



Comparative studies of polyethylene glycol-modified liposomes prepared using different PEG-modification methods

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ABSTRACT

To address the issue of excess polyethylene glycol (PEG)-lipid degradation observed when PEG-modified liposomes are prepared using the pH-gradient method, a concept using a novel PEG-modification method, called the post-modification method, was proposed and evaluated. To assess the proof concept, a preservation–stability study and a pharmacokinetic study were performed that compared the conventional PEG-modification method, called the pre-modification method, with the post-modification method. The results show that PEG-lipid degradation could be markedly inhibited in the post-modification method. Furthermore, the post-modification method could be used without any manufacturing process difficulties, especially with high PEG-lipid content. In addition, a higher blood circulation capability was observed in the post-modification method. Through comparative studies, it was found that the post-modification method was advantageous compared to the pre-modification method. In conclusion, the post-modification method has the potential to be a novel PEG-modification method that can achieve a higher preservation stability of PEG-lipid, a greater ease of manufacturing, and a higher blood circulation capability, especially in the manufacturing of pH-gradient liposomal products.

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1. Introduction

Drug delivery system studies currently focus on achieving selective delivery and distribution of drugs to the target disease sites to enhance safety and effectiveness. Therefore, the feasibility of various particulate systems, such as liposomes, emulsions, lipid microspheres, and polymeric nanoparticles, has been evaluated as potentially effective drug delivery systems [1–3].

To selectively deliver an encapsulated drug to the target site, the control of its pharmacokinetics is essential. The clearance of liposomes is thought to occur through their capture by the mononuclear phagocyte system (MPS), which takes up and removes liposomes circulating in the blood stream. To prolong circulation time in the blood, it is especially important to avoid capture by the phagocytic cells in the liver and spleen. Various characteristics of liposomes affect circulation time in the blood, including chain length, unsaturation of lipids, lipid composition, size, and Zeta-potential [4–6]. Among these, liposome membrane surface modification using monosialoganglioside GM1 or polyethylene glycol (PEG)-conjugated lipid (PEG-lipid) has been shown to greatly improve blood circulation capability [7,8]. Based on these findings, various surface modification approaches have been investigated to develop effective liposome-based drug delivery systems.

Since PEG is a highly hydrophilic polymer with very low toxicity, PEG and its derivatives have been widely used to improve the stability and pharmacokinetics of drug carriers and parent drugs [9]. In liposomal

drug delivery, PEG-lipid has been widely used for liposome surface modification (PEGylation), and this technique has already been used for the preparation of liposomal drug delivery systems, which are known as PEGylated liposomes [10–23]. Specifically, doxorubicin-loaded PEGylated liposomes (Doxil®) have a strong pharmacological effect and low toxicity. Therefore, these have been widely used in clinical applications and are approved in more than 80 countries for cancer treatment [24,25]. To manufacture this product, a unique technology known as the pH-gradient method has been utilized to achieve high drug loading efficiency [26–29].

To achieve high blood circulation capability using PEGylated liposomes, it is important to maintain the physiological and physicochemical stability of PEG-lipid. To our knowledge, however, excess PEG-lipid degradation has been observed, especially in PEGylated liposomes prepared using the pH-gradient method. In general, phospholipids are a component of PEG-lipid and this group is easily hydrolyzed in a bell-shaped manner, with greatest stability at around neutral pH [30]. Unfortunately, PEG-lipid located on the inner surface of liposomes is ineffective and its hydrolysate may cause an increase in membrane permeability. However, this has not been well studied. As described, PEG-modification and the pH-gradient method are both essential for liposomal drug delivery, and these technologies are often used together, such as with Doxil®. Therefore, these issues need to be resolved.

In this study, we propose a new concept (the post-modification method) to avoid excess PEG-lipid degradation, which is observed when PEGylated liposomes are prepared using the pH-gradient method. The advantage of this method is that it modifies the PEG-lipid only on the outer surface of liposomes, thereby avoiding exposure of the PEG-lipid to low pH conditions.

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The main purpose of this study was to assess the proof of concept for our novel preparation method. The post-modification method and conventional PEG-modification method, called the pre-modification method, were compared in terms of stability, ease of manufacturing, and blood circulation capability, using doxorubicin and vincristine as model drugs. The results are discussed with regard to the shielding effect of PEG-lipid, PEG-lipid location, PEG-lipid degradation ratio and pharmacokinetics.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) was obtained from Lipoid GmbH (Ludwigshafen, Germany), cholesterol (Chol) was obtained from Solvay Pharmaceuticals (Nieuweweg, Netherlands), methoxypolyethyleneglycol (Mw 5000)-DSPE (PEG-lipid) was obtained from NOF Corporation (Tokyo, Japan), doxorubicin hydrochloride (DXR) was obtained from RPG Life Science Limited (Maharashtra, India), and vincristine sulfate (VCR) was obtained from Changzhou Leo Chemical (Jiangsu, China). All other chemicals used were of analytical grade.

2.2. Preparation of DXR liposomes by the pre- and post-modification methods

2.2.1. DXR liposomes prepared using the pre-modification method

PEGylated liposomes composed of HSPC and Chol (molar ratio, 54:46) and a given mol% of PEG-lipid (0.5, 1.0, 1.5, and 2.0 mol%) were prepared as follows. HSPC (0.7 g), Chol (0.3 g) and PEG-lipid (0.05–0.20 g) were dissolved in ethanol (1 ml) at 68 °C and hydrated in a 250 mM aqueous solution of ammonium sulfate (9 ml) for 15 minutes at 68 °C to yield crude liposomes. The obtained crude liposomes were extruded through 2 stacked polycarbonate membranes with pore sizes of 200 nm and 100 nm using Extruder T10. DXR loading into liposomes was performed at a DXR/total lipid molar ratio of 0.16 and incubated at 65 °C for 30 minutes according to previous reports [31,32]. Unloaded DXR was removed using the Sepharose 4 Fast Flow column (GE Healthcare, UK) with 10% sucrose and 10 mM histidine solution (pH 6.5) as an eluent. Sterile filtration using a 0.2- μ m membrane filter (Minisart Plus; Sartorius, Goettingen, Germany) was then carried out for PEGylated liposomes loaded with DXR.

As described above, in the pre-modification method, the PEGylation process was conducted concurrently with the downsizing process. Conversely, in the post-modification method, PEGylation was conducted after the downsizing process, so that the applied processing condition could directly affect the success or failure of PEGylation [31].

2.2.2. DXR liposomes prepared using the post-modification method

Bare liposomes (non-PEGylated liposomes) composed of HSPC and Chol (molar ratio, 54:46) were prepared as follows. HSPC (7.0 g) and Chol (2.9 g) were dissolved in ethanol (10 ml) at 68 °C and hydrated in a 250 mM aqueous solution of ammonium sulfate (90 ml) for 15 minutes at 68 °C to yield crude liposomes. The obtained crude liposomes were extruded through 2 stacked polycarbonate membranes with pore sizes of 200 nm and 100 nm using Extruder T100.

PEG-lipid aqueous solution (36.74 mg/ml) was then added to the liposome suspension and heated at 65 °C for 30 minutes to yield PEGylated liposomes with the desired PEG-lipid mol% (0.25, 0.5, 0.75, 1.0, and 2.0 mol%). It was confirmed that PEG-lipid molecules were completely incorporated into the liposomal membrane under the experimental conditions. DXR loading into liposomes was performed at a DXR/total lipid molar ratio of 0.16 and incubated at 65 °C for 30 minutes according to previous reports [31,32]. Unloaded DXR was removed as described in Section 2.2.1.

2.3. Preparation of DXR and VCR liposomes for stability and pharmacokinetic study

2.3.1. PEGylated liposomes prepared using the pre-modification method

HSPC (0.7 g), Chol (0.3 g), and PEG-lipid (0.15 g) (molar ratio, 54:46:1.5) were dissolved in ethanol (1 ml) at 70 °C and hydrated in a 250 mM citrate buffer sucrose solution (pH 2.5, 500 mOsm) (9 ml) to yield crude liposomes. The same process described in Section 2.2.1 was then conducted.

2.3.2. PEGylated liposomes prepared using the post-modification method

HSPC (0.7 g) and Chol (0.3 g) (molar ratio, 54:46) were dissolved in ethanol (1 ml) at 70 °C and hydrated in a 250 mM citrate buffer sucrose solution (pH 2.5, 500 mOsm) (9 ml) to yield crude liposomes. The same process described in Section 2.2.2 was then conducted.

2.3.3. DXR- and VCR-loaded-PEGylated liposomes

DXR loading into PEGylated liposomes was performed at a DXR/HSPC weight ratio of 0.14 and incubated at 65 °C for 30 minutes according to previous reports [31,32]. VCR loading into PEGylated liposomes was performed at a VCR/HSPC weight ratio of 0.22. Unloaded DXR and VCR were removed as described in Section 2.2.1.

2.4. Characterization of various liposomes

2.4.1. Determination of DXR concentration

The total DXR concentration in liposomes was determined by high performance liquid chromatography (HPLC). Briefly, 100 μ l of DXR liposomes was completely dissolved in 2 ml of methanol to analyze total DXR concentration. A designated amount of DXR was dissolved separately in methanol to make solutions of varying concentrations for the preparation of the standard curve. For these prepared solutions, analysis of DXR was performed according to the following: column, Inertsil ODS-2 (4.6 \times 250 mm, 5 μ m, GL Science); column temperature, 40 °C; mobile phase, phosphate solution containing sodium lauryl sulfate/ acetonitrile (1/1); and flow rate, approximately 1.0 ml/minute. An encapsulation efficiency of DXR was calculated from these results according to the following formula:

$$\text{Encapsulation efficiency of DXR(\%)} = [\text{Total DXR concentration}/\text{HSPC concentration}]/0.14 \times 100$$

2.4.2. Determination of VCR concentration

The total VCR concentration in liposomes was determined by HPLC. Briefly, 100 μ l of VCR liposomes were completely dissolved in 2 ml of methanol to analyze total VCR concentration. A designated amount of VCR was dissolved separately in methanol to make solutions of varying concentrations for the preparation of the standard curve. For these prepared solutions, analysis of VCR was performed according to the following HPLC conditions: column, Inertsil C8 (4.6 \times 250 mm, 5 μ m, GL Science); column temperature, 40 °C; mobile phase, phosphate solution/diethylamine/methanol (59/1/140); flow rate, approximately 1.0 ml/min. An encapsulation efficiency of VCR was calculated from these results according to the following formula:

$$\text{Encapsulation efficiency of VCR(\%)} = [\text{Total VCR concentration}/\text{HSPC concentration}]/0.22 \times 100$$

2.4.3. Determination of the lipid component in liposomal preparations

HSPC, Chol, and PEG-lipid were analyzed by HPLC [31]. Briefly, the liposomal sample was dissolved in a mixture of water, chloroform, and 2-propanol. Standard solutions of HSPC, Chol, and PEG-lipid were prepared separately. The lipid component of the prepared solutions was determined using the following HPLC conditions: column, Inertsil Ph

column (4.6×250 mm, 5 μm, GL Science); mobile phase, acetate buffer/methanol/ethanol; detector, refractive index detector (Shimadzu, Japan); flow rate, approximately 1 ml/min.

The PEG-modification ratio was calculated according to the following formula:

$$\text{PEG-modification ratio (mol\%)} = \frac{\text{measured PEG-lipid (mol)}}{[\text{measured HSPC (mol)} + \text{measured Chol (mol)}] \times 100}$$

2.4.4. Other methods

HSPC concentration was determined using the Phospholipid C-test Wako® (Wako Pure Chemicals Ltd., Tokyo, Japan). Particle size and Zeta-potential were determined using Zetasizer 3000HS (Malvern Instruments, UK). To measure particle size (nm), 20 μl of liposomes was diluted with 3 ml of physiological saline solution, and 20 μl of liposomes and PBS were diluted with 2.5 ml of purified water to measure Zeta-potential.

2.5. Comparison studies to compare the pre- and post-modification methods

2.5.1. Accelerated preservation stability study

The liposomal dispersions were stored at 40 °C and 75% relative humidity (RH) for 2 weeks, and the accelerated preservation stability and Zeta-potential were measured at different time periods. The liposomal dispersions were stored at 25 °C for 4 weeks for the accelerated preservation stability, and the residual ratio of HSPC and PEG-lipid were measured at different time periods.

2.5.2. Pharmacokinetics study

This study was conducted at Terumo Corporation (Kanagawa, Japan) and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Male Sprague-Dawley rats aged 6 weeks were purchased from Charles River Co., Ltd. (Kanagawa, Japan). Animals were housed in rooms controlled between 23 ± 1 °C and 55 ± 5% RH, and animals had free access to water and food during acclimatization. To administer the drug at a concentration of 1.0 μmol/ml, DXR liposomes (pre- and post-modification method) and VCR liposomes (pre- and post-modification method) were diluted with 10% sucrose and 10 mM histidine solution (pH 6.5) before administration. These diluted DXR (pre- and post-modification method) and VCR liposomes (pre- and post-modification method) were injected into the tail vein of the rat at a dose of 1.45 mg/kg (2.5 μmol/kg) for DXR (*n* = 3) and 2.31 mg/kg (2.5 μmol/kg) for VCR (*n* = 3). At 0.25, 1, 2, 4, 6, 8, 12, and 24 hours after administration, 0.2 ml of blood was taken with a heparinized syringe and centrifuged for 10 minutes at 5000 rpm and 4 °C to obtain plasma. The plasma (40 μl) was added to methanol (100 μl), and the solution was centrifuged for 10 minutes at 3500 rpm and 4 °C. The supernatant was used for the determination of drug concentration in plasma, and was kept at −20 °C or lower until use. DXR and VCR concentrations in the plasma were determined by HPLC using only results for total drug concentration, which is a summation of drug loaded into the liposomes and free drug in the plasma. A fluorophotometer (Shimadzu, Japan) was used as the detector to analyze DXR concentration in plasma samples, and an ultraviolet absorption photometer (Shimadzu, Japan) was used to analyze VCR concentration in plasma samples.

The area under the plasma concentration-versus-time curve (AUC) was calculated using the trapezoidal rule, and other pharmacokinetic parameters (half-life [*t*_{1/2}], volume of distribution [Vd], and clearance [CL]) were calculated with WINNON LIN using one-compartment model.

2.5.3. Statistical analysis

For group comparisons, one-way analysis of variance (ANOVA) with duplication was applied. Significant differences in the mean values were evaluated using the Student's unpaired *t*-test. A *p*-value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Optimization of temperature conditions for the post-modification process

Before starting the main series of experiments, the influence of temperature conditions on PEGylation was investigated to find a feasible temperature and processing time to ensure the PEG-lipid was modified onto bare liposomes. As shown in Fig. 1, the PEG-modification ratio was plotted as a function of processing time at different temperatures. Above the phase transition temperature of the liposomal membrane (55 °C and 65 °C), PEG-lipid was incorporated onto bare liposomes within 10 minutes. No modification occurred below the phase transition temperature (25 °C), and modification proceeded slowly near the phase transition temperature (35 °C). These results suggest that the temperature of the PEGylation process must be above the phase transition temperature to achieve a high PEG-modification ratio. Consequently, the temperature condition for the PEGylation process was set at 65 °C for 30 minutes.

In this study, designated amounts of PEG-lipid solutions were added to bare liposomes for PEGylation (PEG-lipid additive amount). Unmodified PEG-lipids were eliminated by gel permeation chromatography after PEGylation, and the actual amount of modified PEG-lipid was evaluated from the PEGylated liposomes that were obtained. Fig. 2 shows the relationship between the additive amount and the modified amount of the PEG-lipid in the post-modification method. As seen in Fig. 2, a high linear correlation was observed with an additive amount of PEG-lipid of between 0 and 2.7 mol%, with a linear slope of 0.89 and a multiple correlation coefficient of 0.997.

In previous studies, we found that PEGylated liposomes prepared by the pre-modification method did not pass through the filter pores owing to their high viscosity when a high concentration of PEG-lipid (more than 2.0 mol%) was added [31]. This is a serious issue in the manufacturing process. Conversely, in the post-modification method, the liposomes were guaranteed to pass through the filter pores because high viscosity PEG-lipid was not used. From a practical point of view, the post-modification method is more advantageous compared to the pre-modification method because of the ease of manufacturing of PEGylated liposomes, especially when PEG-lipid contents with higher viscosity are added.

These results suggest that the proposed PEGylation method, the post-modification method, may be a simple and reliable manufacturing technology.

3.2. Characterization of various DXR liposomes for the preservation-stability study

Various DXR liposomes were prepared using the pre- or post-modification method. The Zeta-potential and particle size results are

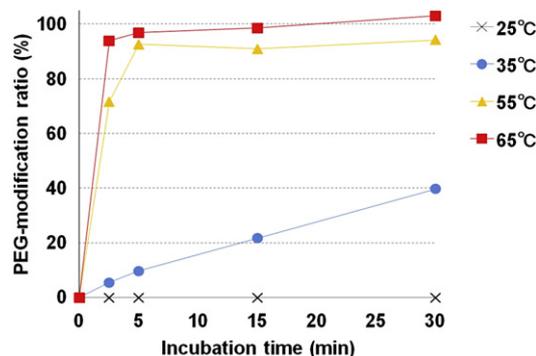


Fig. 1. PEGylation process involving the post-modification method at various temperatures. PEG solution was added in the liposomal suspension to attain a concentration of 0.75 mol% against total lipid (HSPC and Chol), and incubated at 25 °C (×), 35 °C (○), 55 °C (Δ), and 65 °C (□).

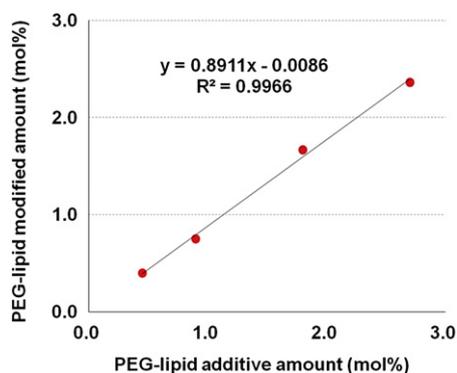


Fig. 2. The relation between applied PEG-lipid and modified PEG-lipid. Different amounts of PEG-lipid solution were added to bare liposomes for PEGylation, and unmodified PEG-lipids were eliminated by gel permeation chromatography after PEGylation.

shown in Fig. 3(A) and Fig. 3(B), respectively. As seen in Fig. 3(A), the Zeta-potential decreased with increasing PEG-lipid content to a constant level of approximately -4 mV. This negative charge density came from the PEG-lipid and is thought to be balanced by the charge-shielding effect of PEG-chains. No clear differences in the change in Zeta-potential between the pre- and post-modification methods were observed. In contrast, as seen in Fig. 3(B), particle size increased with increasing PEG-lipid content of up to approximately 130 nm in the post-modification method. However, no clear changes were observed in the pre-modification method. This behavior is reasonable because the sizing process is initiated after PEGylation in the pre-modification method, and the final particle size is thereby adjusted to the intended size, irrespective of the PEG-lipid content. Conversely, the sizing process is initiated before PEGylation in the post-modification method, and the final particle size is thereby directly affected by the PEG-lipid content. Considering that the chain length of the PEG-lipid that was used (MW: 5000) is approximately 5 nm [17], the observed increase in particle size could be further evidenced, showing that the added PEG-lipids were modified onto the liposomal membrane.

Particle size is a main factor affecting pharmacokinetics. As seen in Fig. 3(B), the observed increase in particle size is due to an increase in the thickness of the PEG layers. The diameter of liposomes without PEG layers (bare liposomes), rather than the diameter of liposomes with PEG layers, are able to affect pharmacokinetics. Therefore, the increase in the liposomal diameter after the PEGylation process does not affect the pharmacokinetics of liposomes.

3.3. Time-dependent change in Zeta-potential during the preservation-stability study

It is generally known that HSPC is partly hydrolyzed into lysophosphatidylcholine (Lyso-PC) and fatty acids at high temperatures and at low pH [30]. Therefore, it is assumed that the negative Zeta-potential of bare liposomes decreases with time owing to HSPC hydrolysis. In contrast, it is assumed that PEGylated liposomes provide lower negative Zeta-potential values than bare liposomes because of the charge-shielding effect provided by hydrated PEG chains covering the negatively charged surface [33,34].

To examine the difference in the charge-shielding effect, the time-dependent change in Zeta-potential values during preservation was performed at 40 °C and 75% RH using various liposomal formulations with different PEG-lipid contents. As shown in Fig. 4, the changes in Zeta-potential values with PEG-lipid content were compared separately between liposome formulations prepared using the pre- or post-modification method at 0, 1, and 2 weeks after storage. Immediately after preparation, all formulations provided only low Zeta-potential (approximately -4 mV), and there was no clear difference in Zeta-potential between the 2 preparation methods, irrespective of PEG-lipid content [Fig. 4 (A)], suggesting that liposomal surface charge is essentially the same at week 0. However, after 1 week of storage, a drastic change in the Zeta-potential was observed in bare liposomes, which decreased to -17 mV, and the Zeta-potential decreased with increasing PEG-lipid content up to a constant level of approximately -5 mV. [Fig. 4(B)]. This indicates that the incorporation of the PEG-lipid effectively shielded the negative charge generated by the hydrolysis of HSPC. This effect was more pronounced in the post-modification method than in the pre-modification method. After 2 weeks of storage, the shielding effect was more clearly observed [Fig 4(C)], with the further increasing negative charge density of bare liposomes effectively suppressed by the introduction of a small amount of PEG-lipid. It was also noted that the apparent suppressing effect of the post-modification method was superior to the pre-modification method (almost double). This finding strongly supports our hypothesis that the PEG-lipid is distributed only on the outer surface of the liposomes in the post-modification method, whereas it is equally distributed on both the inner and outer surfaces in the pre-modification method. Considering that the distribution of PEG-lipid on the liposomes directly contributes to the charge-shielding effect, the post-modification method is thought to be more feasible. As shown in Fig. 4(A) and Fig. 4(B), the charge-shielding effect of the liposomes with 0.75 mol% of PEG-lipid prepared using the post-modification method was almost equivalent to the liposomes with 1.5 mol% of PEG-lipid prepared using the pre-modification method.

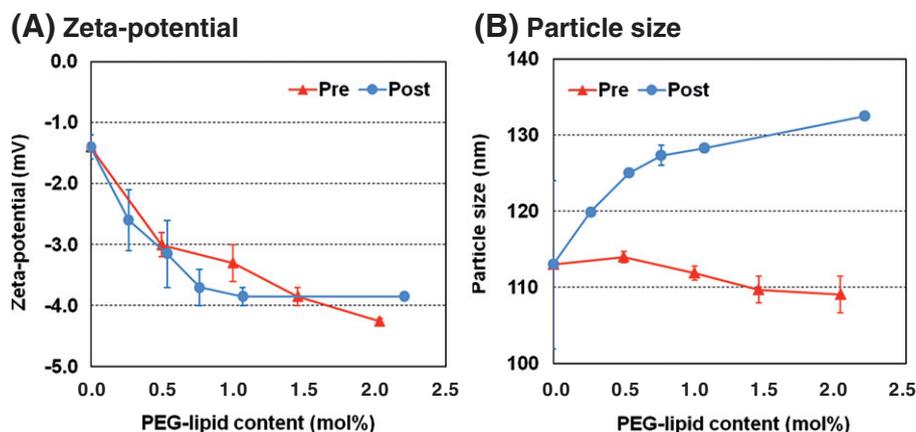


Fig. 3. Characterization of DXR liposomes in relation to PEG-lipid content. (A): Zeta-potential and (B): Particle size. PEGylated liposomes prepared using the pre-modification method (Δ) and the post-modification method (\circ). Data are represented as the mean ($n=3$).

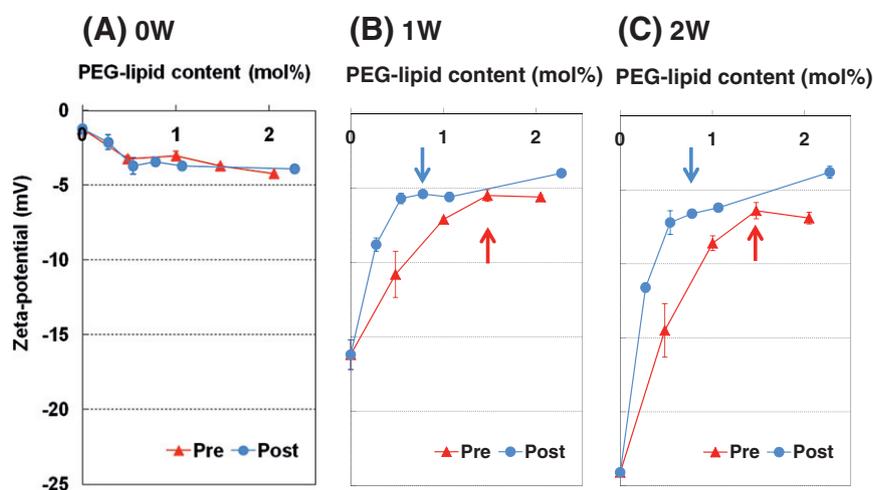


Fig. 4. Time-dependent change in Zeta-potential in PEGylated liposomes prepared using the pre- (Δ) and post-modification method (\circ). Preservation–stability study was conducted at 40 °C and 75% RH, and stored for (A) 0 weeks, (B) 1 week, and (C) 2 weeks. Data are represented as the mean ($n=3$).

3.4. Characteristics of DXR and VCR liposomes for stability and pharmacokinetic study

PEGylated liposomes of DXR and VCR were prepared for the stability and pharmacokinetic study using the pre- and post-modification methods. Composition, drug content, encapsulation efficiency, and particle size were analyzed and are shown in Table 1. From the results of the above-mentioned charge-shielding study, the molar ratio of PEG-lipid was adjusted to 1.5 mol% and 0.75 mol% for the pre- and post-modification methods, respectively, so that the amount of PEG-lipid attached to the outer surface of the liposomes could be equalized. The particle size analysis provided a higher value (approximately 20 nm) in the post-modification method than the pre-modification method, as expected from Fig. 3(B).

3.5. Preservation–stability study

To examine the influence of the preparation method on physico-chemical stability, a preservation–stability study was performed at 25 °C for 4 weeks for the DXR liposomes prepared using the pre- or post-modification method (Table 1). The results are shown in Fig. 5. As seen in Fig. 5(A), the degradation of HSPC after 2 weeks was approximately 10% in both liposomes, but further degradation was not observed until 4 weeks after.

As seen in Fig. 5(B), approximately 20% of the PEG-lipid in the liposomes prepared using the pre-modification method was degraded after 2 weeks, whereas no degradation was observed in the post-modification method after 4 weeks.

The degradation (hydrolysis) rate of PEG-lipid on the inside of the liposomal membrane is expected to be much faster than that of PEG-lipid on the outside of liposomes, because the pH of the internal aqueous phase is kept considerably low when the conventional pH-gradient method is utilized.

Table 1

Characterization of DXR and VCR liposomes. PEGylated liposomes prepared using the pre- and post-modification methods.

PEG-modification method	HSPC:Chol:PEG (molar ratio)	Drug	Encapsulation efficiency (%)	Drug conc. (mg/ml)	Particle size (nm)
Pre	54:46:1.5	DXR	95.7	1.15	91.7
Pre	54:46:1.5	VCR	66.8	1.27	89.0
Post	54:46:0.75	DXR	92.1	1.29	114.6
Post	54:46:0.75	VCR	75.0	1.37	113.9

HSPC is thought to distribute equally to the inside and outside of the lipid bilayer in both methods. Therefore, it is reasonable that no differences in HSPC degradation behavior were observed. Conversely, clear differences in the stability of the PEG-lipid were observed between the pre- and post-modification methods, which strongly suggest that the distribution of PEG-lipid is different between the 2 methods. As mentioned above, in the pre-modification method, PEG-lipid may distribute equally to the inner and outer surfaces of the liposomal membrane, subjecting the internal PEG-lipid to hydrolysis due to the low pH of the internal aqueous phase. However, in the post-modification method, PEG-lipid is exclusively located on the outer surface of the liposomes, thus avoiding hydrolysis. Since this is an important finding to support our hypothesis, further study was conducted using a lower molecular weight PEG-lipid (PEG₂₀₀₀-lipid), and it was confirmed that both HSPC and PEG-lipid degradation occurred in the same manner, even though the PEG molecular weight was lower (data not shown).

3.6. Pharmacokinetic studies

To compare the influence of liposomes prepared using the pre- or post-modification method on blood circulation capability, pharmacokinetic studies were performed using DXR and VCR liposomes (Table 1). The drug concentration profiles in plasma after intravenous administration are shown in Fig. 6(A) for DXR liposomes and in Fig. 6(B) for VCR liposomes. As seen in both figures, both the DXR and VCR liposomes prepared using the post-modification method provided higher drug concentrations in plasma than those prepared using the pre-modification method. The calculated pharmacokinetic parameters are shown in Table 2. There were significant differences in AUC and Vd between liposomes prepared using the pre- and post-modification methods ($p<0.05$), although statistically significant differences in $t_{1/2}$ were not seen. These findings suggest that PEGylated liposomes prepared using the post-modification method are superior to those prepared using the pre-modification method in terms of blood circulation capability.

Interestingly, the CL values for DXR liposomes prepared using the pre- and post-modification methods were the same. DXR can be stably retained into liposomes because of the formation of a rigid complex between DXR and ions in the inner aqueous compartment [35–37]. The CL of DXR may be due to the clearance of liposomes, and there may be no significant differences in the clearance of PEGylated liposomes between the pre- and post-modification methods, although initial distribution immediately after administration is different because of the observed significant differences in Vd between the methods. Although the reason has not yet been elucidated, it may be because of the uniformity of

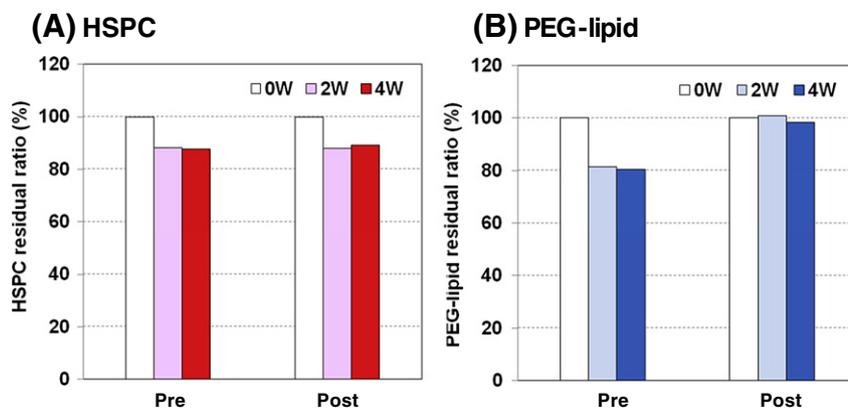


Fig. 5. Percent of remaining HSPC and PEG-lipid in PEGylated liposomes prepared using the pre- or post-modification method after storage at 25 °C for 0, 2, and 4 weeks. (A) Remaining HSPC and (B) remaining PEG-lipid.

PEG-lipid on PEGylated liposomes [31]. On the other hand, there are significant differences in the CL of VCR liposomes between the pre- and post-modification methods. Compared to DXR, VCR does not form rigid ion complexes with the ions in the inner aqueous compartment. This means that VCR is easily released from liposomes, and this hypothesis may be supported by the observed lower CL with VCR than with DXR. From these considerations, the post-modification method might give a rigid membrane structure and show less drug release from liposomes, since the CL value is significantly less than with the pre-modification method.

It is known that various liposome characteristics can affect the blood circulation capability, including particle size, surface charge, and surface modifications [4–6]. However, there were no clear differences in particle size, Zeta-potential, and PEG charge-shielding effect for all liposomes used in this study. Under such circumstances, the observed results were considered to be quite unusual and it is hypothesized that a more complicated mechanism may be the cause of this phenomenon. There are 2 possible reasons for this, and further studies investigating these may be warranted. The first possible reason may be the difference in membrane permeability induced by the amount of PEG modification. In a recent report, the drug release rate from vesicles increased with increasing Chol-PEG content [38]. In the pre-modification method, twice the amount of PEG-lipid is required to achieve the same charge-shielding effect and, therefore, membrane permeability would be higher than that in the post-modification method. The second possible reason may be the content uniformity of PEG-lipids in PEGylated liposomes. In our recent study, it was suggested that PEGylated liposomes prepared using the pre-modification method had more heterogeneous PEG surface properties than the post-modification method [31]. In

the current study, therefore, it may be possible that a smaller amount of PEG-lipid-modified liposomes and an excess amount of PEG-lipid-modified liposomes might be intermingled (heterogeneous sample), even if the average PEG-lipid-modified ratio is adequate. In this case, a smaller amount of PEG-lipid-modified liposomes would not provide the expected blood circulation capability.

Although various other unknown factors may also be involved in this phenomenon, the present pharmacokinetic study demonstrated that the post-modification method has a higher blood circulation capability compared to the pre-modification method. Further experiments are being conducted to investigate these possibilities.

4. Conclusions

Through a series of comparative studies, it was shown that the newly proposed post-modification method could selectively modify PEG-lipid exclusively on the outer surface of liposomes. It was also demonstrated that the post-modification method effectively enabled a charge-shielding effect even by the addition of a small amount PEG-lipid, and that this method could markedly inhibit PEG-lipid degradation, thereby showing high stability. Furthermore, the post-modification method was used without any manufacturing process difficulties, especially with high PEG-lipid content. In addition, a higher blood circulation capability was observed in the post-modification method. Through comparative studies, it is thought that the post-modification method would be more advantageous than the pre-modification method.

In conclusion, the post-modification method has the potential to be a novel PEG-modification method that can achieve higher preservation stability of PEG-lipid, a greater ease of manufacturing, and a

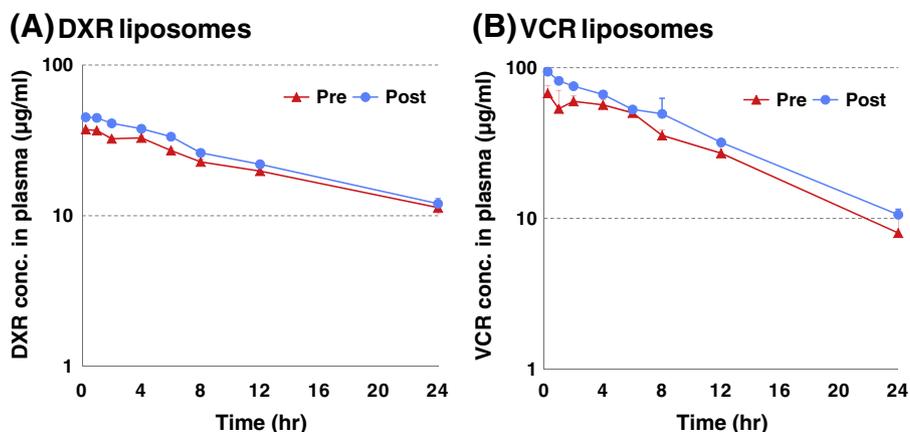


Fig. 6. The drug concentration profiles in plasma after intravenous administration of (A): DXR liposomes and (B) VCR liposomes. PEGylated liposomes prepared using the pre-modification method (Δ) and PEGylated liposomes prepared using the post-modification method (\circ). Data are presented as the mean \pm standard deviation ($n=3$).

Table 2

Pharmacokinetic parameters following intravenous administration of DXR and VCR liposomes to male rats. Data are presented as the mean \pm standard deviation ($n = 3$).

	DXR liposomes (Pre)	DXR liposomes (Post)	VCR liposomes (Pre)	VCR liposomes (Post)
AUC (mg hour/l)	683.4 \pm 18.7	766.3 \pm 17.9 ^a	754.8 \pm 61.5	937.7 \pm 49.3 ^b
$t_{1/2}$ (hour)	13.0 \pm 1.3	11.6 \pm 0.2	7.9 \pm 0.8	7.7 \pm 0.4
Vd (ml/kg)	38.3 \pm 1.6	31.3 \pm 1.1 ^a	32.4 \pm 2.6	24.8 \pm 1.4 ^b
CL (ml/hour/kg)	2.05 \pm 0.13	1.87 \pm 0.03	2.86 \pm 0.37	2.23 \pm 0.09 ^b

^a DXR liposomes (pre) vs DXR liposomes (post), $p < 0.05$.

^b VCR liposomes (pre) vs VCR liposomes (post), $p < 0.05$.

higher blood circulation capability, especially in the manufacturing of pH-gradient liposomal products.

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