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COMMUNICATION

Comparative Evaluation of Targeting Efficiency of Charged and Neutral Liposomes of 5-Fluorouracil

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ABSTRACT

Liposomes were prepared from egg lecithin by the modified ether injection technique using stearylamine and dicetyl phosphate as the charge-inducing agents. Comparative evaluations of charged and neutral liposomes for drug entrapment, size distribution, in vitro drug release, and in vivo drug targeting were made. The charged liposomes were larger in size and showed better drug entrapment efficiency than the neutral liposomes. However, no difference in the pattern of in vitro drug release was observed. Charged liposomes were found entrapped more in the organs of the reticulo-endothelial system than the neutral liposomes.

INTRODUCTION

Liposomes offer the possibility of target selectivity through the choice of appropriate size or surface characteristics, to affect targeting of the drug and also minimize systemic toxicity. The advantage of formulating drugs in liposomes is that liposomes are physically trapped in the capillary bed of organs of the reticulo-endothelial system (RES) and slowly release the drug there, so that a high concentration of the drug is maintained locally (1).

5-Fluorouracil (5-FU) belongs to the pyrimidine group of antitumor agents. It is administered intravenously to treat some kinds of cancer including small cell lung cancer. 5-FU interferes with the growth of cancer cells, which are eventually destroyed. Since side effects of 5-FU are frequently dose related, the toxicity may be minimized by selective targeting of the drug using liposomes (2).

This study presents a comparative evaluation of the targeting efficiency of charged and neutral liposomes in the organs of the reticulo-endothelial system using Swiss albino mice in vivo.

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MATERIALS AND METHODS

Materials

The active substance 5-FU was received as a gift from Tamilnadu Dadha Pharmaceutical Ltd., Madras, India.

Distearoyl phosphatidylcholine (Sigma) and cholesterol were used as the matrix materials. Dicetyl phosphate (Sigma) and stearylamine (Sigma) were used as charge-inducing agents. The other chemicals used (diethyl ether, methanol, acetate buffer, ethyl acetate, and perchloric acid) were of analytical grade.

Methods

Preparation of Large Unilamellar Vesicles (LUV)

Ether Injection Method

The liposomes were prepared by ether injection following the procedure given by Deamer and Bangham (3) as follows: A solution of lipid in the solvent mixture diethyl ether:methanol was prepared by dissolving distearoyl phosphatidylcholine and cholesterol in a molar ratio of (9:10). One milliliter of this solution was introduced into 4 ml of aqueous solution containing 5 mg of 5-FU at a rate of 0.2 ml/min at 55°C, placed in a glass vial, and closed with a silicone rubber injection cap.

Modified Ether Injection Method

The liposomes were prepared by modified ether injection method following the procedure given by Pourkavoos (4).

The lipids were dissolved in chloroform and dried under nitrogen to a thin film in a round-bottom flask. The lipid film was dissolved in the solvent mixture. diethyl ether:methanol (10:2) and 1 ml of this solution was injected at a rate of 0.2 ml/min into the solution of 5 mg of 5-FU in 4 ml of aqueous solution at 60°C. Three formulations were prepared following the method described above (see Table 1).

Estimation of Liposome Drug Encapsulation Level

After the separation of free 5-FU fraction, the amount of drug encapsulated was determined as follows: 3.5 ml of a mixture of dichloromethane:methanol (2:1) was added to the pelleted liposomes to destroy the phospholipid bilayer structure, freeing the drug. This organic solution was poured into a long-necked 10 ml quickfit round-bottom flask and made up to 10 ml with methanol. This solution was analyzed spectrophotometrically at 266 nm (see Table 1) (5).

Analysis of Liposome Size Distribution

The size distribution was studied using a scanning electron microscope by freeze fracture technique, by pipetting the liposome onto gold studs and rapidly freezing with liquid nitrogen-cooled Freon 22 (see Table 1 and Figs. 1–3) (6).

In Vitro Drug Release from Liposomes

The liposome pellets were made up to 5 ml using buffer solution (pH 7.4) and subjected to dialysis. The dialysis donor compartment was a Spectrapor membrane

Table 1

Composition, Size Distribution, and Encapsulation of 5-FU in Neutral and Charged Liposomes

Sample No.	Formulation	Chemical	Amount of 5-Fu Loaded (in μg)	Size[n] (μm)
1	Formulation 1	Distearoyl phosphatidylcholine: cholesterol (7:1)	95.02 (1.759%)	0.52 ± 0.04
2	Formulation 2	Distearoyl phosphatidylcholine: cholesterol:stearylamine (7:1:2)	112.40 (2.24%)	0.77 ± 0.04
3	Formulation 3 (Negative)	Distearoyl phosphatidylcholine: cholesterol:dicetyl stearylamine (7:1:2)	108.02 (2.18%)	0.82 ± 0.03

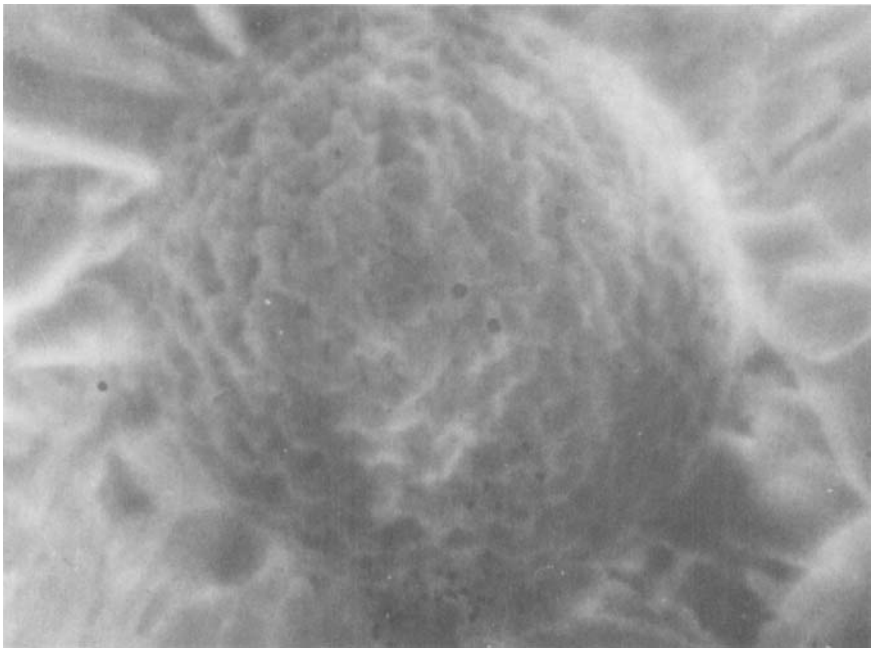

Figure 1. Scanning electron micrograph of liposome prepared by freeze fracture technique.

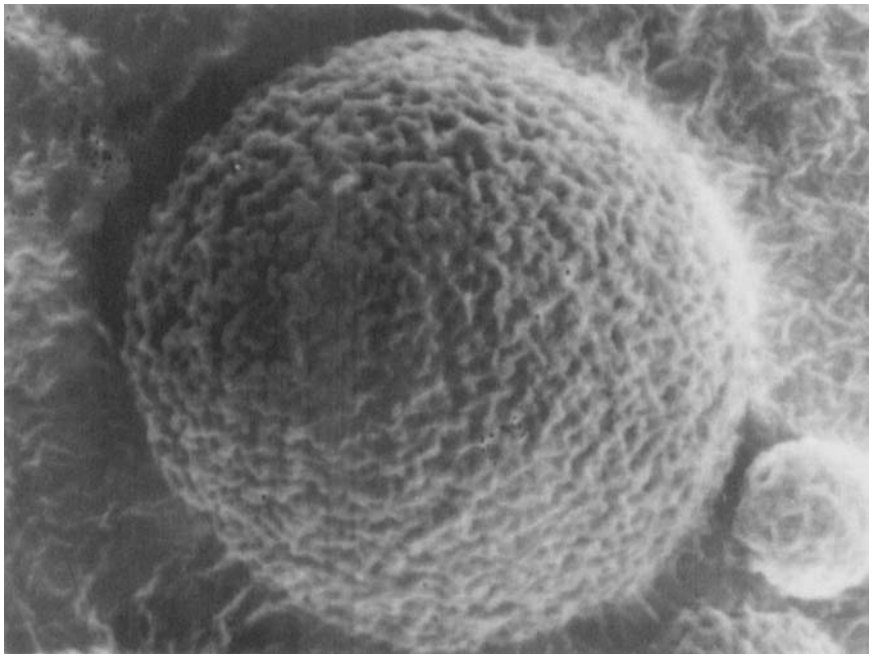

Figure 2. Scanning electron micrograph of liposome prepared by freeze fracture technique.

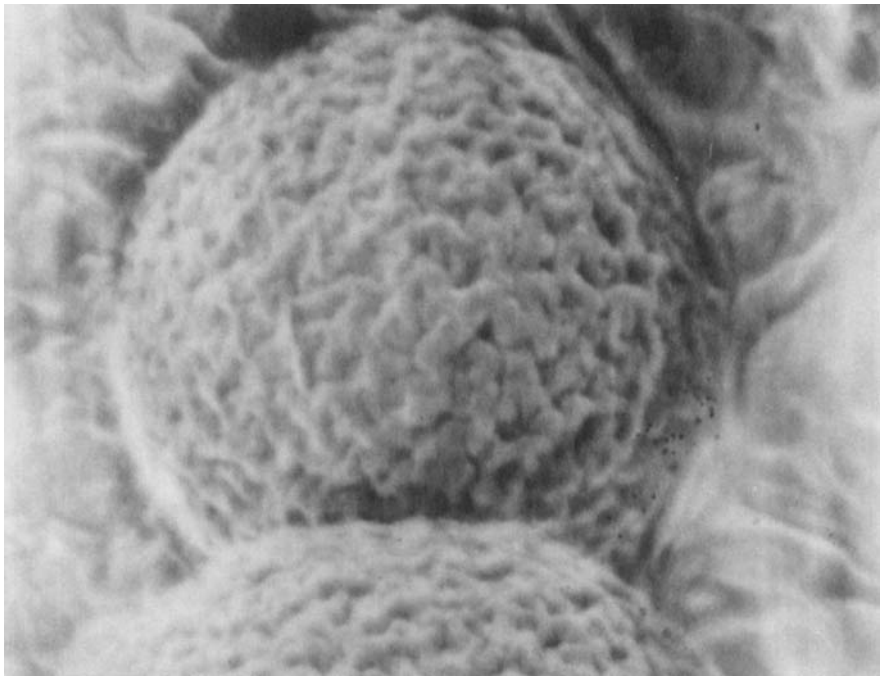

Figure 3. Scanning electron micrograph of liposome prepared by freeze fracture technique.

with a diameter of 49 mm. The dialysis bag was immersed into the receiver compartment containing 5 ml of phosphate buffer solution (pH 7.4). Experiments were performed at 37°C in a thermostatically controlled water bath. At predetermined time intervals, the samples were removed from the receiver compartment and the drug content was determined by HPLC analysis (see Table 2) (2).

Analysis of 5-FU by HPLC

The chromatographic system consisted of a solvent delivery pump, Rheodyne no. 7125 injector with a 20-μl loop, and a variable-wavelength UV-Vis detector operated at 270 nm. Chromatography was carried out on a Hypersil C18 (3 μ) (50 μm × 4.6 mm i.d.) analytical column. The mobile phase, methanol:0.01 × M acetate buffer, pH 4.0, (8:92) was delivered at 1.5 ml/min.

Table 2

In Vitro Release of 5-FU From Charged and Neutral Liposomes

Sample No.	Formulation Number	Cumulative % Release of 5-FU at Different Time Intervals (hr)											
		1	2	3	4	5	6	7	8	9	10	11	12
1	Formulation 1	18.06 ±0.45	32.94 ±0.05	47.47 ±0.73	57.71 ±0.98	67.14 ±0.51	74.30 ±0.10	80.64 ±0.73	85.68 ±1.04	89.34 ±0.51	91.91 ±0.99	94.35 ±0.28	95.81 ±1.06
2	Formulation 2	17.06 ±1.13	30.71 ±0.88	42.81 ±0.71	51.86 ±0.54	60.43 ±1.24	66.91 ±1.19	73.22 ±0.52	78.20 ±0.71	82.78 ±0.98	86.57 ±1.04	90.41 ±0.96	93.31 ±0.97
3	Formulation 3	16.38 ±0.66	30.50 ±0.77	43.85 ±0.51	55.38 ±0.59	64.78 ±0.89	72.67 ±1.54	78.67 ±0.67	82.38 ±0.87	85.87 ±0.12	88.61 ±0.82	90.74 ±1.39	92.38 ±0.86

[a] $n = 3$.

Table 3

Percentage of 5-FU Accumulated in Various Organs of Mice

Sample No.	Drug Distribution in Organs	Percentage of 5-Fluorouracil Accumulated[a] Formulation 1 (Neutral)	Formulation 2 (Positive charge)	Formulation 3 (Negative Charge)	Control
1	Liver	18.84 ±0.56	15.36 ±0.50	21.19 ±0.18	10.27 ±0.43
2	Lungs	13.66 ±0.59	20.23 ±0.12	20.34 ±0.16	9.82 ±0.54
3	Kidney	4.90 ±0.39	4.37 ±0.23	3.68 ±0.10	2.86 ±0.10
4	Spleen	7.37 ±0.42	6.51 ±0.30	7.16 ±0.12	3.57 ±0.19

[a] $n = 3$.

The samples obtained from in vitro studies at different time intervals and samples obtained from in vivo studies were analyzed for 5-FU using HPLC (see Tables 2 and 3) (7).

In Vivo Targeting of 5-FU-Loaded Liposomes

Procedure for In Vivo Studies

Twelve healthy adult mice (23–30 g) were selected and fasted for 12 hr. These were divided into four groups of three each. Group 1 received 100 μg each of free drug (5-FU), which was dispersed in normal saline and administered intravenously through tail vein.

The other three groups received any one of the three different liposome formulations equivalent to 100 μg of 5-FU, respectively. After 3 hr, the mice were sacrificed, and the various organs (liver, lungs, kidneys, and spleen) were isolated and analyzed for the drug.

Procedure for In Vivo Drug Analysis

The various organs (liver, lungs, kidneys, and spleen) were homogenized (using a tissue homogenizer) with water, mixed with 0.1 ml of 60% perchloric acid, and centrifuged at 1200 rpm for 10 min. The supernatant was mixed with an aqueous volume of ethyl acetate and the aqueous layer was analyzed for 5-FU by HPLC using a C18 column (see Table 3) (8).

RESULTS AND DISCUSSION

Drug-loaded neutral liposomes were prepared both by ether injection and modified ether injection methods. These liposomes were analyzed for drug loading. The liposomes prepared by ether injection method were found to entrap 1.28% of 5-FU, whereas the liposomes prepared by modified ether injection method were found to entrap 1.759% of 5-FU. Hence, modified ether injection method was followed for further formulations.

Neutral liposomes had an average diameter of 0.52 μm, positive liposomes had an average diameter of 0.77 μm, and negative liposomes had an average diameter of 0.82 μm. The inclusion of charge-inducing agents increased the size of the vesicles. Also, the charge-inducing agents were found to significantly enhance the amount of drug entrapped in the liposomes.

In vitro drug release studies of the various formulations have indicated the release of 5-FU from all of the formulations to be sustained, following first-order kinetics. The results have shown that the liposomes retain the drug for a sufficiently long period to enable the entrapment of liposomes in the target organ without significant leakage of the drug into the general circulation.

The distribution of 5-FU in the organs of the RES was significantly higher from the liposomes than that of the control. Charged liposomes were found distributed in the organs to a higher extent than the neutral liposomes. The negatively charged liposomes contained 52.37% of the total encapsulated drug and were distributed in the organs of the RES, which is the highest of all the formulations. Due to their charge and larger size, the negatively charged liposomes were found to be entrapped more in the lungs than in the other organs analyzed. These liposomes were also found to be entrapped in the liver to a significant level. The positively charged liposomes were well distributed in the lungs and com-

paratively less in the liver. Since neutral liposomes were smaller in size, they were distributed to a higher extent in the liver than in the other organs. There was no significant difference between the charged and the neutral liposomes in the distribution of the drug to spleen and kidneys.

These studies have shown that the charge-inducing agents increased both the size and encapsulation efficiency of the liposomes. However, adequate selectivity to any of the organs of the RES studied was not observed. The target selectivity of these liposomes may be further improved by the inclusion of specific monoclonal antibodies.

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