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# pH-sensitive, serum-stable and long-circulating liposomes as a new drug delivery system

Myo-Sook Hong, Soo-Jeong Lim, Yu-Kyoung Oh and Chong-Kook Kim

**Abstract**

The lack of stability in blood and the short blood circulation time of pH-sensitive liposomes are major drawbacks for their application in-vivo. To develop pH-sensitive, serum-stable and long-circulating liposomes as drug delivery systems, the impact of polyethylene glycol-derived phosphatidylethanolamine (DSPE-PEG) on the properties of pH-sensitive liposomes was investigated. pH-sensitive liposomes were prepared with dioleoylphosphatidylethanolamine (DOPE) and oleic acid (DOPE/oleic acid liposome) or DOPE and 1,2-dipalmitoylsuccinylglycerol (DOPE/DPSG liposome). The inclusion of DSPE-PEG enhanced the serum stability of both DOPE/oleic acid and DOPE/DPSG liposomes, but also shifted the pH-response curve of pH-sensitive liposomes to more acidic regions and reduced the maximum leakage percentage. The impact of DSPE-PEG, however, was much lower in the DOPE/DPSG liposomes than in the DOPE/oleic acid liposomes. In tumour tissue homogenates, where the pH is lower than normal healthy tissues, the pH-sensitive DOPE/DPSG liposomes released the entrapped markers rapidly, in comparison with pH-insensitive dipalmitoylphosphatidylcholine/cholesterol/DSPE-PEG liposomes. Moreover, the release rate was not affected by the content of DSPE-PEG. The blood circulation time of methotrexate incorporated in DOPE/DPSG liposomes was significantly prolonged with increasing content of DSPE-PEG. Taken together, the liposomes composed of DOPE, DPSG and DSPE-PEG (up to 5%) were pH sensitive, plasma stable and had a long circulation time in the blood. The complete destabilization of the liposomes at tumour tissues suggests that the liposomes might be useful for the targeted delivery of drugs such as anticancer agents.

College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, South Korea

Myo-Sook Hong, Soo-Jeong Lim, Chong-Kook Kim

Department of Microbiology, College of Medicine, Pochon CHA University, South Korea

Yu-Kyoung Oh

**Correspondence:** C.-K. Kim, College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, South Korea. E-mail: ckkim@plaza.snu.ac.kr

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## Introduction

pH-sensitive liposomes which release entrapped drugs at acidic environments have been studied as delivery systems of therapeutic agents (Cordeiro et al 2000; Drummond et al 2000). In particular, pH-sensitive liposomes have been reported to have possible clinical implications for delivering drugs to target sites such as primary tumours and inflammation sites where the pH could be less than physiological (Yatvin et al 1980; Cazzola et al 1997). However, the lack of stability in blood and the short blood circulation time of pH-sensitive liposomes have remained as major drawbacks for their application in-vivo.

Recently, inclusion of polyethylene glycol (PEG)-derived lipids in liposomal bilayers has been shown to inhibit the rapid uptake of liposomes by the reticuloendothelial system, resulting in much longer circulation half-lives in-vivo. This has encouraged attempts to modify classical pH-sensitive liposomes by incorporating PEG-derived lipids in the liposomal membranes (Kono et al 1997;

Slepushkin et al 1997). Slepushkin et al (1997) reported that the addition of PEG-derived lipids to the pH-sensitive liposomes composed of dioleoylphosphatidylethanolamine and cholesteryl hemisuccinate could significantly prolong the blood circulation time, but accompanied a significant decrease in the pH-sensitive release of encapsulated agents.

pH-sensitive liposomes can be made with various compositions of lipids (Hafez et al 2000; Liang et al 2000). Therefore, it needs to be investigated whether PEG-derived lipids could affect the pH sensitivity and the serum stability of pH-sensitive liposomes. In this study, we formulated various liposomes containing a pH-sensitive fluorescent dye and its quencher, and examined the effect of distearoyl phosphatidylanolamine (DSPE)-PEG on the serum stability and pH sensitivity. We then encapsulated methotrexate in the liposomes and investigated the pharmacokinetic profile of liposomal methotrexate to evaluate the prolongation effect of PEG-derived lipids included in pH-sensitive liposomes.

## Materials and Methods

### Materials

Dioleoylphosphatidylethanolamine (DOPE), dipalmitoylphosphatidylcholine (DPPC), cholesterol and oleic acid were purchased from Sigma Chemical Co. (St Louis, MO). 1,2-Dipalmitoylsuccinylglycerol (DPSG) and distearoyl phosphatidylethanolamine-*N*-poly(ethylene glycol) 2000 (DSPE-PEG) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS), and xylene-*bis*-pyridium bromide (DPX) were from Molecular Probes, Inc. (Eugene, OR). Sepharose CL-4B was obtained from Pharmacia LKB Biotech (Uppsala, Sweden). Methotrexate was kindly supplied from Choong-Wae Pharm. Co. (Seoul, Korea). All other reagents were of reagent grade and used without further purification. Lewis-lung carcinoma cell line obtained from ATCC (Manassas VA, USA) was maintained as a solid tumour in C57BL/6 mice by biweekly subcutaneous implantation of the tumour fragments ($2 \times 2 \times 2$ mm$^3$).

### Preparation of liposomes

Liposomes containing ANTS/DPX or methotrexate were prepared using the method of Szoka & Papahadjopoulos (1978) with slight modification. In brief, 10 $\mu$mol of lipid mixtures composed of DOPE/oleic acid or DOPE/DPSG (molar ratio 7:3) with different amount of DSPE-PEG were dissolved in 1 mL of chloroform. The organic solvent was removed on a rotary evaporator under reduced pressure. A dried thin film of the desired lipid composition was dissolved again in 3 mL of organic solvent mixture (isopropyl ether–chloroform, 2:1) and then either 1 mL of ANTS and DPX solution (12.5 mM ANTS, 45 mM DPX, and 68 mM NaCl in 2 mM Tris-HCl buffer, pH 9.0) or 1 mL of methotrexate solution (10 mg mL$^{-1}$) was added dropwise. The resulting mixture was vortexed and the suspension was sonicated in a bath-type sonicator at 37°C for 1 min. The organic solvents were removed under reduced pressure until a clear suspension of liposomes was obtained. To the suspension was then added 0.2 mL of Tris-buffered solution (2 mM Tris, 148 mM NaCl, 0.2 mM EDTA, pH 8.0), and the residual organic solvents were further eliminated under highly reduced pressure. The liposomes were extruded three times through a 0.2-$\mu$m polycarbonate membrane filter (Nucleopore, Costar, MA) and loaded onto a Sepharose CL-4B column ($1 \times 30$ cm) to remove unencapsulated ANTS, DPX or methotrexate, and stored at 4°C until use.

### Measurement of encapsulation efficiency and size determination

A sample (10 $\mu$L) of liposomes containing methotrexate was diluted with 10 mL of phosphate-buffered saline (PBS). Then, 100 $\mu$L of the diluted liposome was mixed with 250 $\mu$L of acetonitrile. After centrifugation, the amount of methotrexate in the supernatant was assayed by HPLC using a cationic exchange column (Partsil 10 SCX, $4.6 \times 250$ mm, GL Science, USA) equipped with a guard column (CX-300, $3.1 \times 30$ mm, Applied Biosystems, USA). As a mobile phase, a mixture of 0.02 M ammonium phosphate and acetonitrile (95:5 v/v, pH 2.0) was used. The eluent was detected at 313 nm.

The size of liposomes was examined by photon correlation spectroscopy as described previously (Park et al 1999). Liposomes were placed in a disposable cuvette and photon counts were measured in a photon correlator at 25°C. A laser particle analyser (LPA-3000, Otsuka Electronics, Japan) was used for the size distribution data.

### Determination of release of ANTS/DPX from liposomes

For the evaluation of pH sensitivity and stability of liposomes containing ANTS/DPX, the increase of fluorescence intensity as a result of leakage of the

quenched liposomal ANTS into the external medium was measured as described in our previous report (Kim et al 1994). For the evaluation of pH sensitivity, liposomes (50 $\mu$L) were added to 1.95 mL of solutions with media of various pH and incubated at room temperature for 30 min. PBS buffer was used for the various pHs (except for medium of pH 5 where citrate buffer (0.01 M) was used). The solution was then passed through a Sepharose CL-4B column. The fluorescence intensity of ANTS in the eluent was monitored using an excitation wavelength of 360 nm and an emission wavelength of 540 nm. The percentage of leakage was calculated using equation 1.

$$\% \text{ Leakage} = \frac{(Ft/F) \cdot Fx - Fo}{Ft - Fo} \times 100 \quad (1)$$

where Fo is the fluorescence intensity before incubation of liposomes at pH 7.4, Ft is the fluorescence intensity after the addition of Triton X-100 to liposomes, Fx is the fluorescence intensity after the incubation of liposomes at various pHs, and F is the fluorescence intensity after the incubation of liposomes at various pHs and treatment with Triton X-100. Ft/F was introduced for the experimental pipetting errors.

For stability studies, human serum was obtained from healthy donors. A sample (500 $\mu$L) of ANTS/DPX liposomes was added to 500 $\mu$L of PBS or serum prewarmed to 37°C. The mixture was then incubated at 37°C in a water bath and then a 50-$\mu$L sample was collected at each time point. The increased fluorescence intensity as a result of lipsomal destabilization was measured and the percentage of ANTS leakage was calculated according to equation 1.

One gram of Lewis-lung carcinoma tumour fragments was homogenized with 4 volumes of saline and then incubated with liposomes containing ANTS/DPX. The mixture of tumour homogenate (1.95 mL) and 50 $\mu$L of the liposome suspension was placed in a dialysis bag. The bag was then placed immediately into 150 mL of PBS medium and incubated at 37°C. At indicated time points, 40-$\mu$L samples were collected. In some cases, the liposome suspension was incubated with normal rat liver homogenates. The increased fluorescence intensity was measured and the percentage of ANTS leakage was calculated as described above.

### Evaulation of circulation longevity of liposomal methotrexate in-vivo

Sprague–Dawley rats (200–300 g) were obtained from the Experimental Animal Breeding Center of Seoul National University (Seoul, Korea). All experiments were performed according to the Seoul National University guidelines of experimental animal care. The rats were fed with commercial rodent chow (Samyang Co., Seoul, Korea) and allowed free access to tap water. The rats were fasted overnight, and the femoral artery and vein were cannulated with a polyethylene tubing (PE-50, Becton Dickinson, NJ) under light anaesthesia with diethylether. A single dose of liposomal methotrexate (8 mg $kg^{-1}$) was intravenously injected into the femoral vein. Blood samples were collected from the femoral artery at each time point, centrifuged to obtain plasma, and stored at $-20$°C until HPLC analysis of methotrexate.

### Pharmacokinetic analysis

The area under the drug concentration–time curve (AUC), area under the first moment plasma concentration–time curve (AUMC), mean residence time (MRT), time-averaged total body clearance (CL) and apparent volume of distribution at steady state (Vss) were calculated by the trapezoidal rule-extrapolation method (Gibaldi & Perrier 1982).

### Statistics

Analysis of variance was used to compare the difference among the group. A $P$ value of less than 0.05 was termed significant. Duncan's multiple range test was used as a post-hoc test.

## Results and Discussion

### Effect of DSPE-PEG on the stability of pH-sensitive liposomes

It would be desirable for liposomes to be stable in the blood circulation, for minimal side effects, and to be completely destabilized at the target tissues, releasing therapeutic agents. To test whether the impact of DSPE-PEG on the properties of pH-sensitive liposomes depended on other lipid compositions of liposomes, we formulated two kinds of pH-sensitive liposomes with various contents of DSPE-PEG : DOPE/oleic acid liposomes and DOPE/DPSG liposomes. Their pH sensitivity has been demonstrated in previous studies (Leventis et al 1987; Lui & Huang 1990). The stability study was performed with liposomes containing ANTS and DPX. Without incorporation of DSPE-PEG, DOPE/oleic acid liposomes were relatively stable in

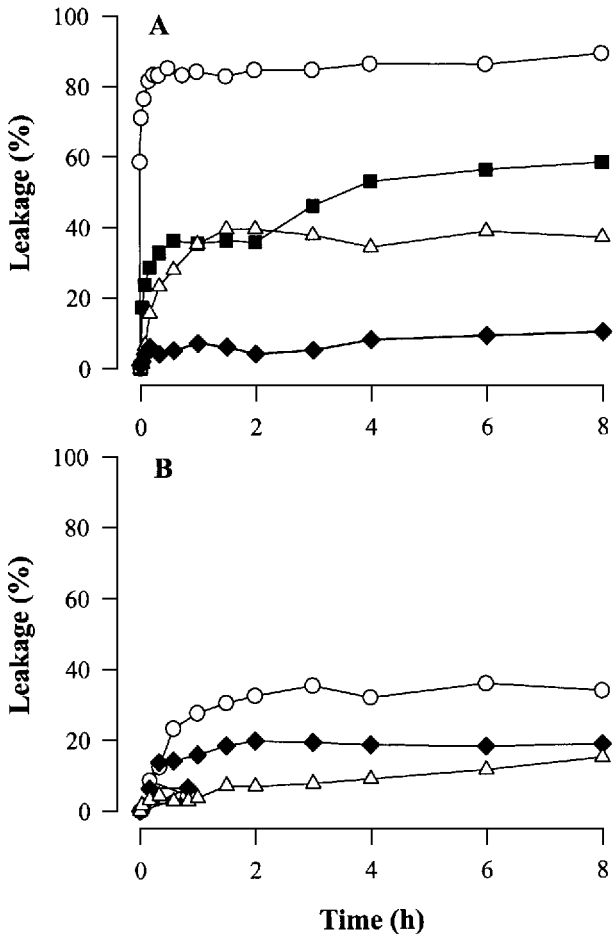


**Figure 1** Effect of DSPE-PEG on the stability of liposomes in serum or PBS. A. DOPE/oleic acid liposomes incorporating 0% (○), 1% (■) or 5% (△) DSPE-PEG incubated in serum; DOPE/oleic acid liposomes without DSPE-PEG incubated in PBS (◆). B. DOPE/DPSG liposomes incorporating 0% (○), 3% (◆) or 5% (△) DSPE-PEG. The liposomes containing ANTS and DPX were added to serum and incubated at 37°C. Samples were collected at each time point and diluted with PBS for the measurement of fluorescence intensity. Data points are the mean values of 4 separate experiments.

PBS (Figure 1A). DOPE/oleic acid liposomes showed the leakage of less than 10% even after 8 h of incubation in PBS. However, they became unstable in serum, resulting in release of about 80% of the contents within 1 h (Figure 1A). In contrast, the presence of DSPE-PEG stabilized the DOPE/oleic acid liposomes. The extent of leakage was less than 55 and 35% even after 8 h of incubation of DOPE/oleic acid liposomes with 1% and 5% DSPE-PEG, respectively.

DOPE/DPSG liposomes were relatively more stable in serum regardless of content of DSPE-PEG (Figure 1B). After 1 h of incubation in serum, less than 30% of liposomal contents were released from DOPE/DPSG liposomes without DSPE-PEG. However, similar to DOPE/oleic acid liposomes, as the content of DSPE-PEG increased, the leakage rates of DOPE/DPSG liposomes in serum gradually decreased. The liposomes composed of DOPE, DPSG and 3 or 5% of DSPE-PEG showed less than 18 and 15% of leakage, respectively, even after 8 h of incubation in serum. Taken together, our results suggest that inclusion of DSPE-PEG increases the stability of pH-sensitive liposomes.

### Effect of DSPE-PEG on the pH sensitivity of liposomes

Since our study showed that stability in serum was increased by inclusion of DSPE-PEG in both DOPE/oleic acid liposomes and DOPE/DPSG liposomes, we investigated the impact of DSPE-PEG on the pH sensitivity of both liposomes containing ANTS/DPX. As a negative control, pH-stable liposomes composed of DPPC/cholesterol were used. DPPC/cholesterol did not show the pH-sensitive release pattern of ANTS over the range of pH values (Figure 2A). In DOPE/oleic acid liposomes, the incorporation of DSPE-PEG significantly reduced the pH sensitivity over the range pH 5–8 (Figure 2A). At pH 5, the leakage was greatly decreased by the addition of only 1% of DSPE-PEG: more than 90% of the contents were released from liposomes without DSPE-PEG whereas only 23% was released from liposomes incorporating 1% of DSPE-PEG. Although the pH sensitivity was slightly lower than DOPE/oleic acid liposomes (Figure 2B), DOPE/DPSG liposomes showed a much less gradual shift of pH sensitivity profile to more acidic regions as the contents of DSPE-PEG increased. In other words, the pH-dependent leakage from DOPE/DPSG liposomes was less affected by the inclusion of DSPE-PEG. At pH 5.25, the leakage from DOPE/DPSG liposomes was about 65 and 50% from liposomes incorporating 0 and 3% DSPE-PEG, respectively. DOPE/DPSG liposomes incorporating DSPE-PEG up to 5 mol% still retained partial acid sensitivity ($>37\%$ leakage at pH 5.25).

These results indicate that although DSPE-PEG influenced the pH sensitivity of liposomes, the impact relied on the lipid composition of the liposomes. Such impact of DSPE-PEG might have resulted from the fact that pKa values of the lipids with pH-sensitive head groups decreased with the change of electrical field by DSPE-PEG. The pH sensitivity of liposomes has been reported to be due to protonation of pH-sensitive lipid head groups (Hazemoto et al 1993). The higher impact of DSPE-PEG on the pH-sensitivity of DOPE/oleic

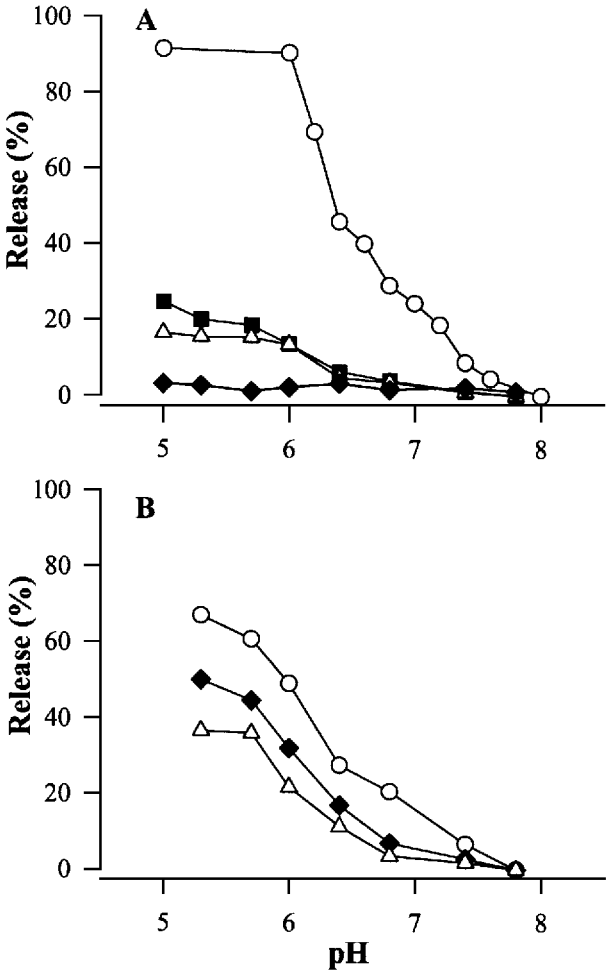


**Figure 2** Effect of DSPE-PEG on the pH sensitivity of liposomes. A. DOPE/oleic acid liposomes incorporating 0% (○), 1% (■) or 5% (△) of DSPE-PEG. pH-insensitive DPPC/cholesterol liposomes (◆, molar ratio 10:5) were used as a control. B. DOPE/DPSG liposomes incorporating 0% (○), 3% (◆) and 5% (△) of DSPE-PEG. The liposomes were incubated in media of various pHs and the leakage of the liposomal content, ANTS, was measured by a fluorometer. Data points are the mean values of 4 separate experiments.

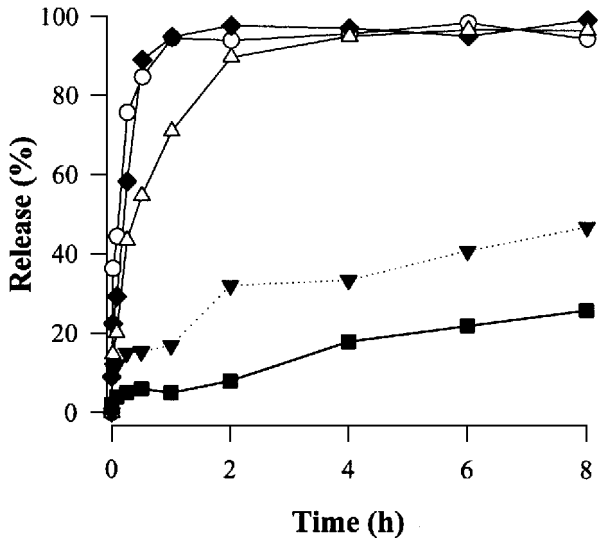


**Figure 3** Effect of DSPE-PEG on the release of markers incorporated in liposomes in tumour or normal tissue homogenates. DOPE/DPSG liposomes with 0% (○), 3% (◆) or 5% (△) of DSPE-PEG, or DPPC/cholesterol liposomes with 3% DSPE-PEG (▼) were added to tumour tissue homogenates and incubated at 37°C. DOPE/DPSG liposomes with 5% of DSPE-PEG (■) were added to the rat normal liver tissue homogenates and incubated at 37°C. Samples were collected at each time point and diluted with PBS for measurement of fluorescence intensity of ANTS. Data points are the mean values of 4 separate experiments.

**Table 1** Methotrexate encapsulation efficiency and size distribution of pH-sensitive liposomes.

| Liposome composition (lipid molar ratio) | Efficiency (%) | Size (nm) |
|---|---|---|
| DOPE/DPSG (7:3) | 1.48±0.16 | 371±48 |
| DOPE/DPSG/DSPE-PEG (7:3:0.31) | 2.21±0.08 | 148±14 |
| DOPE/DPSG/DSPE-PEG (7:3:0.53) | 2.51±0.06 | 143±6 |

Data are presented as means±s.d.

acid liposomes could be explained by the possibility that DSPE-PEG might hinder the protonation of the head group of oleic acid to a greater extent.

Cancer tissue generally has a lower pH than healthy tissue (Hunt et al 1986). Stubbs et al (2000) demonstrated the acidic pH of 6.8 in tumours and the neutral pH of 7.4 in normal tissues using $^{31}P$ magnetic resonance spectroscopy. Martin & Jain (1994) demonstrated a pH value of 6.75 in tumour tissues and 7.2 in normal tissues by fluorescence ratioing microscopy. Therefore, we investigated and compared the release of fluoresecent markers from various liposomal formulations after incubation in cancer tissue homogenates. In tumour tissue homogenates, DSPE-PEG did not significantly influence the stability profiles of the liposomes (Figure 3). Rather, the presence of DOPE and DPSG, which provide pH-sensitivity to the liposomes, appeared to determine the stability of the liposomes in tumour tissue homogenates. While pH-insensitive liposomes composed of DPPC/cholesterol/DSPE-PEG (molar ratio 10:5:0.46, 3 mol% DSPE-PEG) did not completely destabilize even after 5 h, pH-sensitive DOPE/DPSG liposomes released 100% of entrapped ANTS within 2 h regardless of

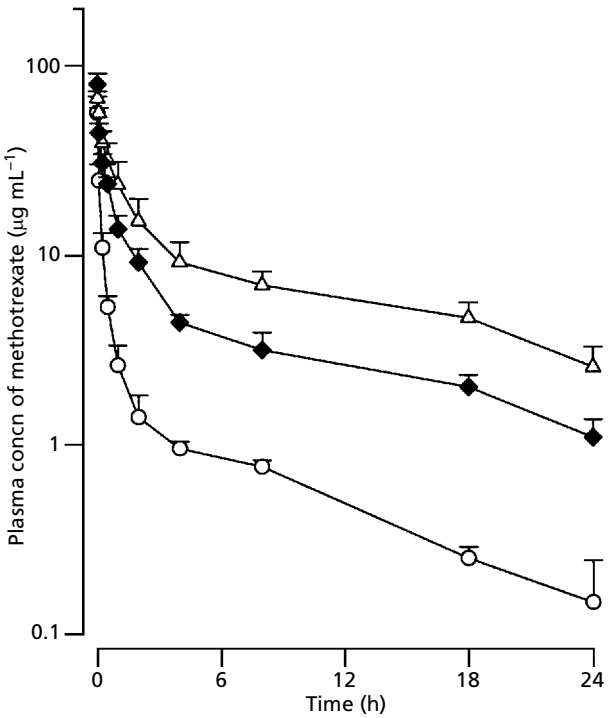


**Figure 4** Plasma concentration versus time profiles of liposomal methotrexate. A single dose of methotrexate (8 mg $kg^{-1}$) in DOPE/DPSG liposomes incorporating 0% (○), 3% (◆) or 5% (△) of DSPE-PEG was intravenously injected into the femoral vein of the rats. Blood samples were collected from the femoral artery at each time point. The amounts of methotrexate were analysed by HPLC. The results are expressed as the mean±s.d., n = 5.

DSPE-PEG content. Unlike tumour tissue homogenates, the normal rat liver homogenates showed that DOPE/DPSG liposomes with 5% of DSPE-PEG liberated less than 30% of the entrapped compounds even after 8 h of incubation (Figure 3). These results indicate that pH-sensitive DOPE/DPSG liposomes with DSPE-PEG are stable in serum, but destabilized at the tumour tissues, releasing entrapped compounds rapidly.

### Methotrexate encapsulation efficiency and size distribution of liposomes

Based on the pH sensitivity (Figure 2) and the stability data (Figure 1), which indicated that DOPE/DPSG/DSPE-PEG liposomes are more pH sensitive and plasma stable than DOPE/oleic acid/DSPE-PEG liposomes, we chose DOPE/DPSG/DSPE-PEG liposomes to encapsulate methotrexate, the anticancer agent. It has been known that the blood circulation time and biodistribution of liposomes could depend on the lipid dose and the size of liposomes (Gregoriadis & Ryman 1972; Gabizon & Papahadjopoulos 1988). In this regard, the determination of methotrexate encapsulation efficiency and the size of the liposomes would be important in developing long-circulating liposomes. DSPE-PEG affected methotrexate encapsulation efficiency of pH-sensitive liposomes. As the content of DSPE-PEG increased, the encapsulation efficiency was enhanced (Table 1). Currently, it is not clear by which mechanism DSPE-PEG increased the encapsulation efficiency of methotrexate. However, we can not exclude the possibility that there is an interaction between methotrexate and DSPE-PEG.

The size distribution pattern was also influenced by DSPE-PEG. In the presence of DSPE-PEG, the average size of liposomes became smaller and the distribution ranges became narrower (Table 1). Such a narrower size distribution of liposomes by DSPE-PEG is consistent with our previous study which showed the narrower size distribution of PEG-derived lipid-containing liposomes compared with the liposomes without PEG-derived lipids (Hong et al 2001). It is thought that the electrostatic repulsion among the liposomes with DSPE-

**Table 2** Pharmacokinetic parameters of liposomal methotrexate.

| Parameter | Liposome composition | | |
|---|---|---|---|
| | DOPE/DPSG | DOPE/DPSG/3% DSPE-PEG | DOPE/DPSG/5% DSPE-PEG |
| AUC ($\mu$g h $mL^{-1}$) | 23.6±0.3 | 92.9±10.1* | 169.0±30.0*† |
| AUMC ($\mu$g $h^2$ $mL^{-1}$) | 110±9 | 490±80 | 1070±192*† |
| MRT (h) | 4.54±0.45 | 5.26±0.50 | 6.35±0.69*† |
| Vss (mL $kg^{-1}$) | 1500±220 | 456±48* | 311±88* |
| CL (ml $h^{-1}$ $kg^{-1}$) | 330.0±23.0 | 86.9±9.5* | 48.6±9.8* |

Data are presented as means±s.d. *$P < 0.05$, compared with DOPE/DPSG liposomes. †$P < 0.05$, compared with DOPE/DPSG/3% DSPE-PEG liposomes.

PEG might reduce the feasible aggregation of liposomes after extrusion, resulting in the smaller average size. Given that the small size of liposomes elevated the blood levels of the liposomes (Gabizon & Papahadjopoulos 1988), the smaller size of pH-sensitive liposomes incorporating DSPE-PEG might contribute to prolonging the blood circulation time.

### Effect of DSPE-PEG on the blood circulation time of liposomal methotrexate

For the effective delivery of drugs to the lower-pH tissues, in addition to liposomes being stable enough not to release drugs in the blood, liposomes themselves should reside in the blood circulation for a prolonged time by avoiding uptake by the reticuloendothelial system. Therefore, we further examined the impact of DSPE-PEG on the circulation longevity of liposomal drugs by monitoring the pharmacokinetic profile of methotrexate in DOPE/DPSG/DSPE-PEG liposomes. Methotrexate administered in pH-sensitive DOPE/DPSG liposomes without DSPE-PEG showed rapidly declining plasma concentrations followed by a slower elimination phase (Figure 4). However, methotrexate administered in pH-sensitive liposomes incorporating DSPE-PEG showed higher plasma concentrations over the period tested. Moreover, the plasma levels of methotrexate increased as the content of DSPE-PEG increased up to 5%. It needs to be noted that the plasma concentrations of methotrexate might not directly indicate the liposomal methotrexate concentrations in Figure 4. Rather, it is likely that the concentrations may reflect the sum of the leaked free methotrexate and liposomal methotrexate. Particularly for the liposomes uncovered with PEG, due to the instability of the liposomes in serum (Figure 1), a substantial fraction of the total methotrexate concentration in serum may be accounted for by the leaked free methotrexate. Of the pharmacokinetic parameters, AUC and MRT increased with the content of DSPE-PEG, whereas Vss and CL decreased (Table 2). The serum-stability of the liposomes incorporating DSPE-PEG may account, in part, for the prolonged circulation of methotrexate.

### Conclusions

Our study suggests the possibility that the limitations of pH-sensitive liposomes can be overcome by including a sterically stabilizing component, DSPE-PEG. Moreover, we demonstrated that the DOPE/DSPE-PEG liposomes incorporating DPSG were more pH sensitive and serum stable than those incorporating oleic acid, leading to prolonged circulation and rapid leakage of the entrapped compounds in tumour tissues. Taken together, DOPE/DPSG/ DSPE-PEG liposomes might be useful for the selective delivery of anticancer drugs to target tumour tissues.

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