

Release of Drugs from Liposomes Varies with Particle Size

Masahiro YAMAUCHI,* Kyoko TSUTSUMI, Masayuki ABE, Yoichi UOSAKI, Masashi NAKAKURA, and Noboru AOKI

Drug Formulation Research Laboratories, Pharmaceutical Research Center, Kyowa Hakko Kogyo Co., Ltd.; 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411–8731, Japan.

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The efficacy of many drugs is improved by liposomal formulations. The greatest improvements in therapeutic benefits are achieved if the drug is retained in the liposomes for several hours after administration. Many basic drugs can be concentrated efficiently into liposomes in response to a transmembrane pH gradient. However, the rate of release from liposomal formulations is drug-dependent; for example, doxorubicin is released slowly from liposomes whereas vincristine leaks out rapidly. The aim of this study was to identify the causes of the rapid release of drugs from liposomes and then to apply this knowledge to the development of more stable formulations. Our initial focus was to explore the influence of liposomal size on the rate of release of drugs. The retention of doxorubicin within liposomes was independent of the particle size as far as this experimental condition was concerned. However, the rate of release of vincristine varied in relation to the particle size of the liposomes; vincristine was retained more effectively in larger liposomes. Experimental data generated using ^{31}P -NMR analysis and trap volume measurements, indicated that the number of lipid bilayers in liposomes increased as the particle size was increased. Additional lipid bilayers are likely to present a more effective barrier thereby slowing the release of drugs.

Key words liposome; release; delivery; drug; size

Administration of liposomes loaded with active drugs can result in enhanced therapeutic activity^{1,2)} and reduced toxic side effects.^{3–5)} For example, liposomes are widely used to improve the delivery of many anticancer, antibiotic and antifungal drugs, such as doxorubicin, epirubicin, vincristine and ciprofloxacin.^{6–10)} The effectiveness of this formulation approach is dependent on the rate of drug release from the liposomes. Liposomes that rapidly release their contents *in vivo* will not improve delivery of drugs to target sites²⁾; for therapeutic value, it is important that drugs are retained in liposomes *in vivo* for an appropriate time.^{11,12)} Weakly basic drugs can be actively concentrated inside liposomes using a transmembrane pH gradient^{13–15)} or an ammonium sulfate gradient.¹⁶⁾ However, the retention of drugs in liposomes is drug-dependent and can vary dramatically. For example, the anticancer drugs doxorubicin and epirubicin are well retained inside liposomes,^{17–19)} whereas the anticancer drug vincristine and the antibiotic ciprofloxacin tend to leak out rapidly.^{14,19,20)}

In order to obtain homogenous preparations, liposomes are often extruded through polycarbonate filters of 0.4, 0.2 and 0.1 μm pore size. A high proportion of liposomes that are passed through 0.2 μm filters remain as multilamellar vesicles. On the other hand, extrusion of liposomes through 0.1 μm filters produces mainly unilamellar vesicles.²¹⁾ Zhang *et al.*²²⁾ reported that the release of the amphiphilic drug 5-carboxyfluorescein (CF) was greater from unilamellar liposomes than from multilamellar liposomes of similar particle size. The curvature of small unilamellar vesicles (SUVs) is greater, and packing between lipids in the membranes is looser, compared with large unilamellar vesicles. For this reason, SUVs are believed to release drugs more readily.^{23,24)}

The aim of the present study was to identify the causes of the rapid release of drugs, such as vincristine, from liposomes and then to apply this knowledge to the development of more stable formulations. Initially, we investigated the ef-

fect of particle size on the leakage of drugs from liposomes incubated in fetal bovine serum (FBS); for these studies, doxorubicin and vincristine were used as examples of well-retained and readily released drugs, respectively.

MATERIALS AND METHODS

Materials Egg yolk phosphatidylcholine (EPC) was purchased from Nippon oil and fat (Tokyo, Japan). Vincristine and doxorubicin were acquired from Sigma-Aldrich (St. Louis, MO, U.S.A.) and Kyowa Hakko (Tokyo, Japan), respectively. FBS was obtained from GIBCO BRL (Grand Island, NY, U.S.A.). Nuclepore polycarbonate filters and Sepharose CL-6B were purchased from Corning (Acton, MA, U.S.A.) and Amersham Pharmacia Biotech (Uppsala, Sweden), respectively. All other chemicals were of analytical grade quality.

Preparation of Liposomes EPC liposomes were formed by hydrating the lipid with the following buffers: (i) 100 mmol/l citric acid (pH 4.0), (ii) 10 mmol/l Tris-HCl (pH 7.3) containing 1 mmol/l calcein or (iii) 50 mmol/l Tris-HCl (pH 7.0). Calcein, a fluorescent dye, was added to permit the determination of the 'trap volume' (see below). The resulting liposomes were extruded several times through two stacked Nuclepore polycarbonate membrane filters with pore sizes of 0.2, 0.1, 0.08 or 0.05 μm to obtain liposomes of corresponding sizes. The liposomes were diluted with the respective buffer to give a lipid concentration of 62.5 mg/ml in (i), 12.5 mg/ml in (ii) and 30.1 mg/ml in (iii), respectively. Lipid concentrations were determined using the phosphorus assay (Wako, Tokyo, Japan).

Liposome Size Determination The particle size of the vesicles was measured by dynamic light scattering (ELS-800, Otsuka Electronics, Osaka, Japan). Average diameters were evaluated as a Z-average using the monomodal method (a cumulant analysis).

* To whom correspondence should be addressed. e-mail: masahiro.yamauchi@kyowa.co.jp

Drug Uptake into Liposomes Drugs were added to the liposome preparations (EPC 62.5 mg/ml) in 100 mmol/l citric acid (pH 4.0) to give a drug-to-lipid w/w ratio of 0.02 (molar ratio 0.0267 for doxorubicin, 0.0167 for vincristine). The exterior solutions were then titrated to pH 7.4 with 1.0 mol/l NaOH, thus creating a pH gradient (acidic inside) across the vesicles.

In order to determine encapsulation efficiency, aliquots of the liposomal preparations were centrifuged for 1 h at approximately $110000\times g$ to precipitate the liposomes. The supernatant was removed and the drug content was analyzed to provide an estimate of unencapsulated drug. Doxorubicin, was determined by measurement of the absorbance at 496 nm after the addition of Triton X-100 (1 vol%). For analysis of vincristine, samples were diluted with 2 volumes of 2-propanol and centrifuged. Portions (50 μ l) of the supernatants were analyzed by isocratic high-performance liquid chromatography (HPLC) using an AM-312 ODS column (150 $\times$ 6.0 mm I.D., S-5 mm, 120 Å) (YMC, Kyoto, Japan) eluted with acetonitrile–0.1% triethylamine in 0.05 mol/l phosphate buffer (pH 7.3; 50:50, v/v) at 1 ml/min. Vincristine eluted at a retention time of 15 min and was quantified by measurement of the absorption at 300 nm.

Determination of Trap Volume The volume of the aqueous compartment of the liposomes, the “trap volume”, was determined by measuring the amount of calcein in the liposomes, as described elsewhere.²⁵ Calcein was detected using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) with the excitation and emission monochrometers set at 490 nm and 520 nm, respectively. An aliquot (40 μ l) of liposomal suspension (EPC 12.5 mg/ml) in Tris–HCl buffer (pH 7.3) containing 1 mmol/l calcein was diluted to 2 ml with Tris–HCl buffer and the fluorescence of the suspension was measured after addition of 20 μ l of Cobalt(II) chloride (10 mmol/l). This fluorescence measurement (F_{in}) provides an estimate calcein in the internal compartment plus the unquenched fraction in the external compartment. Subsequently, 20 μ l of 20% Triton X-100 was added and the fluorescence measured again. The detergent destroys the integrity of the liposomes and the resultant fluorescence intensity (F_{totq}) represented the equilibrium concentration of free calcein. Another aliquot (40 μ l) of liposomal suspension was diluted to 2 ml with Tris–HCl buffer and the fluorescence of the suspension was measured after (F_{tot}) addition of 20% Triton X-100 but without $CoCl_2$. F_{tot} was the fluorescence of total calcein present. Thus, the extent of quenching was obtained directly from the sample itself as F_{totq}/F_{tot} . The trap volume was calculated from:

$$\frac{(F_{in} \times 2.06 - F_{totq} \times 2.08) / (F_{tot} \times 2.02 - F_{totq} \times 2.08) \times 100}{= \text{trap volume (\% of total)}} \quad (1)$$

Determination of Liposomal Lamellarity by ^{31}P -NMR The lamellarity of the liposomal preparations was determined using ^{31}P -NMR spectroscopy according to the experimental protocol published elsewhere.²⁶ Liposomal samples containing 39 mmol/l (30.1 mg/ml) lipid in 40% D_2O were subjected to ^{31}P -NMR. Measurements were performed both with and without the addition of an external shift reagent, aqueous praseodymium chloride (39 mmol/l in Tris–HCl buffer). Empty liposomes in Tris-buffer (pH 7.0) were used instead of vincristine-encapsulated liposomes in citrate-

buffer because vincristine changed the permeability of Pr^{3+} and the interaction between citric acid and Pr^{3+} made the liposomes aggregate. ^{31}P -NMR analysis was performed using a Bruker DRX500 spectrometer (Karlsruhe, Germany) with a ^{31}P -probe, at 202.46 MHz. Experimental conditions were: Acquisition time, 0.81 s; spectrum width, 40650 Hz; data size, 65 K complex; 90° pulse width, 18 microseconds; relaxation delay, 2 s; number of acquisitions, 2580; chemical shift reference, external H_3PO_4 ; temperature, 60 °C. Liposome lamellarity was calculated from the ratio of intensities of shifted *versus* non-shifted ^{31}P -resonances following the addition of Pr^{3+} as reported earlier.²⁶ The number of bilayers was calculated by:

$$L = \text{peak area of both peaks} / (2 \times \text{peak area of shifted peak}) \quad (2)$$

Drug Release Experiments Samples (50 mg/ml, 65 mmol/l EPC) were diluted 1:9 with FBS and incubated at 37 °C. Aliquots were removed at various times and, after removal of unencapsulated drug by size exclusion chromatography (Sephacrose CL-6B columns), the concentrations of encapsulated drugs were determined as described before.

RESULTS

Preparation of Liposomes Containing Doxorubicin and Vincristine Typical encapsulation efficiencies were between 95% and 100%. The average size of the liposomes was not affected by the uptake of the drugs.

Determination of Trap Volume The trap volume of liposomes was calculated assuming that all the liposomes were unilamellar. It was assumed that the occupancy volume per phospholipid molecule was 1.253 nm³ and that the thickness of the lipid bilayer was 3.7 nm.²⁷ The molecular weight of EPC is 773. The units of lipid concentration (a) and particle size (b) are mg/ml and nm, respectively; the trap volume was calculated using the following equations:

$$\begin{aligned} \text{the number of lipids per 1 ml} \quad (c) &= \frac{a}{773} \times 10^{-3} \times 6.02 \times 10^{23} \\ \text{the number of lipids per liposome} \quad (d) &= \frac{\frac{4}{3} \pi \left[\left(\frac{b}{2} \right)^3 - \left(\frac{b}{2} - 3.7 \right)^3 \right]}{1.253} \\ \text{the number of liposomes per 1 ml} \quad (e) &= \frac{c}{d} \\ \text{trap volume (ml) per 1 ml} \quad (f) &= e \times \frac{4}{3} \pi \times \left(\frac{b}{2} - 3.7 \right)^3 \times 10^{-21} \\ \text{trap volume (\%)} &= \frac{f}{1} \times 100 = \frac{\left(\frac{b}{2} - 3.7 \right)^3 \times a \times 9.76 \times 10^{-2}}{\left(\frac{b}{2} \right)^3 - \left(\frac{b}{2} - 3.7 \right)^3} \quad (3) \end{aligned}$$

According to Eq. 3, trap volume is proportional to particle size. However, the results of the experiments performed in this study revealed that the trap volume increased with increasing particle size when the particle size was less than 120 nm but remained constant in larger liposomes (Fig. 1). Therefore, these data suggested that liposomes were multilamellar when the particle size was larger than 120 nm.

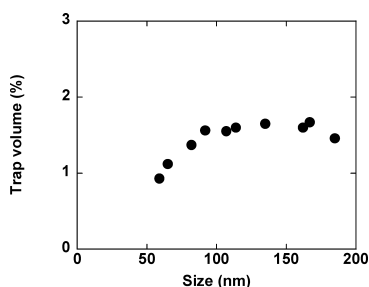


Fig. 1. Relationship between Particle Size and Trap Volume of Liposomes

EPC liposomes were prepared by extrusion through different filters. The trap volume of the preparations was calculated according to the method described in the text.

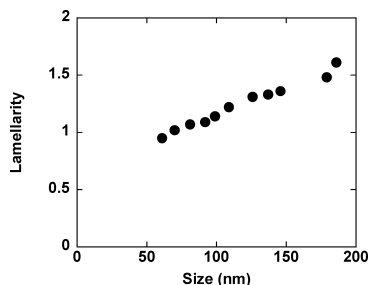


Fig. 2. Relationship between Particle Size and Liposomal Lamellarity

EPC liposomes were prepared by extrusion through different filters. Lamellarity of the preparations was determined using ^{31}P -NMR analysis with Pr^{3+} as shift reagent as described in Materials and Methods.

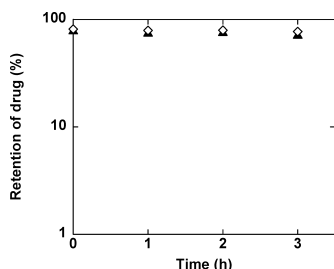


Fig. 3. Representative Retention Profiles of Doxorubicine in Liposomes after Incubation with FBS

Retention of doxorubicin in 99 nm (▲) and 171 nm (◇) EPC liposomes (6.5 mmol/l lipid concentration). Retention was measured in the presence of 90% FBS at an incubation temperature of 37 °C.

Determination of Liposomal Lamellarity by ^{31}P -NMR

The lamellarity of liposomes was measured directly using ^{31}P -NMR but as discussed later, lamellarity determined by NMR probably underestimates the actual value.²⁶⁾ Lamellarity increased with increasing liposomal particle size (Fig. 2) and exceeded unity when the size was larger than 120 nm. Taking into account the results from the determination of trap volume as well as the investigation of lamellarity, most liposomes larger than 120 nm were probably vesicles with two or more membranes.

Release of Drugs from Liposomes The rate of release of doxorubicin from liposomes during 3 h incubation at 37 °C in 90% FBS was independent of the size of the liposomes (Fig. 3). Negligible release of doxorubicin was observed from both 99 nm and 171 nm liposomes during 3 h incubation. However, the retention of vincristine was highly dependent on the particle size (Fig. 4). The rate of release of encapsulated vincristine can be treated as an apparent first-order reaction, therefore:

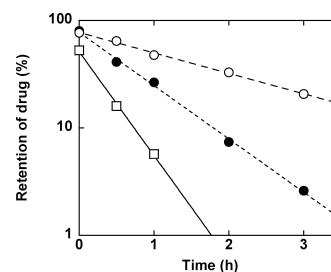


Fig. 4. Representative Retention Profiles of Vincristine in Liposomes after Incubation with FBS

Retention of vincristine in 76 nm (□), 91 nm (●) and 157 nm (○) EPC liposomes (6.5 mmol/l lipid concentration). Retention was measured in the presence of 90% FBS at 37 °C. The solid, dotted and dashed lines are regression curves for retention ratio = $51.4 \times e^{(-2.23 \times \text{time})}$, $r^2 = 0.999$; retention ratio = $77.7 \times e^{(-1.14 \times \text{time})}$, $r^2 = 0.996$; and retention ratio = $77.4 \times e^{(-0.44 \times \text{time})}$, $r^2 = 0.994$, respectively.

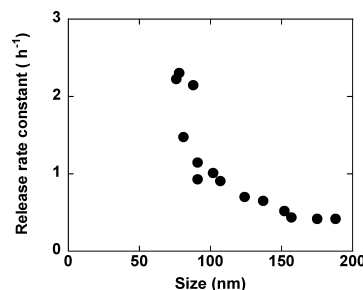


Fig. 5. Relationship between Mean Particle Size and Release of Vincristine from EPC Liposomes (6.5 mmol/l Lipid Concentration)

Release was measured in the presence of 90% FBS at 37 °C. Release rate constants were determined by fitting the experimental data as shown in Fig. 4.

$$-d[X]/dt = k[X] \quad (4)$$

where $[X]$ is the effective concentration of vincristine in liposomes and k is the apparent first-order rate constant. Integrating Eq. 4 with respect to time gives:

$$[X] = [X]_0 \exp(-kt) \quad (5)$$

where $[X]_0$ is the initial concentration of vincristine in liposomes. The rate constants were determined by fitting the experimental data as shown in Fig. 4. The rate constants for the release of vincristine from liposomal preparations of varying particle size were determined and plotted (Fig. 5). Vincristine was released rapidly from small vesicles during incubation in FBS at 37 °C but drug retention was increased in larger liposomes.

DISCUSSION

The rate of release from liposomal formulations is drug-dependent; for example, previous studies have demonstrated that doxorubicin is released slowly^{17–19)} whereas vincristine leaks out rapidly¹⁹⁾ from liposomes. The rate of release could depend on the precipitation of drugs in liposome interior.^{19,20)} The aim of the present study was to identify the alternative causes of the rapid release of some drugs from liposomes. We studied the influence of particle size of liposomes on drug release using doxorubicin and vincristine as examples of well-retained and readily released drugs, respectively. The results confirmed that doxorubicin was only slowly released from liposomes during *in vitro* incubation in FBS, and that the rate of drug release was unaffected by particle size as far

as this experimental condition was concerned (Fig. 3). However, vincristine was poorly retained within liposomes during incubation in FBS and the rate of release of the drug was greatly influenced by the size of the liposomes. The retention of vincristine was improved significantly in larger liposomes (greater than 120 nm).

In small liposomes the curvature of the vesicle is greater, and consequently the packing between lipids in the membranes is looser, compared with larger liposomes. For this reason, smaller liposomes are believed to release drugs more readily.²³ This is likely to be the explanation for the rapid release of vincristine from small liposomes, approximately 50 nm, observed in the present study. However, it is doubtful whether this is the only reason for the increased retention of vincristine in large liposomes; therefore, the relationship between drug retention and particle size was probed further. The correlation between particle size and lamellarity was investigated indirectly by measuring the trap volume of the liposomes. If all the liposomes were composed only of unilamellar vesicles, the trap volume would increase lineally with increasing particle size. However, there was a large discrepancy between the calculated and measured value of the trap volume; the measurements showed that the trap volume remained constant when the particle size was larger than 120 nm. Therefore, these data suggest that liposomes are mainly unilamellar vesicles when the particle size is less than 120 nm but most liposomes are composed of multilamellar vesicles when the particle size is larger than 120 nm.

The lamellarity of liposomes was also measured directly using ³¹P-NMR and these measurements revealed that lamellarity increased with increasing particle size. Others have reported that lamellarity determined by cryo-electron microscopy is higher than suggested by ³¹P-NMR measurements²⁶; mean lamellarity determined by cryo-electron microscopy and by ³¹P-NMR was 1.9 and 1.35, respectively.²⁶ Cryo-electron microscopy shows the actual native structure of vesicles whereas ³¹P-NMR only determines the ratio of the outer to inner phospholipids. Thus, it is likely that the lamellarity data determined by NMR reported here is an underestimate of the actual value.

The conclusion from both the indirect and direct assessment of lamellarity is that in liposomes smaller than 120 nm, most liposomes are unilamellar vesicles but that the proportion of oligolamellar vesicles increases with increasing particle size; in liposomes larger than 120 nm, most of the liposomes are composed of vesicles with two or more membranes.

It is probable that the presence of multiple lipid bilayers presents a more effective barrier than a single membrane.²² Therefore, drugs are likely to be retained more efficiently inside oligolamellar liposomes.

In summary, modest increases of particle size significantly improved the retention of vincristine in liposomes, which can mainly be attributed to the increased number of lipid bilayers in large (>120 nm) liposomes. Thus, it is predicted that drugs, such as vincristine, that are released rapidly from conventional liposomes could be formulated more effectively

using larger liposomes leading to significant improvements in clinical efficacy.

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