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Thermodynamic Properties of Calcium-containing DPPA and DPPC Liposomes Made by Novel Nanotechnology

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KEYWORDS

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ABSTRACT:

The work is about the preparation of calcium-containing 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA)and their calorimetric study. We determined the possible structure of calcium-containing liposomes made by our new technology and determined their thermostability. The paper provides calculations showing how many phospholipid molecules are required to make a 200 nm diameter liposome. Calculations showed that 33x103 lipid molecules are needed to prepare one DPPA and DPPC liposome. Based on the calorimetric experiments, we determined that the structure of uncomplexed DPPA liposomes is unilaminar (one double layer), while DPPC liposome is a nanoparticle with a multilaminar (multilayer) structure. This was determined by the cooperativity of the heat absorption peak. Calorimetric studies of calcium liposomes made by our technology showed that calcium ions are placed in the multilaminar structure of the DPPC liposome. Calcium ions also formed a complex in the DPPA liposome structure, moreover, calcium made the DPPA liposome multilaminar, since the cooperative narrow heat absorption peak was transformed into a three-peak heat absorption peak. Since both types of liposomes in complex with calcium ions present a multilaminar structure, where the number of lipid heads in one particle is large, the number of calcium ions in one particle will also be increased. That makes it possible to use these nanoparticles as transporters of a large amount of calcium ions in a living organism.

1. Introduction

One of the main goals of pharmaceutical companies in the world is to improve the results of treatment by taking into account many points of view, including enhancing the therapeutic effectiveness of the drug, as well as minimizing the side effects of the drug [1]. For treatments to be effective, especially when using toxic drugs that cure the disease but damage other tissues, scientists must develop drug carriers that deliver the drug to the target site in the required concentration. In addition, it was necessary that drugs with such high toxicity did not touch other organs and the transferred substance (drug) had to be protected from the destructive action of enzymes. For this purpose, scientists have created various types of bio-nano-transporters, such as dendrimers, liposomes, and others [2]. The lipid composition of liposomes is almost identical to the composition of the cell membrane, and their degradation occurs in the same way as biological membranes. It should also be noted that they do not cause an allergic reaction in the body. The aim of the presented work is to prepare calcium-containing

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liposomes that prevent the side effects caused by hypo/hypercalcemia. It is known that calcium ions play an important role in the human body, in particular they participate in various cellular processes [3],[4].

It should be noted that 99% of the amount of calcium in the body is in the bones, the remaining small amount found in the intracellular and extracellular areas also plays an important role in the functioning of the body [5]. At the same time, an increase in the amount of calcium in the blood (so-called hypercalcemia) can have a negative impact on human health. Hypercalcemia can cause pancreatitis, high blood pressure, kidney stones, severe hypercalcemia inhibits neuromuscular and myocardial depolarization, causing muscle weakness and arrhythmia. Also, the increased concentration of calcium in the blood leads to a decrease in the volume of erythrocytes, which leads to anemia [6]. Another adverse event associated with hypercalcemia is the fact that red blood cells actively promote blood coagulation and thrombus formation, which is initiated by calcium [7],[8].

On the other hand, it should be noted that the low content of calcium ions (hypocalcemia) is also a great danger for the body. It should be noted that calcium levels naturally decrease with age and as a result, the body begins to fill it by removing calcium from the bones. For this reason, people take calcium orally to treat and prevent low calcium levels, muscle cramps, osteoporosis, softening of the bones, as these problems cannot be resolved when in the body there is calcium deficiency. Placing calcium ions in nanoparticles will increase the effectiveness of the drug incorporated into the drug delivery system and, accordingly, the dose of the drug will decrease. For this purpose, it is necessary to select the appropriate chemical composition and structure of the drug-delivery nanoparticles during the preparation of the complex nanoparticle. It is clear that drug-delivery nanoparticles must be made of molecules that are not toxic to living organisms. order for the advantage of nanoparticles to be fully used, it is necessary to minimize immune sensitivity to nanoparticles. In addition, it is important that these nanoparticles have the ability to interact with membranes and be able to enter the cytoplasm through the membrane.

2. Motivation

The goal of the presented work is the production of calcium-containing complex liposomes from natural phospholipids, because such systems will not increase the concentration of calcium in the blood and therefore avoid the complications caused by hypercalcemia. Due to the enzymes in the cell, the nanoparticles that have entered the cytoplasm undergo structural breakdown, they are broken down into their component lipids, as a result of which the calcium ions placed in the nanoparticle are found inside the diseased cell with a large concentration [9],[10].

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 the polar, hydrophilic head by a glycerol bridge [11],[12],[13]. Currently, the process of making liposomes is carried out using different protocols, according to which it is possible to make liposomes with different chemical composition and physical properties (size, number of lamellae, amount of surface charge, internal aqueous volume, etc.) [14],[15],[16]. Our university also managed to prepare phospholiposomes using our technology, where both hydrophobic and hydrophilic drugs can be incorporated into its structure [17], while the amount of drug placed in the liposome is significantly increased.

Degradation of liposomes with such a property in the cells where it is necessary to deliver this drug occurs in the same way as degradation of biological membranes and most importantly, when they get into the body, they do not cause a negative reaction of the body..

In our case, liposomes made of Dipalmitoyl phosphatidylcholine - DPPC and 1,2-palmitoyl-phosphatidic acid - DPPA phospholipids are used as drug-delivery nanoparticles. By incorporating hydrophobic drugs into liposomes, the final product becomes water soluble, which is also important. It is appropriate to study the structure and stability of complex liposomes containing drugs using differential scanning calorimeter (DSC) [18],[19],[20]. During the preparation of drug and liposome complexes, in order to determine their ratio, the number of lipids needed to

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create a liposome with a diameter of 200 nm is calculated in the paper.

3. Preparation Method of Dppa and Dppc Liposomes

In the experiments, liposomes were used, which were produced using a new, simple technology developed by us [21]. Two types of phospholipids were used to make liposomes1,2-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).The production technology for both lipids was similar. We studied the thermal stability of calcium-containing nanoparticles using a calorimeter. We chose calcium chloride ($CaCl_2$) as a calcium salt, and DPPA and DPPC lipids were used as lipids.

Since the liposome is a spherical lipid bilayer with a thickness of h=5 nm and a diameter of d=200 nm, we calculated the surface area for such a spherical nanoparticle as follows. We have taken 2 concentric spheres, the radii of which are d/2 and (d/2-h) respectively. If we take into account that the total surface area of the spherical liposome, which consists of two outer and inner layers, will be calculated by the formula $S=4\pi(\frac{d}{2})^2+4\pi(\frac{d}{2}-h)^2$.

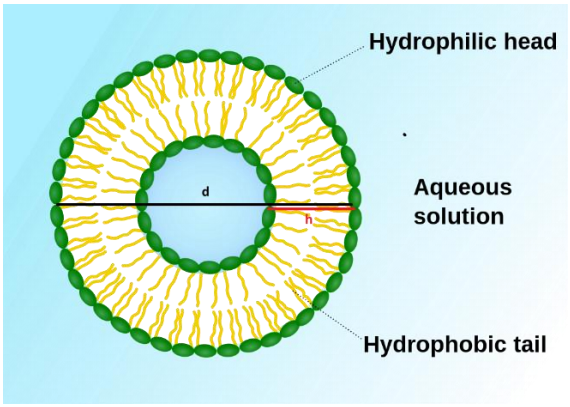

Fig. 1. Liposome structure

To calculate how many lipids are needed to make one liposome with a diameter of 200 nm, we need to divide this area by the area of one lipid, which according to the literature is equal to a = 0.71×10^(-18) m^2. Accordingly, the formula that will be used to calculate the amount of lipids required for the preparation of liposomes with a diameter of 200 nm has the following form:

$$N=\frac{4\pi(\frac{d}{2})^2+4\pi(\frac{d}{2}-h)^2}{a}$$

Taking these data into account, we can write

$$N=\frac{4\times3.14(\frac{200\times10^{-9}}{2})^2+4\times3.14(\frac{200\times10^{-9}}{2}-5\times10^{-9})^2}{0.71\times10^{-18}}\approx 330\,000\approx3.3\times10^5 \quad (2)$$

As confirmed by the obtained calculations, 3.3×10^5 lipids are needed to make 1 liposome with a thickness of 5 nm and a diameter of 200 nm.

We used the calorimetric method to determine the thermodynamic parameters of liposomes, both uncomplexed and complex, calcium-containing DPPA and DPPC liposomes, as drug-delivery nanoparticles. The thermodynamic study of liposomes was carried out using a precise DASM-4 microcalorimeter (Pushchino, Russia), which belongs to high sensitivity type heat flow calorimeters (Privalov & Potekhin, 1986).

4. Results

Initially, calorimetric experiments were performed on uncomplexed, pure DPPA and DPPC liposomes prepared by our new nanotechnology. For this purpose, in the case of both lipids, 1 mg of lipid was used.

A temperature range of $0\text{-}100^0$C was selected for calorimetric measurements of liposomes, with a temperature scan rate of 2^0C/min.

Fig.2 shows the calorimetric curve of liposomes made from DPPA lipids.

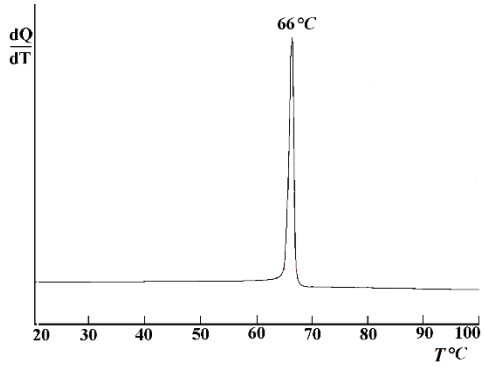

Fig. 2. Temperature(T) dependence of liposome suspension's heat capacity(dQ/dT), temperature scanning speed V=2°K/min

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As can be seen from the curve, a single cooperative narrow absorption peak with a temperature maximum at 66^0C is obtained, indicating that one type of bond breaking occurs in the liposomes.

The calorimetric study of DPPC liposomes is shown in Fig. 3

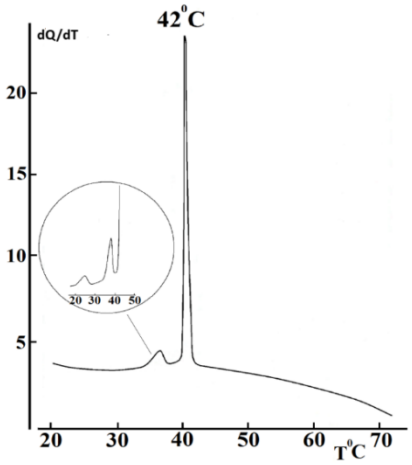

Fig. 3. Temperature(T) dependence of liposome suspension's heat capacity(dQ/dT), temperature scanning speed V=2°K/min

According to the graph, the ordered structure of DPPC liposomes undergoes thermal decomposition in several temperature ranges. Also, DPPC liposomes are less thermostable than DPPA liposomes.

We note that the difference between the heights of the heat absorption peaks at 24^0C and 42^0C degrees is large, and this heat absorption peak (at 24^0C) can be detected only by selecting high concentration and high sensitivity parameters of the instrument.

In the case of DPPC liposome on the calorimetric curve, the presence of so-called pre-peaks should be related to the complex structure of the liposome.

In addition to the mentioned pure DPPA and DPPC liposomes, calcium- containing complex DPPA and DPPC liposomes were prepared and measured by us. The amount of $CaCl_2$ required for their preparation was selected according to the above calculations. However, it should be noted that the amount of calcium we took is more than the amount of calcium obtained by calculation, because calcium may be placed not only at the lipid heads, but also in the free volume of liposomes. We also consider that $CaCl_2$ dissociates into ca and cl ions, and only Ca binds in the liposome structure, which is why the amount of $CaCl_2$ during the formation of complexes was 0.4 mg, and the amount of lipids was 1 mg.

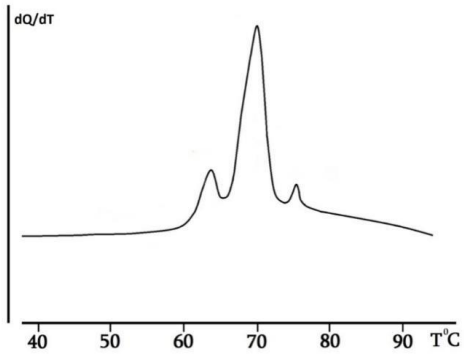

Fig. 4. Temperature(T) dependence of complex DPPA/$CaCl_2$ liposomes suspension's heat capacity (dQ/dT), temperature scanning speed V=2°K/min.

As can be seen from the calorimetric record, the heat absorption peak shape of the complex liposome is completely different from the heat absorption peak shape of pure DPPA liposomes (see Fig. 2) It is clear that the reason for such a radical change in the nanoparticle structure is the participation of calcium ions in the formation of DPPA phospholipid liposomes. Moreover, we think that the reason for such a change in structure must be in our technology of preparation of nanoparticles.

Using a similar method, we prepared complex liposomes from $CaCl_2$ and DPPC lipids, the calorimetric curve of which is shown in Fig. 5.

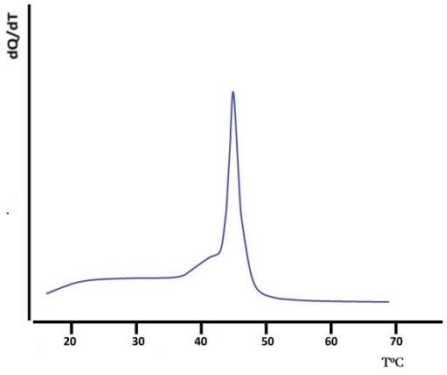

Fig. 5. Temperature (T) dependence of complex DPPC/$CaCl_2$ liposomes suspension's heat capacity (dQ/dT), temperature scanning speed V=2°K/min.

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The heat absorption peak shape of complex DPPC/CaCl2 obtained by calorimetric experiments is completely different from the heat absorption peak shape of pure DPPC liposomes (see Fig. 3). The heat absorption peak shape of complex DPPC/$CaCl_2$ obtained by calorimetric measurements is completely different from the heat absorption peak shape of pure DPPC liposomes (see Fig. 3).

5. Discussion

According to literature, thermostability of liposomes, including liposomes derived from DPPA and DPPC lipids, is different. It is known that the longer the hydrocarbon chains in the molecule, the higher the phase transition temperature. During the phase transition, changes occur in the lipid bilayer, in particular, in the gel state, the fatty chains of lipids are closer to each other. As the temperature rises, the fatty chains become more mobile and the lipid bilayer becomes thinner. [22]

It is important to know the thermostability of liposomes as drug- delivery nanosized particles in order to determine the feasibility of their use in living systems. Liposomes with a phase transition temperature lower than the phase transition temperature of a living system cannot be used as drug carriers. The phase transition temperature of the liposomes we use is higher than the temperature of a living organism, which allows us to use them as drug carriers. Our research shows that DPPA liposome is about 25^0C more thermostable than DPPC liposome (see Fig. 2, 3). Since the chemical composition of the hydrophobic tail of liposomes made of DPPA and DPPC lipids is practically the same, their hydrophobic interactions in aqueous solution will be the same. The difference exists in the interaction between the hydrophilic heads of the lipids in the liposome. It is the weak hydrogen bonds between the phospholipid heads that give rise to the heat absorption peaks that are observed in calorimetric experiments. In the case of DPPC liposomes, the presence of a choline group in the hydrophilic head of the lipid decreases the hydrogen bond energy compared to the hydrogen bond energy present in DPPA. This explains the temperature difference in heat absorption between DPPC and DPPA liposomes. In addition, DPPA lipids are more tightly packed and form additional intermolecular bonds with water.[23]

In addition, according to our calorimetric experiments, DPPA and DPPC-liposomes differ not only in thermostability, but also in the shape of the calorimetric peak. In particular, during the calorimetric measurement of DPPA liposomes, only one cooperative heat absorption peak was observed, which is why we can conclude that these liposomes are unilamellar. In the case of DPPC liposomes, the cooperative heat absorption peak is preceded by two more, smaller heat absorption peaks, so-called pre-transition peaks, respectively - the first at 24^0C, and the second at 37^0C temperature maxima (see Fig. 3), which indicates that the structure of liposomes made of DPPC is not simple and it should be multilamellar, that is, the liposome should contain multiple bilayer structures separated from each other by water layers. Here we would like to mention that the water layer between the bilayers inside the liposome should be thin, the structure of which will be more ordered (so-called structural water) than free water, and therefore the connections between the lipid heads of the bilayer will be changed.

It is generally known that the presence of Ca^{2+} in the extracellular space strengthens and regulates the lipid bilayer. Calcium in DPPC lipids may bind to the phosphate group of DPPC lipids as well as to the oxygen of the carbonyl group. It should also be noted that 20% more calcium ions are bound to the phosphate group of DPPC lipids than to the carbonyl group[24],[25]. It is also known that at high calcium concentrations, the calcium ion can bind to every third lipid [24],[25]. After calcium binds to DPPC lipids, the lipid heads gradually increase in size and the thickness of the DPPC bilayer increases. At low concentrations, most of the calcium ions are present deep in the lipid bilayer. Also, the average number of water molecules in the vicinity of phosphate and carbonyl groups of lipids decreases with increasing $CaCl_2$ concentration [24],[25] and their place is taken by calcium ions. Quite interesting is the result that was revealed during the calculation of the amount of heat that was released as a result of the impact of temperature, both during the destruction of the structure of the pure liposome and calcium-containing complex liposomes. It was found that the amount of heat released during the breakdown of the complex DPPC liposome structure containing calcium is less than the amount of heat released during the breakdown of the structure of pure DPPC

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liposomes, because the concentration of water around the liposomes decreases, less hydrogen bonds are established. As for DPPA liposomes, they have fully saturated acyl chains[23] and smaller heads than DPPC molecules, allowing for stronger binding. In addition, PA groups can form intermolecular hydrogen bonds with water. After Ca interacts with DPPA liposomes, calcium is located on the surface of liposomes, so that DPPA liposomes have the ability to form additional intermolecular hydrogen bonds with water, which is also shown by the experimental results. In particular, compared to pure DPPA liposomes, the amount of heat released as a result of melting of calcium-containing complex DPPA liposomes is large.

The structure of the liposome made of pure DPPA lipids, as previously mentioned, is unilamellar, as indicated by a single cooperativity curve undergoing a structural breakdown at 66^0C. However, the calorimetric curve of DPPA complex liposomes containing $CaCl_2$ is absolutely different from the pure liposome curve. On the calorimetric curve of the complex liposome, it can be clearly seen that the ordered structure of the complex liposomes undergoes thermal destruction in several temperature intervals near the temperature of 62^0C, 69^0C and 76^0C (see Fig. 3). Therefore, we can conclude that the structure of the complex liposomes formed by the interaction of DPPA liposomes and Ca is not simple and it should be multilamellar,so , the complex liposome should contain multi-layered structures separated from each other by water layers. We think that CaCl2 dissociated into Ca and Cl ions. Here we note that water is contained in the inner volume of the nanoparticle, which is hermetic from the environment of the nanoparticle. When the sample was heated in the calorimeter ampoule, the hermetically sealed water began to expand, which caused an increase in the size of the nanoparticles and a stretching of the lipid bilayers. It is clear that depending on the multilaminar structure and geometry of the particles, the lipid bilayers will be stretched by different forces, in particular, nanoparticle bilayers with smaller diameters will be stretched by more forces than those with larger diameters. As a result, under certain stresses, it is possible for a small diameter lipid bilayer to fuse/integrate with a neighboring bilayer. As already mentioned, when multilamellar complex liposome is heated, the water contained in its internal volume begins to expand, as a result the membrane expands, when the tension reaches a critical limit, the bonds between lipids in the membrane are broken. That is why we think that this should be the reason for the presence of pre-transition peaks, which appear on the temperature dependence curve of heat absorption of DPPA / CaCl2 complex liposomes. The calorimetric curve also allows us to determine that during the preparation of liposomes complexed with DPPC lipids and calcium salts, the divalent calcium ion creates electrostatic bonds between the layers of DPPC multilaminar liposomes, which is revealed by the change of low-temperature calorimetric heat absorption peaks. In particular, unlike the calorimetric curve of the pure liposome, where two small peaks appear before the main transition peak, these small peaks disappear in the calorimetric curve of the complexed calcium-containing liposome. Which gives us the possibility to assume that the calcium ions are connected with the layers in the inner volume of the multilamellar liposome, which caused the aggregation of the small liposomal bilayers inside the liposome and, accordingly, the change of the low temperature peaks.

6. Conclusion

In the initial stage of liposome preparation, phospholipids and calcium chloride are intensively mixed, where the formation of ionic bonds between phospholipid and calcium occurs (see the protocol of the described method). The consequence of this is that, unlike pure DPPA liposomes, which are obtained with a unilaminar structure, calcium-containing liposomes are multilaminar. This is confirmed by the formation of additional small heat absorption peaks instead of the cooperative heat absorption peak.The addition of calcium ensures the formation of a multilamellar structure of complex nanoparticles (in the case of DPPA liposomes), which indicates that a large amount of calcium ions can be found in these nanoparticles. As for the liposome made of DPPC lipids, they are already multilamellar, therefore, when calcium is added, a large amount of calcium ions will be found in them. Thus, we can conclude that the multilamellar structure helps the nanoparticle to contain as many calcium ions as possible in the structure of both DPPC and DPPA liposomes. This also means that liposomes made of DPPA and DPPC lipids can be used as good calcium transporting nanoparticles.

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