Preparation, Characterization, and in Vitro/in Vivo Evaluation of Paclitaxel-Bound Albumin-Encapsulated Liposomes for the Treatment of Pancreatic Cancer

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ABSTRACT: Paclitaxel (PTX)-loaded liposomes were developed with the goal of enhancing the effects of cancer treatment. Although loading substances into the lipid membrane of liposome cause some destabilization of the lipid membrane, PTX was nearly exclusively embedded in the lipid membrane of liposomes, due to its low water solubility. Hydrophobic drugs can be encapsulated into the inner core of bovine serum albumin (BSA)-encapsulated liposomes (BSA-liposome) via noncovalent binding to albumin. Since PTX is able to noncovalently bind to albumin, we attempted to prepare PTX-loaded BSA-liposome (PTX–BSA-liposome). The amount of PTX loaded in the BSA-liposome could be increased substantially by using ethanol, since ethanol increases PTX solubility in BSA solutions via prompting the binding PTX to BSA. On the basis of the results of transmission electron microscopy and small-angle X-ray scattering, PTX–BSA-liposome formed unilamellar vesicles that were spherical in shape and the PTX was encapsulated into the inner aqueous core of the liposome as a form of PTX–BSA complex. In addition, the PTX–BSA-liposome, as well as nab-PTX, showed cytotoxicity against human pancreatic cancer cells, AsPC-1 cells, in a PTX concentration-dependent manner. The in vivo antitumor effect of PTX–BSA-liposomes was also observed in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells by virtue of its high accumulation at the tumor site via the enhanced permeability retention effect. These results suggest that PTX–BSA-liposomes have the potential for serving as a novel PTX preparation method for the treatment of pancreatic cancer.

INTRODUCTION

Paclitaxel (PTX), which promotes the polymerization of microtubule proteins and inhibits their disassembly, has been clinically used for the chemotherapy of pancreatic cancer.† Since the clinically available PTX preparation, Taxol, contains Cremophor EL as a solubilizing agent due to its low water solubility (less than 2 μg/mL), patients who are administered Taxol often develop Cremophor EL-derived side effects, such as hypersensitivity (breathing difficulty, reduced blood pressure, and tachysystole) and neurotoxicity, thus incentivizing research into the development of alternative PTX preparations that do not involve the use of Cremophor EL. Liposomes offer significant potential for overcoming this issue because they can be loaded with both hydrophilic and lipophilic drugs. Furthermore, it is well known that liposomes with diameter less than 200 nm are able to passively accumulate in solid tumors via the enhanced permeability retention (EPR) effect.‡,§ On the basis of these facts, numerous attempts have been made to develop PTX-load liposomes for pancreatic cancer treatment in the basic research field.∥,‖ However, PTX can stably exist, not in the inner core (aqueous area) but in the membrane (lipophilic space of a liposome) because of the hydrophobic properties of PTX even though the substances to be loaded into the lipid membrane cause destabilization of the lipid membrane.¶,‖

In a previous study, we reported on the design of a novel liposome, a bovine serum albumin (BSA)-encapsulated liposome (BSA-liposome), with the objective of encapsulating hydrophobic drugs into the inner aqueous core of the liposome on the basis of the increased water solubility of hydrophobic drugs as the result of being noncovalently bound to BSA.‖ This novel liposome was superior due to the encapsulation of hydrophobic drugs into the inner aqueous core of its structure with good stability and biocompatibility compared with the liposome without BSA. In addition, BSA-liposomes were observed to accumulate in an inflammatory lesion in the colon of the experimental colitis model mice induced by dextran sulfate sodium,† in indicating that the BSA-liposome meets criteria for the EPR effect.¶ Since PTX can noncovalently bind to albumin,‖ BSA-liposome would be expected to
encapsulate PTX into the inner aqueous core of the liposome and selectively deliver PTX to solid tumor sites via the EPR effect. However, there were some unfavorable results associated with our previous study, in that it was not possible to load a sufficient amount of tacrolimus in the BSA-liposomes, which were used as a model of an albumin-bound hydrophobic drug, to exert a therapeutic effect in vivo, indicating that a novel method for increasing the amount of hydrophobic drug loaded into the BSA-liposomes will be required for the development of a PTX-loaded BSA-liposome (PTX-BSA-liposome) that is capable of exerting an adequate therapeutic efficacy in vivo.

In this study, we report on the preparation of PTX-BSA-liposome, which contains an amount of PTX needed for cancer treatment, and evaluation of the antitumor efficacy of PTX-BSA-liposome against pancreatic cancer. For this purpose, we prepared PTX-BSA-liposomes using ethanol that can promote the binding of hydrophobic drugs to albumin. We subsequently examined the physicochemical properties and structure of PTX-BSA-liposome. Finally, we compared the antitumor effects between the PTX-BSA-liposome and a commercially available PTX formulation, nab-PTX, against pancreatic cancer.

RESULTS

Solubility of PTX. As a result of comparison of the solubility of PTX in ultrapure water, in 20% ethanol, and in a BSA solution ([BSA] = 200 mg/mL), the solubility of PTX in 20% ethanol and the BSA solution was approximately 20 and 69 times higher than that for ultrapure water (Figure 1).

Furthermore, the solubility of PTX in a BSA solution containing 20% ethanol ([BSA] = 180 mg/mL) was synergistically increased compared to that in a 20% ethanol and BSA solution (approximately 150-fold and 43-fold for the 20% ethanol and BSA solution, respectively).

Effect of Ethanol on BSA Structure. Structural changes in BSA in 20% ethanol were evaluated by far-UV circular dichroism (CD) spectra and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Figure 2a, the secondary structure in the case of BSA in 20% ethanol changed slightly compared to that of BSA in ultrapure water (α-helix content: 67.7 and 61.6%, β-sheet: 8.8 and 9.4%, for BSA in ultrapure water and BSA in 20% ethanol, respectively). In SDS-PAGE, a strong single band for BSA in ultrapure water and BSA in 20% ethanol, respectively. In SDS-PAGE, a strong single band for BSA in ultrapure water and BSA in 20% ethanol, respectively. According to the results of PTX solubility, as shown in Figure 1, the loading efficiency (LE) of PTX was dramatically increased in the PTX-saturated BSA solution in 20% ethanol compared to that in the PTX-saturated 20% ethanol and the BSA solution (Table 1). In addition, the particle size and ζ-potential of the preparations were also well-regulated with a good polydispersity index (PDI) in PTX-BSA-liposome prepared by the PTX-saturated BSA solution in 20% ethanol (Table 1). On the basis of these results, we used PTX-BSA-liposomes prepared by the PTX-saturated BSA solution in 20% ethanol in further experiments.

Preparation of PTX-BSA-Liposome. The liposomes encapsulating PTX were prepared by the thin-film hydration method using PTX-saturated 20% ethanol, BSA solution, and BSA solution in 20% ethanol, and the amount of PTX loading in the liposome was compared. In accordance with the results of PTX solubility, as shown in Figure 1, the loading efficiency (LE) of PTX was dramatically increased in the PTX-saturated BSA solution in 20% ethanol compared to that in the PTX-saturated 20% ethanol and the BSA solution (Table 1). Table 1. In addition, the particle size and ζ-potential of the preparations were also well-regulated with a good polydispersity index (PDI) in PTX-BSA-liposome prepared by the PTX-saturated BSA solution in 20% ethanol (Table 1). On the basis of these results, we used PTX-BSA-liposomes prepared by the PTX-saturated BSA solution in 20% ethanol in further experiments.

Morphology Analysis. Transmission electron microscopy (TEM) images confirm that the PTX-BSA-liposome were unilamellar vesicles with a spherical shape (Figure 3a). Furthermore, SAXS profiles of the PTX-BSA-liposomes appeared as a convex curve (Figure 3b), indicating that unilamellar vesicles had been produced. For PTX-BSA-liposomes, a system composed of some PTX-BSA molecules entrapped in a template liposome is considered. For a template liposome, the layers of the inner water phase, the inner PEG chain, the inner head group of the lipid, the inner cholesterol, the alkyl chain, the outer cholesterol, the outer head group of the lipid, and the outer PEG chain are considered. Thus, we set k = 8 of the multilayer spherical model to describe the unilamellar PEG-liposomes containing cholesterol (Figure S2). The experiment data were well fitted (Figure 3b), and the obtained parameters are summarized in Table 2. Furthermore, the calculated values for the volume fraction of BSA (ηBSA) and PTX-BSA (ηPTX-BSA) were 0.1212 and 0.0488, respectively, indicating that the BSA and PTX concentrations in the inner aqueous phase of the liposome are estimated to be 1.74 and 0.23 mM.

Each parameter was defined as below: r1, inner diameter of the liposome; r2 and rφ, PEG chain width; r3 and rη, the head group width; r4 and rω, cholesterol width; r5 + r6 + rφ alkyl chain width; nine electron densities (ρ1, ρ2 ... ρ9); ρω, solute density; ρ1 = ρφ, ρ3 = ρη, and ρ4 = ρω due to

![Figure 1. Solubility of PTX in different solvents. Each column represents the mean ± SD (n = 3). **p < 0.01.](image1)

![Figure 2. Effect of 20% ethanol on the structure of BSA. (a) Far-UV CD spectrum of BSA in water and 20% ethanol. Results are shown for BSA in water (solid line) and BSA in 20% ethanol (gray solid line). (b) SDS-PAGE. lane 1: BSA in water, lane 2: BSA in 20% ethanol.](image2)
Table 1. Physicochemical Properties of PTX–BSA-Liposomes Prepared by Different Hydration Solutions

| hydration solution | particle size (nm) | PDI | \(\zeta\)-potential (mV) | LE* (%) |
|--------------------|-------------------|-----|------------------------|--------|
| 20% ethanol        | 152.4 ± 5.7       | 0.026 ± 0.005 | 1.22 ± 1.3             | 0.0066 ± 0.00023 |
| BSA                | 174.1 ± 6.6       | 0.042 ± 0.02  | 0.03 ± 1.9             | 0.0061 ± 0.0011  |
| BSA + 20% ethanol  | 146.1 ± 5.2       | 0.06 ± 0.005  | -2.6 ± 4.5             | 0.14 ± 0.058**  |

*Loading efficiency (LE) is calculated by the following equation: LE (%) = [PTX in liposome (mg/mL)/total amount of egg PC and cholesterol (mg/mL)] × 100. All liposomes are composed of hydrogenated egg yolk phosphatidylcholine (HEPC)/cholesterol/N-(carboxyl-methoxypoly-(ethyleneglycol) 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG\(_{2000}\)) at a molar ratio 12:6:1. All values represent the mean ± SD (n = 3). **\(p < 0.05\) as compared with BSA.

Figure 3. Morphology analysis of PTX–BSA-liposome. (a) TEM images of PTX–BSA-liposomes. (b) SAXS profiles of PTX–BSA-liposome. Gray dots and the black line indicate experimental data and the fitting curves, respectively.

Symmetrical bilayer structures and \(\rho_s\) the electron density of the inner phase, which includes BSA.

**Intracellular Uptake of PTX–BSA-Liposome.** To evaluate the intracellular uptake of PTX–BSA-liposomes, the BSA and the lipid membrane in BSA-liposome were labeled with fluorescein isothiocyanate (FITC) and Cy5, respectively. Furthermore, dansyl-L-asparagine (DNSA), which is a fluorescent probe for albumin and binds to site I of albumin as well as PTX,\(^{13}\) was used as a model drug instead of PTX because PTX is nonfluorescent and a cytotoxic drug. After incubating fluorescently labeled BSA-liposomes with AsPC-1 cells for 4 h, all of the fluorescent substances inside the cell were observed to be highly fluorescent (Figure 4).

**In Vitro Antitumor Study.** The antitumor effects of PTX–BSA-liposomes against AsPC-1 cells were evaluated in vitro. For comparison, nab-PTX, a commercially available albumin-PTX nanoparticle, was used. As shown in Figure 5, both the PTX–BSA-liposomes and nab-PTX exhibited concentration-dependent cytotoxicity against AsPC-1 cells and nab-PTX exhibited a lower value of IC\(_{50}\) than PTX–BSA-liposome (IC\(_{50}\) PTX–BSA-liposome and nab-PTX, for 37.6 and 29.4 nM, respectively, p < 0.01).

**In Vivo Tumor Accumulation of PTX–BSA-Liposome.** The Cy5-labeled PTX–BSA-liposomes were accumulated in the tumors in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells. The Cy5-labeled PTX–BSA-liposomes were accumulated in the tumors in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells (AsPC-1 cells). As seen in Figure 7a, PTX–BSA-liposomes suppressed tumor growth with strong fluorescence intensity observed at 24 h after administration (Figure 6a). Furthermore, the results of ex vivo measurements of the fluorescence intensity in the organs showed that the PTX–BSA-liposomes were highly distributed in tumors compared with other organs, except for the spleen, at 24 h after administration (Figure 6b), indicating that the BSA-liposomes are capable of delivering PTX to the tumor site.

**In Vivo Antitumor Effect of PTX–BSA-Liposome in a Mouse Model That Had Been Subcutaneously Inoculated with Pancreatic Cancer Cells.** Since high accumulation of PTX–BSA-liposomes in pancreatic cancer was confirmed, the antitumor effect of PTX–BSA-liposomes was investigated in a mouse model subcutaneously that had been inoculated with pancreatic cancer cells (AsPC-1 cells). As seen in Figure 7a, PTX–BSA-liposomes suppressed tumor growth.

Table 2. SAXS Parameters of PTX–BSA-Liposomes

| \(r_1\) (nm) | \(r_2\) (nm) | \(r_3\) (nm) | \(r_4\) (nm) | \(r_5\) (nm) | \(\rho_s\) (e nm\(^{-3}\)) | \(\rho_\infty\) (e nm\(^{-3}\)) | \(\rho_{\infty}\) (e nm\(^{-3}\)) | \(\rho_{\infty}\) (e nm\(^{-3}\)) | \(\rho_{\infty}\) (e nm\(^{-3}\)) | \(\rho_{\infty}\) (e nm\(^{-3}\)) |
|-------------|-------------|-------------|-------------|-------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 57.5        | 3.5         | 0.9         | 0.4         | 2.3         | 348.3         | 337            | 400            | 320            | 270            | 334            |
Table 3. Effect of PTX–BSA-Liposomes and nab-PTX on Hematology in a Mouse Model That Had Been Subcutaneously Inoculated with Pancreatic Cancer Cells

|          | WBC (10^3/μL) | RBC (10^4/μL) | PLT (10^3/μL) |
|----------|---------------|---------------|---------------|
| saline   | 30.7 ± 13.9   | 677.0 ± 87.7  | 54.0 ± 36.5   |
| PTX–BSA  | 47.0 ± 9.7    | 813.0 ± 44.4  | 97.1 ± 13.3   |
| nab-PTX  | 39.0 ± 6.6    | 731.6 ± 137.1 | 72.6 ± 28.1   |

*All values are represented as the mean ± SD (n = 3–5).

**DISCUSSION**

In the present study, the amount of PTX loaded into the inner aqueous core of BSA-liposome was increased when PTX in conjunction with a BSA solution containing 20% ethanol as a hydration solution was used. Since PTX can theoretically exist in the inner aqueous core of liposomes in the form of a PTX–BSA complex, the following possibility would explain the role of contribution of ethanol in increasing the amount of PTX loaded into BSA-liposomes. A possible reason for this is that ethanol increased the solubility of PTX, resulting in enhancing the degree of binding of PTX to BSA in a BSA solution because PTX needs be present in an aqueous phase for binding to BSA. On the basis of this principle, ethanol is generally used in protein-binding experiments as a solubilizing agent for hydrophobic drugs.\(^{14}\) The results reported in this study (Figure 1) are consistent with this hypothesis: (i) the solubility of PTX in 20% ethanol was approximately 20 times higher than that for ultrapure water, and (ii) the amount of PTX bound to BSA increased from 0.5 to 24% when the solvent changed from a BSA solution in water to a BSA solution containing 20% ethanol, resulting in the amount of PTX in the BSA solution containing 20% ethanol being dramatically increased. Thus, the increase in the solubility of PTX in an aqueous phase by ethanol can be mainly attributed to improving the binding PTX to BSA, resulting in increased amounts of PTX being present in the BSA-liposomes.

Although ethanol can improve the amount of PTX loaded into BSA-liposomes, a concern exists that the changes in the albumin structure, fragmentation, and aggregation may have been caused by the presence of ethanol.\(^{15,16}\) In industry, the method of Cohn is classically used as an ethanol-based manipulation (the use of 40% ethanol) for purifying albumin from plasma,\(^{17,18}\) suggesting that albumin would have tolerance in ethanol solution up to 40%. In fact, the present study showed that the secondary structure of BSA was nearly completely preserved without any aggregations and fragmentations when a 20% ethanol solution was used (Figures 2 and S1). Furthermore, it is also a concern that the presence of ethanol may have an effect on the binding of PTX to BSA. In a previous study, Li et al. reported the possibility that ethanol (0.7–24.7% vol/vol) may have had an effect on the interaction of ochratoxin A with albumin.\(^{19}\) Since ochratoxin A and PTX both bind to site I of albumin, the presence of ethanol may also affect the interaction between PTX and BSA.\(^{20}\) Further studies related to this issue will be needed. In addition, it is also possible that ethanol may have had an effect on lipid bilayer formation in the liposome due to the fact that phospholipids and cholesterol are soluble in ethanol. On the basis of morphological observations by TEM, PTX–BSA-liposomes prepared using a BSA solution containing 20% ethanol had a spherical shape and contained unilamellar vesicles (Figure 3a). Furthermore, SAXS analysis data were well-fitted with the 8-layer model (the layers of the inner water phase, inner PEG chain, inner head group of lipid, inner cholesterol, alky1 chain, outer cholesterol, outer head group of lipid, and outer PEG chain) (Figure 3b and Table 2), corroborating the conclusion that PTX–BSA-liposomes form not multilamellar vesicles but unilamellar vesicles that are spherical in shape. These findings compared with saline (\(p < 0.01\)) and nab-PTX (no significance). In addition, no decrease in body weight was observed in any of the mice groups throughout the experiment (Figure 7b). Moreover, no change in the number of blood cells (white blood cell (WBC), red blood cell (RBC), and platelet (PLT)), which reflects the myelosuppression of PTX side effects, was observed in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells that had been administered to PTX–BSA-liposomes and nab-PTX (Table 3).

**Figure 6.** Tumor accumulation of Cy5-labeled PTX–BSA-liposome in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells (AsPC-1 cells). (a) Fluorescence images that were taken at 0, 0.5, 6, 12, and 24 h after the injection of Cy5-labeled PTX–BSA-liposomes. (b) Fluorescence intensity of isolated tissues at 24 h after Cy5-loaded PTX–BSA-liposome injection. Each column represents the mean ± SD (\(n = 5\)).

**Figure 7.** In vivo antitumor efficiency in a mouse model that had been subcutaneously inoculated with pancreatic cancer cell mice that were treated with saline (open circle), PTX–BSA-liposome (closed circle), and nab-PTX (gray closed circle). (a) Relative tumor volume profiles in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells. (b) Body weight profiles in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells. Data are represented as the mean ± SD (\(n = 3–5\)). *\(p < 0.05\), **\(p < 0.01\).
suggest that ethanol would have negligible effects on lipid bilayer formation in PTX−BSA liposomes. We also evaluated the antitumor effect of PTX−BSA-liposomes against pancreatic cancer in vitro. As a comparison, we used nab-PTX (Abraxane)21,22 and albumin-PTX nanoparticles that were approved by the FDA in 2013 for the treatment of metastatic pancreatic cancer when used with gemcitabine.23,24 In the in vitro study, PTX−BSA-liposomes showed concentration-dependent cytotoxicity against AsPC-1 cells but the IC₅₀ value of PTX−BSA-liposome was higher than that of nab-PTX (Figure 5). Chatterjee et al. reported that pancreatic cancer cells recognize albumin and this facilitates the uptake of nab-PTX via caveolin-1.25 In addition, Davidson et al. reported that pancreatic cancer cells take up albumin more efficiently than normal pancreatic cells to consume albumin as a source of nutrients.26 On the other hand, PEG-modified liposomes, like PTX−BSA-liposomes, have a hydration layer on the surface of the liposome, which results in inhibiting cellular uptake.27,28 In the present study, confocal images clearly showed that DNSA−BSA-liposomes are taken up by AsPC-1 cells in the form of intact BSA-liposomes (Figure 4). In addition, the pretreatment of cytochalasin D (an inhibitor of microinocytosis pathway) resulted in significant inhibition of the cellular uptake of Cy5-labeled BSA-liposomes by AsPC-1 cells but a filipin pretreatment (an inhibitor of the caveolae-mediated pathway) did not (Figure S5). Considering these findings, nab-PTX was rapidly taken up by cells via the caveolae-mediated pathway whereas PTX−BSA-liposome was taken up, not via the caveolae-mediated but by the microinocytosis pathway due to the inhibition by the hydration layer that was formed by the PEGylation of the surface, thus causing a higher IC₅₀ value for the PTX−BSA-liposomes.

Although PTX−BSA-liposomes showed lower antitumor effects against AsPC-1 cells than nab-PTX in the in vitro study, the in vivo study showed that the PTX−BSA-liposomes had a higher antitumor efficiency compared with nab-PTX in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells (Figures 6a and 7a). It was reported that macromolecules, including PEG-liposomes, passively accumulate in solid tumors via the EPR effect,2,3 indicating that PTX−BSA-liposomes would be expected to accumulate in tumors by virtue of this effect. To achieve a strong EPR effect, macromolecules are required to meet the following three criteria: (i) molecular size with a negative charge (less than 200 nm in diameter), (ii) biocompatibility, and (iii) adequate retention in the systemic circulation.29 In the case of PTX−BSA-liposomes, the physicochemical properties of the PTX−BSA-liposomes were well controlled at ca. 150 nm with slight negative charge (Table 1). In addition, our previous study showed that BSA-liposomes possess good biocompatibility with nonhematologic toxicity and hepatic and renal injury associated with them.30 Furthermore, on the basis of SAXS analyses, we estimate that the surfaces of BSA-liposomes are completely covered with PEG chains. As a result, the BSA-liposomes can avoid being captured and degraded by the mononuclear phagocyte system and acquire a stealth effect (the long blood retention).31 These characteristics would be expected to confer on PTX−BSA-liposomes a high accumulation property at the tumor site via the EPR effect in AsPC-1 bearing mice. In fact, the in vivo accumulation of PTX−BSA-liposomes in the tumor site was observed starting 6 h after administration in AsPC-1 bearing mice (Figure 6a). On the other hand, it was reported that blood retention for nab-PTX was insufficient due to the fact that its particle morphology was unstable in vivo,32 indicating that nab-PTX would be limited to tumor accumulation via the EPR effect. On the basis of these facts, it would be likely that PTX−BSA-liposomes exerted a higher therapeutic efficiency against pancreatic cancer in vivo than nab-PTX.

There are differences between the present study and the report by Wei et al., which is a research concept similar to the present study.31 They designed thermosensitive liposomes encapsulating both an antitumor drug (PTX)−albumin complex and an antipancreatic stellate cell drug−albumin complex, which they referred to as TSL/HSA-PE. This liposome showed good tumor penetration properties in a three-dimensional tumor spheroid containing pancreatic cancer in vitro and a strong antitumor effect in pancreatic cancer-bearing mice. As compared with the report by Wei et al., the present study revealed the BSA structure after encapsulation into liposomes and the structure of the PTX−BSA liposome was fully characterized. Furthermore, the PTX−BSA-liposomes developed in the present study exhibited in vivo antitumor effects when a 10 times less amount of PTX dose than TSL/HSA-PE (0.5 PTX mg/kg twice a week and 5 PTX mg/kg every 3 days, for PTX−BSA-liposome and TSL/HSA-PE, respectively) was administered. However, experimental conditions in our study were not the same as those in the study conducted by Wei et al., such as the kind of pancreatic cancer cells and lipid components of liposome. Since both liposomes are promising carriers of PTX for the treatment of pancreatic cancer, further evidence will need to be collected.

In conclusion, the amount of PTX loaded in the inner aqueous core of BSA-liposomes can be increased by using ethanol as a solubilizing agent by virtue of enhancing the degree of noncovalent binding of PTX to BSA. In addition, PTX−BSA-liposomes have antitumor efficacy against pancreatic cancer with high tumor accumulation in a mouse model that had been subcutaneously inoculated with pancreatic cancer cells. Thus, the preparation method of PTX−BSA-liposomes established in this study would be useful information on the development of a novel type of liposomes that contains both encapsulated drug and BSA.

**METHODS**

**Chemicals.** HEPC and DSPE-PEG2000 were obtained from NOF Co. (Tokyo, Japan). Cholesterol was obtained from Nacalai tesque (Kyoto, Japan). BSA was purchased from Sigma Chemical Co. (St. Louis, MO). PTX and FITC were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cy5-cholesterol was synthesized according to a previously published procedure.9

**CD Spectra.** Far-UV CD spectra (200−250 nm) were recorded with a Jasco-720 spectropolarimeter (Tokyo, Japan), as reported previously.8

**SDS-PAGE.** SDS-PAGE analysis was performed as reported previously.8 Samples (10 μL, [BSA] = 100 μg/mL) were loaded into a 12.5% polyacrylamide gel and detected by staining with Coomassie Blue R-250. Gel images were recorded using a Bio-Rad GS-800 calibrated densitometer.

**Evaluation of PTX Solubility in Different Solutions.** The solubility of PTX in 20% ethanol solution and a 200 mg/mL BSA solution containing 20% ethanol was evaluated, as previously reported with minor modifications.8 A saturated solution of PTX in 100% ethanol (2 mL) was added dropwise
into either ultrapure water (8 mL) or to a 200 mg/mL solution of BSA (8 mL), resulting in the final concentration of ethanol in each solution of 20%. These solutions were stirred at room temperature for 5 min and then centrifuged at 20000g for 10 min to remove the deposited PTX. The supernatant in the BSA solution was deproteinized using acetonitrile and centrifuged at 20000g for 10 min. The PTX concentration in both solutions was analyzed using high-performance liquid chromatography (HPLC), as reported previously.6

Preparation of PTX−BSA-Liposome. PTX−BSA-liposomes were prepared by the thin-film hydration method, as reported previously, but with minor modifications.5 In short, the thin film composed of HEPC, cholesterol, and DSPE-PEG2000 with a molar ratio of 12:6:1 was hydrated with PTX−BSA solution containing 20% ethanol ([BSA] = 180 mg/mL). The liposome size was regulated by passing the particles through polycarbonate membranes (400 and 100 nm). Unencapsulated PTX and BSA were removed by ultracentrifugation (150 000g for 2 h). The physicochemical characteristics (particle size, PDI, and ζ-potential) were determined by dynamic light scattering (ELS-Z2; Photal Otsuka Electronics, Osaka, Japan). The concentration of PTX in the PTX−BSA-liposome was determined for use in subsequent experiments as follows: the PTX−BSA-liposome solution was deproteinized using acetonitrile and centrifuged at 20000g for 10 min. The concentration in the supernatant was determined using HPLC, as reported previously.5

TEM. Liposomes were stained by 2% (w/w) ammonium molybdate. TEM images were observed with a FEI Titan Themis at 200 kV, as previously reported.32

SAXS. SAXS measurements were carried out at BL-40B2 of the synchrotron radiation facility SPring-8 (Hyogo, Japan) with a 1 m camera using a Rigaku imaging plate (30 × 30 cm2, 3000 × 3000 pixels) as the detector. The exposure time was 300 s, and the wavelength of the beam was 1 Å. A quartz capillary cell (2 mm diameter; Hilgenberg GmbH) was used for all measurements. A model of a PTX−BSA-liposome is considered wherein a number of PTX−BSA molecules are entrapped in liposomes as a template, as illustrated in Figure S1.33 A PTX−BSA-liposome is modeled with the following equations

\[
P(q) = P_{\text{lip}}(q) + N_{\text{BSA}} \times P_{\text{BSA}}(q) + N_{\text{PTX−BSA}} \times P_{\text{PTX−BSA}}(q)
\]

\[
N_{\text{BSA}} = \frac{V_{\text{aqueous}}}{V_{\text{BSA}}} \eta_{\text{BSA}}, \quad N_{\text{PTX−BSA}} = \frac{V_{\text{aqueous}}}{V_{\text{PTX−BSA}}} \eta_{\text{PTX−BSA}}
\]

where \(N\), \(v\), and \(η\) are the number of molecules in a liposome, the volume of one molecule, and the volume fraction of molecules in an inner phase of a liposome, respectively. \(P_{\text{lip}}(q)\), \(P_{\text{BSA}}(q)\), and \(P_{\text{PTX−BSA}}(q)\) are the form factor of a liposome, BSA, and PTX−BSA, respectively, and given as described in the Supporting Materials and Methods.

Cell Experiments. AsPC-1 cells from a human pancreatic cancer cell line were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin). Cells were maintained at 37 °C under an atmosphere of 5% CO2. For evaluating the intracellular uptake of drug-loaded BSA-liposome, BSA and the lipid bilayer of BSA-liposome were labeled with FITC and Cy5-cholesterol, respectively. In addition, DNSA, a fluorescent probe of albumin, was used as a model of an albumin-bound drug. For the cellular uptake test of DNSA−BSA-liposomes, AsPC-1 cells were seeded in poly-lysine-coated glass bottom culture dishes (\(q = 35 \text{ mm};\) Matsunami Glass Ind., Ltd.) at a cell density of \(2 \times 10^5\) cells per dish and cultured for 24 h. The cells were then incubated with DNSA−BSA-liposomes ([DNSA] = 4 μg/mL) at 37 °C for 4 h. The culture medium was then removed and cells were washed thrice with cold PBS (−) and then fixed with 4% paraformaldehyde for 5 min at room temperature. Confocal images were obtained by confocal laser fluorescence microscopy (LSM510META, ZEISS, Germany).32 Excitation and emission wavelengths were used as follows: 405 and 420−480 nm for DNSA, 488 and 530−600 nm for FITC, and 633 and 636−690 nm for Cy5, respectively. For evaluating the antitumor effect of PTX−BSA-liposome and nab-PTX, the AsPC-1 cells were seeded in a 96-well cell culture plate at a cell density of 5000 cells per well and cultured overnight. The cells were then incubated with PTX−BSA-liposomes ([PTX] = 0.1−1000 nM) and nab-PTX ([PTX] = 0.1−1000 nM) for 72 h. Cell viability was determined using a cell counting kit-8 (Dojindo molecular technologies, Inc., Japan), and the half maximal inhibitory concentration (IC\(_{50}\)) was calculated. The cell viability (%) was calculated using the following equation

\[
\text{cell viability(%) = } \frac{A_{\text{sample}}}{A_{\text{saline}}} \times 100
\]

Animals. BALB/cSlc-nu/nu mice (male, 5 weeks) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in a room in which stable conditions were maintained (18−24 °C, 40−70%) with a standardized light/dark cycle. They were allowed free access to food and drinking water. The animals were treated in accordance with the NIH guidelines. All animal experiments were reviewed and approved by the Animal Care and Use committee of Sojo University (Permit #: 2017-P-014).

In Vivo Biodistribution of PTX−BSA-Liposomes in a Mouse Model That Had Been Subcutaneously Inoculated with Pancreatic Cancer Cells. Five BALB/cSlc-nu/nu mice were inoculated with \(2 \times 10^6\) AsPC-1 cells subcutaneously into the left back of the mice. When the tumor volume reached about 200 mm\(^3\), mice were injected with Cy5-labeled PTX−BSA-liposome (0.5 mg PTX/kg) via their tail vein. After a predetermined time (30 min, 6, 12, and 24 h), biodistribution images were acquired using an IVIS Lumina XR instrument (PerkinElmer Inc., Waltham, MA). After the final imaging, the mice were sacrificed and organs (tumor, liver, kidneys, spleen, heart, lungs, and pancreas) collected and the ex vivo fluorescence intensity of Cy5 in the organs measured. The tumor volume (\(V\)) was calculated according to the formula \(V = 0.5 \times L \times W^2\), where \(L\) is the largest tumor diameter and \(W\) is the smallest tumor diameter.35

In Vivo Antitumor Study of PTX−BSA-Liposome in a Mouse Model That Had Been Subcutaneously Inoculated with Pancreatic Cancer Cells. The mouse model was subcutaneously inoculated with pancreatic cancer cells (tumor volume; 50−100 mm\(^3\)). They were randomly assigned into three groups and intravenously administered by either saline, PTX−BSA-liposome (0.5 mg PTX/kg), or nab-PTX (0.5 mg PTX/kg) twice a week, and the body weight and tumor volume were monitored during the course of the treatment. At 64 days after the first treatment, the mice were sacrificed and their blood collected. The blood cell numbers (WBC, RBC, and PLT) were then assessed using an animal blood cell counter (MEK-6458; NIHON KOHDEN Corp., Tokyo, Japan).
Statistics. All data are expressed as the mean ± SD statistical analyses for multiple comparisons in the study were determined by the analysis of variance (two-way ANOVA) followed by the Bonferroni analysis. A probability value of $p < 0.05$ was considered to be significant.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00537.

SAXS profiles of BSA in a 20% EtOH solution (Figure S1); schematic illustration of a model for a liposome as a template containing BSA molecules (Figure S2); SAXS profiles of BSA (top) and PTX−BSA (bottom), and their best fitting curves (black lines) (Figure S3); SAXS profiles of PEG-liposome containing BSA (gray dot) and their best fitting curves (black lines) (Figure S4); cellular uptake of Cy5-labeled PTX−BSA-liposomes into A549 cells under an inhibitor treatment (Figure S5); SAXS parameters of BSA and BSA-PTX (Table S1); SAXS parameters of BSA-liposomes (Table S2) (PDF)

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Y.O., K.T., M.S., and S.I. performed the experiments and analyzed the data. Y.O., K.T., and M.O. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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