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Interliposomal transfer of crystal violet dye from DPPC liposomes to magnetoliposomes

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Abstract

Magnetoliposomes offer new challenges in the field of modern biotechnology and biomedicine. To investigate the important mechanism of interliposomal transfer of encapsulated substances, we investigated in the present work magnetoliposomes and liposomes containing the dye crystal violet. Our study of transmembrane transport showed that the dye transfer from DPPC liposomes to magnetoliposomes was temperature dependent.

Keywords: Nanoparticles; Transmembrane transfer; Magnetoliposomes; Dye crystal violet; Interliposomal transfer; Dipalmitoylphosphatidylcholine (DPPC)

1. Introduction

Liposomes are biocompatible colloids that show promise as a vehicle for drug delivery. In 1988, the so-called "magnetoliposomes" were developed [1]. This new type of vesicle consisted of nanosized magnetic particles wrapped in a phospholipid bilayer and offered new challenges in the field of modern biotechnology and biomedicine [2,3]. For these purposes it is very important to know the transport properties of the membrane-impermeant substances into cells. The list of substances whose injection has been attempted includes antitumor drugs, enzymes, hormones, clot lysis drugs, anti-inflammatory drugs and more. Assessment of the transport properties of the cell membrane is commonly made by measurement of the kinetics of transmembrane transport of various tracers. Tracers in this case mean substances that can be located inside or outside the cell and can be quantified or have their transmembrane movement monitored, either qualitatively or quantitatively. Transmembrane transport studies can be performed either on artificial systems (pure lipid bilayers) or cellular membranes that typically contain 30–40% protein by weight.

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In the present work, the magnetoliposomes consisted of lipid mixture dipalmitoylphosphatidylcholine (DPPC) with nanosized magnetic particles and liposomes wrapped dye crystal violet were used for the study of the transmembrane transport. The lipid bilayer of the used DPPC provides a simplified model of cellular membrane, because any ''impurities'' in the form of proteins are missing in the lipid bilayer. Crystal violet was chosen as a tracer because it is hydrophilic, represents a polar drug that does not perturb the phase transition behaviour of phosphatidylcholines and has an absorption maximum at 590 nm. Leakage from liposomes could thus be easily confirmed by spectrophotometry.

2. Experimental methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma. The dye crystal violet (synonym Gentian Violet, formula: $C_{25}H_{30}ClN_3$, $F_w = 408$) was obtained from Fluka. Other chemicals were of analytical grade. The water was double distilled before use.

2.2. Preparation of magnetic carrier

The synthesis of our aqueous magnetic fluid was based on coprecipitation of Fe^{2+} and Fe^{3+} salts by NH_4OH at 60 °C. In a typical synthesis to obtain 1 g of Fe_3O_4 precipitate, 0.86 g of $FeCl_2 \cdot 4H_2O$ and 2.35 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 40 ml of deionized water by vigorous stirring, such that $Fe^{3+}/Fe^{2+} = 2$. As the solution was heated to 80 °C, 5.6 ml of a = 25% NH_4OH was added. The precipitate was isolated from the solution by magnetic decantation without washing with water. To obtain an acidic sol, the precipitate was mixed with 1 ml concentrated perchloric acid and peptization was accomplished by adding water. The magnetic properties were estimated by magnetization measurement using a vibrating sample magnetometer (VSM), and the size and morphology of the particles were determined by transmission electron microscopy (TEM) and atomic force microscopy (AFM) working in taping mode. The AFM image of nanosized magnetite particles was taken from a 1×1 mm area at a scan rate of 1.498 Hz. The total area of the sample was approximately 10×10 mm.

The examination of the nanosized magnetic particles by TEM was done using a Tesla BS 500 microscope normally operated at 90 kV and 100,000 × magnification by replication technique. A drop of magnetic fluid sample containing 5.2×10^{14} particle/cm^3 was deposited on the 400 mesh copper grid and dried with ambient air before the pictures were taken.

As Fig. 1 shows, the sample represents a well-dispersed system of magnetic particles in carrier fluid.

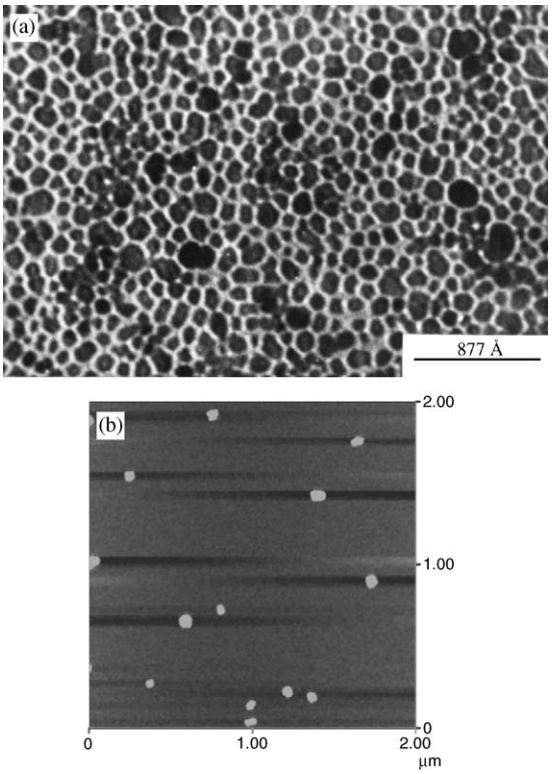

Fig. 1. Transmission electron microscopy of pure magnetic particles (a) and Atomic force microscopy image of pure magnetite (b).

2.3. Preparation of magnetoliposomes

It seems that the use of a larger amount of magnetite in the liposomes would allow more effective fixing of the liposomes by magnetic force. Therefore, it is necessary to know how much magnetite can be incorporated into liposomes. The amount of magnetite in liposomes was examined as a function of the initial amount of magnetite while the amount of lipid was fixed (1.36 μmol). Magnetoliposomes were prepared by mixing lipid solution (1.36 μmol) with various concentrations of magnetic particles, in an effort to obtain magnetoliposomes with maximum concentration of magnetic particles inside the liposomes.

To estimate the entrapment efficiency of magnetite in liposomes, the phospholipids and Fe_3O_4 contents in magnetoliposomes were measured spectrophotometrically using a JENWAY 6300 instrument. The determination of the phospholipids content, according to the method of Vaskovsky [4], involved a prior, complete thermal digestion of a phospholipids sample at 180 °C for 45 min, in the presence of 70% perchloric acid; the resulting inorganic orthophosphate was then reacted with acidic molybdenum to form a blue complex with an absorption maximum at 820 nm. The determination of the encapsulated Fe_3O_4 amount was based on the determination of ferrous ion using *o*-phenantroline [5], as follows. A liposomal sample (0.1 ml) was mixed with 0.1 ml of Triton X-100 solution (5%), then the magnetite was ionized by adding 0.5 ml concentrated HCl, and 1.0 ml of hydroxylamine hydrochloride solution (10%) was added to reduce ferric ions. After 15 min, 1.0 ml of *o*-phenantroline solution (0.5%) was added, the mixture was neutralized with 0.5 ml of 12 N NaOH, and the pH was adjusted at about 4.0 with sodium citrate solution (30%). The volume of the sample was adjusted to 10 ml and the absorbance was determined at 509 nm. The optimal amount of the magnetite per DPPC unit was highest in the case when 1.83 mg of magnetite was mixed with 1.36 μmol of lipid, as shown in Fig. 2. Therefore, in the following experiments the liposome suspension prepared with 1.83 mg of magnetite in 1.36 μmol of lipids was used as a standard.

The prepared magnetoliposomes were also observed using Transition electron microscopy (Fig. 3). The samples were prepared by replication technique, a carbon-coated copper grid was

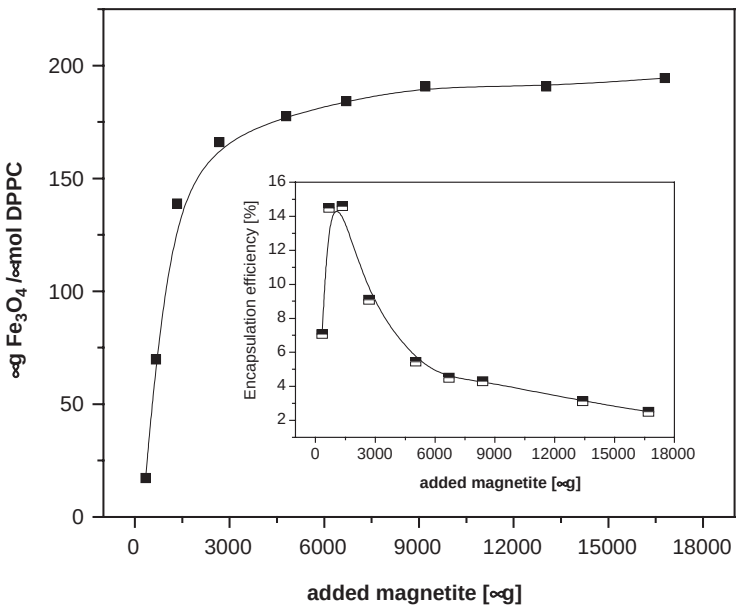

Fig. 2. Study of encapsulation efficiency of magnetic particles in DPPC liposomes.

immersed in the sample and allowed to dry in the air before introduction into the electron microscope.

2.4. Preparation of phospholipids with marker

Phospholipid liposomes were prepared by dissolving DPPC in chloroform at a concentration 1.36 μM/ml and incubation of 15 min at 50 °C. Crystal violet (CV) with a concentration 30 nM/ml was added to the lipid solution, the mixture was sonicated for 5 min and consequently incubated for 15 min at 50 °C. After the evaporation of organic solvent, a thin film was formed on the inner surface of a glass flask. To obtain a clear homogeneous dispersion, the dried lipids were sonicated for 10 min in a small amount of water. To find out the maximum amount of CV encapsulated in phospholipid DPPC, several samples with lipid concentration 1.36 μmol and various concentrations of crystal violet were prepared and spectrophotometrically analysed. Free CV was removed by washing and centrifugation till the supernatant showed zero absorbance at 590 nm. The results are shown in Fig. 4. The entrapment amount of the CV per unit of lipid was highest in the case of the preparation of liposomes with 50 μg of CV in 1.36 μmol of lipid.

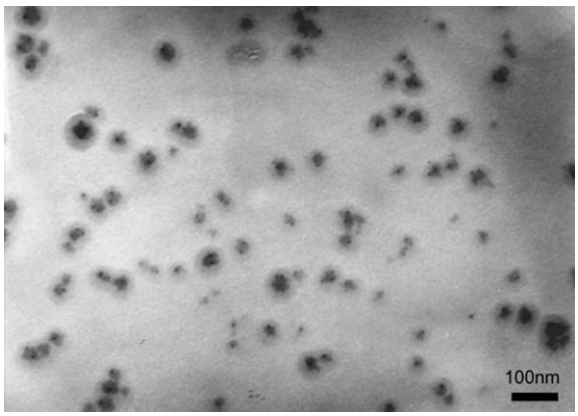

Fig. 3. TEM image of magnetoliposomes.

2.5. Differential scanning microcalorimetry studies

To study intermembrane transfer, it is important to know the interaction of encapsulated tracer with lipid membrane. In the microcalorimetric measurements the differential adiabatic scanning

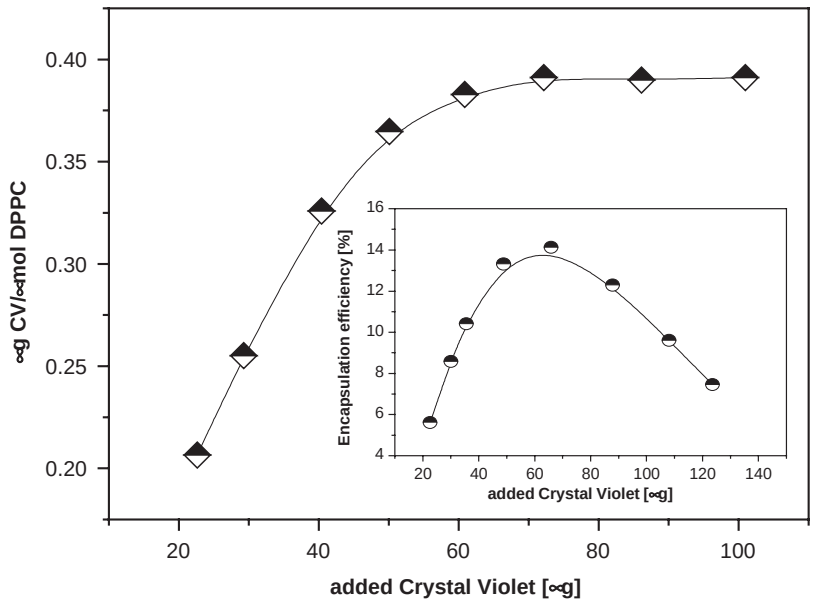

Fig. 4. Effect of the amount of dye crystal violet on the encapsulation ratio in unit lipid.

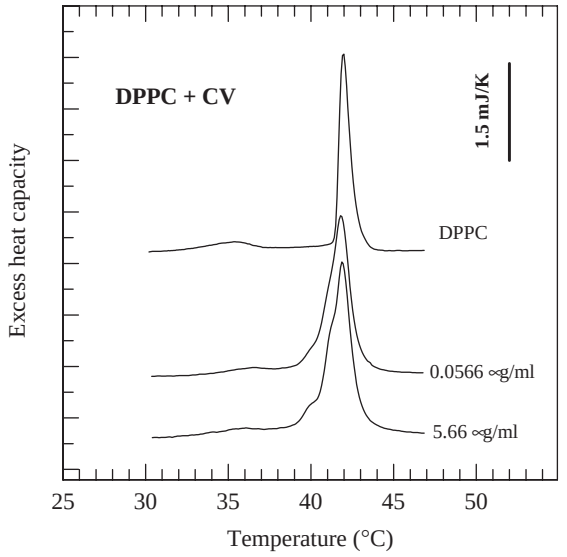

Fig. 5. Microcalorimetric measurements of pure DPPC (0.2 mg/ml) liposomes and DPPC with various concentrations of CV.

microcalorimeter DASM 4 was used. The volumes of the experimental and reference cells were 0.4789 and 0.4779 cm^3, respectively. To prevent gas bubbles formation at heating, a constant extra pressure is applied to the external inlet of the cells (~150 kPa). The experimental temperature interval was 20–50 °C and the heating rate was 1 °C/min. The samples for microcalorimetric study were prepared as follows: DPPC liposomes were incubated for 1 h at 50 °C with crystal violet and were shaken several times during the incubation to form multilamellar liposomes with wrapped crystal violet. Then the samples were equilibrated for at least 2 h at room temperature. Fig. 5 shows the DSC calorimetric profiles of the thermotropic gel–to–liquid crystaline transition of DPPC and DPPC mixtures with various concentrations of crystal violet. The addition of CV broadens the main phase transition of DPPC while the T_m remains essentially unchanged.

3. Results and discussion

In the performed experiments, the ionic stabilized magnetic fluids with concentration of magnetic particles 33.5 mg/ml and corresponding saturation magnetizations $I_s = 2.7$ mT were used. The magnetic log-normal particles size parameters, obtained by means of Chantrell et al. [6] technique from VSM measurements, were $D_V^{MAG} = 9.2$ nm, the standard deviation $\sigma^{MAG} = 0.15$. The size distribution of colloidal particles in magnetic fluid was characterized by TEM and AFM too. Fig. 1 shows that the sample represents a well-dispersed system of particles. The particle size polydispersity was captured by the log-normal distribution law [7] from the measurement of 450 particles from TEM micrograph. The obtained values of the mean diameter and standard deviation were $D_V^{TEM} = 10.3$ nm and $\sigma^{TEM} = 0.25$, respectively. Similar results were obtained from AFM, $D_V^{AFM} = 9.6$ nm and $\sigma^{AFM} = 0.23$. It can be seen that the calculated mean magnetic diameter is smaller than the values calculated from AFM and TEM [8]. The differences of the determination of particle size distribution were discussed in work [9], for example.

3.1. Transfer of dye from liposomes to magnetoliposomes

The transfer of dye from liposomes to magnetoliposomes was studied in physiological solution at 1.36 μM/ml liposome and magnetoliposome

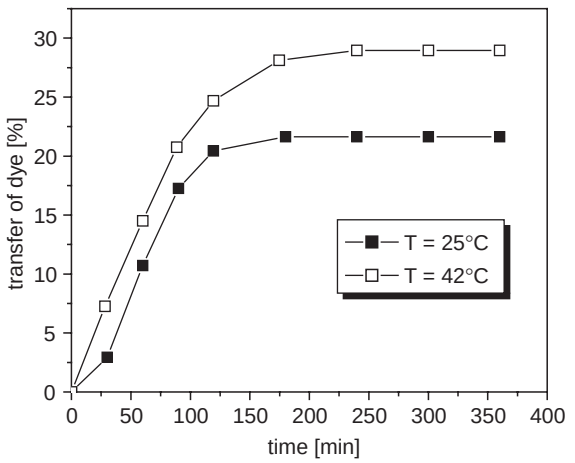

Fig. 6. Transfer of the water soluble dye crystal violet from DPPC liposome to magnetoliposomes at room temperature and at 42 °C.

concentrations (300 μg of magnetite in 1.36 μmol of lipids). As shown in Fig. 6, the saturated amount of transferred dye was reached within 240 min; at 25 °C the saturated value was 21% and at 42 °C the amount of transferred dye was 28%, respectively. To confirm that the increased dye content in the magnetoliposomes was not due to the simple dye binding to the magnetic particles during the experiment, the phospholipids and iron content in every retained sample was also measured. Within the time course of the experiment (6 h), no change in the phospholipids/magnetite ratio was seen, indicating that the observed increase of the dye content in retained fractions was not due to the binding of liposome to magnetoliposomes, but resulted uniquely from transfer movement.

In conclusion, it can be said that the magnetoliposomes with 300 μg magnetite and liposomes with 7 μg crystal violet in 1.36 μmol DPPC were prepared. In both cases approximately 14% of encapsulation efficiency was achieved. Spectral measurements of magnetoliposomes as well as DSC measurements of DPPC liposomes with crystal violet showed, that Tm of the main transition of DPPC remains unchanged after addition of CV. Finally the temperature-dependent membrane transport of dye CV from DPPC to magnetoliposomes was determined and the saturated amount of transferred dye was reached within 240 min. At 25 °C the saturated value was 21% and at 42 °C the amount of transferred dye was 28%, respectively.

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