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HEPATIC UPTAKE AND TISSUE DISTRIBUTION OF LIPOSOMES: INFLUENCE OF VESICLE SIZE

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ABSTRACT

The size-dependent disposition of liposomes in rats was studied. Liposomes consisting of phosphatidylcholine, cholesterol, dicetylphospate and alpha-tocopherol in a molar ratio of 4:4:1:1, containing a trace of [^{14}C]-labeled cholesterol as a marker of the lipid phase, were prepared and sized by extruding through polycarbonate membrane. [^{3}H]-inulin was used as a marker of the aqueous phase. *In situ* liver perfusion in rats showed that hepatic extraction of liposomes was significant for multilamellar vesicles (MLVs) larger than 0.4 μm (0.40, 0.82 and 1.31 μm) and small unilamellar vesicles (SUV), but negligible for 0.25 μm MLV. Pharmacokinetic analysis after intravenous

(i.v.) injection showed that the area under the plasma elimination curve (AUC) was significantly higher, but the volume of distribution (Vd) and the elimination rate constant (ke) were significantly lower for the 0.25 μm than for the 1.31 μm liposomes. Comparing the distribution of 1.36 and 0.25 μm MLVs after i.v. injection, the 1.31 μm MLV showed a significantly higher concentration in liver and spleen, but lower concentration in plasma and kidney, than the 0.26 μm in terms of dose percent. These results suggest that size is one of the important factors affecting the fate of liposmes *in vivo*. There must be a minimun size for effective uptake of liposomes by the reticulo-endothelial system. If below the minimum effective uptake size, the MLV should remain in higher concentration in circulation.

INTRODUCTION

Liposomes are lipid vesicles dispersed in aqueous media. The vesicles consist of one or more concentric spheres of lipid bilayers separated by a water or aqueous buffer compartment. Their characteristics of trapping various solutes and interaction with cells by endocytosis or fusion have led to their application as a vehicle for intracellular delivery[1]. Therapeutic drugs can be entrapped in the enclosed aqueous phase or lipid bilayers, depending on their hydro- or

lipophylicity. The use of liposomes as effective drug carriers is currently of great interest[2]. It has become apparent that a substantial fraction of intravenously injected liposomes is taken up by the reticuloendothelial system (RES)[3]. However, many factors such as lipid composition[4], surface charge[5] and vesicle size[6-8] may influence the disposition of liposomes. In the present study, a size range extended to larger than 1 um was studied. The influence of liposomal vesicle size on hepatic retention and tissue distribution was investigated. Correspondence of size-dependent elimination from the perfusate in *in situ* hepatic perfusion experiments to size-dependent tissue distribution after intravenous injection of liposomes was also examined.

MATERIALS AND METHODS

Chemicals and Reagents

Egg L-α-phosphatidylcholine (PC), phosphatidylcholine dipalmitoil (PD), dicetylphosphate (DP) and phosphate buffered saline (PBS) were purchased from Sigma (St. Louis, MO). Cholesterin Krist and cholesterol (CH) were from Merck (Darmstadt, Germany). DL-α-tocopherol was from Nacalai tesque (Kyoto, Japan). ^{14}C-cholesterol, ^{3}H-inulin, and Aquasol-2, used as lipid marker, aqueous marker and liquid scintillation cocktail, respectively, were from Du Pont (Boston, USA).

Preparation of Liposomes

Multilamellar liposomes (MLV) were prepared by the technique of Bangham[9]. Briefly, a chloroform solution containing PC, CH (cold, with a trace of ^{14}C), DP and DL-α-tocopherol in the molar ratio 4:4:1:1 was dried in a rotation evaporator. The lipids were dispersed in PBS by vortexing, and MLV was formed.

Homogenous vesicle size was obtained by extruding the liposomes through a polycarbonate (PC) membrane (Nucleopore, CA, USA) of uniform pore diameter[10]. To obtain the small vesicle (nano) size, the liposomes were extruded through a series of PE membranes in a sequence of decreased pore diameters. Size distribution of the liposomal vesicles was measured with a Photal particle counter (LPA-3000, Otsuka Electronics, Japan).

Small unilamellar vesicles (SUV) were formed by intermittent sonication of the MLV dispersion at 20 amplitude microns output power setting of a sonifier (Soniprep 150, MSE, England) until the mixture became translucent. During sonification the vial containing the lipid dispersion was kept in an ice-water bath.

The total phospholipid concentration of the prepared liposomes was assayed by extraction with chloroform and measuring the absorbance at 485 nm using a spectrometer[11].

Liver Perfusion Study

Liver perfusion was performed by the method of Tyrrel[12]. Male Long-Evans rats, 300-350 g body weight,

were used as liver donors for the *in situ* perfusion study. The rat was anesthetized with pentobarbital intraperitoneal (i.p.) injection. The portal vein and the inferior vena cava were cannulated with polyethylene (PE)-100 and PE-200 tubings, respectively. The liver was perfused from the portal vein cannula at a rate of 6.3 ml/min by the use of a peristaltic pump (IKA-Schlauchpumpe, Germany). The perfusion medium consisted of Hanks' balanced saline (HBS), pH 7.4, which has been saturated with O_2/CO_2 (95/5) and warmed to 37°C. The initial outflow (about 50 ml) of the perfusate was allowed to pass through the liver directly to waste, thereby flushing all blood from the organ. The outflow was then directed to a fractional collector (Gilson, USA) as soon as 0.1 ml liposome (lipid concentraiton 5 mg/ml) with ^3H inulin added as a tracer was injected rapidly into the perfusion line at the site of portal vein. The perfusion was continued and the outflow was collected for determination of ^{14}C and ^3H radioactivities. The total recovery of inulin in the outflow served as an indicator for checking leakage of the perfusion system.

At the end of perfusion, hepatocyte viability of the treated liver was checked by trypan blue exclusion test[13].

Calculation

The recovery of liposomes from hepatic perfusion was estimated by the following equation:

$$\text{Recovery} = \frac{\sum_{i=1}^{n} FL(i)}{\sum_{i=1}^{n} FI(i)} \quad (1)$$

where n represents the total number of collected fractions taken into calculation; and FL(i) and FI(i), the dose percent of liposomes and inulin in the ith fraction, respectively.

Mean transit time (Tr) was expressed as follows:

$$Tr = \frac{\int_{o}^{\infty} t * C(t)dt}{\int_{o}^{\infty} C(t)dt} \quad (2)$$

Equation 2 can be modified to equation 3 for calculation of mean effluent fraction (MEF) of liposomes during liver perfusion.

$$MEF = \frac{\sum_{i=1}^{n} i * F(i)}{\sum_{i=1}^{n} F(i)} \quad (3)$$

where F(i) represents the dose percent in fraction i.

Plasma Elimination and Tissue Distribution Study

Male Long-Evans rats, 300-350 g body weight, were anesthetized with pentobarbital i.p. injection. The carotid artery was cannulated with a PE-50 tubing for blood sampling. A bolus dose of liposome 0.1 ml (containing lipid 10 mg/ml) per kg body weight, was injected into the femarol vein. Blood samples were collected on a time schedule up to 60 min. The blood

concentration time profiles were fitted to an one- and a two- compartment models using PCNONLIN program[14]. The model suitable for each profile was selected according to AIC value[15].

Immediately after the last blood sampling (60 min) animals were sacrificed by exsanguination. Organs or tissues were excised, soaked in saline, blotted with filter paper, weighed and finally homogenized to determine liposomal lipid.

Determination of Radioactivity

The liver perfusion outflow, the plasma samples and the tissue homogenates were counted for radiolabel in a Beckman LS-5801 liquid scintillation counter after mixed with aquasol-2.

RESULTS

Size Determination of Liposome Vesicles

Passing through a PC membrane of a selected pore size, liposomes of homogenous vesicle size around the correspondent membrane pore diameter with a narrow distribution band were obtained (Table 1). The results validated the use of PC membrane for preparation of homogenous liposomes of expected vesicle size. The SUV, without extruding through PC membrane, showed 21 $\pm$ 3 nm in vesicle size.

Recovery of Liposomes from Liver perfusion

Fig. 1 shows the outflow profiles of inulin and liposomes after liver perfusion. In all the

TABLE 1

Size Distribution of Liposomal Vesicles After Extruding Through PC Membrane.

Membrane Pore Diameter μm	Vesicle Diameter μm (SD)
2.0	1.31 (0.082)
1.0	0.82 (0.108)
0.4	0.40 (0.049)
0.2	0.25 (0.016)

experiments, more than 90% inulin dose was presented in the initial 20 fractions of the outflow, and the liposomes in the outflow waned to less than 1% dose after the 15th fraction. Accordingly, the initial 20 fractions should cover almost a complete run of a perfusion dose, and therefore 20 fractions (n = 20) were taken for calculation of recovery and MEF, applying equations (1) and (3). The results are shown in Table 2. The recoveries of liposomes from liver perfusion were significantly less than the given doses except in the case of that perfused with 0.25 μm liposome, where no significant difference showed between the dose and the recovery. The diminished fraction of liposomes through liver perfusion indicated the hepatic uptake of liposomes.

The MEF value representd a theoretical mean residence time period, in term of fractions, for a substance passing through the perfusion system, i.e.,

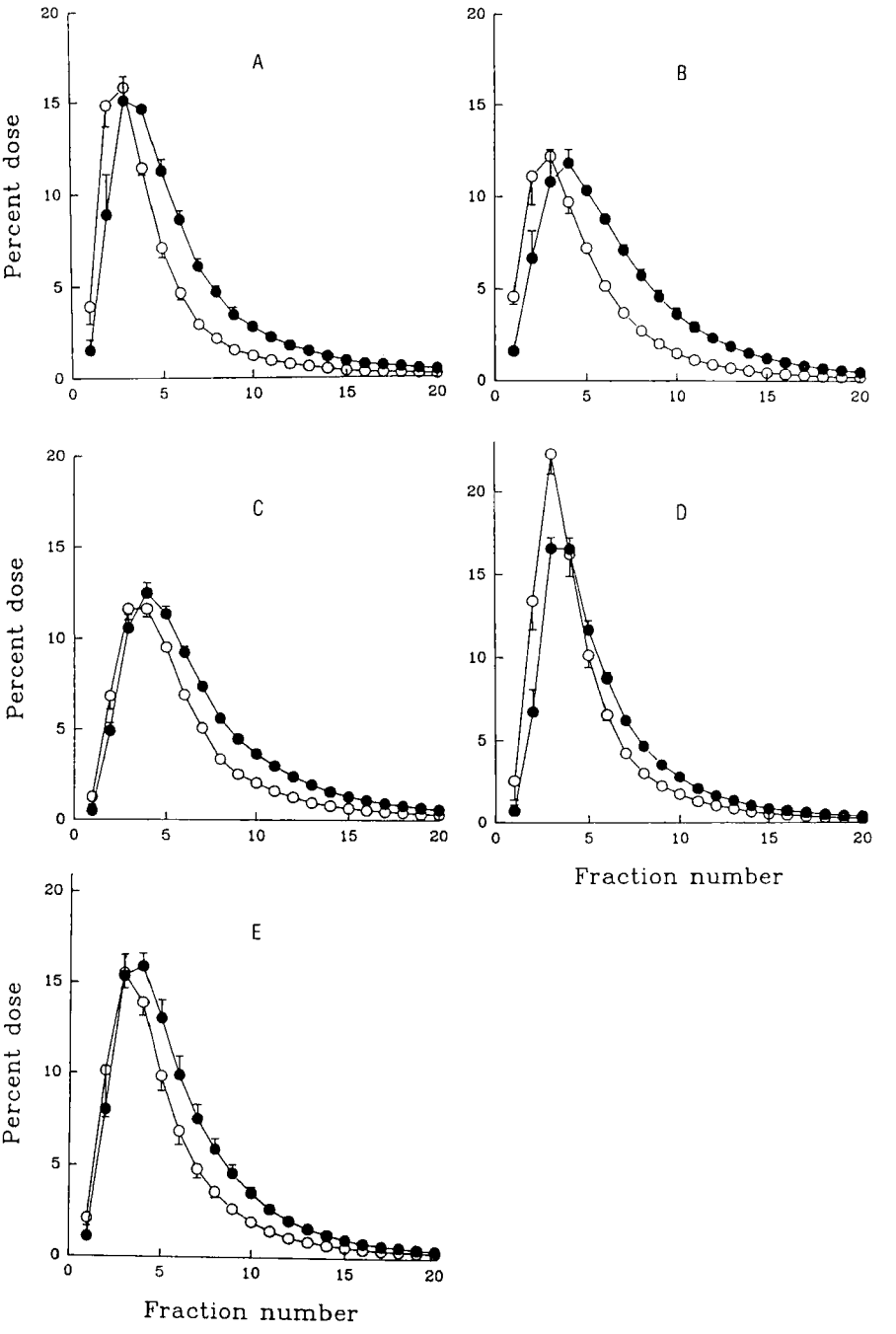

FIGURE 1

Outflow profiles of inulin and liposomes of different vesicle size after liver perfusion. (A) 1.3 μm liposome; (B) 0.8 μm liposome; (C) 0.4 μm liposomes; (D) 0.25 μm liposomes and (E) SUV. key: (○), empty liposomes; (●) free inulin.

TABLE 2

The Recovery and The MEF of Lipoosmes From Liver Perfusion.

Liposome Diameter μm	Recovery Dose %	MEF Liposomes	MEF Inulin
1.3	80.1 (1.40)a	4.7 (0.16)b	6.1 (0.13)
0.8	76.6 (2.40)a	5.0 (0.23)b	6.6 (0.26)
0.4	80.6 (0.46)a	5.7 (0.15)b	6.8 (0.16)
0.25	100.7 (1.78)	4.8 (0.14)b	5.9 (0.11)
SUV	81.0 (2.16)a	5.3 (0.24)b	6.1 (0.27)

Data were mean (SE) of 4 to 6 determinations.
a. $p < 0.01$, significantly different from the given dose by Student's t-test.
b. $p < 0.01$, significantly different from inulin by Student's t-test.

the liver. In all cases, liposomes passed faster than inulin.

Effects of Liposomal Vesicle Size on Disposition of Liposomes

Liposomes of different vesicle size (0.25 and 1.31 μm) showed significantly different plasma level-time profiles (Fig. 2) in rats following i.v. injection. By fitting the plasma data to a conventional pharmacokinetic model using PCNONLIN, pharmacokinetic parameters were estimated (Table 3). The AIC values indicated that the plasma elimination of 0.25 μm liposome was monophasic while that of 1.31 μm liposome was biphasic. The plasma elimination of 0.25 μm liposome was significantly slower than that of 1.31 μm. The AUC and the k_{10} of the 0.25 um liposome were about

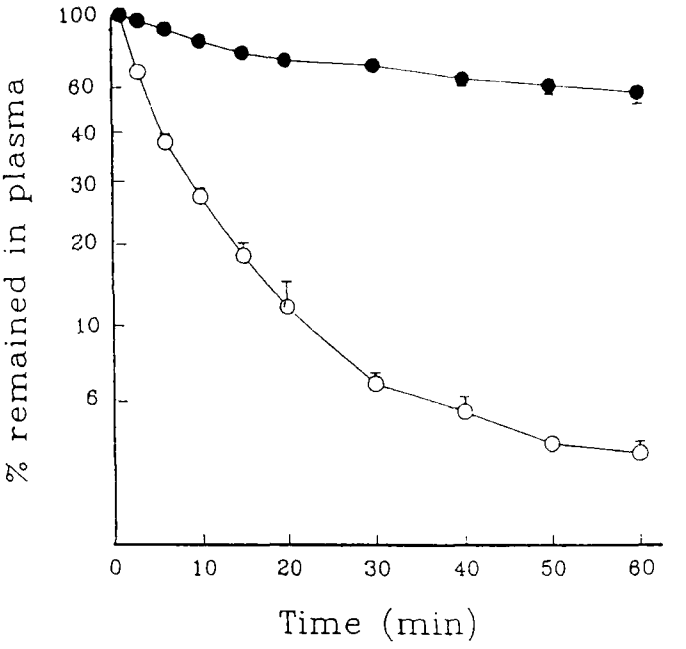

FIGURE 2

Plasma elimination of MLVs after i.v. injection in rats. The radio-activity of liposome in plasma for each rat was normalized using plasma concentration at 1 min after i.v. injection as 100%. key: (●) 0.25 μm, (○) 1.31 μm.

nine-fold and one twelfth, respectively, of that of the 1.31 μm liposome.

Influence of Liposome Size on Tissue Distribution

The distribution of liposomes in kidney, lung, liver and spleen 60 min after i.v. injection are shown in Table 4. Significantly ($p < 0.01$) higher dose fractions in liver and spleen, but lower in kidney in the animals dosing with 1.31 um rather than 0.25 μm liposomes were observed. However, on a tissue weight

TABLE 3

Relative Values[a] of Pharmacokinetic Parameters of Liposomes After i.v. Injection in Rats.

	0.26 μm	1.31 μm	Tcal	p
AUC	9473 ± 1293	1005 ± 45	6.54	< 0.01
k_{10}	0.011 ± 0.002	0.135 ± 0.012	10.18	< 0.01
k_{10}-$T_{1/2}$	68.8 ± 9.3	5.2 ± 0.5	6.04	< 0.01
C_{max}	95.3 ± 1.34	134.8 ± 9.69	4.03	< 0.01
V_d	1.14 ± 0.02	2.86 ± 0.07	24.49	< 0.01
A		102.0 ± 8.40		
B		32.9 ± 7.66		
α		0.40 ± 0.11		
β		32.9 ± 7.66		
k_{12}		0.17 ± 0.07		
k_{21}		0.14 ± 0.04		
n	5	4		

Data are mean ± SE.

a, The radio-activity of liposome-dose (1 mg liposomal lipid/kg body weight) and plasma concentrations for each rat were normalized using plasma concentration at 1 min after i.v. injection as 100%; and time in minute.

TABLE 4

Distribution of Liposomes 60 min Following i.v. Injection in Rats.

	0.25 μm	1.31 μm	Tcal	p
	% Dose/A Whole Organ			
Kidney	0.93 (0.07)	0.16 (0.01)	10.23	< 0.01
Liver	21.36 (2.20)	53.73 (2.90)	8.9	< 0.01
Lung	0.95 (0.07)	0.90 (0.08)		
Spleen	8.19 (0.81)	14.11 (1.10)	4.28	< 0.01
	% Dose/g Tissue			
Kidney	0.40 (0.03)	0.08 (0.01)	11.67	< 0.01
Liver	2.25 (0.30)	4.91 (0.34)	5.97	< 0.01
Lung	0.69 (0.07)	0.66 (0.06)		
Pancreas	0.20 (0.02)	0.07 (0.003)	6.19	< 0.01
Plasma	5.11 (0.63)	0.13 (0.01)	7.95	< 0.01
Spleen	14.35 (1.40)	24.40 (2.24)	3.81	< 0.05
n	6	6		

Data are mean (SE).

TABLE 5

Tissue-to-Plasma Concentration Ratio of Liposomes at 60 min After Intravenous Injection into Rats.

Size μm	A 0.25	B 1.31	Tcal	P	B/A
Kidney	0.08(0.01)	0.68(0.06)	9.547	< 0.01	8.4
Liver	0.49(0.09)	42.33(6.63)	6.305	< 0.01	86.7
Lung	0.14(0.01)	5.59(0.88)	6.183	< 0.01	40.5
Pancreas	0.04(0.01)	0.63(0.08)	7.784	< 0.01	14.3
Spleen	3.11(0.63)	174.23(26.83)	6.377	< 0.01	56.1

Data are mean (SE) of 6 determinations.

basis, distribution in the pancreas was highest among those tissues.

Tissue to plasma concentration of liposomes showed a pronounced differences between the 0.25 and 1.31 μm MLVs (Table 5). Sixty min after i.v. injection, the 1.31 μm MLV showed higher concentrations in liver, lung and spleen than in plasma. As many as 174 times higher in spleen, and 42 times higher in liver, than in plasma were observed. On the contrary, the 0.25 μm MLV showed lower concentrations in liver and lung than in plasma.

DISCUSSION

The lipid composition of liposomes chosen for this study was reported to form stable liposomes in buffer solution and in serum[16].

The extrusion of liposomes through PC membranes achieved a defined and well-characterized size

distribution of vesicles. Discrepancies between the diameters of membrane pores and the treated vesicles were observed. The membrane pore diameters larger than 0.4 μm produced slightly smaller vesicles, while those smaller than 0.4 μm produced slightly larger vesicles than the corresponding pore diameters. The magnitude and the tendency of such discrepancies have been observed in other laboratories[10].

Results of the liver perfusion study provided the following information: a) the mean residence time of MLV and SUV in liver is shorter than that of aqua media, regardless of the vesicle size and b) there is a size-threshold of liposome for the hepatic extraction of MLV, although the threshold is not clear-cut. The hepatic extraction of liposomes from the hepatic perfusate is almost equivalent among 0.4 to 1.3 μm liposomes but different from that of 0.25 μm liposome. A comparable 'size-threshold' pattern has been observed in mice where the disposition of 0.46 and 0.16 μm liposomes was quite similar, but both were significantly different from that of 0.058 μm liposomes[7].

The hepatic uptake of SUV and MLV are by different mechanisms. MLV interacts mainly with Kupffer cells while SUV, with parenchyma cell[17-21]. The clearance of SUV from liver perfusate must be due to parenchyma cells but not Kupffer cells.

Particles which are smaller than a certain size, e.g. 0.25 μm in rats as shown in the present study, seem less detectable by Kupffer cells and could pass freely through sinosoid. On the other hand the SUVs (0.021 μm), although too small to be recognized by Kupffer cells, should be able to pass through the pores between the Kupffer cells and contact parenchyma cells[1].

The plasma level decline of liposomal radioactivity in rats given 0.25 μm liposome was significantly slower, and the volume of distribution (Vd) was significantly smaller, than for those given 1.32 μm liposome. This result, combined with the result of hepatic perfusion study, suggests that the uptake of 0.25 μm liposome by RES is remarkably less than that of the larger liposomes. The results of tissue distribution study verifies such speculation. The liposomal radioactivity in the liver and the spleen of rats given 1.31 μm liposome was about two-fold that of rats given 0.25 μm liposome. The liver- and the spleen-to-plasma radioactivity ratios for rats given 1.31 μm liposome was 87 and 56 times that given 0.25 μm liposomes.

In the present experiment, 0.25 μm MLVs were found to be maintained higher concentration in circulation for a longer period. The size-threshold for escaping RES uptake may be different among animal species, and

needs to be assessed further. However, sizing liposomes to an appropriate diameter to minimize RES uptake can be a simple method for maintaining higher plasma concentration and prolonging plasma elimination time of liposomes, which is beneficial for liposome targeting to other tissues.

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