BPB Reports

Report

Triggered Release of Paclitaxel from PEG-Modified Liposomes by Sorbitan Ester-Based PEG-Modified Niosomes

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Received April 14, 2026; Accepted May 14, 2026

Liposomes have been widely utilized as drug carriers to achieve the efficient tumor accumulation of anticancer drugs through the enhanced permeability and retention effect. However, due to their rigid lipid membranes, drug release from liposomes is limited in tumor tissues, thereby compromising anti-tumor efficacy. Therefore, the development of technologies that selectively promote drug release from liposomes in tumors is required. We previously demonstrated that polyethylene glycol (PEG)-modified sorbitan monooleate (Span 80) niosomes (PEG-Span 80 niosomes) enabled the triggered release of doxorubicin from the aqueous core of PEG-modified liposomes (PEG-liposomes). In the present study, we investigated whether PEG-Span 80 niosomes also promote the release of paclitaxel (PTX) from the lipid bilayer of PEG-liposomes. In addition, the triggering effect of PEG-modified sorbitan trioleate (Span 85) niosomes (PEG-Span 85 niosomes) on PTX release from PEG-liposomes was evaluated. PTX release from PEG-liposomes was significantly enhanced from early time points in the presence of PEG-Span 80 niosomes, and the amount released at 48 h was approximately 3.9-fold larger than that in the absence of PEG-Span 80 niosomes. Moreover, PEG-Span 85 niosomes promoted PTX release from PEG-liposomes, and the amount of PTX released in the presence of PEG-Span 85 niosomes was approximately 2.1-fold larger than that in the presence of PEG-Span 80 niosomes. In addition, the triggered release of PTX from PEG-liposomes induced by PEG-Span 85 niosomes was partially attributable to membrane fusion between these particles. These results provide valuable information for the realization of safe and effective liposome-based cancer chemotherapy.

Key words PEG-liposome, triggered release, paclitaxel, Span 80 niosome, Span 85 niosome

INTRODUCTION

Liposome-based drug delivery systems offer a number of advantages for cancer chemotherapy, such as the dispersal of lipophilic drugs without a harmful solubilizer.[1,2] Moreover, liposomes enable not only the control of the non-specific biodistribution of anticancer drugs, but also enhanced tumor accumulation through the enhanced permeability and retention effect.[3,4] Regarding the design of the liposomal lipid composition, to minimize the leakage of the incorporated anticancer drugs into the bloodstream, the lipid bilayer of liposomes is often intended to form a rigid structure. Although this property of liposomes contributes to a reduction in the systemic side effects of anticancer drugs, it also limits drug release in tumor tissues,[5] resulting in poor therapeutic efficacy.[6,7] Therefore, to achieve effective cancer therapy using liposomal anticancer drugs, it is necessary to develop strategies to trigger drug release from liposomes locally in tumor tissues.[8,9]

We previously revealed that doxorubicin release from polyethylene glycol (PEG)-modified liposomes (PEG-liposomes) was promoted via interactions with PEG-modified sorbitan monooleate (Span 80) niosomes (PEG-Span 80 niosomes).[10] Moreover, we demonstrated that the anti-tumor efficacy of doxorubicin-loaded PEG-liposomes was significantly augmented through the sequential administration of PEG-Span 80 niosomes. Therefore, a niosome-based triggering system for the release of hydrophilic drugs from the intraliposomal aqueous phase may be successfully established. On the other hand, it remains unclear whether this system is applicable to promoting the release of lipophilic drugs from the lipid bilayer of PEG-liposomes.

In the present study, PEG-liposomes loaded with paclitaxel (PTX), a lipophilic anticancer drug, were prepared, and the effects of Span 80 niosomes on the PTX release property of PEG-liposomes were examined. In addition, with the aim of the more efficient release of PTX from PEG-liposomes, we prepared PEG-niosomes composed of sorbitan trioleate (Span 85) instead of Span 80, and investigated their triggering effect on PTX release from PEG-liposomes.

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MATERIALS AND METHODS

Preparation of PTX-Loaded PEG-Liposomes and PEG-Span Niosomes PTX-loaded PEG-liposomes and PEG-Span niosomes were prepared according to our previous method.[10,11] In brief, phospholipids (NOF Inc., Tokyo, Japan) or Span (Fisher Scientific UK Ltd., Loughborough, UK), cholesterol (Fisher Scientific UK Ltd.), 1,2-distearoylphoshphatidyl-ethanolamine-N-[methoxy(polyethyleneglycol)-2000] (PEG_{2000}-DSPE) (NOF Inc.), and PTX (100 mmol/mol lipid) were mixed in chloroform at the molar ratios listed in Table 1. The mixture was dried by evaporation under reduced pressure. The resulting thin film was hydrated with PBS under mechanical agitation. The PTX-loaded PEG-liposomes and PEG-Span niosomes obtained were sonicated for 10 min using a probe-type sonicator. Unencapsulated PTX was then removed by gel filtration with a Sephadex G-25 column (PD-10, Cytiva, Marlborough, MA, USA). The particle sizes of PTX-loaded PEG-liposomes and PEG-Span niosomes were measured using Zetasizer Pro (Malvern Instrument, Worcestershire, UK).

***In Vitro* PTX Release from PEG-Liposomes** PTX release from PEG-liposomes in the presence or absence of Span niosomes was evaluated by the dialysis method. PTX-loaded PEG-liposomes were mixed with an equal volume of PBS, PEG-Span 80 niosomes, or PEG-Span 85 niosomes at a molar ratio of 1: 3, 1: 6, or 1: 10 and loaded into a dialysis tube (Spectra/Por4 molecular membrane tubing, MWCO: 12,000-14,000, Spectrum Laboratories Inc., Breda, the Netherlands). The dialysis tube was placed into 3% bovine serum albumin in PBS and incubated at 37°C. The amount of PTX released was measured at predetermined time points by HPLC.

Analytical Method for the Quantification of PTX PTX was assessed using reversed-phase HPLC (Shimadzu, Kyoto, Japan) with an ultraviolet detector (SPD-20A) and COSMOSIL $5C_{18}$-MS-II column (4.6 mm i.d. × 150 mm; Nacalai Tesque, Kyoto, Japan). The column temperature was 30°C, and the injection volume was 20 μL. The mobile phase (0.1% phosphoric acid: acetonitrile = 1: 1) was delivered at 1.1 mL/min. PTX was detected at a wavelength of 227 nm.

Fluorescence Resonance Energy Transfer (FRET) PTX-loaded PEG-liposomes were double-labeled with 0.4 mol% of *N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine (NBD-PE) (Avanti Polar Lipids, Alabaster, AL, USA) and 0.2 mol% of Rhodamine-PE (Avanti polar lipids) for FRET measurements. Fluorescence-labeled PTX-loaded PEG-liposomes (final total lipid concentration: 3.3 mM) were mixed with PEG-Span 85 niosomes (final total lipid concentration: 20 mM) at 37°C, and changes in the fluorescence of NBD and Rhodamine were then observed with the fluorescence spectrophotometer, F2500 (Hitachi, Ltd., Tokyo, Japan). Lipid mixing was assessed by the energy transfer efficiency (*ET*) obtained using the equation below:

$$ET = \frac{I_{Rho}}{I_{NBD}}$$

where I_{Rho} and I_{NBD} indicate the fluorescence intensities derived from Rhodamine (Ex/Em = 472/584 nm) and NBD (Ex/Em = 472/527 nm). The energy transfer ratio (*ET* ratio), a quantitative index for lipid mixing, was calculated by the following equation:

$$ET\ ratio = \frac{ET_0 - ET_t}{ET_0 - ET_{max}}$$

where ET_0 indicates the ET value just before adding PEG-Span 85 niosomes to fluorescence-labeled PTX-loaded PEG-liposomes, ET_t indicates the value at time *t* after mixing, and ET_{max} is the value after the addition of Triton X-100 (5%) to the mixture.

Statistical Analysis Results are presented as the mean ± standard deviation (SD) of three experiments. Multiple comparisons between control groups and other groups were performed using Dunnett's test.

RESULTS AND DISCUSSION

Effects of PEG-Span 80 Niosomes on PTX Release from PEG-Liposomes We investigated the capacity of PEG-Span 80 niosomes to trigger the release of anticancer drugs from not only the aqueous core, but also the lipid bilayer of PEG-liposomes. PTX was previously shown to be encapsulated in the lipid bilayer of liposomes.[12] The particle size of PTX-loaded PEG-liposomes was approximately 100 nm (Table 1). The PTX encapsulation efficiency of PEG-liposomes was 87.1 ± 1.7%. As shown in Figure 1, PTX release from PEG-liposomes was significantly enhanced in the presence of PEG-Span 80 niosomes, even at early time points, and the amount released at 48 h was approximately 3.9-fold larger than that in the absence of PEG-Span 80 niosomes. Span 80 vesicles have been shown to easily interact and hemifuse with liposomal membranes.[13] Moreover, we previously demonstrated that PEG-Span 80 niosomes also fused with PEG-liposomal membranes, and consequently increased the membrane fluidity of PEG-liposomes.[10] In addition, we observed that PTX release from PEG-Span 80 niosomes was faster than that from PEG-liposomes.[14] Based on these findings, the increase in PEG-liposomal membrane fluidity induced by the fusion with PEG-Span 80 niosomes may partly contribute to the enhanced release of PTX from the bilayer of PEG-liposomes.

Table 1. Composition and Physicochemical Properties of PTX-Loaded PEG-Liposomes and PEG-Span Niosomes

	Composition (molar ratio)	Average particle size (nm)
PTX-loaded PEG-liposomes	HSPC: cholesterol: PEG_{2000}-DSPE (85.5: 9.5: 5.0)	104.6 ± 10.2
PEG-Span 80 niosomes	Span 80: cholesterol: PEG_{2000}-DSPE: DCP (46.5: 46.5: 5: 2)	139.6 ± 21.8
PEG-Span 85 niosomes	Span 85: cholesterol: PEG_{2000}-DSPE: DCP (46.5: 46.5: 5: 2)	98.9 ± 10.2

Each value represents the mean ± SD (*n* = 3).
HSPC: hydrogenated soybean phosphatidylcholine, DCP: dicetylphosphate.

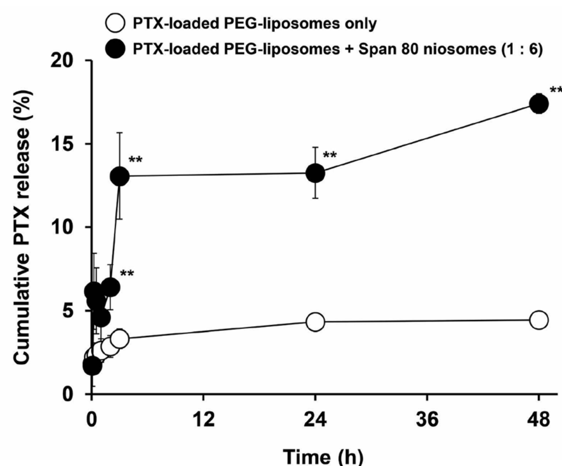

Fig. 1. Triggered PTX Release from PEG-Liposomes by PEG-Span 80 Niosomes

PTX-loaded PEG-liposomes were mixed with PBS or PEG-Span 80 niosomes at a molar ratio of 1: 6 in a dialysis bag and were incubated at 37°C for 48 h. The amount of PTX released was measured at predetermined time points. Each value represents the mean ± S.D. (n = 3). **p <0.01, significantly different from PTX-loaded PEG-liposomes only.

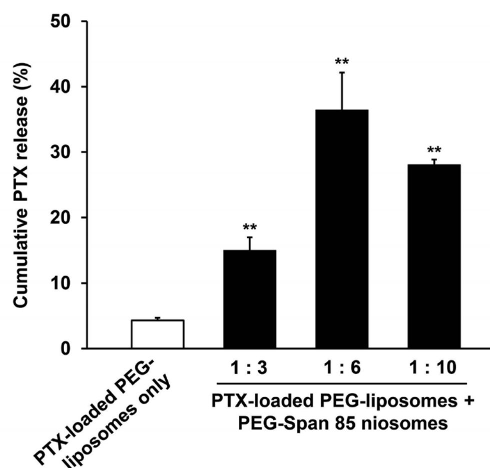

Fig. 2. Effects of PEG-Span 85 Niosomes on PTX Release from PEG-Liposomes at Different Molar Ratios

PTX-loaded PEG-liposomes were mixed with PBS or PEG-Span 85 niosomes at a molar ratio of 1: 3, 1: 6, or 1: 10 in a dialysis bag and were incubated at 37°C. The amount of PTX released was measured at 48 h. Each value represents the mean + S.D. (n = 3). **p <0.01, significantly different from PTX-loaded PEG-liposomes only.

Effects of PEG-Span 85 Niosomes on PTX Release from PEG-Liposomes Unsaturation in lipids is known to play an important role in fusion between lipid bilayers.[15-17] Lipid nanoparticles containing unsaturated cationic lipids were found to efficiently fuse with the endosomal membrane and release their payloads into the cytoplasm, whereas lipid nanoparticles containing saturated cationic lipids hardly escaped from the endosome.[18] Based on these findings, we hypothesized that PEG-niosomes composed of Span 85, which contains three unsaturated bonds, may promote the release of PTX from PEG-liposomes more efficiently than PEG-niosomes composed of Span 80, which contains only one unsaturated bond. Therefore, we investigated the triggered release of PTX from PEG-liposomes by PEG-Span 85 niosomes. As shown in Figure 2, PTX release from PEG-liposomes was approximately 4.4% at 48 h in the absence of PEG-Span 85 niosomes, while approximately 17.0% of PTX was released in the presence of PEG-Span 85 niosomes at a molar ratio of liposomes to niosomes = 1: 3. Moreover, PTX release from PEG-liposomes increased to approximately 36.4% when PEG-Span 85 niosomes were added at a molar ratio of 1: 6. This value was 2.1-fold greater than that triggered by PEG-Span 80 niosomes (approximately 17.4% at 48 h), as shown in Figure 1. Therefore, the more efficient triggering of PTX release from PEG-liposomes may be obtained by using PEG-Span 85 niosomes. On the other hand, the triggered release of PTX from PEG-liposomes was not increased at a molar ratio of PEG-liposomes to PEG-Span 85 niosomes = 1: 10 compared with that at a molar ratio of 1: 6. This result suggests that the triggering effect of PEG-Span 85 niosomes on PTX release from PEG-liposomes is saturated at a molar ratio of 1: 6. Promotion of PTX release from PEG-liposomes by PEG-Span 85 niosomes requires direct contact between their membranes. At a molar ratio of 1: 6, the liposomal membrane surface available for niosome binding was likely to be saturated, and therefore, further increases in the concentration of PEG-Span 85 niosomes would not lead to additional triggering.

Membrane Interaction Between PEG-Liposomes and PEG-Span 85 Niosomes To elucidate the mechanisms underlying enhanced PTX release from PEG-liposomes triggered by PEG-Span 85 niosomes, lipid mixing between PEG-liposomes and PEG-Span 85 niosomes was examined by FRET. As shown in Figure 3, the *ET* ratio rapidly increased immediately after the addition of PEG-Span 85 niosomes to PEG-liposomes, indicating that membrane fusion occurred between these two nanoparticles. This result strongly suggests that PEG-Span 85 niosomes promoted PTX release from PEG-liposomes via a similar mechanism to that of PEG-Span 80 niosomes. Previous studies reported no significant differences in the release profiles of encapsulated contents between Span 80 niosomes and Span 85 niosomes.[19,20] Based on these findings, the membrane fluidity of Span 85 niosomes is considered to be similar to that of Span 80 niosomes. Therefore, the more efficient triggering effect induced by PEG-Span 85 niosomes relative to PEG-Span 80 niosomes is attributable to their superior membrane fusion capacity rather than to a greater increase in membrane fluidity.

In conclusion, we herein demonstrated that PEG-Span 80 niosomes enabled the triggered release of hydrophobic PTX from PEG-liposomes, in addition to hydrophilic doxorubicin. Moreover, PEG-Span 85 niosomes possessed a greater capacity to promote PTX release from PEG-liposomes than PEG-Span 80 niosomes. In addition, the present results showed that the enhanced release of PTX from PEG-liposomes induced by PEG-Span 85 niosomes was attributable to lipid membrane fusion between these particles. These results will make important contributions to the realization of safer and more effective liposome-based cancer chemotherapy.

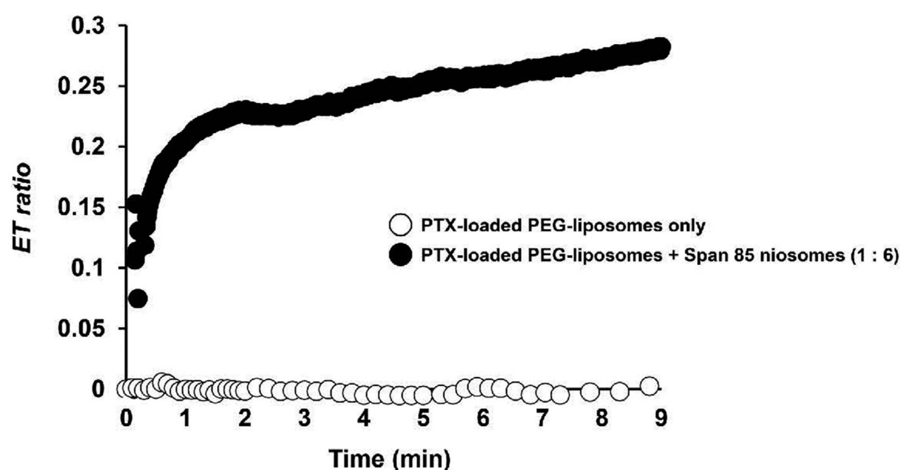

Fig. 3. Evaluation of Lipid Mixing Between PTX-Loaded PEG-Liposomes and PEG-Span 85 Niosomes by the FRET Method

PTX-loaded PEG-liposomes double-labeled with NBD-PE and Rhodamine-PE were mixed with PBS or PEG-Span 85 niosomes at a molar ratio of 1: 6. The result shown in the figure is a representative of three independent experiments.

Conflict of interest The authors declare no conflict of interest.

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