Novel technology for the fast production of complex nanoliposomes

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Incorporation of different chemical substances, including medicines, into liposomes has been increasingly evaluated in recent years due to their targeted drug delivery potential. Current technologies of nanoparticle-incorporating liposome preparation are relatively complex. We present a novel method of incorporating complex hydrophobic and hydrophilic molecules as well as simpler substances into dipalmitoylphosphatidylcholine (DPPC) or dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA) liposomes. In order to prepare these complex liposomes, for the first stage it is necessary for ligands and lipids to directly interact, followed by the addition of pH-buffered water or solvent at temperatures slightly above the liposome phase transition temperature. The resulting mixture is briefly but vigorously shaken and then transformed into liposomes of the desired size using an extruder. Particle sizing and calorimetry were used to evaluate liposome formation. This technology is simple, fast and economical, requiring no more than 30 min to prepare the complex liposomes—faster (and less expensive) than current methods. This technology can be usefully added to the armamentarium available to pharmacologists who desire to prepare complex liposomes.

Keywords: calcium, gold nanoparticles, liposome preparation, novel nanotechnology

1. INTRODUCTION

Scientists continue to strive to create medications that have a selective effect on targeted sites (organs, tissues, cells etc.) only. Drug packaging into nanoparticles of different selectivity may provide one of the solutions to this challenge, due to the improved side-effect and toxicity profiles of these formulations. Nanoparticle-enclosed medications do not undergo structural changes and are delivered to damaged tissues without any chemical modification [1–4]. Moreover, different nanoparticles allow the enclosure of a wide variety of chemicals and biologically active molecules (hormones, proteins, DNA, antibodies etc.). Some of these currently in use clinically show remarkable treatment efficacy [5–8]. Phospholiposomes, due to their biosafety (no negative effects on humans), are frequently used in nanosystems [9–11].

Published literature indicates that encapsulated drugs are more effective than the medicine alone. For example, experiments carried out with animals with cancer revealed that liposome-encapsulated doxorubicin circulates in an organism unchanged for extended intervals. The liposome-encapsulated doxorubicin also yielded decreased lung cancer growth and metastasis when used in a clinical setting. Using the liposomes allows treatment to be more effective, with less toxicity [12–15].

Different methods currently used to make liposomes allow their preparation with different chemical compositions and physical properties (e.g., size, number of lamellae, electrostatic surface potential etc.) [16]. Incorporation of both hydrophobic and hydrophilic medications into lipid vesicles can be achieved [17–19]. Degradation of liposomes occurs in the same way as biological membranes, without having negative effects on the host organism.

Passive and active methods of drug incorporation into lipid vesicles are well known. The passive method of complex liposome preparation involves using an organic solvent to dissolve the lipids and drug molecules. After shaking and evaporation of the solvent, a thin lipid–drug layer is formed, which is then dispersed in water and the dispersion extruded to create vesicles of the desired size, with the drug molecules incorporated within them [20–22]. With the active method, medications are incorporated into preformed liposomes. For this method, however, it is necessary to choose liposomes able to absorb drug molecules [23].

In this paper we present a novel technology by which it is possible to incorporate a wide variety of chemical substances into liposomes, in order to facilitate their delivery to target sites. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA) phospholipids were used as the drug delivery systems. With a simple adjustment, this technology allows incorporation of small as well as large hydrophobic and hydrophilic molecules into a liposome. Additionally, the technique is faster and less expensive than those already well established. The technology presented is based on calorimetric evaluation after temperature-induced structural changes of the DPPC and DPPA liposomes (vesicles).

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doi: 10.4024/19MD16A.jbpc.16.04

For preparation of artificial vesicles it needs to mention about usage of cholesterol molecules, which are included in structure of almost all natural cell membrane. Cholesterol gives the rigidity of natural membrane, occupying the space between the hydrophobic tails of the lipids molecule and not allow them to bend. Also cholesterol prevents movement of polar molecules through the cell membrane, which is very important for preparing a complex liposomes.

2. MATERIALS AND METHODS

The mixture of hydrophobic biologically active substances (the water-insoluble drugs, cholesterol) and lipids—DPPC and DPPA (Lipoid, Newark, New Jersey)—(the quantity of which is about 3 mg) should be dissolved in 50 μL ethanol (at room temperature) followed by intensive shaking. In the case of hydrophilic biologically active substances (the water-soluble drugs, metal ions) they and the lipids (about 3 mg) should be dissolved in 50 μL water (at room temperature) followed by intensive shaking. In next step, in both cases, 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 (Fig. 1).

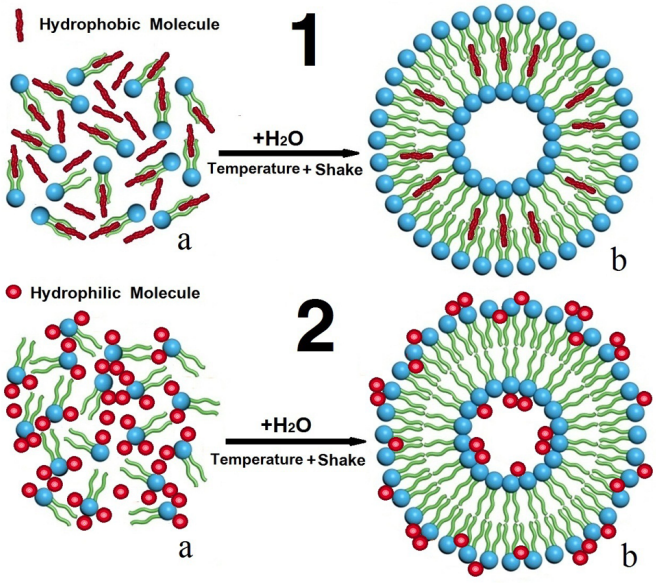

Figure 1. Schemata of the liposome preparation technology:
1. Incorporation of hydrophobic molecules into the liposomes: a, mixture of hydrophobic molecules and DPPC in organic solvent; b, final complex vesicles.
2. Incorporation of hydrophilic molecules into the liposomes: a, mixture of hydrophilic molecules and DPPC in water; b, final complex vesicles.

Calorimetric measurements were carried out with a DASM-4A (Pushchino, Russia) instrument.

Vesicle size measurements were carried out with a (Zetasizer (Malvern Instruments Ltd, Malvern, UK).

In order to evaluate the technology, cholesterol molecules, calcium ions and gold nanoparticles were incorporated into the liposomes. Since cholesterol is almost insoluble in water, it requires several steps to incorporate it into the structure of the liposomes. For this purpose we prepared combined liposomes: 3.1 mg of DPPC and 0.8 mg of cholesterol (molar ratio is 2:1) were dissolved in 50 μL ethanol, followed by intensive shaking, and then 1 mL of water heated to 70 °C was added. After vigorous shaking for 2 min, a liposomal suspension was obtained without cholesterol aggregates. The suspension was extruded through a 200 nm porous membrane to obtain vesicles of desired dimensions, viz. of the optimal size for effective uptake by cells.

In order to incorporate Ca into the vesicles, $CaCl_2$ was chosen as the calcium-containing salt and DPPA as the lipid due to its higher thermal stability. 0.8 mg $CaCl_2$ and 3.1 mg DPPA (molar ratio is 1:1.6) were dissolved in water at 70 °C, heated to 80 °C, and vigorously shaken for 2 min, followed by extrusion of the liposome suspension through a 200 nm membrane filter.

For preparing liposomes with incorporated gold nanoparticles, the method of Turkevich et al. and Frens [31, 32] was used to prepare 24 nm gold nanoparticles beforehand. 3 mg DPPA was added to 1 mL of a 0.54 μM suspension of gold nanoparticles of diameter 24 nm. The obtained suspension was heated in an aqueous bath at 80 °C for 2 min with vigorous shaking, and then extruded through a 200 nm porous membrane. The colour of gold nanoparticles depends on their diameter: small particles (< 40 nm) are red, and larger ones (> 50 nm) are blue [27, 29, 30]. At the end of our process the colour of the suspension had become blue, indicative of the incorporation of gold nanoparticles into the liposomes.

3. RESULTS AND DISCUSSION

Preparation of 200 nm complex DPPC–cholesterol liposomes takes less than 30 min (instead of the 3.5 hours required with the already established methods).

Published data suggest that the phase transition of pure DPPC liposomes takes place at approx. 42 °C [24–26]. Fig. 2 shows our calorimeter date from pure DPPC liposomes, showing their change of heat capacity (ΔC_p) during the phase transition. The existence of ΔC_p during the phase transition indicates that the vesicle structure is opened. The change of C_p can be interpreted as a consequence of the entry of water molecules into the hydrophobic part of the liposome. This mechanism

should provide a pathway allowing a target hydrophobic molecule to be incorporated into the hydrophobic part of the liposome. After cooling the suspension back to room temperature, the vesicles revert to their original structure, trapping the target molecules inside the liposome. During the first step it is impossible to get DPPC–cholesterol liposomes without using an organic solvent. The calorimetric curve obtained using water as a solvent (Fig. 3, curve a) is almost the same as for pure DPPC (Fig. 2), i.e. particularly narrow and sharp, which indicates that the incorporation of cholesterol molecules did not occur, while the calorimetric curve of complex DPPC–cholesterol liposomes prepared by using an organic solvent (Fig. 3, curve b) is indicative of incorporation, showing the necessity of organic solvent during preparation of the liposomes.

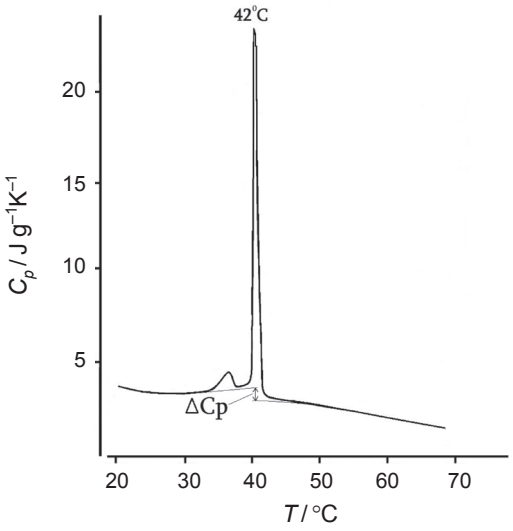

Figure 2. Dependence of heat capacity on temperature for pure DPPC liposomes.

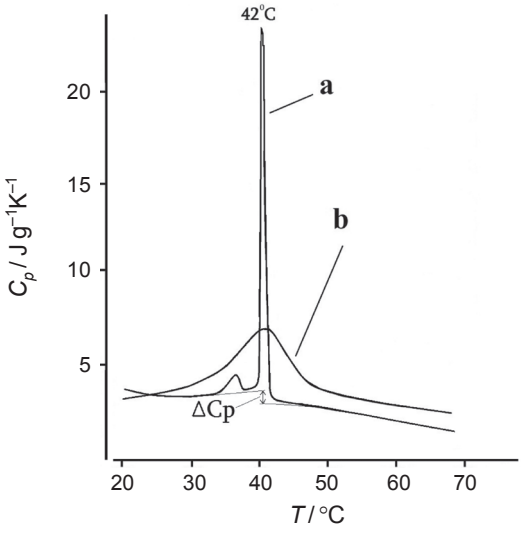

Figure 3. Dependence of heat capacity on temperature for DPPC–cholesterol liposomes: a, DPPC–cholesterol liposomes prepared in water; b, DPPC–cholesterol liposomes prepared using organic solvent (ethanol).

Fig. 4, curve a shows the calorimetric trace of DPPA–cholesterol liposomes prepared in water during melting; we see that the phase transition occurs at 66 °C. However, during the melting of the DPPA–cholesterol (molar ratio 3:1) suspension, in which the liposomes were first dissolved in organic solvent, calorimetry revealed that the cholesterol molecules are incorporated into the DPPA liposomes at 61 °C (Fig. 4, curve b).

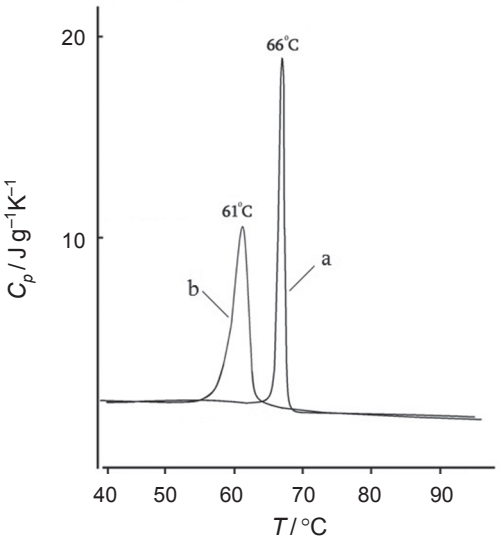

Figure 4. Dependence of heat capacity on temperature for DPPA–cholesterol liposomes: a, DPPA-cholesterol liposomes prepared in water; b, DPPA–cholesterol liposomes prepared using organic solvent (ethanol).

It is easier to package hydrophilic substances into liposomes by this technology, since the interaction between the lipids and chemicals of a polar nature occurs in an aqueous environment and there is no need to use organic solvent. Considering that in recent times calcium-containing medications are used very frequently, it may be serviceable to incorporate hydrophilic calcium-containing molecules into liposomes. Our experiments with $CaCl_2$ and DPPA show that the heat absorption peak is different from that of pure DPPA liposomes (Fig. 5A), which indicates the presence of calcium inside the DPPA liposomes (Fig. 5B).

When discussing novel methods of preparation of these types of liposomes, it is important that they are able to incorporate substances not only based on their physical properties but also based on their size. Nanoparticles made from gold, silver etc. are already used for the treatment of various diseases. Gold nanoparticles incorporated into liposomes are more effective for both diagnostics and treatment compared to gold nanoparticles alone [27, 28]. The enhanced treatment effectiveness may be because the gold nanoparticles incorporated in liposomes are better able to penetrate through damaged organ cell membranes.

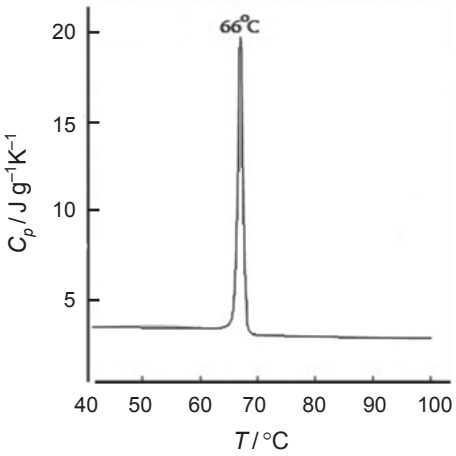

Figure 5A. Temperature dependence of the specific heat capacity of pure DPPA liposomes suspended in water. Heating rate 2 K min^{-1}.

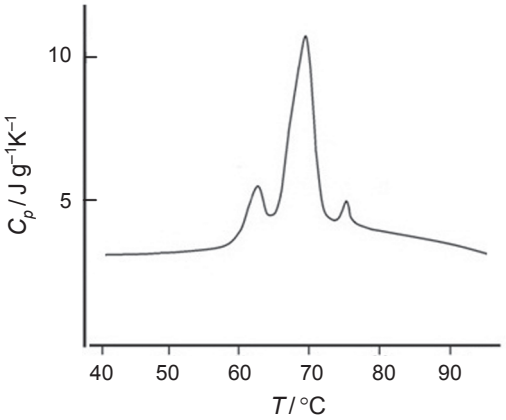

Figure 5B. Temperature dependence of the specific heat capacity of pure DPPA–$CaCl_2$ liposomes suspended in water. Heating rate 2 K min^{-1}.

Our method also allows incorporation of gold, silver and presumably other nanoparticles into lipid vesicles. Fig. 6 shows the dimensions of the DPPA–gold nanoparticle liposomes in suspension. Calorimetric measurements confirm the existence of complex DPPA–gold nanoparticle liposomes (Fig. 7). The peak of heat capacity on the calorimetric curve of complex DPPA–gold nanoparticle liposomes significantly differs from the peak for pure DPPA liposomes (Fig. 5A).

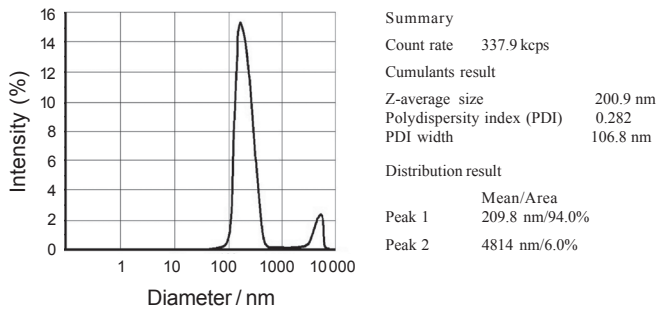

Figure 6. Size estimation from the Zetasizer for complex DPPA–gold nanoparticle liposomes.

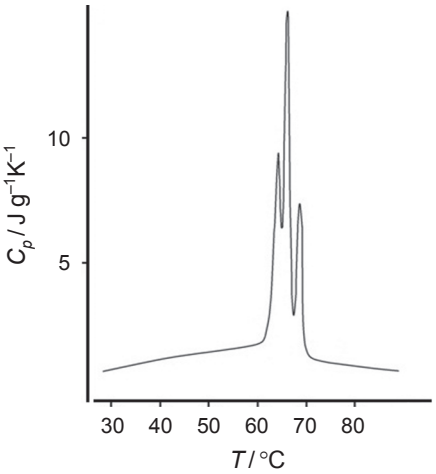

Figure 7. Calorimetric curve of complex DPPA–gold nanoparticle liposomes. Heating rate 2 K min^{-1}.

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