO_3$ as a calcium compound, which is a white, odorless, water-insoluble substance.It is used in medicine to improve low calcium levels in the blood.

Calcium carbonate is an ionic compound used as a calcium supplement or an antacid used to relieve heartburn, stomach acid [1, 4, 19]. Therefore, it is important to study complex nanoparticles containing calcium.It is appropriate to study the structure and stability of complex liposomes containing drugs using differential scanning calorimeter (DSC) [11, 24, 34].

Preparation method of DPPA and DPPC liposomes

In the experiments, we used liposomes, which were produced using a new, simple technology [35].Two types of phospholipids were used to make liposomes: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA). DPPA and DPPC lipids were purchased from Lipoid (Newark, New Jersey).In the case of both lipids, the liposome production technology was similar [39].Since nanoparticles with a diameter of 200 nm are effective for targeted delivery of drugs, we made liposomes with a thickness of 5 nm and a diameter of 200 nm.As a result of mathematical calculation, we determined that 3.3×10^4 lipids are needed to make one liposome with a thickness of 5 nm and a diameter of 200 nm.In order to determine the thermal stability of liposomes as drug- delivery nanoparticles, we used the calorimetric method in the case of pure and complex calcium-containing DPPA and DPPC liposomes. DASM-4 micro calorimeter (Pushchino, Russian Federation) is used in the presented work, which belongs to highly sensitive micro calorimeters.

Experimental part

Calorimetric experiments were performed in water and 20 % glycerol solvent on both pure liposomes made of DPPC and DPPA lipids, as well as complex liposomes containing calcium ions.In order to determine the thermal stability, the temperature interval from 0 to 100^0C and the speed of temperature scanning – 20ºC/min were selected. Calorimetric measurements were performed on DPPA liposomes prepared both in water (see Fig. 1) and in 20 % glycerol solvent (Fig. 2).

Based on the calorimetric measurements, we calculated the amount of heat released during melting of DPPA liposomes. It was found that the amount of heat released during melting of DPPA liposomes prepared in water is 21.2 MJ, and the amount of heat released during melting of DPPA liposomes prepared in glycerol solvent is 25.3 MJ.

We also conducted experiments on calcium-containing complex DPPA liposomes prepared both in water and in glycerol solvent (Figs. 3 and 4). The temperature dependence curves of the heat capacity of liposomes allow us to talk about the structure of the liposome. A calorimetric study of both pure and calcium-containing liposomes prepared from DPPA lipids in 20 % glycerol solvent is so interesting. As can be seen from the calorimetric records, the structure of pure liposomes prepared in glycerin solvent is multi-layered, multilamellar, which is indicated by the presence of two peaks on the calorimetric record. In contrast to the pure liposome, the small peak disappears in the calcium-containing complex liposomes that indicates that the calcium ions have indeed bound to the DPPA lipids (see Fig. 4).

Based on calorimetric measurements, we calculated the amount of heat released as a result of melting of calcium-containing DPPA liposomes prepared in both water and 20 % glycerol. In this case, a different amount of heat was released as a result of melting. It was found that the amount of heat released during melting of complex calcium-containing liposomes prepared in water is 18.6 MJ, and the amount of heat released as a result of melting of complex calcium-containing liposomes prepared in glycerol solvent is 13.5 MJ. We also performed experiments on DPPC lipids prepared in water and in 20 % glycerol solvent, and

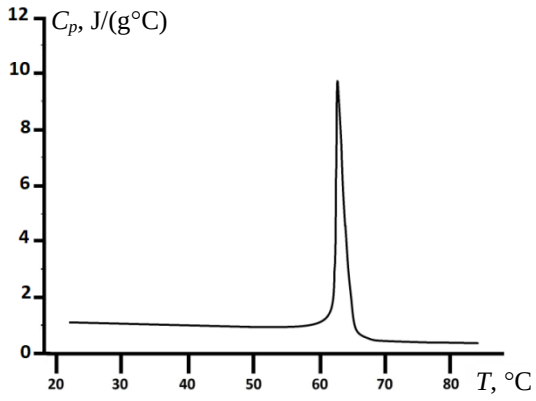

Fig. 1. Temperature dependence of heat capacity of DPPA liposomes prepared in water. Temperature scanning speed V = 2°C/min

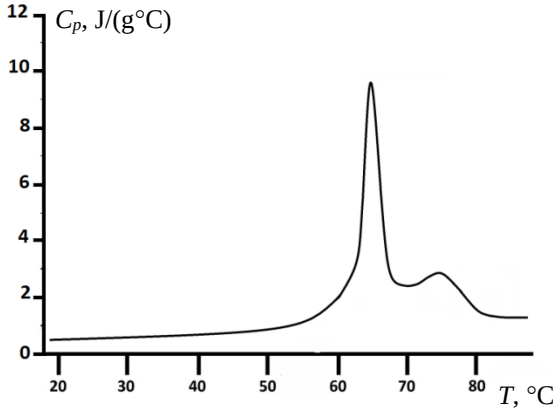

Fig. 2. Temperature dependence of heat capacity of DPPA liposomes prepared in 20 % glycerol. Temperature scanning speed V = 2°C/min

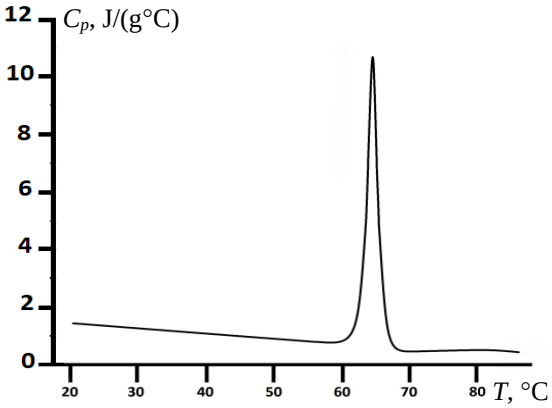

Fig. 3. Temperature dependence of heat capacity of calcium-containing DPPA liposomes prepared in water. Temperature scanning speed V = 2°C/min

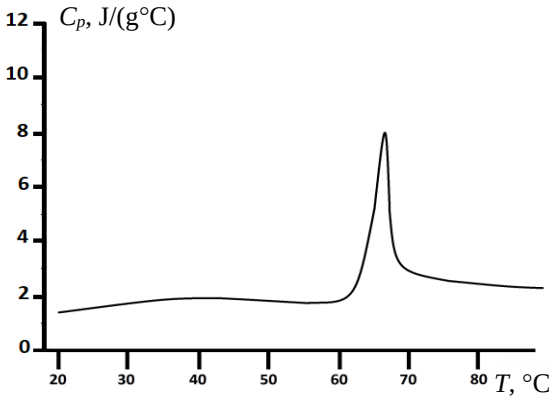

Fig. 4. Temperature dependence of heat capacity of calcium-containing DPPA liposome prepared in 20 % glycerol. Temperature scanning speed V = 2°C/min

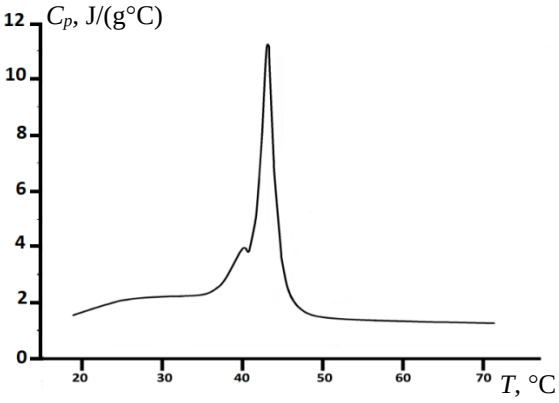

Fig. 5. Temperature dependence of heat capacity of DPPC liposome suspensions prepared in water. Temperature scanning speed V = 2°C/min

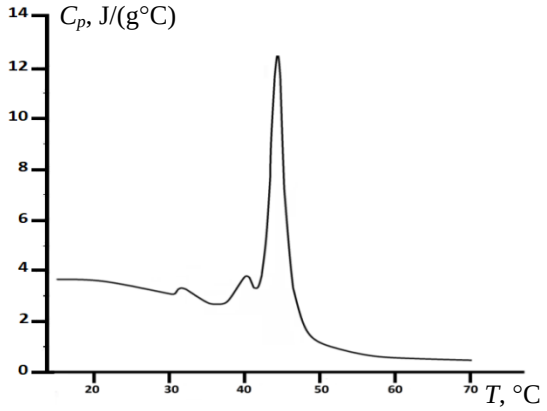

Fig. 6. Temperature dependence of heat capacity of DPPC liposome suspension prepared in 20 % glycerol. Temperature scanning speed V = 2°C/min

prepared both pure and $CaCO_3$ containing complex liposomes.

Calorimetric measurements were performed on DPPC liposomes prepared both in water (see Fig. 5) and in 20 % glycerol solvent (Fig. 6). Based on calorimetric measurements, we calculated the amount of heat released during melting of liposomes prepared from DPPC lipids.

It was found that the amount of heat released during melting of DPPC liposomes prepared in water is 23.6 MJ, and the amount of heat released during melting of DPPC liposomes prepared in glycerol solvent is 25.4 MJ.

We also conducted experiments on calcium-containing complex DPPA liposomes prepared both in water and in glycerol solvent (Figs. 7 and 8). The temperature dependence curves of the heat capacity of liposomes allow us to talk about the structure of the liposome. Calorimetric studies showed that DPPC liposomes prepared in water and in glycerol solvent, both pure and calcium-containing complex liposomes maintain a multilamellar structure (Figs. 7 and 8).

Based on calorimetric measurements, we calculated the amount of heat released during melting of DPPC liposomes and calcium-complex liposomes. It was found that the amount of heat released during melting of calcium-containing liposomes prepared in water is 15 MJ, and the amount of heat released during melting of calcium-containing liposomes prepared in glycerin solvent is equal to 18 MJ. The fact that calcium ions really bind to the structure of liposomes prepared from DPPA and DPPC lipids, both in water and in glycerol solvent, was also demonstrated by spectrophotometric studies. Fig. 9 shows the absorption wavelength dependence curves of both pure DPPA liposomes and calcium-containing DPPA liposomes prepared in water. We also performed spectrophotometric measurements on DPPC liposomes prepared in water. Fig. 10 shows the absorption wavelength dependence curves of both pure DPPC liposomes and calcium -containing DPPC liposomes prepared in water.

On the spectrophotometric curve, in the case of pure liposomes, a small absorption peak will appear at a wavelength of 235 nm, which disappears in the case of calcium-containing complex liposome, which also indicates that calcium ions bind to both DPPA and DPPC lipids in complex liposomes.

Discussion

It should be noted that the recording of calorimetric peaks occurs as a result of the breaking of hydrogen bonds, which are formed between the hydrophilic heads of phospholipids. namely, between the O=P–OH group of one phospholipid and the C=O group of another phospholipid. From our studies, it can be seen that the breakdown temperature of the ordered structure of DPPA liposome is 24^0C higher (66^oC) than that of pure liposome prepared from DPPC lipids (42^0C) (see Figs. 1, 2, 5, 6). Considering that DPPC and DPPA phospholipids have the same tail length and composition, it is clear that the difference in transition temperatures must be due to the different interaction forces between the lipid heads. The energy of hydrogen bonds between the heads of DPPA lipids is greater than the energy of hydrogen bonds between the heads of DPPC lipids. Because the DPPA lipid heads are smaller in size than the DPPC lipid heads, this allows the hydrophilic DPPA lipid heads to come closer together and therefore fit more tightly. This is due to the fact that the length of the hydrogen bonds between the DPPA lipid heads is shorter and therefore stronger than the hydrogen bonds between the hydrophilic heads of DPPC lipids. At the same time, strong hydrogen bonds are the reason that the structure of pure DPPA liposomes will be single-layer, i.e. unilaminar, which is confirmed by the calorimetric record of pure DPPA liposome prepared in water, which is narrow and cooperative (Fig. 1). As for DPPC liposomes, its structure can be multi-layered, i.e. multi-laminar, where it becomes possible to form new hydrogen bonds between the layers in the inner volume of the liposome. This is confirmed by the shape of the calorimetric peak of heat absorption, which in the case of DPPC liposome is not cooperative and contains so-called pre-peaks before the main transfer peak, which in our opinion is due to the presence of a multilamellar structure of the liposome (see Fig. 5).

The hydrogen bond energy of DPPA and DPPC liposomes prepared in the presence of glycerol is greater than the hydrogen bond energy of DPPA and DPPC liposomes prepared in water because glycerol can form additional hydrogen bonds with the heads and tails of DPPA and DPPC lipids, because of this bonds, they can form a multi-layered, multi-laminal structure and establish bonds between neighboring layers.

The calorimetric curve of DPPA liposomes prepared in glycerol becomes non-cooperative instead of cooperative and additionally a small peak appears in the region of 75 °C (see Fig. 2). This may be due to the additional hydrogen bonds that glycerol forms with DPPA lipids. These formed bonds are stronger than the hydrogen bonds between lipids. It is also known from the literature that upon addition of glycerol, the tilt angle of the DPPC lipid tails changes so that they become parallel to each other [16, 32],which allows them to, come closer to each other and therefore form stronger hydrogen bonds than DPPC lipids prepared in water,which also determines the amount of heat released during melting.

It is known from the literature that the calcium ion binds to the phosphate group of all types of phospholipids and forms a bond with them [5, 18, 25, 26, 36, 37]. which competes with the hydrogen bonds between phospholipids, so not all phospholipids are able to form hydrogen bonds with each other [13, 17]. As a result, the amount of heat released during melting of calcium-containing liposomes is smaller than the amount of heat released as a result of melting of pure liposomes.

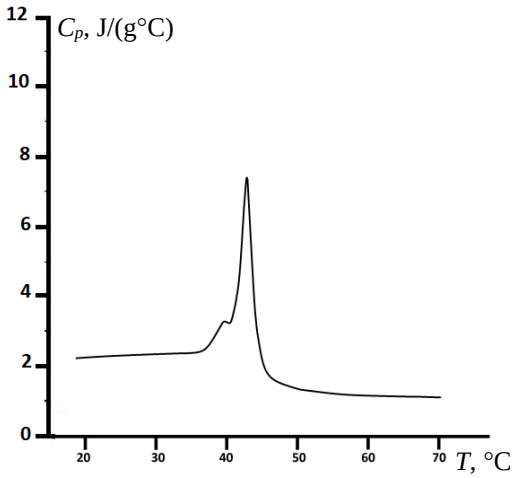

Fig. 7. Temperature dependence of the heat capacity of calcium-containing DPPC liposomes prepared in water. Temperature scanning speed V = 2°C/min

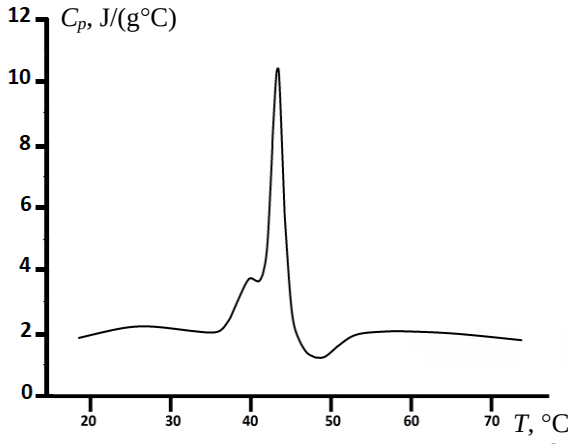

Fig. 8. Temperature dependence of heat capacity of calcium-containing DPPC liposome prepared in 20 % glycerol. Temperature scanning speed V = 2°C/min

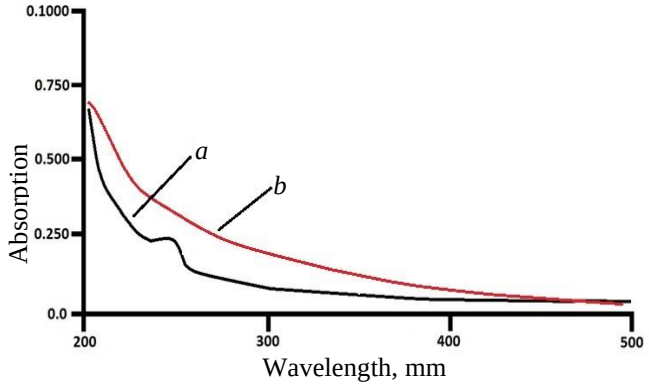

Fig. 9. Absorption dependence curves on wavelength of: *a* – pure DPPA liposomes; *b* – calcium-containing complex DPPA liposomes in water

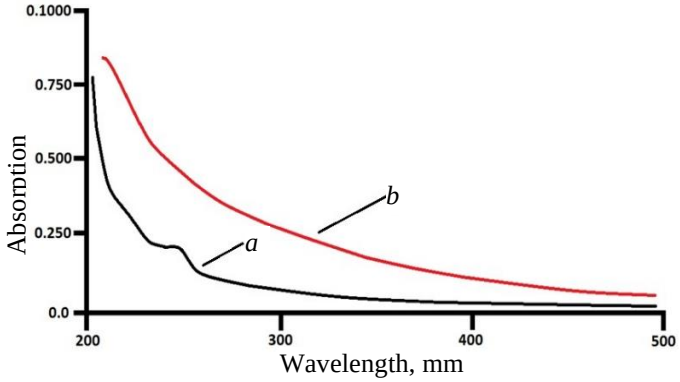

Fig. 10. Absorption dependence curves on wavelength of: *a* – pure DPPC liposomes; *b* – calcium-containing complex DPPC liposomes in water

The structure of calcium-containing complex liposomes prepared in glycerin is changed compared to pure liposomes prepared in glycerin, because calcium competes with the additional hydrogen bonds that glycerol forms with DPPA and DPPC lipids, and the resulting structure has a reduced number of phospholipid layers for both DPPA lipids and DPPC lipids, as confirmed by calorimetric experiments (see Figs. 4 and 8). According to the calorimetric experiments, we can see 1 peak on the calorimetric curve of the complex calcium-containing DPPA liposomes prepared in glycerin, in contrast to the pure DPPA liposomes prepared in glycerin (Fig. 2). As a result, we can conclude that unlike pure DPPA lipids prepared in glycerol solvent, complex calcium-containing liposomes form a unilamellar structure (Fig. 4). As for the calcium-containing complex DPPC liposomes prepared in glycerol solvent (Fig. 8), in contrast to pure, uncomplexed DPPC liposomes prepared in glycerol (Fig. 7), we have only one additional pre-peak in the calorimetric record, as a result, we can assume that the number of layers, decreased in the case of complex liposome.

In addition to calorimetric experiments, the formation of a complex between liposomes and calcium ions is also confirmed by spectrophotometric experiments. On the spectrophotometric curve, in the case of pure liposomes, a small absorption peak will appear at a wavelength of 235 nm, which disappears in the case of calcium-containing complex liposome, which also indicates that calcium ions bind to both DPPA and DPPC lipids in complex liposomes.

Conclusion

The calorimetric method is not a structural method, however it is possible to talk about the ordered structure of macromolecules by the shape of the heat absorption peak obtained during melting of macromolecules. In particular, the cooperative and narrow phase transition peak of melting of the liposome indicates that one type of bonds are broken in the structure. Whereas, several peaks and/or an increase in the width of the temperature transition at half the height of the heat absorption peak indicate that we are dealing with a non-cooperative process. Unlike uncomplexed, pure DPPA liposomes in experiments, whose temperature breakdown is cooperative (transition width at half height of the peak is 1.8 degrees), the peak shape of calcium-

containing DPPA liposomes during the temperature transition process is multi-peaked, therefore we have a non-cooperative process. All this allows us to conclude that uncomplexed DPPA liposomes should be simple, single bilayer, unilaminar nanoparticles. Accordingly, the structure of calcium-containing DPPA liposomes should be multi-layered, as evidenced by thefact that the number of heat absorption peaks is already two instead of one. Accordingly, we can conclude that the structure of calcium-containing DPPA liposomes is multi-layered, multi-laminar. Multilaminar structure of liposomes allows to incorporate a large amount of calcium into it. Therefore, the therapeutic use of such nanoparticles should be effective without any side effects.

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