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Biophysical study of calcium-containing DPPC liposomes prepared in water and a glycerol aqueous solution

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Abstract. In this study, both pure and calcium-containing complex liposomes made from DPPC phospholipids were investigated using calorimetric and spectrophotometric methods. Liposomes were prepared using a new technology in both water and a 20% glycerol aqueous solution. Glycerol allows drug-containing DPPC liposomes to penetrate the dermis of the skin through the epidermis. Calorimetric and spectrophotometric experiments show that calcium ions are incorporated into the structure of DPPC liposomes. Consequently, these nanoparticles can be used to treat diseases that require a significant amount of calcium, ensuring that the calcium within the liposomes will not cause side effects when it enters the bloodstream. Through the conducted experiments, we examined the structure and thermal stability of calcium DPPC liposomes prepared in water and glycerol, which is essential for their effective practical use. We found that the structure of all complex liposomes is multilamellar, which enables them to incorporate a larger amount of calcium ions than unilamellar liposomes. Based on the calorimetric experiments conducted, we identified a new approach to determine the maximum amount of drug, including calcium, that can be incorporated into nanoparticles, which is a crucial factor.

Key words: DPPC liposomes — $CaCl_2$ with liposome complex — Differential scanning calorimetry method — Spectrophotometric method

Introduction

Elderly individuals often suffer from bone-related diseases such as osteoporosis and osteopenia. Effective and timely treatment of these conditions is challenging, resulting in prolonged treatment periods that negatively impact patients' quality of life and significantly increase healthcare costs (Bose and Tarafder 2012; Balmayor and van Griensven 2015; Carrick and Costner 2018). These diseases are typically treated with medications that target the condition but may also cause side effects. Drug transporters offer a solution by minimizing drug toxicity and delivering the medication directly to the site of injury. This targeted approach can make treatment faster and more effective, as it increases the concentration of the drug in the affected area while protecting surrounding tissues and organs from potential damage. Nanoparticles containing drugs can be considered as carriers of such drugs, because in the structure of one nanoparticle it is possible to place a drug in a large concentration, including drugs of a toxic nature for the body. Such nanoparticles can be made of phospholipids that make up cell membranes of living organisms. Therefore, such phospholipids are not toxic to the human body. At the same time, drug-containing nanoparticles can be delivered directly to the healing organ, where their structure will be broken down and from their structure, the drug is released in a large concentration. For example, calcium ions can be encapsulated in phosphatidylcholine liposomes (Shin et al. 2020). Moreover, researchers

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have developed calcium-responsive liposomes that release their contents in response to elevated calcium levels at target sites (Lou and Best 2020).

The presented work discusses the possible use of nanoparticles containing calcium ions in the treatment of diseases caused by calcium deficiency in the body. It is known that 99% of the amount of calcium in the body is in the bones, and the remaining amount is in the blood, where its normal concentration should not exceed 2.1–2.55 mmol/l (Schnellmann 2010). Therefore, the increase in the concentration of calcium in the blood causes a number of diseases, such as pancreatitis, high blood pressure, kidney stones (Bilezikian et al. 2016). Severe hypercalcemia impairs neuromuscular and myocardial depolarization, leading to muscle weakness and arrhythmias. Also, the increased concentration of calcium in the blood causes a decrease in the volume of erythrocytes, which in turn causes anemia. 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 (Carrick and Costner 2018; John 2018). On the other hand, it should be noted that the low amount of calcium ions in the body (hypocalcemia) is also a great danger, because during hypocalcemia the number of fractures increases (mostly in older people) (Tinawi 2021), the cause of which is osteoporosis disease (Vo et al. 2012), and this number reaches 8.9 million *per* year (Gass and Dawson-Hughes 2006; Pharande et al. 2014). For this reason, people take calcium orally to treat and prevent low calcium levels, muscle cramps, osteoporosis, softening of the bones (Salgado and Coutinho 2004), as these problems cannot be resolved when the body is deficient in calcium. The essence of placing calcium in nanoparticles lies in the fact that, unlike the use of calcium drugs in their pure form, it is possible to increase the amount of the drug in the destination where there is a calcium deficiency. Therefore, in the presented work, calcium-containing liposomes prepared from phospholipids included in the membranes of living human cells have been studied as calcium transporters. Our simple new technology makes it possible to produce calcium liposomes that can be used as calcium transporters in calcium-deficient areas of the human body (Shekiladze et al. 2017). Such complex liposomes have a number of advantages. On the one hand, the use of phospholipids eliminates the toxic effect of liposomes (Shaheen et al. 2006), and such liposomes are carriers of a large amount of calcium drugs, so that any side effects of hypercalcemia in the body are excluded. Both pure DPPC liposomes and complex DPPC liposomes containing calcium chloride were studied in this manuscript, both deionized water and 20% glycerol aqueous solution were used as solvents. It was decided to use glycerol because with glycerol it is easier to deliver calcium through the epidermis of the skin, since it is known from the literature that glycerol together with the drug has the ability to cross the skin barrier (Björklund et al. 2013). In order to use drug-containing nanoparticles as a means of transporting drugs in a living system, it is important that their dimensions are such that, on the one hand, it is possible to place a certain amount of drugs in its structure, and they can penetrate into tissues and cells. Because of this, it is accepted that there are some effective sizes for drug-carrying nanoparticles, ranging from a few units to 250 nanometers in diameter (Cullis and Kruijff 1979; Singh and James 2009). At the same time, drug-containing nanoparticles should not be prepared from toxic molecules, their structure should be stable under certain conditions. Therefore, thermodynamic and spectrophotometric studies of nanoparticles will answer many questions (Mabrey and Sturtevant 1976; Mdzinarashvili et al. 2016, 2023). Calorimetric studies are not directly a structural method, but depending on the structure of the studied object with its thermodynamic properties (specific heat capacity and the amount of absorbed/released heat), we can judge the structure of the object. We hope that research of drug nanoparticles by thermodynamic and spectrophotometric methods will provide an opportunity to find out the structure of calcium liposomes and their stability depending on temperature and time (Shekiladze et al. 2017).

Materials and Methods

Liposomes used in this study were made of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). DPPC lipids were purchased from Lipoid (Newark, New Jersey). Both pure and calcium-containing liposomes were prepared using our new technology, which is faster than the existing technology and is cost-effective (Mdzinarashvili et al. 2016; Shekiladze et al. 2017). In brief, the mixture of hydrophilic biologically active substances $CaCl_2$ and DPPC lipids should be dissolved in 50 μl water or in 50 μl 20% glycerol at room temperature, followed by intensive shaking. DPPC lipids were used at a concentration of 1 mg/ml, while calcium chloride was used at two concentrations – 0.3 mg/ml and 5 mg/ml, considering the requirement to prepare complex liposomes in which the added calcium chloride would be completely incorporated (see calculations below). In next step, 1 ml of water heated to 70°C is added to the above-mentioned mixtures, and after 2–3 min vigorous mechanical shaking the complex liposomes are formed. At this stage the sizes of the liposomes are not uniform. In order to equalize the liposome diameters, as a final step the suspension of complex liposomes is extruded through a nanoporous membrane.

Thermodynamic studies of liposomes were performed on a highly sensitive differential scanning calorimeter DASM-4 microcalorimeter (Pushchino, Russia). Spectrophotometric

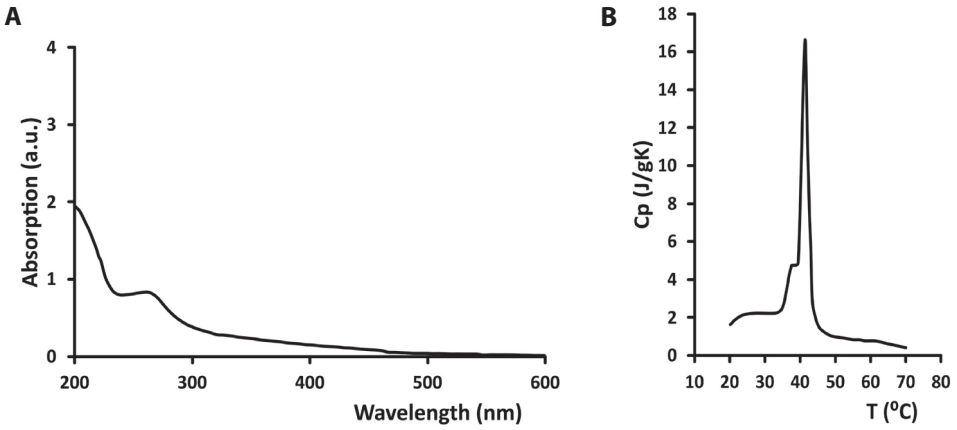

Figure 1. Spectrophotometric and differential scanning calorimetric measurements of DPPC liposomes prepared in deionized water. **A.** Absorption spectrum of pure DPPC liposomes. **B.** Temperature dependence curves of the specific heat capacity (Cp) of pure DPPC liposomes.

studies of liposomes and calcium liposomes were performed using an Ultraviolet/Visible High Sensitivity Spectrophotometer UVS-3800 (USA). Zetasizer Nano ZS from Malvern Instruments Ltd. (Malvern, UK) is used to study the sizes and zeta potential.

Results

It should be mentioned that in all calorimetric experiments carried out in this work, the temperature scanning speed was V = 2 K/min.

Figure 1A shows the optical spectrum of liposomes prepared with our new technology from 1 mg/ml DPPC lipids in deionized water. The presented spectrum confirms the formation of DPPC liposomes. In particular, the formation of liposomes is confirmed by the slope of the spectrum in the high wavelength region (200–500 nm) with respect to the light wavelength axis. In this wavelength region, liposomes are scattered by the Rayleigh scattering law (Strutt 1871). A small peak near 250 nm should be due to the presence of a phosphocholine chemical group in the DPPC lipid head.

A differential scanning calorimeter study was conducted on the mentioned liposome suspension, the result of which is shown in Figure 1B. As can be seen from the obtained graph, the thermal degradation of pure DPPC liposomes is multistep and the phase transition of the liposome structure is in the interval of 35–50°C, with the maximum peak at 42.4°C.

Pure DPPC liposomes using the new technology were also prepared in 20% glycerol aqueous solution. The optical spectrum of liposomes is shown in Figure 2A. According to the spectrum, we also have light scattering according to Rayleigh's law, which unambiguously confirms the presence of DPPC liposomal vesicles in 20% glycerol aqueous solution. However, the short-wavelength peak observed in pure DPPC liposomes prepared in water disappears in the optical spectrum of pure DPPC liposomes prepared in 20% glycerol solution since glycerol binds to both the phosphate group and the choline group (Terakosolphan et al. 2018). A microcalorimetric experiment was performed on these liposomes, the result of which is shown in Figure 2B. There are many differences between the calorimetric curves of DPPC liposomes prepared in 20% glycerol aqueous solution and in water (see Fig. 2B). There is also a difference between the temperature intervals of the phase transition. In particular, the phase transition interval of DPPC liposomes prepared in 20% glycerol is increased (30–50°C), compared to the phase transition temperature interval of DPPC liposomes

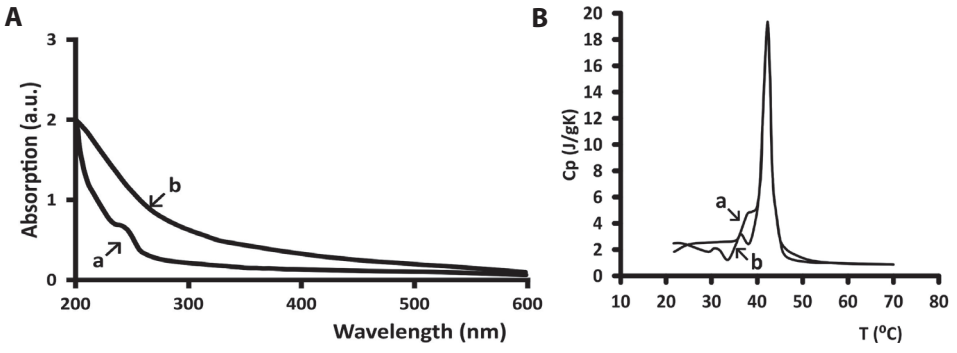

Figure 2. Spectrophotometric and differential scanning calorimetric measurements of DPPC liposomes. **A.** Absorption spectrum of DPPC liposomes prepared in deionized water (a) and in 20% glycerol aqueous solution (b). **B.** Temperature dependence curves of the specific heat capacity of DPPC liposomes prepared in deionized water (a) and in 20% glycerol aqueous solution (b).

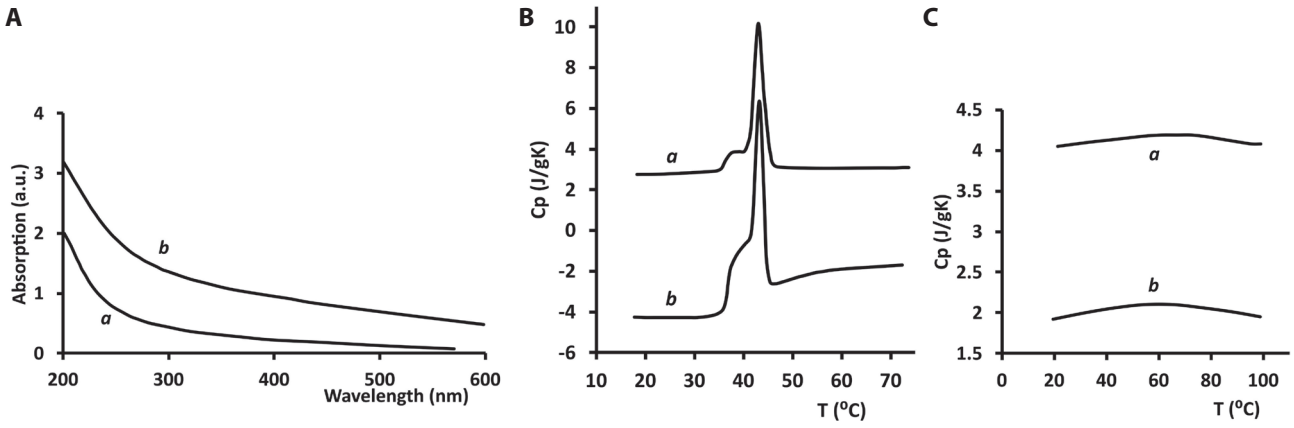

Figure 3. Spectrophotometric and differential scanning calorimetric measurements of DPPC liposomes. **A.** Absorption spectrum of calcium-containing of DPPC liposomes prepared with 0.3 mg/ml (a) and 5 mg/ml (b) of $CaCl_2$ in water. **B.** Temperature dependence curves of the specific heat capacity of calcium-containing DPPC liposomes prepared with 0.3 mg/ml (a) and 5 mg/ml (b) of $CaCl_2$ in water. **C.** Temperature dependence curves of the specific heat capacity of pure water (a) and of 5 mg/ml of $CaCl_2$ aqueous solution (b).

prepared in deionized water (35–50°C). An additional phase transition prepeak also appeared at 32°C and the maximum peak is increased to 42.5°C for liposomes prepared in 20% glycerol. Also the glycerol aqueous solution is increasing the heat absorption from 23.5 J/g to 35.6 J/g.

Regarding the preparation of calcium-containing liposomes, two concentrations of calcium chloride were selected for experiments: 0.3 mg/ml and 5 mg/ml of $CaCl_2$, which were incorporated into DPPC liposomes. In both cases, liposomes were prepared in deionized water as well as in 20% glycerol aqueous solution. Calculations are made for a unilamellar liposome, which requires 3.3×10^5 lipids to produce. We performed these calculations to obtain the minimum amount of calcium that would be incorporated into the liposome and form a complex. The resulting liposomes were measured using a Zetasizer, which revealed that, compared to pure liposomes with a size of 200 nm, the size of the complex liposomes increased to 300 nm. Additionally, the zeta potential, which was −10.5 mV for pure liposomes, increased to +25 mV for the complex liposomes. Calcium-containing DPPC liposomes prepared in deionized water, in the presence of different concentrations of $CaCl_2$ salt were measured spectrophotometrically and calorimetrically (Fig. 3). The spectrum shows light scattering according to Rayleigh's law, which unambiguously confirms the presence of calcium-containing DPPC liposomal vesicles in water. However, the short-wavelength peak observed in pure DPPC liposomes prepared in water (Fig. 1A) disappeared for calcium-containing complex DPPC liposomes prepared in water (Fig. 3A). A microcalorimetric experiment was performed on complex DPPC liposomes made with 0.3 mg/ml of $CaCl_2$ molecules and 5 mg/ml of $CaCl_2$ in water (Fig. 3B). As shown in the obtained calorimetric curve, the thermal effect of melting for calcium-containing DPPC liposomes (Fig. 3B) is similar to that of pure liposomes prepared in water (Fig. 1B), although a noticeable difference remains. In particular, the maximum value of the peak of the phase transition of the calcium-containing DPPC liposomes is reduced, and accordingly, the specific heat of the phase transition is also decreased from 23.5 J/g to 19.9 J/g in case of complex DPPC liposomes compared to pure liposomes.

Most notably, for DPPC liposomes containing 5 mg/ml of $CaCl_2$, the specific heat capacity decreased sharply at room temperature and became negative (Fig. 3B). This result can be explained by the fact that, with a large amount of calcium, only some of the calcium ions are incorporated into the inner volume of the DPPC liposomes. The excess calcium ions that cannot be incorporated into the structure of liposomes remain in the solvent outside the liposomes. This result can be explained by assuming that the heat capacity of the $CaCl_2$ solution is lower than that of pure water. Differential measurement means that the absolute heat capacities of the samples are not measured; instead, the difference in heat capacities between the samples is determined. In our case, the specific heat capacity of a suspension of complex DPPC liposomes containing 5 mg/ml of $CaCl_2$ was measured relative to pure water. This explains our result: since the specific heat capacity of the calcium-containing liposome suspension is lower than the specific heat capacity of pure water, the difference is negative. In response to the above, we conducted calorimetric experiments to determine how the specific heat capacity of the $CaCl_2$ solution (recalculated to concentration of $CaCl_2$) changes with concentrations of calcium chloride. As shown in Figure 3C, the specific heat capacity of calcium

chloride is lower than the heat capacity of water. Therefore, since the complex liposomes were measured against water in the calorimetric experiments, their curves deviate from the calorimetric curve of pure liposomes. This allows us to conclude that some of the calcium remains outside the complex liposome in the aqueous solution, leading to a decrease in heat capacity relative to that of water. Since, in our opinion, a significant portion of the 5 mg/ml calcium chloride is located outside the nanoparticles in the aqueous environment, we dialyzed this sample against 300 ml of pure water and analyzed the resulting sample calorimetrically (Fig. 4). According to the calorimetric data, the heat capacity at room temperature was again measured as a positive value of approximately 2.1 J/(g×K).

The experiments proceeded with the preparation of complex liposomes in a 20% glycerol aqueous solution. Both calorimetric and spectrophotometric experiments were conducted. Spectrophotometric measurements revealed that the spectra of 0.3 mg/ml and 5 mg/ml of $CaCl_2$ liposomes were practically indistinguishable from each other, although differences were observed in comparison to pure liposomes. The difference between the spectra allows us to conclude that calcium is incorporated into liposomes. Comparison of the absorption spectra indicates that calcium-containing DPPC liposomes are likely larger in diameter than the pure DPPC liposomes, this is confirmed by the increase in the slope of the curve with respect of the wavelength axis (Fig. 5A).

As for the calorimetric experiments, the calorimetric records of both samples are similar (Fig. 5B), differing only in their initial heat capacities. This indicates that some of the calcium is incorporated into the liposome, while some remains in the 20% glycerol aqueous solution. This affects the overall heat capacity of the solution, resulting in a graph that begins with a negative value. The negative specific heat capacity observed for calcium-containing DPPC liposomes prepared in pure water, attributed to the presence of free calcium ions in the liposome suspension (Fig. 5B), can

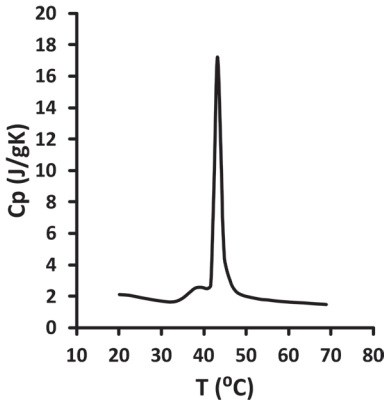

Figure 4. Differential scanning calorimetric measurements of the temperature dependence of the specific heat capacity of the complex of DPPC liposomes prepared with 5 mg/ml calcium salt $CaCl_2$ and then dialyzed against to water.

similarly be applied to the suspension of DPPC liposomes prepared in an aqueous glycerol solution containing calcium. In this case as well, the observed negative specific heat value is likely associated with the presence of free calcium salt in the aqueous glycerol solution at the room temperature. We calorimetrically measured the specific heat capacity of a solution containing 5 mg/ml of $CaCl_2$ dissolved in a 20% aqueous glycerol solution, relative to that of pure 20% glycerol. Measurements showed that the specific heat capacity of the calcium-containing liposome was reduced by 4.5 J/(g×K) at temperatures around 40°C. That is, only a part of the calcium ion was placed in the structure of DPPC liposomes prepared in the 20% aqueous glycerol solution, while the rest was in pure form in the 20% aqueous glycerol solution. This reduces the specific heat capacity of the liposome suspension to a negative value.

The calorimetric curve shows that the heat of melting for DPPC liposomes containing 0.3 mg/ml of calcium prepared in 20% glycerol is similar to that of liposomes prepared in

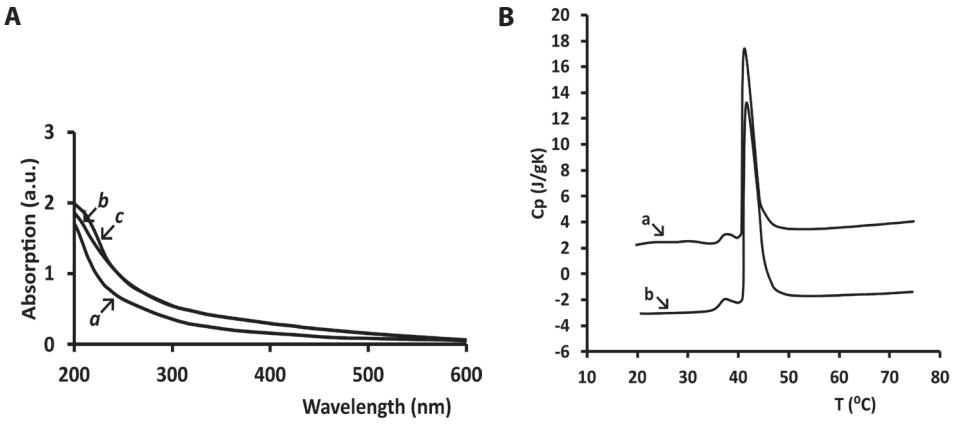

Figure 5. Spectrophotometric and differential scanning calorimetric measurements of DPPC liposomes. **A.** Absorption spectrum of DPPC liposomes prepared in 20% glycerol without calcium (a), in aqueous glycerol solution with 0.3 mg/ml $CaCl_2$ (b) and in aqueous glycerol solution with 5 mg/ml $CaCl_2$ (c). **B.** Temperature dependence curves of the specific heat capacity of calcium-containing DPPC liposomes prepared with 0.3 mg/ml (a) and 5 mg/ml (b) of $CaCl_2$ in aqueous glycerol solution.

deionized water with the same amount of calcium (Fig. 3B and 5B). However, the difference is still noticeable. In particular, the maximum value of the phase transition peak has increased. Difference is also observed between the calorimetric specific heat capacities and the specific heats of absorption. The specific heat of absorption of liposomes containing 0.3 mg/ml of calcium chloride prepared in 20% glycerol (15.2 J/g) is lower than the specific heat of absorption of liposomes containing 0.3 mg/ml of calcium chloride prepared in water (19.9 J/g). However, it is noteworthy that the phase transition temperature does not change.

Discussion

By analyzing the calorimetric results, we can conclude that all the liposomes we prepared – both pure and calcium-containing, whether in water or in 20% glycerol – are multilamellar, meaning their structure consists of multiple nested bilayers. This idea arose from the observation of non-cooperative phase transitions in the calorimetric record, which should be attributed not to the breaking of bonds of the same type but to the disruption of bonds of different types. This occurs due to the breaking of bonds with different energies between the layers in multilamellar liposomes, leading to the formation of prepeaks.

Significant differences were observed between pure DPPC liposomes prepared in water and those prepared in 20% glycerol. In particular, spectrophotometric experiments show that the peak in the optical spectrum of pure DPPC liposomes prepared in water, which appears in the wavelength range of 200–300 nm, disappears in the case of pure liposomes prepared in glycerol. This can be explained by the fact that glycerol binds to the phosphocholine group of DPPC lipids, which is responsible for the formation of the optical absorption peak in the 200–300 nm wavelength range. As for calcium-containing complex liposomes, the goal was to obtain calcium chloride in an amount that would fully bind to the DPPC ligands and facilitate the formation of calcium-containing complex liposomes. To do this, we performed calculations for a unilamellar liposome with a diameter of 200 nm. 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:

$$S = 4\pi\left(\frac{\mathrm{d}}{2}\right)^2 + 4\pi\left(\frac{\mathrm{d}}{2} - \mathrm{h}\right)^2$$

where, d is the dimeter of the liposomes, h is the thickness of the liposomes.

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 $a = 0.71\times10^{-18}$ 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:

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

where, d is the dimeter of the liposomes; h is the thickness of the liposomes, and a is area of one lipid.

Taking these data into account, we can write $N \approx 330\,000 \approx 3.3\times10^5$ lipids.

It is known from the literature that one calcium ion binds to one lipid; however, this ratio may vary depending on environmental conditions and other factors (Zander et al. 2020). We also considered the possibility that some of the calcium might be incorporated into the internal free volume of the liposome within the aqueous environment. Based on these calculations, we used a calcium chloride concentration of 0.3 mg/ml. To ensure that no additional calcium would bind to the DPPC lipids, we also used a higher calcium concentration of 5 mg/ml. However, it should also be noted that since our experiments clearly demonstrated the multilamellar nature of the prepared liposomes, this structure allows for the binding of additional calcium ions. In addition, calcium is also present in the internal volume of the liposome. However, determining the number of layers and quantifying the calcium ions within the internal volume using calorimetric and spectrophotometric methods is not feasible. Consequently, it is impossible to precisely determine the amount of calcium contained in a single liposome.

An interesting result was observed on the calorimetric curve of liposomes made from 1 mg/ml of DPPC lipids and 0.3 mg/ml of $CaCl_2$ prepared in deionized water: the specific heat capacity of calcium-containing DPPC liposomes at room temperature is nearly the same as that of pure DPPC liposomes (Fig. 1B and Fig. 3B). Thus, we can conclude that the structure of the calcium-containing complex liposomes did not change thermodynamically, indicating that the incorporated calcium ions are fully integrated into the liposome structure. As for the liposomes prepared from 5 mg/ml $CaCl_2$ and DPPC lipids, in both water and a 20% glycerol aqueous solution, calorimetric experiments revealed that the specific heat capacity of the complex liposome at room temperature is negative. This result can be explained by the fact that the differential measurement of calcium liposomes was performed

relative to pure water, which has a significantly higher heat capacity than the suspension of calcium DPPC liposomes. As a result, we can conclude that only a portion of the 5 mg of $CaCl_2$ formed a complex with DPPC liposomes, while the rest remains in the water. This reduces the heat capacity of the suspension, leading to a negative heat capacity value in the differential measurement. To confirm these findings, we conducted an experiment in which we passed an aqueous solution of complex liposomes through a dialysis bag placed in a 300 ml beaker of water and measured the resulting suspension in comparison to the aqueous solution outside the dialysis bag. The obtained results are consistent with the calorimetric measurements of the 0.3 mg/ml calcium chloride solution. However, compared to pure liposomes, a difference was noted in the heat associated with the phase transition of the liposomes. This means that calcium only changes the structure of the liposome. Calorimetrically, the temperature dependence of the specific heat during the melting of the liposome is determined. The specific heat of melting for pure liposomes is 25 J/g, while for calcium-containing liposomes, it is 15 J/g. This result indicates that the ionic bonds between calcium ions and the hydrophilic heads of DPPC lipids reduce the number of hydrogen bonds among the lipids in the liposome. As noted in the literature, calcium in DPPC lipids may bind to both the phosphate group and the oxygen of the carbonyl group. Additionally, it has been observed that 20% more calcium ions are associated with the phosphate group of DPPC lipids than with the carbonyl group (Melcrová et al. 2016). Calcium binding increases the size of DPPC liposomes and thickens the lipid bilayer. At low concentrations, most calcium ions are located within the deeper regions of the lipid bilayer. Additionally, the average number of water molecules around the phosphate and carbonyl groups of lipids decreases as the $CaCl_2$ concentration increases (Chiu and Prenner 2012; Szcześ 2016). Calcium ions replace these water molecules, leading to a reduction in the number of hydrogen bonds and that is why the heat of phase transition of calcium liposomes is reduced. It is also noteworthy that for complex liposomes prepared in water, the small peak observed in pure liposomes disappears, and the inclination angle relative to the wavelength axis increases, which is a consequence of the increase in liposome size. This is further confirmed by Zetasizer measurements, which show that, compared to the pure liposome with a diameter of 200 nm, the size of the complex liposome increases to 300 nm. As for the complex liposomes prepared in glycerol, their spectrum is nearly identical to that of the liposomes prepared in water. However, there is a difference in the calorimetric record at room temperature, the specific heat capacity of the complex liposome containing 5 mg/ml $CaCl_2$ becomes negative. This, similar to the calorimetric record of the complex liposome prepared in water, is explained by the fact that part of the calcium is located outside the liposome in the aqueous glycerol solution. It is also noteworthy that for the calcium-containing liposomes prepared in both water and 20% aqueous glycerol solution, the nanoparticle size increased from 200 nm to 300 nm compared to the pure liposome. Also, the zeta potential confirms that we have a suspension of nanoparticles. In the case of pure liposomes, the zeta potential was −10 mV, while for the complex liposome, it increased to +25 mV.

In addition, for all concentrations of both, pure and complex liposomes, prepared in water and glycerol, the phase transitions are the same, and the bonds that stabilize the liposomes remain unchanged. The difference is that the specific heat capacity during the phase transition in glycerol has increased due to a more cooperative peak, even though the overall specific heat capacity remains the same. In the case of pure liposomes in both water and glycerol, the amount of heat is in the range of 20–35 J/g. For liposomes containing 0.3 mg of calcium chloride, the heat is in the range of 15–20 J/g, while for those incorporating 5 mg/ml of calcium chloride, it ranges from 10–20 J/g in both water and glycerol. This means that the hydrogen bonds between the lipids of liposomes prepared in glycerol are similar to those in liposomes prepared in water.

Conclusion

In all experiments, both pure liposomes and calcium-containing liposomes, prepared in either 20% glycerol or water, were produced using the new technology described in the literature (Mdzinarashvili et al. 2016). The spectrophotometric curves of all DPPC liposomes, measured in the ultraviolet region from 200 to 600 nm, show a tilted spectrum with respect to the wavelength axis (Fig. 1A, 2A, 3A, 5A). This effect is attributed to Rayleigh scattering by nanoparticles in the suspension. These results confirm that the described technology can be used to prepare both pure and calcium-containing complex liposomes, whether in water or in 20% glycerol. It should be highlighted that DPPC liposomes prepared in water exhibit a characteristic spectrum in the 200–300 nm range. However, this spectrum changes for DPPC liposomes prepared in 20% glycerol (compare Fig. 1A and Fig. 2A), likely due to an interaction between glycerol and the phosphocholine group of the DPPC lipids.

For calcium-containing liposomes, the spectrum also undergoes a change. Specifically, as shown in Figure 1, pure liposomes prepared in water exhibit a light absorption peak around 250 nm, attributed to the presence of the phosphocholine group in the lipid heads. This peak disappears in the optical spectrum of the complex liposomes prepared in water, suggesting that calcium binds to the liposome head and indicating the formation of a calcium-DPPC liposome

complex. Since the phase transition peak of pure liposomes prepared in glycerol became more cooperative compared to those prepared in water, we conclude that the heat capacity of the phase transition increased in glycerol. However, the total heat remained unchanged, indicating that the hydrogen bonds between lipids were unaffected by the addition of glycerol.

Thus, spectrophotometric and calorimetric experiments confirmed that the new technology we developed produces both pure and calcium-containing complex DPPC liposomes. Although the calorimetric method is not a structural method, it still allows us to infer whether the liposome structure is single-layered or multi-layered. Calorimetric experiments indicated that glycerol increased the number of liposome layers, making them more multi-layered. Since calcium ions are incorporated into the phospholipid layers, the presence of multiple layers in a liposome increases the calcium ion content within a single liposome, thereby enhancing the effectiveness of the drug and delivering more calcium to calcium-deficient areas.

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