RESEARCH PAPER

Accepted 18 June 2000
Revised 30 May 2000
Received 25 June, 1999
Indian J. Pharm. Sci. 2000, 62 (5) 360-363

Studies on the Preparation and Characterization of Thermosensitive Liposomes

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Thermosensitive liposomes prepared from synthetic lipids such as dipaltmitoyl phosphatidyl choline (DPPC), distearoyl phosphatidyl choline (DSPC) and cholesterol (CH) have been tried for local drug release in response to hyperthermia for achieving tumor drug targeting. Herein we report the use of natural lipid, soyabean lipid (SPC) and cholesterol (CH) to prepare thermosensitive liposomes. When liposomes are prepared by reverse evaporation method, it was found that most of the particles are multilamellar small vesicles. *In vitro* dialysis method was used to study the behavior of the liposomes made from different ratios of SPC and CH after the anticancer drug [2,4 (1H, 3H)-pyrimidine] (5-FU) fluorouracil was entrapped. From the result, it was found that, when the ratio of SPC and cH is proper (8:1), the liposomes made from them release most of the entrapped drug at 40° and the differential scanning calorimetry (DSC) showed that the liquid crystalline transition temperature (T_m) of the liposomes is about 40° too.

Liposomes are colloidal particles, used as targeted drug-delivery systems. Enhancement of therapeutic efficacy and reduction of toxicity of a variety of drugs have been demonstrated with liposome-encapsulated dosage forms[1,2]. However, the drawback is short half-life and poor targeting when administered intravenously[3]. To design thermosensitive liposomes, which can release entrapped drug as their T_m and increase the drug concentration at the target site to reduce toxicity to normal tissues, it is a general practice to use synthetic lipid such as DPPC (T_m=43)[4-6]. However, based on previous work from our laboratory, we used natural soyabean phosphatidyl choline (SPC) and cholesterol (CH) to prepare thermosensitive liposomes (T_m=39.9°).

5-Fluorouracil (5-FU) belongs to the pyrimidine group of antitumor agents. It interferes with the growth of cancer cells and is administered intravenously to treat cancer. 5-FU is selected as the entrapped drug to indicate the leakage of liposome, because it is hydrophilic and does not affect the phase-transition behavior of the liposome.

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MATERIALS AND METHODS

Soyabean phosphatidyl choline was purchased from Fats Co. Ltd. (Shanghai, China) and cholesterol was of analytical grade. 5-FU was purchased from HuaLian Co. Ltd. (Shanghai, China) and was used as a solution in phosphate buffer (PBS pH 8.0 and 198 mosm) at a concentration of 8 mg/ml. The organic solvents used were of HPLC grade (China).

Liposome preparation:

Liposomes were prepared with SPC/CH by modified reverse-phase evaporation method. At the beginning, SPC and CH in different molar ratios (16:1) and (8:1) and (4:1) were dissolved in isopropyl ether/chloroform (1:1 v/v). After 5-FU solution (8 mg/ml PBS) was added, the mixture was sonicated to give a w/o emulsion. The organic solvents in the emulsion were removed under reduced

pressure, resulting in the formation of a viscous gel-like phase. Then PBS was added, was continued, under reduced pressure to form liposome dispersion. Selected samples were subjected to negative stain scanning electron microscopic (SEM) observation for visualization of the structure and the size distribution.

5-FU encapsulation:

The amount of liposomally entrapped 5-FU was determined using a HPLC method. The liposomal suspension that was passed through a Sephadex G-25 column was equilibrated with PBS buffer and untrapped 5-FU was removed. The total amount of 5-FU and untrapped one were determined by HPLC.

Analysis of 5-FU by HPLC:

The chromatographic set-up consisted of a Waters model 510 pump with a Rheodyne no 7725i injector, A Waters 490 programmable multiwavelength detector was set at 265 nm and a CkChrom Chromatography from American Hi-Tech Co. The column used was 25 cm x 0.46 cm I.D. Spherisorb C_{18}. The mobile phase was 0.05 M phosphate buffer (pH=4.0) containing 5% of methanol, pumped at a flow-rat of 1.0 ml/min. The chromatogram of 5-FU was shown in Fig. 1.

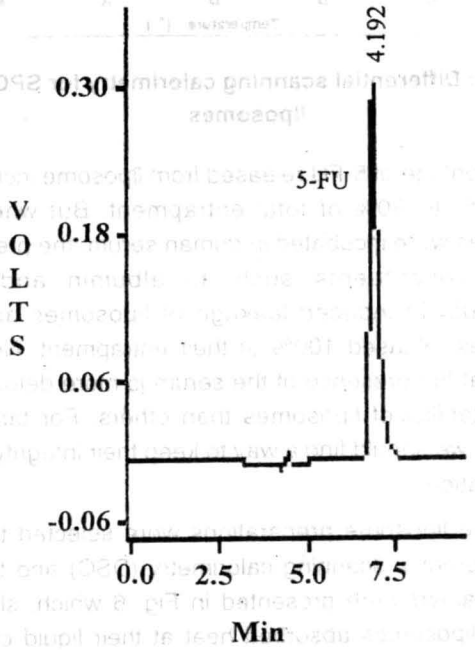

Fig. 1 : HPLC chromatogram of 5-FU.
The mobile phase was 0.05 phosphate Buffer (pH 4.0) containing 5% of methanol, pumped at a flow rate of 1.0 ml/min. The peak of 5-FU is the higher one

In vitro drug release from liposome:

The liposomal suspension was made up to 5 ml using PBS solution and 0.5 ml was subjected to dialysis bag (mw, 100 100) with a diameter of 21 mm to dialysis. The dialysis bag was immersed into the receiver compartment containing 50 ml PBS solution (pH 8.0) or 0.9% saline solution or human serum. Experiments were performed at different temperature as 30°, 35°, 37°, 39°, 41°, 43°, 45° and 50° in a thermostatically controlled water bath. At each temperature, the samples were allowed to stay for 5 min, after which, the samples were removed from the receiver compartment and the drug released was determined by HPLC analysis. Aliquots (0.2 ml) of the medium from the receiver compartment were collected under different temperatures and the drug released was determined using the HPLC method.

RESULTS AND DISCUSSION

The liposomes prepared by modified REV method were found to entrap 12% of 5-FU. Most of the liposomes are multilamellar small liposomes. which can be seen from Fig. 2 and the size is about 0.2~1.0 μm. 5-FU was analyzed by HPLC, the peak of 5-FU is single and sharp, the retention time is about 6 min.

Fig. 2 : SEM photograph of liposomes
The picture depicts negatively stained multilamellar vesicles (MSVs). The particle diameter was in the range 100-200 nm. (x 100 000; bar = 1 μm)

When we compared Fig. 3 with Fig. 4 and Fig. 5, there are similarities and differences between them. The behaviors of the liposomes with different compositions

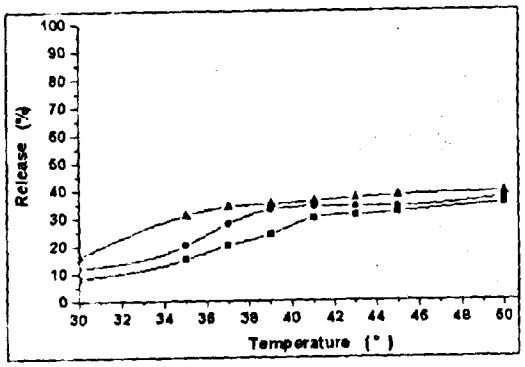

Fig. 3 : The percentage of 5-FU release from liposomes in PBS medium

-■- the concentration of CH in liposome is high.
-●- the concentration of CH in liposome is medium.
-▲- the concentration of CH in liposome is low.

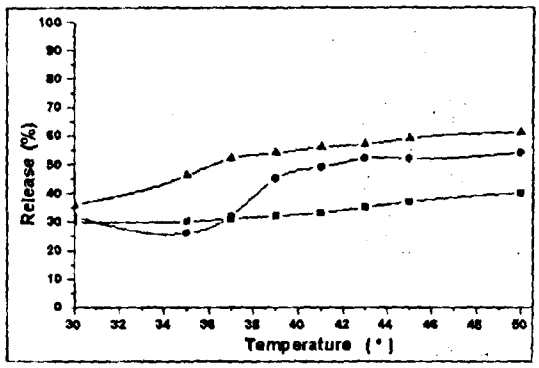

Fig. 4 : The percentage of 5-FU release form liposomes in saline medium

-■- the concentration of CH in liposome is high.
-●- the concentration of CH in liposome is medium.
-▲- the concentration of CH in liposome is low.

in different media are the same. The concentration of CH is the most important element, which affects the behaviour of a liposome. The liposome becomes stable as the content of CH increases and the phase transition temperature increases as a result. But if the content of CH is low, the phase transition temperature is lower than 37°, which dose not with the requirement of hyperthermia therapy. Therefore, we choose the molar ratio of SPC and CH is 8:1. The liposomes of these compositions show their sharp release at about 40°, which is most desirable.

There are a lot of elements, which affect the behaviour of liposomes such as osmolarity or human serum. Compare Fig. 3 with Fig. 4 When the osmolarity of the receptor medium changed from 198 mosm to 298 mosm,

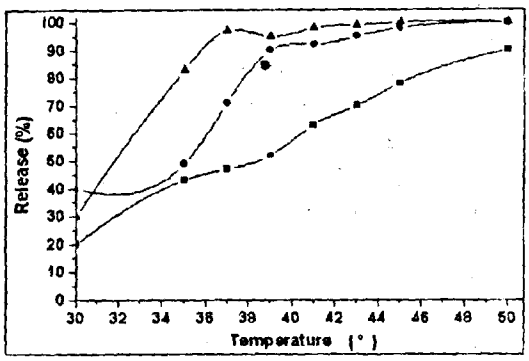

Fig. 5 : The percentage of 5-FU release form liposomes in human serum medium

-■- the concentration of CH in liposome is high.
-●- the concentration of CH in liposome is medium.
-▲- the concentration of CH in liposome is low.

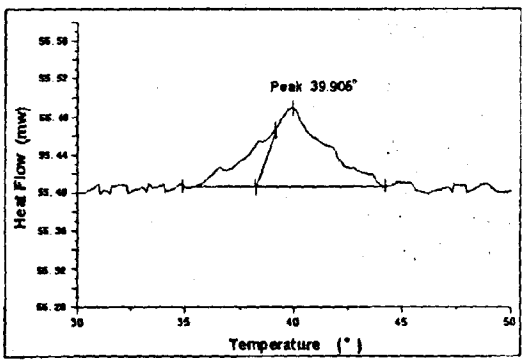

Fig. 6 : Differential scanning calorimetry for SPC/CH liposomes

the percentage of 5-FU released from liposome increased from 30% to 50% of total entrapment. But when the liposomes were incubated in human serum, the presence of the constituents such as albumin and α-2 macroglubulin induced leakage of liposomes and the liposomes released 100% of their entrapment. Now we know that the presence of the serum is more detrimental to the stability of liposomes than others. For targeting purpose, we should find a way to keep their integrity while in circulation.

Some liposome preparations were selected to perform differential scanning calorimetry (DSC) and the results obtained were presented in Fig. 6 which, showed that the liposomes absorbed heat at their liquid crystalline temperature, which is about 40°. The result agrees with that obtained in the *in vitro* test.

In Fig. 3, 4 and 5, it can be observed that the

liposomes release about 10-40% 5-FU of their entrapments at 30°, indicating that the liposomes are unstable at different receptor media at room temperature. Next, it is essential to find a modification that would improve the stability of liposomes and maintain their integrity in circulation resulting in an increased targeting to tumor cells. On conclusion the results of this investigation clearly suggest that thermosensitive liposomes can be prepared.

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