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Biotin-Conjugated Block Copolymeric Nanoparticles as Tumor-Targeted Drug Delivery Systems

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Abstract: To achieve targeted drug delivery for chemotherapy, a ligand-mediated nanoparticulate drug carrier was designed, which could identify a specific receptor on the surfaces of tumor cells. Biodegradable poly(ethylene oxide)/poly(ε-caprolactone) (PEG/PCL) amphiphilic block copolymers coupled to biotin ligands were synthesized with a variety of PEG/PCL compositions. Block copolymeric nanoparticles harboring the anticancer drug paclitaxel were prepared via micelle formation in aqueous solution. The size of the biotin-conjugated PEG/PCL nanoparticles was determined by light scattering measurements to be 88-118 nm, depending on the molecular weight of the block copolymer, and remained less than 120 nm even after paclitaxel loading. From an *in vitro* release study, biotin-conjugated PEG/PCL nanoparticles containing paclitaxel evidenced sustained release profiles of the drug with no initial burst effect. The biotin-conjugated PEG/PCL block copolymer itself evidenced no significant adverse effects on cell viability at 0.005-1.0 μg/mL of nanoparticle suspension regardless of cell type (normal human fibroblasts and HeLa cells). However, biotin-conjugated PEG/PCL harboring paclitaxel evidenced a much higher cytotoxicity for cancer cells than was observed in the PEG/PCL nanoparticles without the biotin group. These results showed that the biotin-conjugated nanoparticles could improve the selective delivery of paclitaxel into cancer cells via interactions with over-expressed biotin receptors on the surfaces of cancer cells.

Keywords: nanoparticle, drug delivery, targeting, polyethylene oxide, polycaprolactone.

Introduction

Although there has been significant progress in the development of new anticancer agents and anticancer technology in general, cancer is still a major cause of death. Currently used chemotherapeutic drugs are systemically active and are not selective for cancer cells. Thus, high systemic dosages are required to reach therapeutically effective concentrations at the tumor site, resulting in severe side effects such as the destruction of bone marrow cells, cardiotoxicity, nephrotoxicity, hepatotoxicity, and hematotoxicity.[1,2] The side effects associated with chemotherapy limit the dose (and cumulative dose) that can be administered to patients, leading to relapse of the tumor and, often, the development of drug-resistance. Therefore, significant efforts have been made to develop alternative therapies that improve the therapeutic indices of anticancer drugs both by increasing efficiency and decreasing toxicity.[1-6]

One approach that overcomes this limitation is the active targeting of tumors with particulate drug carriers.[1,2] Polymeric nanoparticles can be delivered to specific sites by size-dependent passive targeting or by active targeting through conjugation of the targeting molecules.[7-19] Nanoparticles conjugated with targeting molecules (e.g., antibodies, sugars, and vitamins) could achieve a high degree of selectivity to specific organs and enhance the internalization of the drug into target cells.[7-11]

Notably, rapidly dividing cells, such as those present in solid tumor cancers, require large amounts of certain vitamins, including folate, vitamin B_{12} and vitamin H; as a consequence, the receptors involved in vitamin uptake are over-expressed on the surfaces of cancer cells.[11,20] Folate has been known as an attractive candidate molecule for targeting cancer cells; many studies show that folate-conjugated drug carriers exhibit high specificity in the receptor-mediated process.[20-23] Though cancerous tumors exhibit higher

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biotin content than normal tissue, few studies have analyzed drug delivery systems using vitamin H (biotin) as a ligand for cancer targeting.

Therefore, in this study, we focused on a tumor-targeted drug delivery system that uses biotin-conjugated block copolymeric nanoparticles. We prepared biodegradable amphiphilic block copolymers composed of poly(ethylene glycol) (PEG) and poly(ε-caprolactone) (PCL), which can self-assemble to form nanoparticles with a core-shell structure and the capacity to load a hydrophobic drug.[24-33] PEG is well known for possessing special physicochemical and biological properties, including solubility in water, hydrophilicity, low cytotoxity, and absence of antigenicity and immunogenicity;[12,14] PEG could also greatly increase the stability of nanoparticles in blood circulation. Hydrophobic PCL is a biodegradable, biocompatible, semicrystalline polymer.[34]

The main objective of this study is to prepare biotin-conjugated PEG/PCL amphiphilic block copolymeric nanoparticles and to investigate their feasibility as ligand-mediated drug carriers for targeting anticancer therapy. Biotin-conjugated PEG/PCL block copolymers were characterized by FT-IR, ^{1}H-NMR, 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay and GPC measurements. The particle size, surface characteristics and loading amount of the anticancer drug were also investigated. The *in vitro* release kinetics of the anticancer drug from the biotin-conjugated PEG/PCL nanoparticles was determined depending on the molecular weight of the copolymers. In addition, an *in vitro* cytotoxicity study was performed using normal and cancer cells, and their biotin ligand-dependent cytotoxicities were compared.

Experimental

Materials. Polyoxyethylene bis(amine) (M_w = 3,350, as determined by the supplier), d-biotin (vitamin H), ε-caprolactone, stannous octoate, *N*,*N*′-dicyclohexylcarbodiimide (DCC), deuterium oxide (atom = 99.9%), and chloroform-d (atom =99 .9%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylsulfoxide (DMSO), dimethylformamide (DMF), pyridine, and toluene were purchased from the Junsei Chemical Co. Ltd. (Tokyo, Japan). Dulbecco's Modified Eagle Medium, Dulbecco's phosphate buffered saline (PBS), RPMI-1640 medium (RPMI), Minimum Essential Medium, penicillin-streptomycin (100 U/mL), trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA tetrasodium), and fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD, USA). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution was obtained from Promega Corporation (Madison, WI, USA). Paclitaxel was purchased from Samyang Genex Corporation (Daejeon, Korea). Paclitaxel solution (Taxol®) was also purchased from Bristol-Myers Squibb (Montreal Quebec, Canada). Distilled and deionized water was prepared using a Mili-Q Plus System (Millipore, Bedford, MA, USA). Spectra/Por membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA and Canada). All other chemicals used were reagent-grade, and used without further purification.

Methods.

Preparation of Biotin-conjugated PEG/PCL Block Copolymers: Polyoxyethylene bis(amine) (PEG; M_w = 3,350; 0.4 g) and biotin (0.09 g) were dissolved in DMSO, and DCC was dissolved in pyridine. DCC solution was added to a solution of polyoxyethylene bis(amine) and biotin. The reaction was continued at room temperature for 24 h under a nitrogen atmosphere.[35] The reaction mixture was dialyzed (dialysis membrane, MWCO 3,500; Spectrum Laboratories, Inc., Rancho Dominguez, CA) for 1 day to remove unreacted biotin and other byproducts such as unconjugated PEG. The biotin-conjugated PEG was then freeze-dried (Ilshin Lab Co. Ltd, Korea).

To prepare biotin-conjugated PEG/PCL block copolymer, the resulting biotin-conjugated PEG was reacted with ε-caprolactone in the presence of stannous octoate in anhydrous toluene at 110 °C for 5 h. Copolymerization was accomplished by varying the feed molar ratios of ε-caprolactone monomer to biotin-conjugated PEG as shown in

Table I. Composition and Molecular Weight of Biotin-Conjugated PEG/PCL Block Copolymers

Sample	Polymer Type	Feed Molar Ratio[a]	Composition Weight (%)[b]	Number-Average Molecular Weight[c]	Polydispersity (M_w/M_n)
		ε-CL/PEG	PEG: ε-CL		
PEG	PEG	0	100 : 0	3,200	1.12
BP	Biotin/PEG	0	100 : 0	3,500	1.04
BPC100	Biotin/PEG/PCL100	100	86 : 14	4,100	1.09
BPC150	Biotin/PEG/PCL150	150	64 : 36	5,400	1.15
BPC300	Biotin/PEG/PCL300	300	49 : 51	7,200	1.71
PC75	PEG-PCL75	75	66 : 34	4,800	1.31
PC150	PEG-PCL150	150	43 : 57	7,500	1.43

[a]Calculated on the basis of the M_w (3,350) of PEG measured by Sigma. [b]Estimated as the difference between the experimental total M_n of copolymer and PEG homopolymer in GPC experiments. [c]Measured by GPC analysis.

Table I. In addition, PEG/PCL triblock copolymers without biotin moiety were synthesized as controls by a similar method, the ring opening polymerization of ε-caprolactone by PEG.

After 24 h, the reaction product was dissolved with THF and then precipitated with diethyl ether to remove unreacted ε-caprolactone. It was then extracted in an excessive amount of methanol to remove PEG homopolymers and residual monomers, and washed several times with diethyl ether. The obtained biotin-conjugated PEG/PCL copolymer was dialyzed for 1 day and then lyophilized.

Preparation of Paclitaxel-loaded Nanoparticles: Nanoparticles of biotin-conjugated PEG/PCL block copolymer containing the anticancer drug were prepared using the dialysis process described in our previous studies.[24-33] We used the hydrophobic anticancer drug paclitaxel. Biotin-conjugated PEG/PCL block copolymer (50 mg) was dissolved in 5 mL of DMF; paclitaxel was then added with various weight ratios to the polymer and stirred at room temperature. To form paclitaxel-loaded nanoparticles with micelle structure and remove free (unloaded) paclitaxel, the mixture was poured into a cellulose dialysis membrane (MWCO 3,500; Spectrum Laboratories, Inc., Rancho Dominguez, CA) and dialyzed against deionized water for 24 h.

The nanoparticle suspension was sonicated (Branson Ultrasonics Co., USA) and then centrifuged (300 rpm × 15 min, Jouan BP403, France) to eliminate unloaded paclitaxel and aggregated particles. The supernatant nanoparticle solution obtained from this process was frozen and lyophilized.

Characterization of Biotin-conjugated PEG/PCL Block Copolymers: The structure of biotin-conjugated PEG/PCL block copolymer was confirmed by FT-IR (Nicolet IR 860 spectrometer, Thermo Nicolet, Madison, WI, USA) and ^{1}H-NMR spectroscopy (Bruker AMX-500, Karlsruhe, Germany) measurements (NMR solvent: dimethyl sulfoxide-d6). The average molecular weight and molecular weight distribution of the block copolymers were determined by gel permeation chromatography (GPC; Waters Model 510 HPLC pump, Milford, MA, USA). Molecular weights were characterized by elution times relative to polystyrene monodisperse standards using GPC in conjunction with a Millenium software program. Three ultrastyragel® tetrahydrofuran (THF) columns (each 7.8 mm inner diameter and 30 cm length, HR-0.5, HR-4, HR-5, all by Waters, Milford, MA, USA) and a Waters R410 differential refractometric detector were used. The mobile phase was THF at a flow-rate of 1 mL/min. The TNBS assay was used to determine quantitatively the conjugation of biotin molecules with PEG, using a previously reported method.[24,36]

In addition, the average particle size and size distribution of the nanoparticles were determined using a Zetasizer (Malvern-zetasizer 3000 hs, Malvern, UK) at 25 °C. The morphology of biotin-conjugated PEG/PCL nanoparticles was investigated using a field emission scanning electron microscope (SEM, JEOL-6430F, Kyoto, Japan) at an operating voltage of 10 kV.

Drug Loading: The paclitaxel-loaded nanoparticles were dissolved in DMF. The amount of paclitaxel loaded in the inner core of biotin PEG/PCL nanoparticles was determined by using a UV-visible spectrophotometer (Shimadzu Model UV 2101PC, Japan). The loading amount of paclitaxel in nanoparticles was calculated from the mass of initial drug-loaded nanoparticles and the mass of the incorporated paclitaxel using the following equation:

$$\text{Drug loading (\%)} = \frac{\text{Amount of paclitaxel in nanoparticles}}{\text{Amount of paclitaxel loaded nanoparticles}} \times 100$$

$$= \frac{\text{Paclitaxel}}{\text{Paclitaxel + Polymer}} \times 100$$

***In vitro* Release Behavior of Paclitaxel from Polymeric Nanoparticles:** Experiments examining the release of paclitaxel from biotin-conjugated PEG/PCL nanoparticles were performed using a dialysis-membrane diffusion technique.[30,31,33] The 4 mg of paclitaxel-loaded nanoparticles were precisely weighed and suspended in 5 mL of PBS (0.2 M, pH 7.4). The solutions were diluted 10-fold with PBS. The 5 mL of diluted solution were then transferred to dialysis membrane tubing, and the membrane bag was immersed in release medium with continuous gentle stirring at 37 °C. The total amount of paclitaxel used for the *in vitro* release test was not more than 0.3 μg/mL, the solubility of paclitaxel in water at 37 °C.[37] At predetermined time intervals, 3 mL aliquots of the aqueous solution were withdrawn from the release medium and replaced with the same volume of fresh medium to maintain nonsaturation (sink) conditions. The amount of paclitaxel released in each time interval was measured by HPLC, which consisted of a Waters 510 pump, a UV-vis detector (Waters-486), and a TMC temperature controller. Analysis was performed on a 5 μm LiChrosorb RP-18 (250 × 4 mm I.D.; Merck Co.). The mobile phase of acetonitrile-water (60 : 40) was pumped at a flow rate of 1.5 mL/min, and paclitaxel eluted from the column was detected at 227 nm.[23]

***In vitro* Cytotoxicity Study:** Human uterine cervix adenocarcinoma (HeLa 229) cells were obtained from the Korea Cell Line Bank. The human fibroblast (HF) cells were aseptically isolated from a foreskin donated by the Urology Department of Hanyang University Hospital in Seoul, Korea. HeLa 229 cells were cultured in RPMI medium with 10% FBS and 100 U/mL of penicillin-streptomycin. The fibroblasts were incubated using a culture medium composed of DMEM, 10% FBS, and 100 U/mL of penicillin-streptomycin. Cell viability was determined using a tetrazolium compound [3(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] assay. The HeLa 229 and HF cells were incubated in conditioned media containing different con-

centrations of paclitaxel-loaded nanoparticles or paclitaxel solution as a control, in 96-well microplates at 37 °C in a humidified atmosphere containing 5% CO_2. After predetermined incubation periods, MTS solution was added to each well. After 4 h of incubation at 37 °C, the MTS and medium mixture were transferred into each well of a 96-well microplate. The absorbance was measured at 490 nm using a microplate reader (EL310, Bio-Teck Instruments Inc., Winooski, VT, USA). Cell viability was expressed as a percentage of a control that had not been treated with nanoparticles, using the following equation:

$$\text{Vability (\%)} = (N_i/N_c) \times 100,$$

where N_i and N_c are the number of surviving cells in the group treated with paclitaxel-loaded nanoparticles and in the untreated cell group, respectively.

***In vitro* Cellular Uptake:** Human breast adenocarcinoma (MCF-7) cells were obtained from the Korea Cell Line Bank. The MCF-7 cells were cultured in RPMI medium with 10% FBS and 100 U/mL of penicillin-streptomycin. For the cellular uptake experiment, MCF-7 cells were seeded in the tissue culture flask. After the cells reached 70-80% confluency, they were incubated with paclitaxel and paclitaxel-loaded PEG/PCL nanoparticles with biotin group (BPC300a) at 0.1 μg/mL paclitaxel concentration in growth medium for 0.5, 1.0, and 4.0 h, respectively at 37 °C.[38] In order to verify the involvement of biotin receptors in nanoparticle uptake, free biotin (5 mM) was co-incubated with nanoparticles. At the designed interval, the sample wells were washed three times with PBS to remove extracellular drug. Cells were scraped from the bottoms of the flasks and quantitatively transferred to conical centrifuge tubes. The resulting cell/saline suspensions were lyophilized. The dried samples were quantitatively transferred from the conical centrifuge tube to microcentrifuge tubes using 0.5 mL aliquot of methanol. The samples were then dried, resuspended in 100 μL of methanol, centrifuged at 12,000 rpm for 5 min, and 5 μL of the supernatant was injected onto the HPLC column. All samples were prepared in triplicate.

Statistical Analysis: Analysis of variance (ANOVA) and Newman-Keuls post-hoc analyses were performed using StatSoft Statistica 5.0 (Tulsa, CA). Statistical significance was determined at a value of $p < 0.05$.

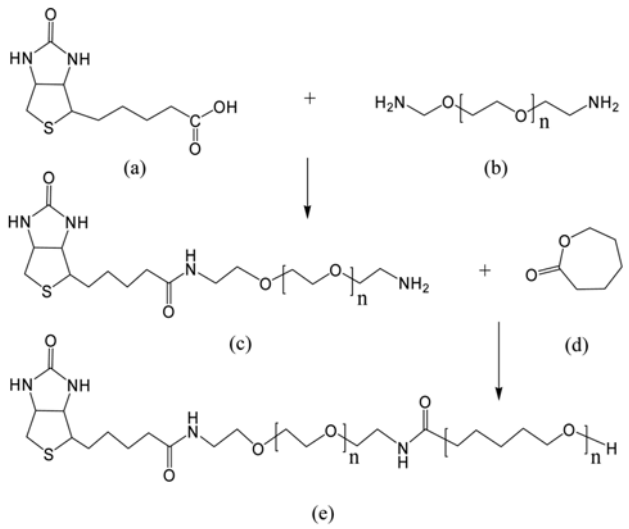

Figure 1. Synthetic scheme of biotin-conjugated amphiphilic poly(ethylene oxide)/poly(ε-caprolactone) block copolymer; (a) biotin, (b) polyoxyethylene bis(amine), (c) biotin-conjugated PEG, (d) ε-caprolactone, and (e) biotin-conjugated PEG/PCL block copolymer.

Results and Discussion

Characterization of Biotin-conjugated PEG/PCL Copolymers. Figure 1 shows the synthetic scheme of biotin-conjugated PEG/PCL amphiphilic block copolymers. Biotin-conjugated PEG polymer, which has one free amine group at the other end, was prepared via a dicyclohexylcarbodiimide (DCC)-mediated coupling of biotin to PEG. One amine group of PEG was coupled with a carboxyl group of biotin, and the other was kept free by controlling the molar ratio of biotin to PEG. The biotin-conjugated PEG/PCL

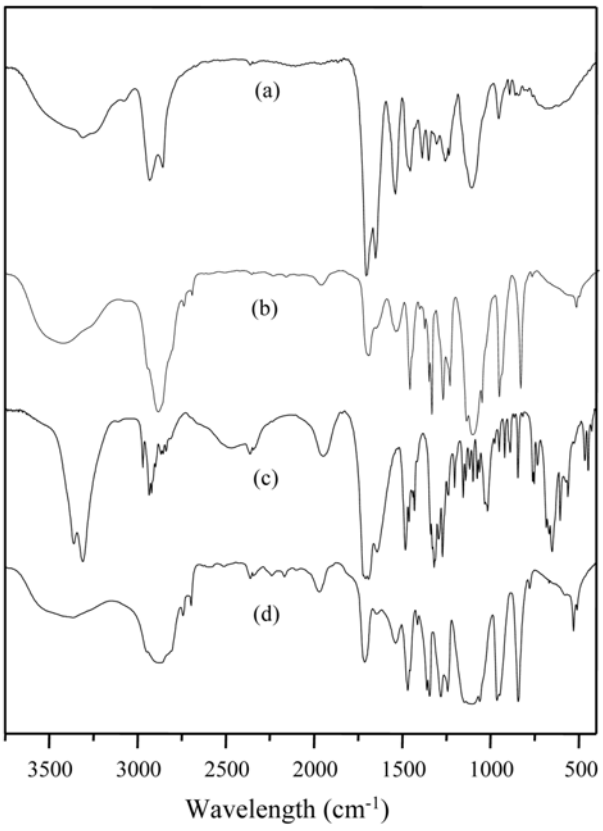

Figure 2. FT-IR spectra of (a) biotin-conjugated PEG/PCL block copolymer, (b) biotin-conjugated PEG, (c) biotin, and (d) PEG.

block copolymers were then synthesized by ring opening polymerization of ε-caprolactone in the presence of biotin-conjugated PEG with stannous octoate. The biotin-conjugated PEG/PCL block copolymers were prepared with varying compositions by varying the feed molar ratio of ε-caprolactone to biotin-conjugated PEG as shown in Table I.

To confirm the synthesis of the copolymer, the FT-IR spectra of PEG, biotin, biotin-conjugated PEG, and biotin-conjugated PEG/PCL block copolymer were compared (Figure 2). For the biotin-conjugated PEG/PCL copolymer, a new strong carbonyl band appears at 1722 cm^{-1} (Figure 2(a)) attributed to the formation of diblock copolymers of PCL and PEG. It was observed that the aliphatic CH-stretching band of ε-caprolactone at 2943 cm^{-1} increased and that the absorption band of the CH-stretching vibration in PEG at 2882 cm^{-1} decreased (Figure 2(a)) compared to biotin-conjugated PEG (Figure 2(b)). The absorption bands at 3427 cm^{-1} were also assigned to the terminal hydroxyl groups in biotin-conjugated PEG/PCL block copolymer.

Figure 3 exhibits the ^{1}H-NMR spectra and chemical shift assignments of biotin-conjugated PEG/PCL block copolymer. The typical signals of the PEG units appear at about 3-4 ppm due to the methylene protons (Figure 3(d)). For biotin-conjugated PEG, the biotin group was identified through two characteristic peaks due to the methane protons (β-1 and β-2) at 4.2 and 4.3 ppm, and two peaks due to the urea protons (α-1 and α-2) at 6.3 and 6.4 ppm (Figure 3(b)). Characteristic peaks of the PCL unit due to the methylene protons are shown at around 2.0 ppm (Figure 3(a)). Synthesized biotin-conjugated PEG and biotin-conjugated PEG/PCL copolymer were also confirmed by a new amide peak at 8.1 ppm.

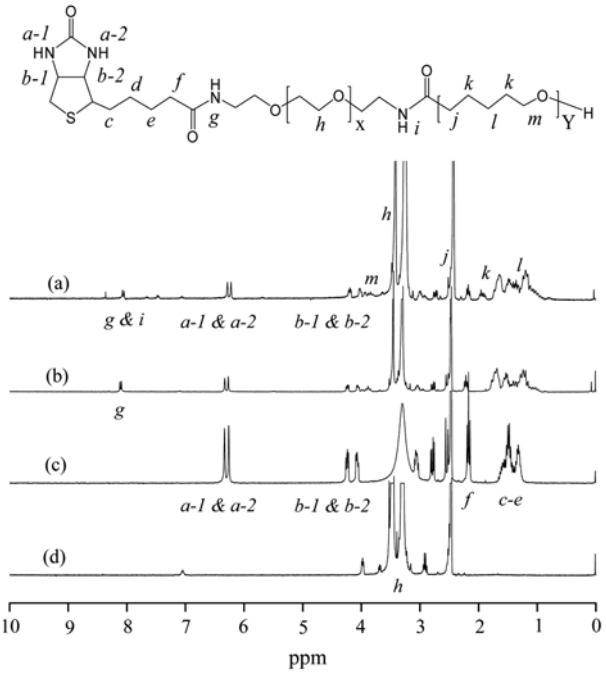

Figure 3. ^{1}H-NMR spectra of (a) biotin-conjugated PEG/PCL block copolymer, (b) biotin-conjugated PEG, (c) biotin, and (d) PEG.

To determine quantitatively the incorporation of biotin molecules into terminal amine groups of the PEG homopolymer, the amine end group was measured by a TNBS assay. The results of the TNBS assay revealed that the degree of substitution of PEG by biotin was 43.1%, which means that 56.9% of terminal amine groups in PEG could participate in the initiation of ring-opening polymerization of ε-caprolactone monomer.

Table I shows the composition and molecular weight of PEG/PCL block copolymers, which were determined by GPC measurement. The molecular weight of the block copolymer could be controlled by adjusting the feed molar ratio of ε-caprolactone to PEG. The copolymer molecular weight increased with the feed molar ratio, as shown in Table I. PEG/PCL block copolymers not conjugated with biotin molecules (PC75 and PC150) had a much greater molecular weight than that of biotin-conjugated copolymers synthesized using the same feed molar ratio of ε-caprolactone monomer. The molecular weight of PC150 (biotin-unconjugated copolymer; the feed molar ratio of ε-caprolactone/PEG = 150) was 7,500, whereas that of BPC150 (biotin-conjugated-PEG/PCL block copolymer; feed molar ratio of ε-caprolactone/PEG = 150) was 5,400. It was suggested that the ring-opening polymerization of ε-caprolactone by biotin-unconjugated PEG occurred at both terminal amine groups of PEG.

Size and Size Distribution of Nanoparticles. The particle size and size distribution of paclitaxel-loaded and unloaded nanoparticles were shown in Table II. For biotin-conjugated PEG/PCL block copolymers, the formed nanoparticles had sizes of about 87-97 nm, depending on the molecular weight of the block copolymers. The size of the biotin-conjugated PEG/PCL nanoparticles increased gradually with the molecular weight of the block copolymers. However, the biotin-

Table II. Particle Size of Drug-Loaded and Unloaded Block Copolymeric Nanoparticles

Sample	Feed Weight Ratio Polymer : Drug[a]	Particle Size (nm)[b]	
		Average	S.D.
BPC100	1.00 : 0.00	88	2.85
BPC150	1.00 : 0.00	91	2.60
BPC300	1.00 : 0.00	98	2.59
PC150	1.00 : 0.00	96	3.39
BPC100a	1.00 : 1.00	90	4.00
BPC150a	1.00 : 1.00	97	2.81
BPC300a	1.00 : 1.00	118	4.44

[a]Paclitaxel. [b]Average nanoparticle sizes were determined using a Zetasizer (Malvern-zetasizer 3000 hs, Malvern, UK) at 25 °C.

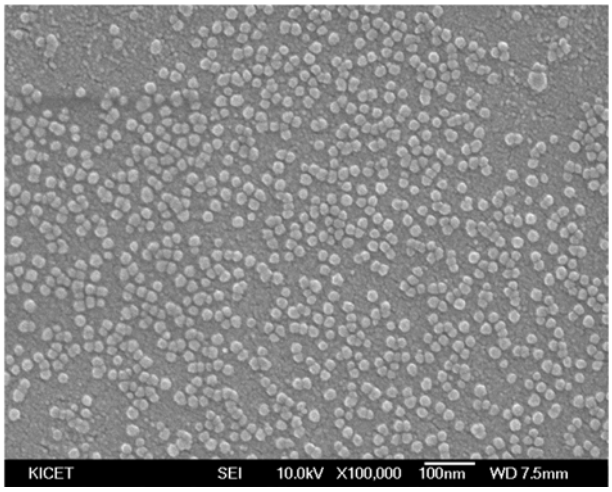

Figure 4. Biotin-conjugated PEG/PCL nanoparticles (BPC150) observed by field emission scanning electron microscopy (FE-SEM).

unconjugated PEG/PCL block copolymer (PC150, molecular weight = 7,500) was smaller size than BPC 300, which has molecular weight 7,200. Note that the biotin-unconjugated PEG/PCL block copolymer with a larger fraction of hydrophobic PCL forms smaller particles by hydrophobic interaction. However, the size data were not statistically different. The paclitaxel-loaded nanoparticles had size 90-118 nm. The size of biotin-conjugated PEG/PCL nanoparticles is suitable for an injectable drug delivery system, especially for tumor chemotherapy. At the tumor level, the mechanism of accumulation of intravenously injected nanoparticles relies on passive diffusion or convection across the leaky, hyperpermeable tumor vasculature as an enhanced permeation and retention effect. In addition, the particle size increased some after the paclitaxel loading, but no significant change in size distribution was observed, as shown in Table II.

Biotin-conjugated PEG/PCL block copolymeric nanoparticles were also observed with a field emission scanning electron microscopy (FE-SEM) measurement. As shown in Figure 4, the particles exhibited were sub-micron in size and had a spherical shape. The size of particles observed by FE-SEM was smaller (about 20-30 nm) than the size of those obtained by light scattering. This is because the diameter of block copolymeric nanoparticles with micelle structure determined by light scattering measurement reflected the hydrodynamic diameter of micelles, which were swollen in aqueous solution, while that measured by FE-SEM was the diameter of dried micelles.

Drug Loading. Table III exhibits the loading amount of paclitaxel in PEG/PCL nanoparticles depending on the molecular weight and the feed weight ratio of drug to copolymer. Paclitaxel-loaded nanoparticles were prepared by varying the initial feed weight ratio of copolymer : paclitaxel from 1.00 : 0.75 to 1.00 : 4.00. As shown in Table III, the drug amount increased with the feed weight ratio of paclitaxel to copolymer (BPC150a, BPC150b, and PBC150c). When the feed weight ratio of paclitaxel to copolymer exceeded 2.00 : 1.00, it was found that an aggregation of unloaded drug occurred during the preparation process of drug-loaded nanoparticles, resulting in a decrease in the yield of paclitaxel-loaded nanoparticles. It implies that the hydrophobic interaction between drug molecules was greater than that between drug and biotin-conjugated PEG/PCL copolymer with increasing the feed amount of paclitaxel with high lipophilic character.

Table III. Drug Loading of Paclitaxel-Loaded Nanoparticles

Sample	Feed Weight Ratio	Drug Loading (%)[b]
	Polymer : Drug[a]	
BPC100a	1.00 : 1.00	2.72 ± 0.64
BPC150a	1.00 : 1.00	11.38 ± 2.03
BPC150b	1.00 : 2.00	33.21 ± 2.85
BPC150c	1.00 : 4.00	34.87 ± 2.15
BPC200a	1.00 : 1.00	33.37 ± 1.98
BPC300a	1.00 : 1.00	46.58 ± 2.50
PC150d	1.00 : 0.75	47.28 ± 3.12

[a]Paclitaxel. [b]Drug loading = (amount of paclitaxel in nanoparticles/amount of paclitaxel-loaded nanoparticles) × 100 = [paclitaxel/(paclitaxel + polymer)] × 100.

As the molecular weight of PCL block in biotin-conjugated PEG/PCL block copolymers increased (feed weight ratio of copolymer : drug = 1.00 : 1.00), the drug loading gradually increased (BPC100a = 2.72 ± 0.64; BPC150a = 11.38 ± 2.03; BPC200a = 22.37 ± 1.98; BPC300a = 46.58 ± 2.50). It indicated that the increase in the hydrophobic chain length of the block copolymer enhances an interaction between paclitaxel, a hydrophobic drug, and PCL, leading to an increase in drug loading.

Drug Release Study. Figure 5 shows the *in vitro* release kinetics of paclitaxel from nanoparticles. The release profiles of paclitaxel, shown in Figure 5(a), depended on the drug loading in nanoparticles. The free paclitaxel, which was not incorporated in nanoparticles, exhibited a rapid release (more than 95% within 12 h) whereas the paclitaxel loaded into the inner core of the nanoparticles exhibited sustained release behavior and was released much slower from nanoparticles containing higher amounts of the drug. It indicated that the increase of paclitaxel-loading in the nanoparticles enhanced an interaction between paclitaxel and copolymers, leading to a decrease in the drug release rate. In addition, Figure 5(b) shows the release kinetics of the drug from biotin-conjugated nanoparticles over eight days. Biotin-conjugated PEG/PCL nanoparticles, BPC150a and BPC300a, showed controlled release profiles and achieved accumulated release percentages of about 89% and 85%, respec-

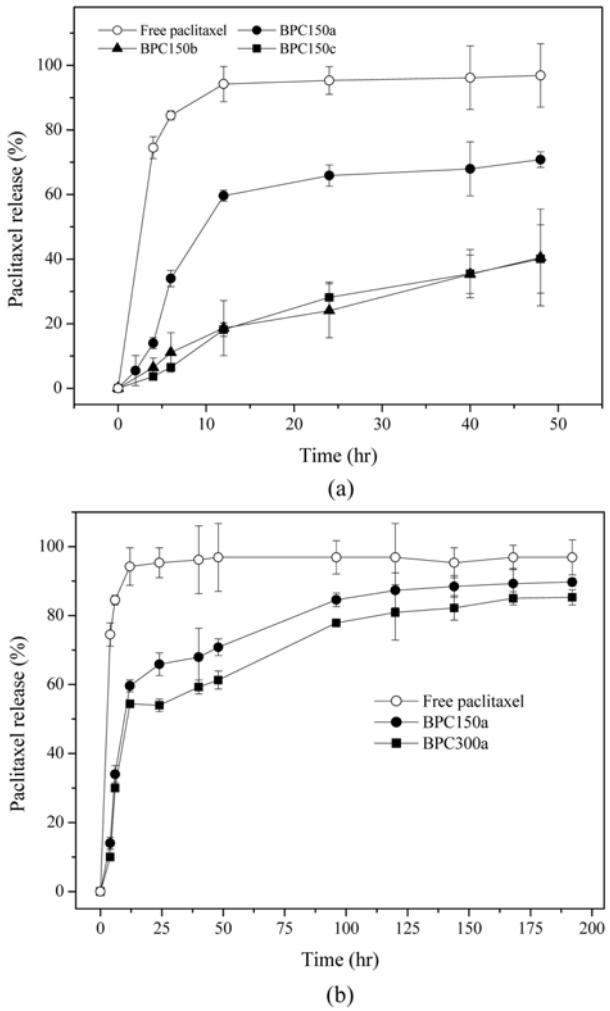

Figure 5. *In vitro* release kinetics of paclitaxel from nanoparticle; (a) release profiles of free paclitaxel, and paclitaxel from nanoparticles (BPC150a, BPC150b, and BPC150c) and (b) release profiles of free paclitaxel, and paclitaxel from BPC150a and BPC300a during 8 days. Each point represents the mean ± S.D. of three samples.

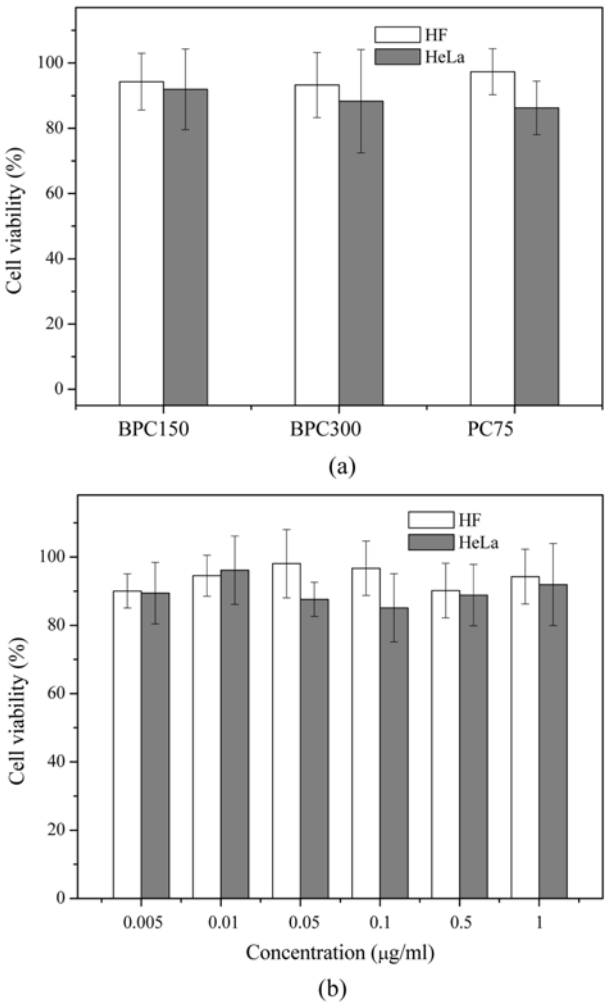

Figure 6. *In vitro* cytotoxicity of biotin-conjugated and -unconjugated amphiphilic block copolymers; (a) cell viability according to block copolymer type (1.0 μg/mL) and (b) cell viability depending on the concentration of BPC150a.

tively. These results suggested that the release of drug from this biotin-conjugated PEG/PCL nanoparticle system was mainly influenced by diffusion not by degradation of PCL block.

Cytotoxicity Study of Biotin-conjugated PEG/PCL Nanoparticles. The biocompatibility of biotin-conjugated and unconjugated PEG/PCL block copolymeric nanoparticles without drug molecules were evaluated by *in vitro* cytotoxicity tests using normal human fibroblasts (HF) and HeLa cells (Figure 6). Figure 6(a) shows the cell viability of HF and HeLa cells after a 24-h incubation with BPC150, BPC300, and PC75 (concentration of nanoparticle suspension = 1.0 μg/mL). Regardless of the conjugation of biotin molecules and the molecular weight of block copolymers, all nanoparticle samples exhibited more than 85% cell viability. In addition, cell viability was not significantly influenced by the concentration of nanoparticle suspension (BPC150) as shown in Figure 6(b).

In addition, the viability of cells treated with paclitaxel-loaded PEG/PCL nanoparticle (both with and without biotin ligand) depended on the concentration of the nanoparticle suspension (0.005-1.0 μg/mL). Figure 7 shows the results of cell viabilities determined by MTS assay after treatment with BPC300a and PC150d nanoparticle suspensions having similar drug loading (BPC300a: 46.58 ± 2.50; PC150d: 47.28 ± 3.12) for 48 h. The cells treated with biotin-conjugated nanoparticles (BPC300a) exhibited lower cell viability than those treated with biotin-unconjugated nanoparticles (PC150d), despite that the drug loading (Table III) and the *in vitro* release kinetics of paclitaxel were similar in those cases (data not shown). This indicated that the paclitaxel that was loaded into the biotin-conjugated PEG/PCL nano-

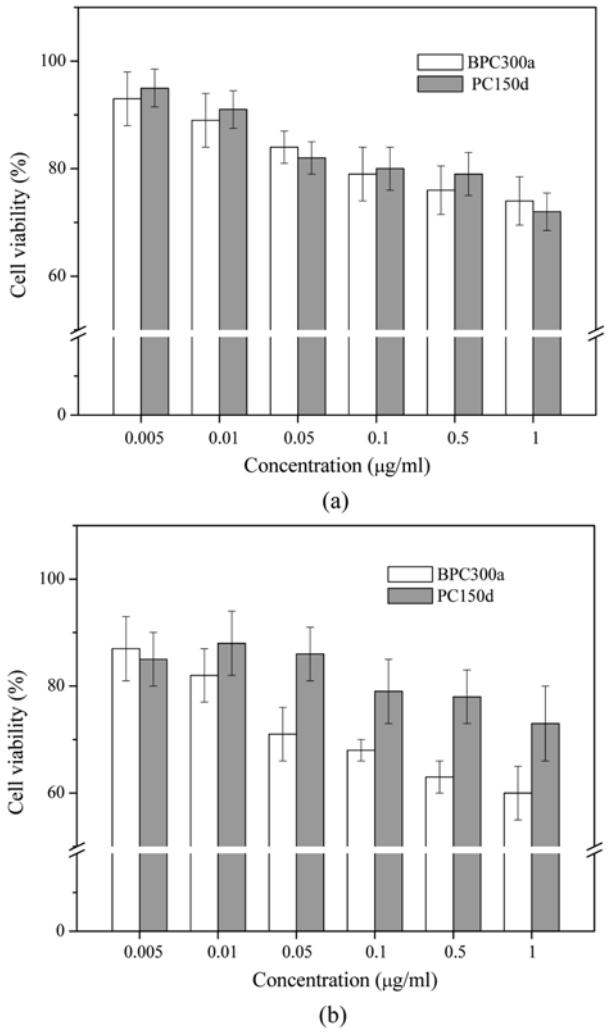

Figure 7. The viability of HF and HeLa cells incubated with the paclitaxel-loaded PEG/PCL nanoparticles with and without biotin group and its dependence on the concentration of the nanoparticle suspension. The cell viability determined by MTS assay after treatment with the nanoparticle suspension for 24 h; (a) HF and (b) HeLa cells.

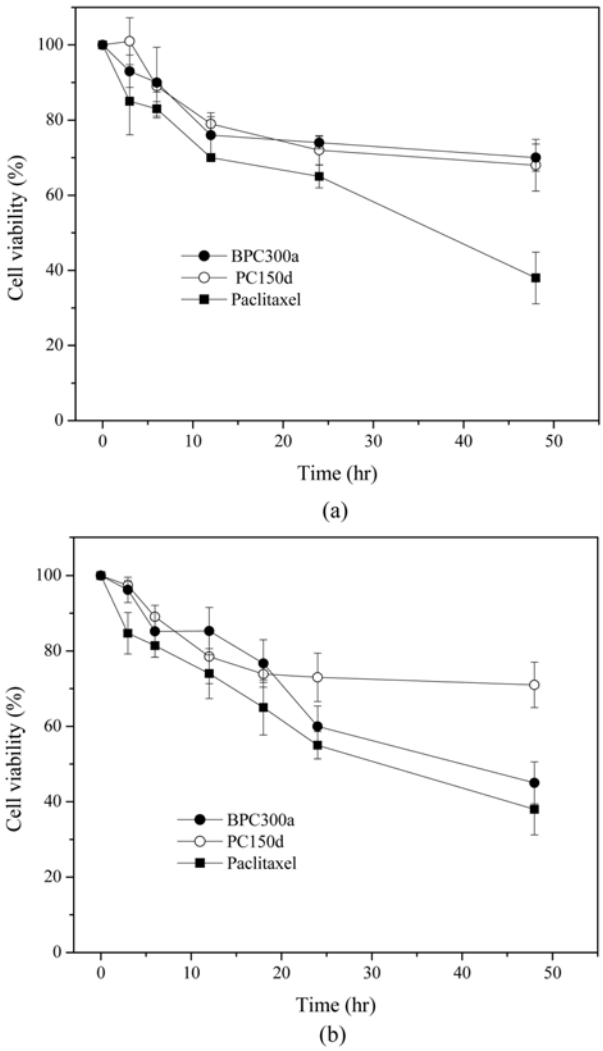

Figure 8. Time course of the viability of cells exposed to paclitaxel-loaded PEG/PCL nanoparticles with biotin group (BPC300a: ●), paclitaxel-loaded PEG/PCL nanoparticles without biotin group (PC150d: ○), and free paclitaxel solution (■); (a) HF and (b) HeLa cells.

particles affected the cells more effectively than those in biotin-unconjugated nanoparticles. Note that the viability of cancer cells (HeLa cells) was much lower than that for normal cells at the same concentration of nanoparticle suspension. The cell viability was statistically different at more than 0.05 μg/mL of concentration ($p < 0.05$). It could be concluded that HeLa cells are more sensitive to biotin-conjugated nanoparticles than normal HF cells.

The viability of HF and HeLa cells that were incubated with 1.0 μg/mL of nanoparticle suspension (BPC300a and PC150d) was also investigated as a function of time. The PC150d without biotin groups exhibited relatively high cell viability, regardless of the cell type (Figure 8). In contrast, biotin-conjugated PEG/PCL nanoparticles (BPC300a) exhibited more severe cytotoxicity against cancer cells than against normal HF ($p < 0.05$). The viability of HF was 70.0% (Figure 8(a)), while the viability of HeLa cells was 45.5% after 48-h incubation (which was expected about 60% drug release from Figure 5) (Figure 8(b)). We noticed that biotin-conjugated PEG/PCL nanoparticles containing paclitaxel could be delivered into cancer cells over-expressing the biotin receptor, resulting in high cytotoxicity for cancer cells.

***In vitro* Cellular Uptake.** The cell uptake characteristics for free paclitaxel and paclitaxel-loaded PEG/PCL nanoparticles with biotin group were shown in Figure 9. For biotin-conjugated PEG/PCL nanoparticles, the time course of uptake of paclitaxel in MCF-7 cells and HeLa cells at 37 °C

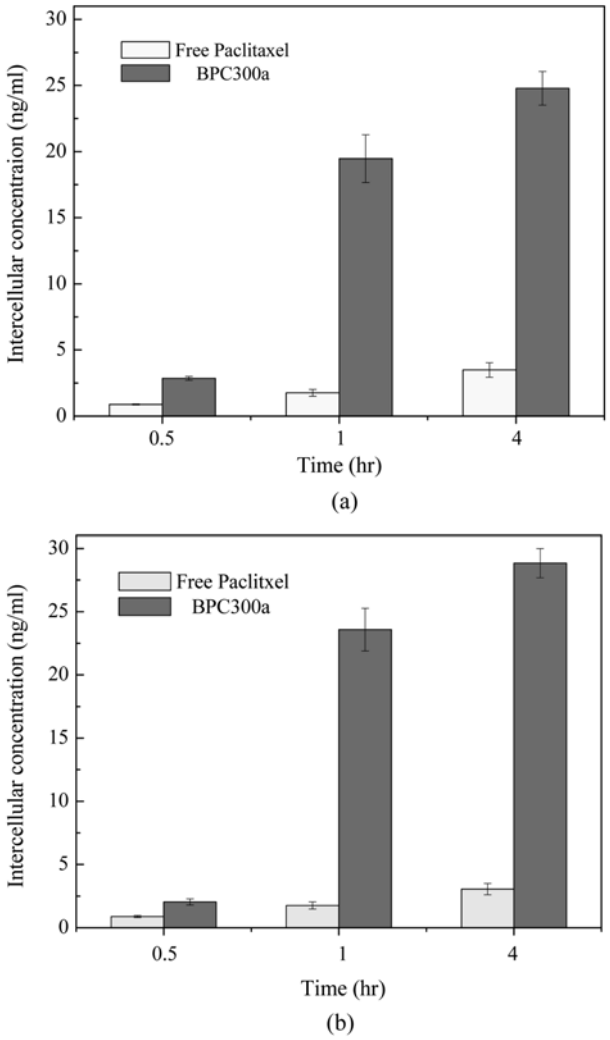

Figure 9. *In vitro* cell uptake of drug in free paclitaxel formulation and biotin-conjugated PEG/PCL nanoparticles containing paclitaxel (BPC300a) as a function of time; (a) MCF-7 cells and (b) HeLa cells. The amounts of paclitaxel taken up by cells were measured after co-incubation of free biotin (5 mM) with biotin-conjugated nanoparticles and free paclitaxel formulation. Each value represents the mean ± S.D. (n = 3).

indicated a progressive increase in uptake. The MCF-7 cells and HeLa cells which incubated with biotin-conjugated PEG/PCL nanoparticles containing paclitaxel (BPC300a) showed enhanced uptake of drug up to 7.1 and 9.4-fold, respectively, compared with the free paclitaxel formulation after 4 h cell culture. Considering the release kinetics of paclitaxel from biotin-conjugated PEG/PCL nanoparticles (shown in Figure 5), it could be expected that biotin-conjugated PEG/PCL nanoparticles were rapidly uptaken into cancer cells before leaking of drug. The enhanced cellular uptake of the drug formulated in biotin-conjugated PEG/PCL nanoparticles compared with free paclitaxel formulation could be attributed to the biotin-receptor mediated endocytosis.

Conclusions

Biodegradable PEG/PCL amphiphilic nanoparticles conjugated with biotin ligand were prepared and used in targeted chemotherapy for cancer treatment. The biotin-conjugated nanoparticles were about 88-118 nm in size and exhibited a narrow size distribution. The loading amount of the hydrophobic anticancer drug paclitaxel was influenced by the chain length of the hydrophobic PCL segment and the feed weight ratio of the drug to the copolymer. The release of paclitaxel from biotin-conjugated PEG/PCL nanoparticles exhibited sustained release kinetics without initial burst effects. The cytotoxicity study showed that biotin-conjugated PEG/PCL nanoparticles admitted relatively high cell viabilities (more than 85%) at 0.005-1.0 μg/mL of concentration. To evaluate the ability of biotin-conjugated nanoparticles to target cancer cells, the viability of human fibroblasts and HeLa cells was also determined for paclitaxel-loaded nanoparticles with and without biotin ligands. Paclitaxel-loaded PEG/PCL nanoparticles with the biotin group exhibited highly selective toxicity for cancer cells, whereas the paclitaxel-loaded nanoparticles without biotin group showed similar cytotoxicity in normal and cancer cells. The MCF-7 and HeLa cells incubated with biotin-conjugated PEG/PCL nanoparticles containing paclitaxel (BPC300a) showed significantly enhanced uptake of drug compared with the free paclitaxel formulation. It suggested that these biotin-conjugated PEG/PCL nanoparticles could be selectively delivered into cancer cells through biotin-receptor mediated endocytosis.

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