Paclitaxel-incorporated nanoparticles using block copolymers composed of poly(ethylene glycol)/poly(3-hydroxyoctanoate)

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Abstract

Block copolymers composed of poly(3-hydroxyoctanoate) (PHO) and methoxy poly(ethylene glycol) (PEG) were synthesized to prepare paclitaxel-incorporated nanoparticle for antitumor drug delivery. In a 1H-NMR study, chemical structures of PHO/PEG block copolymers were confirmed and their molecular weight (M.W.) was analyzed with gel permeation chromatography (GPC). Paclitaxel as a model anticancer drug was incorporated into the nanoparticles of PHO/PEG block copolymer. They have spherical shapes and their particle sizes were less than 100 nm. In a 1H-NMR study in D2O, specific peaks of PEG solely appeared while peaks of PHO disappeared, indicating that nanoparticles have core-shell structures. The higher M.W. of PEG decreased loading efficiency and particle size. The higher drug feeding increased drug contents and average size of nanoparticles. In the drug release study, the higher M.W. of PEG block induced the acceleration of drug release rate. The increase in drug contents induced the slow release rate of drug. In an antitumor activity study in vitro, paclitaxel nanoparticles have practically similar anti-proliferation activity against HCT116 human colon carcinoma cells. In an in vivo animal study using HCT116 colon carcinoma cell-bearing mice, paclitaxel nanoparticles have enhanced antitumor activity compared to paclitaxel itself. Therefore, paclitaxel-incorporated nanoparticles of PHO/PEG block copolymer are a promising vehicle for antitumor drug delivery.

Keywords: Nanoparticles; Block copolymer; Poly(3-hydroxyoctanoate); Drug delivery

Background

Nanoparticles have been extensively investigated in the biomedical field as a drug delivery vehicle due to their properties such as small particle size less than 1,000 nm, favorable biodistribution, and drug targeting possibility [1,2]. Among them, core-shell type nanoparticles have great potentials in the antitumor drug delivery system since they have intrinsic properties, i.e., hydrophobic core as a drug reservoir and hydrophilic outer shell as a protective layer [2,3]. They have superior properties such as extended blood circulation time of drug, passive targeting of solid tumor, dissolution of hydrophobic drugs, and enhanced biodegradation.

Poly(3-hydroxyoctanoate) (PHO) is one of the polyesters derived from bacterial source [4,5]. PHO has attractive properties as a biomedical material such as low melting temperature and crystallinity and excellent elastomeric and mechanical properties compared to other kinds of polyhydroxyalkanoates [4-6]. The drawback of PHO is slower degradation rate and higher hydrophobic properties than other kinds of PHAs [6]. Therefore, various chemical modifications of PHO have been attempted to endow hydrophilic properties and to improve mechanical properties as a biomedical material [5,7].

In this study, we synthesized block copolymers composed of PHO/PEG to make core-shell-typed nanoparticles and to fabricate suitable vehicles for antitumor drug delivery. Since PHO has hydrophobicity, it could be consisted in the core of the nanoparticles while PEG must be hydrated to the direction of aqueous environment. Paclitaxel was selected as an anticancer agent due to its poor aqueous solubility. Characteristics of nanoparticles

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of PHO/PEG block copolymers were analyzed and anti-tumor activity of drug-incorporated nanoparticles was studied in vitro/in vivo.

**Methods**

**Materials**

PHO was produced by *Pseudomonas oleovorans* ATCC 29347 (American Type Culture Collection (ATCC), Manassas, VA 20110, USA) as reported previously [4] and purified from the lyophilized cells by extraction with hot chloroform [8]. The purified PHO analyzed by gas chromatography was consisted of 7% 3-hydroxyhexanoate and 93% 3-hydroxyoctanoate. Methoxy poly(ethylene glycol)-amine (molecular weight (M.W.) = 2,000, 5,000, 12,000 g/mol) was purchased from Sunbio Co. Ltd., Cheonan, Korea. Fluorescein isothiocyanate (FITC), *N*, *N*-dicyclohexyl carbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich Chemical Company (St Louis, MO, USA). The dialysis membranes with molecular weight cut-offs (MWCOs) of 12,000 and 8000 g/mol were purchased from Spectra/PorTM dialysis membrane (Spectrum Laboratories Inc, Rancho Dominguez, CA, USA). Dichloromethane (DCM), dimethylformamide (DMF), tetrahydrofuran (THF), methanol, and acetone were of extra pure grade.

**Synthesis of PHO/PEG block copolymers**

Five hundred grams of PHO were dissolved in 20 ml of THF. Ten milligrams of DCC and 5.5 mg of NHS was added to this solution and magnetically stirred for 6 h. After that, the solution was filtered using filter paper to remove by-products and the solvent was evaporated using rotary evaporator following precipitation into methanol. Precipitates were harvested and dried under reduced pressure for 2 days to obtain NHS-activated PHO as shown in Figure 1. Five hundred milligrams of NHS-activated PHO was dissolved in 20 ml of THF and 2 equivalents of MPEG amine were added. This solution

![Figure 1 Synthesis scheme (A) and 1H-NMR spectra of copolymers (B, C). Synthesis scheme of PHO/PEG block copolymers (A). 1H-NMR spectra of PHO (B) and PHO/PEG 12 K (C) in CDCl3.](image-url)
was stirred for 24 h and then precipitated into excess amount of methanol three times. Precipitates were harvested and dried under vacuum for 3 days.

Characterization of PHO/PEG block copolymer

Characterization of the synthesized polymers was performed in CDCl₃ by 500 mHz ¹H-NMR spectroscopy (500 mHz superconducting FT-NMR spectrometer, Varian Unity Inova 500 MHz NB High Resolution FT NMR; Varian Inc, Santa Clara, CA, USA). Molecular weight of polymers was measured with the gel permeation chromatography (GPC) system (Waters 2690D-2410, Waters, MA, USA) as described previously [5]. GPC was equipped with a Waters 6000 solvent delivery system, RI detector, and a Rheodyne injector. Phenogel columns of 500, 10³, 10⁴, and 10⁵ Å were used. A standard curve was established with standard polystyrene samples.

Preparation of paclitaxel-incorporated nanoparticles using PHO/PEG block copolymer

PHO/PEG block copolymer was dissolved in 5 ml of THF and then paclitaxel dissolved in 2 ml of DMF was added. This solution was slowly dropped into 10 ml of cold deionized water for 15 min and then stirred magnetically for 60 min at 4°C. After that, this solution was dialyzed using a dialysis tube (MWCO = 12,000 g/mol) against deionized water for 1 day. To remove solvent, deionized water was exchanged at every 2-h intervals. After that, dialyzed solution was used for analysis or lyophilization. Empty nanoparticles were prepared in the absence of paclitaxel.

Drug content and loading efficiency were measured using a UV-1201 spectrophotometer (Shimadzu Co. Ltd., Kyoto, Japan) at 273 nm. Empty nanoparticles were used as a blank.

Morphology of the polymeric micelles was observed using a transmission electron microscope (TEM, JEOL JEM-2000 FX II, JEOL, Akishima-shi, Japan). One drop of nanoparticle solution containing phosphotungstic acid 0.05% (w/w) was placed onto a carbon film coated on a copper grid for TEM. Observation was done at 80 kV.

Table 1 Characterization of PHO/PEG block copolymers

|            | M.W. of PEG (g/mol)ᵃ | M.W. by GPC (g/mol) | Particle size (weight average, nm) |
|------------|----------------------|---------------------|-----------------------------------|
|            | Mₘ                   | Mₙ                   | PDIᵇ                              |
| PHO        | -                    | 57,800               | 51,900                            | 1.11 | 97.2  |
| PHO/PEG 2 K| 2,000                | 60,200               | 53,200                            | 1.13 | 78.7  |
| PHO/PEG 5 K| 5,000                | 63,100               | 60,900                            | 1.04 | 72.6  |
| PHO/PEG 12 K| 12,000              | 70,500               | 65,300                            | 1.08 | 62.8  |

ᵃM.W. of PEG was from the manufacturer’s data;ᵇPDI (polydispersity index) was Mₘ/Mₙ.

Drug release study

The release experiment was carried out in vitro as follows: Paclitaxel-incorporated nanoparticle solution was prepared as described above. After the dialysis procedure, the volume of nanoparticle solution was adjusted to 50 ml and then 5 ml of this solution was introduced into a dialysis tube (MWCO = 12,000 g/mol) and then dialysis tube was introduced into a 200-ml bottle with 95 ml of phosphate-buffered saline (PBS, 0.1 M, pH 7.4, 0.1% (w/v) Tween 80). This bottle was placed in a shaking incubator with stirring speed of 100 rpm at 37°C. At specific time intervals, the media were taken for analysis of drug concentration. After that, the whole media was replaced with fresh PBS to prevent drug saturation. The concentration of the paclitaxel released into PBS was evaluated using a high-performance liquid chromatography (HPLC, PerkinElmer, Waltham, MA, USA) using Phenomenex Sphereclone 5 micro ODS(2) 250 mm × 4.6 mm column and 75% methanol as a moving phase. The Flexar HPLC system (PerkinElmer, Waltham, MA, USA) was equipped with a Solvent Manager 5-CH degasser, an autosampler, a quaternary LC pump, a column oven, and a UV-visible detector. The flow rate was set to 1.5 ml/min and the temperature at 50°C. The UV detector (226 nm) was used for the measurement. To prepare the sample for the measurement of HPLC, paclitaxel was dissolved in ethanol, making 20 μl for injection.

Antitumor activity of paclitaxel-incorporated nanoparticles in vitro

Antitumor activity of paclitaxel-incorporated nanoparticles was evaluated using HuCC-T1 human cholangiocarcinoma cells. HCT116 cells were seeded at a density of 2×10³ cells per well in 96-well plates with 10% FBS and incubated overnight at 37°C and 5% CO₂ atmosphere. After that, free paclitaxel, paclitaxel-incorporated nanoparticles and empty nanoparticles were added to 96-well plates at

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Figure 2 (See legend on next page.)
100 μl. For paclitaxel treatment, paclitaxel dissolved in DMSO was diluted 1,000 times with RPMI1640 supplemented with 10% FBS and added to cells. For nanoparticle treatment, paclitaxel-incorporated nanoparticles were diluted with RPMI1640 supplemented with 10% FBS and treated to cells. Controls were treated with 0.1% v/v of dimethyl sulfoxide. For the testing of cellular cytotoxicity of empty nanoparticles, they were diluted with serum-free RPMI1640 media and sterilized with a 0.8-μm syringe filter and added to 2 × 10^6 HCT116 cells. Viable cells were evaluated with MTT cell proliferation assay as described previously [9]. After 3 days of incubation, 30 μl of MTT (5 mg/ml) was added to the 96-well plates and incubated for 4 h. The formazan crystals formed were solubilized with SDS solution (100 μl of SDS-HCl solution (SDS 10% w/v, 0.01 M HCl)) and the absorbance (560-nm test/630-nm reference) was determined using an automated computer-linked microplate reader (Infinite M200 Pro microplate reader, Molecular Device Company, Sunnyvale, CA, USA). Each measurement of drug concentration was obtained as the mean value of eight wells. The amount of formazan present is proportional to the number of viable cells, because only living cells will reduce MTT to blue formazan. The results were expressed as a percentage of the absorbance present in the drug-treated cells compared with that in the control cells.

**Apoptosis/necrosis analysis**

Apoptosis and necrosis of HCT116 cells were analyzed by flow cytometry [9]. FITC-annexin V and PI were employed to analyze apoptosis and necrosis of HCT116 cells, respectively. Cells (1 × 10^6 cells) were exposed to paclitaxel or paclitaxel-incorporated nanoparticles in serum-free RPMI1640 media for 48 h, and cells were washed with phosphate-buffered saline (PBS). The cells were harvested by trypsinization, and cell pellets were resuspended in a binding buffer (pH 7.4, 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2) containing FITC-annexin V (1 μg/ml) and then further incubated for 30 min. Ten minutes prior to termination of incubation, PI (10 μg/ml) were added to stain necrotic cells under dark conditions. Following that, the cells were immediately analyzed using a FACS-can flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA).

**Antitumor activity of paclitaxel-incorporated nanoparticles against animal tumor xenograft**

The tumor xenograft model was employed to assess antitumor activity of paclitaxel or paclitaxel-incorporated PA nanoparticles. HCT116 cells (5 × 10^6) were implanted subcutaneously in the backs of mice (Athymic nude BALB/c male mice, 20 g). When the diameter of tumor mass was approximately 2 ~ 3 mm (10 days after), paclitaxel or paclitaxel-incorporated nanoparticles were injected intravenously via tail vein of the mouse (paclitaxel dose = 10 mg/kg). The treatment group was divided as follows; control - 200 μl of PBS (0.01 M, pH 7.4); paclitaxel treatment - paclitaxel dissolved in Cremophor® EL/ethyl alcohol (5:5, v/v) solution were mixed with aqueous solution of empty nanoparticles; paclitaxel-nanoparticles prepared from PHO/PEG 5 K - lyophilized nanoparticles were reconstituted in deionized water and sterilized with a 0.8-μm syringe filter. Five mice were used for each treatment group. At intervals of 5 days, length and width of solid tumor was measured and tumor volume was calculated with the following equation: Tumor volume (mm^3) = Length × Width^2. Tumor mass was approximately 2 ~ 3 mm (10 days after), paclitaxel or paclitaxel-incorporated nanoparticles were injected intravenously via tail vein of the mouse (paclitaxel dose = 10 mg/kg). The treatment group was divided as follows; control - 200 μl of PBS (0.01 M, pH 7.4); paclitaxel treatment - paclitaxel dissolved in Cremophor® EL/ethyl alcohol (5:5, v/v) solution were mixed with aqueous solution of empty nanoparticles; paclitaxel-nanoparticles prepared from PHO/PEG 5 K - lyophilized nanoparticles were reconstituted in deionized water and sterilized with a 0.8-μm syringe filter. Five mice were used for each treatment group. At intervals of 5 days, length and width of solid tumor was measured and tumor volume was calculated with the following equation: Tumor volume (mm^3) = Length × Width^2. Tumor mass was taken from the back of mice and the weight of tumor was compared between each of the treatment groups 2.5 weeks after drug injection. All animal experiments followed the guidelines of the Committee for the Care and Use of Laboratory Animals of Pusan National University.

**Statistical analysis**

Results of animal experiments were expressed as average ± standard deviation, and statistical significance was evaluated using the Student’s t test (SigmaPlot program

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**Table 2 Characterization of paclitaxel-incorporated nanoparticles of PHO/PEG block copolymer**

| Polymer/drug (mg/mg) | Drug contents (%, w/w) | Particle size (weight average, nm) |
|----------------------|------------------------|-----------------------------------|
|                      |                        | Theoretical | Experimental |
| PHO                  | 45/5                   | 10          | 7.1          | 115.1 |
| PHO/PEG 2 K          | 45/5                   | 10          | 6.8          | 103.1 |
| PHO/PEG 5 K-1        | 45/5                   | 10          | 6.5          | 97.6  |
| PHO/PEG 5 K-2        | 40/10                  | 20          | 11.9         | 110.8 |
| PHO/PEG 12 K         | 45/5                   | 10          | 6.6          | 82.6  |
version 11.2, Systat software, Inc., CA 95110, USA). A p value <0.05 was considered significant.

**Results**

**Characterization of PHO/PEG block copolymer**

As reported previously, PHO was produced from *P. oleovorans* ATCC 29347 using octanoate-supplemented mineral medium [8]. PHO was purified from lyophilized cells by extraction with hot chloroform because cell lysates were not soluble in chloroform and PHO can be solved in it. The purified PHO was composed of 3-hydroxyhexanoate (7%) and 3-hydroxyoctanoate (93%) [8]. To synthesize block copolymers composed of PHO and PEG, carboxylic end group of PHO was activated using DCC and NHS, and then PHO-NHS was reacted with various M.W. of MPEG-amine (Figure 1A). Since MPEG-amine is soluble in methanol, reactants were precipitated into methanol. Final product was analyzed using 1H-NMR spectroscopy in CDCl₃. The chemical structures of PHO/PEG block copolymer were shown in Figure 1B,C. As shown in Figure 1B, typical 1H-NMR spectra of the PHO homopolymer showed specific peaks around 0.9, 1.3, 1.6, 2.56, and 5.2 ppm, respectively. In the PHO/PEG block copolymer as shown in Figure 1C, the ethylene peak of PEG appeared at 3.65 ppm. M.W. of PHO homopolymer and PHO/PEG block copolymer were analyzed using GPC as shown in Table 1. As shown in Table 1, the M.W. of PHO/PEG block copolymer was increased as much as PEG M.W. at each sample even though the gap of M.W. was not exactly the same. These results indicated that MPEG was successfully attached to the carboxylic end of the PHO block.

**Characterization of paclitaxel-incorporated nanoparticles**

Nanoparticles of PHO/PEG block copolymers were fabricated by nanoprecipitation-diafiltration method, i.e. block copolymer dissolved in solvent was precipitated into deionized water to make nanoparticles and solvent was removed by dialysis membrane. To confirm whether or not nanoparticles were formed, nanoparticle solution was analyzed using dynamic light scattering and TEM. As shown in Figure 2A, nanoparticles were successfully fabricated at all of the polymers and their average particle sizes were less than 100 nm (Figure 2B). Furthermore, particle sizes were decreased according to the increase of M.W. PEG block (Table 1), indicating that hydrophilic nature of PEG block might have increased hydrophilicity of block copolymer by higher M.W. of PEG since PEG is a water-soluble polymer. The higher M.W. of PEG in the block copolymer is known to cause decreased particle size, increased colloidal stability, and increased biological properties [10]. Since block copolymers composed of hydrophilic and hydrophobic blocks are normally formed nanoparticles with core-shell structure, nanoparticles of PHO/PEG block copolymer were examined using 1H-NMR spectroscopy with D₂O. As shown in Figure 2C, specific peaks of PEG were only observed when nanoparticles were distributed in D₂O (Figure 2C(f)). When nanoparticles were added to CDCl₃, however, specific peaks both PHO and PEG appeared as shown in Figure 2C(g). These results indicated that nanoparticles of PHO/PEG block copolymers are formed core-shell structures, i.e., PEG block consisted an outer shell of nanoparticles while PHO block consisted a solid inner core of nanoparticles.

Paclitaxel was loaded into nanoparticles by nanoprecipitation and dialysis procedure. Even though direct dialysis was also possible to form nanoparticles, it also induced the increase in particle size higher than 300 nm (data not shown) while nanoprecipitation resulted in reduced particle size. Because DMF is a non-volatile solvent and hard to remove by evaporation, the remaining solvent was removed by dialysis. The results of paclitaxel-incorporated nanoparticles of PHO/PEG block copolymers are summarized in Table 2. As shown in Table 2, the higher M.W. of PEG induced the
decrease in the amount of loaded drug and loading efficiency as well. Furthermore, drug entrapment into nanoparticles induced the increase in particle size compared that into empty nanoparticles. The higher the drug feeding ratio, the higher the drug contents and average size of nanoparticles.

Figure 3 shows paclitaxel release from the nanoparticles of PHO/PEG block copolymer. As shown in Figure 3A, the higher M.W. of PEG block induced increase of faster drug release rate than the lower M.W. of PEG block. The higher PEG M.W. is known to accelerate drug release rate [10]. Initial burst release of drug was observed until 12 h and then drug release profile was relatively continuous manner. Furthermore, increased drug contents induced slower release rate of drug as shown in Figure 3B. Since paclitaxel is hydrophobic drug, hydrophobic interactions might be increased at higher drug content and this phenomenon caused decreased drug release rate. At higher drug contents, hydrophobic drug can be aggregated in the solid core of the nanoparticles and aggregated drug release slowly from the nanoparticles [2,3]. Furthermore, larger particle size at higher drug loading might be the one of reason for slower release [11].

Antitumor activity of paclitaxel-incorporated nanoparticles

The antitumor activity of paclitaxel-incorporated nanoparticles of PHO-PEG block copolymer was tested using HCT116 human colon carcinoma cells in vitro. As shown in Figure 4A(a), viable cells were decreased according to the exposure time of paclitaxel. Especially, paclitaxel-incorporated nanoparticles showed enhanced antiproliferation activity at the same paclitaxel dose while empty nanoparticles did not show anti-proliferation effect against tumor cells as shown in Figure 4A(b). Figure 4B showed cell morphology according to the treatment of paclitaxel or paclitaxel nanoparticles. As shown in Figure 4B, cell density was decreased according to the increase of paclitaxel dose. Cells were crowded at a lower dose of paclitaxel while they were scattered at a higher concentration of paclitaxel.

Figure 5 shows the apoptosis/necrosis analysis of HCT116 cells with treatment of paclitaxel and paclitaxel nanoparticles. As shown in Figure 5, apoptotic cells were dose-dependently increased and, especially, apoptotic cells with treatment of paclitaxel nanoparticles were practically similar to those treated with paclitaxel even though they were slightly higher. These results also supported those of Figure 4.
In vivo antitumor activity of paclitaxel-incorporated nanoparticles against HCT116 cells were evaluated with tumor xenograft model using nude mice. As shown in Figure 6, tumor weight with treatment of paclitaxel nanoparticles were significantly lower than that of control or paclitaxel + empty nanoparticles while paclitaxel + empty nanoparticles were not significantly changed tumor mass compared to control. At least, tumor mass of mice treated with paclitaxel nanoparticles were three times smaller than that of paclitaxel + empty nanoparticles. The antitumor activity of paclitaxel at the animal tumor xenograft model might be due to the sustained release function of nanoparticles. These results indicate that paclitaxel-incorporated nanoparticles are a potent anticancer agent compared to conventional carriers.

**Discussion**

Nanoparticles are regarded as ideal vehicles for anticancer drug targeting due to their intrinsic physicochemical properties [12]. Even though various active targeting using receptor-ligand interaction strategy have been investigated successively [12-15], passive targeting using plain nanoparticles or PEGylated nanoparticles are still preferentially considered for tumor targeting due to the vasculature

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**Figure 5** Flow cytometric analysis of HCT116 cells. Annexin V was used for apoptosis analysis and PI was used for necrosis analysis. HCT116 cells with treatment of paclitaxel, empty nanoparticles, and paclitaxel-incorporated nanoparticles (PHO/PEG 5 K).
permeability of tumor tissue [16,17]. Bae insisted that numerous biological and physiological elimination mechanisms of tumor preferentially decide the fate of nanoparticles prior to their access in the tumor cells [16]. Until the nanocarriers are close to the cell surfaces, no specific interaction between receptor on the tumor cell surface and ligand on the nanoparticle surface happens. At this moment, the preferential translocation of nanoparticles from blood circulation to tumor vasculature (so-called enhanced permeation and retention (EPR) effect) is still considered for a solid tumor targeting [16,17]. Diameter of nanoparticles less than 200 nm are known to have advantages for a solid tumor targeting [16,17]. Otherwise, paclitaxel itself could be absorbed to the serum protein in the blood and rapidly cleared from blood circulation. Then, these factors can induce decrease in opportunities for tumor targeting [22]. Therefore, paclitaxel nanoparticles formed by PHO/PEG block copolymer are a promising vehicle for antitumor drug delivery.

Figure 6 Tumor mass of HCT116 tumor xenograft-induced nude mice. Ten days after tumor implantation, drug solution was injected intravenously (Paclitaxel dose 10 mg/kg). Control - PBS (0.1 M, pH 7.4); Paclitaxel + Empty nanoparticle treatment - paclitaxel dissolved in Cremophor EL/ethanol mixture (5/5, v/v) was diluted ten times with PBS and mixed with aqueous solution of empty nanoparticles (the content of empty nanoparticles in this treatment were adjusted to similar concentration with paclitaxel NP treatment; paclitaxel-incorporated nanoparticles (Paclitaxel NP) - aqueous solution of paclitaxel-incorporated nanoparticles (PHO/PEG 5 K) was sterilized with a 0.8-μm syringe filter.

For antitumor drug delivery, paclitaxel was selected as an anticancer drug because it is commonly used in the clinics and has strong hydrophobic property (aqueous solubility is less than 0.3 μg/ml) and it has a specific interaction with serum proteins [22]. Due to its poor aqueous solubility, specific surfactant/solvent mixtures (Cremophor EL/ethanol mixture) are commercially used. However, due to the intrinsic toxicity of specific surfactant, various formulations for enhancing aqueous solubility of paclitaxel have been investigated such as nanoparticles, liposomes, and polymeric micelles [23]. Since PEG has a protective effect for the adsorption of protein and macrophage cells, PEGylation of nanocarriers may enhance blood circulation time of nanocarriers, unwanted content with serum protein/macroage cells, and then chance to contact tumor tissues in vivo.

Paclitaxel was incorporated into the nanoparticles by nanoprecipitation and dialysis procedure. The size of paclitaxel nanoparticles was slightly increased compared to that of empty nanoparticles. Even though the diameter of nanoparticles was increased with paclitaxel loading, the average size of nanoparticles did not exceed 200 nm. Furthermore, paclitaxel was slowly released from the nanoparticles for over 6 days. In a drug release study, diffusion from nanoparticles must be the preferential mechanism rather than the degradation of polymer because PHO itself has very slow degradation kinetics [6,19]. Nanoparticles showed practically similar anticancer activity against tumor cells as shown in Figure 4. Furthermore, nanoparticles showed practically similar effect on the apoptosis and necrosis of tumor cells as shown in Figure 5. Antitumor activity of paclitaxel nanoparticles in vivo was evaluated using HCT116 tumor-bearing mice as shown in Figure 6. As shown in Figure 6, paclitaxel nanoparticles significantly inhibited growth of tumor mass in vivo the tumor xenograft model as shown in Figure 6 while paclitaxel itself seemed to have minimal anticancer activity. The EPR effect of the nanoparticles can be considered to explain these results, i.e., nanoparticles properly entered into the cells and killed the tumor cells [16,17]. Otherwise, paclitaxel itself could be absorbed to the serum protein in the blood and rapidly cleared from blood circulation. Then, these factors can induce decrease in opportunities for tumor targeting [22]. Therefore, paclitaxel nanoparticles formed by PHO/PEG block copolymer are a promising vehicle for antitumor drug delivery.
Conclusions
Paclitaxel-incorporated nanoparticles were prepared using PHO/PEG block copolymers for antitumor drug delivery. Paclitaxel-incorporated nanoparticles of PHO/PEG block copolymer have spherical morphology with small particle size less than 100 nm. In a $^1H$-NMR study in D$_2$O, the core-shell structure of PHO/PEG nanoparticles was confirmed. The higher M.W. of PEG decreased loading efficiency of drug and particle size. The higher drug feeding resulted in increased drug contents and average size of nanoparticles. The higher M.W. of PEG block accelerated drug release rate while higher drug contents delayed the release rate of drug. In an antitumor activity study using HuCCT1 cells in vitro, paclitaxel-incorporated nanoparticles have similar antiproliferation activity against HCT116 tumor cells. However, paclitaxel nanoparticles showed enhanced antitumor activity against HCT116 tumor cells of mice in vivo.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HYK carried out the fabrication and characterization of nanoparticles. JHR, CWC, and GWS participated in the morphological observation, particle size analysis, and spectroscopical analysis of polymers and nanoparticles. TWK, DHK, CWC, and YHR contributed to the synthesis of microbial polymers. YIJ designed and synthesized block copolymers. DHK and HWK conceived the study and coordination. All authors read and approved the final manuscript.

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