Enhancement of cellular binding efficiency and cytotoxicity using polyethylene glycol base triblock copolymeric nanoparticles for targeted drug delivery

Jaemoon Yang,[1] Eun-Jin Cho,[2] Sungbaek Seo,[1] Jae-Won Lee,[1] Ho-Geun Yoon,[3] Jin-Suck Suh,[2] Yong-Min Huh,[2] Seungjoo Haam[1]

[1]*Department of Chemical Engineering, College of Engineering, Yonsei University, Seoul 120-749, South Korea*
[2]*Department of Radiology, College of Medicine, Yonsei University, Seoul 120-752, South Korea*
[3]*Department of Biochemistry and Molecular Biology, Center for Chronic Metabolic Disease Research, College of Medicine, Yonsei University, Seoul 120-752, South Korea*

Received 29 September 2006; revised 12 January 2007; accepted 23 January 2007
Published online 9 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31312

Abstract: Folate (FA) conjugated tri-block copolymers were prepared by bioconjugation of poly ε-caprolactone diol and various molecular weights of diamine polyethylene glycol. The synthetic tri-block copolymers were characterized by ^{1}H-NMR. Three types of nanoparticles were prepared by nanoprecipitation. Their size and morphology were verified by laser scattering and transmission electron microscopy, respectively. The colloidal stability of the nanoparticles was evaluated by turbidity test. The anticancer drug doxorubicin (DOX) was encapsulated in the nanoparticles during preparation. Drug loading amounts and release behavior from prepared nanoparticles were investigated. Fluorescent-activated cell sorting analysis and epi-fluorescencic microscopic imaging of prepared nanoparticles exhibited good cellular uptake against target cells. FA receptor expressed OVCAR3 cells that showed higher mean fluorescence intensity than FA receptor defect A549 cells at specific polyethylene glycol chain lengths. The cell cytotoxicity of prepared nanoparticles was evaluated for receptor mediated drug delivery. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 84A: 273–280, 2008

Key words: folate; polyethylene glycol; poly ε-caprolactone diol; doxorubicin; nanoparticle

INTRODUCTION

Receptors have site-specific properties and increase cellular uptake within target cells in natural metabolism.[1–3] These properties may allow for the selective targeting of imaging and therapeutic agents to tumor tissue, reducing the side effects of drugs.[4–6] Folate (FA), one of the well known receptor mediated targeting moieties, shows high binding affinity to tumor cells using the specific ligand targeting model.[7,8] FA receptor is overexpressed on the surfaces of human ovarian, brain, endometrial, kidney, head, lung, and breast cancer cells in particular.[9,10] A number of studies have investigated FA conjugated with drug carriers such as liposomes, linear polymers, and polymeric micelles.[11–13] In addition, researchers have used specific organic polymers such as poly (D,L lactide-*co*-glycolide) (PLGA), polylactide (PLA), polycaprolactone (PCL), polyalkylcyanoacetate (PACA), and its copolymers, because of their biocompatibility and biodegradability as well as their low toxicity.[14–16] PCL is an inexpensive biodegradable polyester and is easy to manipulate and copolymerize with other polymers. In addition, it does not generate an acid environment, unlike PLA, PGA, and their copolymer, PLGA. The acidic conditions could cause deformation of the release pattern or drug effects. In addition, the degradation of PCL by hydrolysis is very slow because of high crystallinity.[14] This property can be modified by increasing the hydrophilicity and biodegradability of PCL through copolymerization with polyethylene glycol (PEG).[17] PEG is a hydrophilic and biocompatible polymer that induces longtime circulation through prevention of protein adhesion and the enhanced permeation and retention (EPR) effect.[18,19]

Correspondence to: S. Haam; e-mail: haam@yonsei.ac.kr
Contract grant sponsor: KOSEF (National Core Research Center for Nanomedical Technology); contract grant numbers: R15-2004-024-00000-0, R01-2006-000-10023-0
Contract grant sponsor: National R&D Program for Cancer Control
Contract grant sponsor: Ministry of Health and Welfare, Republic of Korea; contract grant number: 0620190-1
Contract grant sponsor: Yonsei University Research Fund of 2006

The amphiphilic block copolymers of polyester and PEG can form spherical nonionic micelles that increase the solubility of organic materials.[16,20] In addition, these copolymer micelles have attracted much attention because of their biodegradability, improved biocompatibility, and potential applications in drug (or gene) delivery.[17]

The amphiphilic block copolymers can be synthesized by polymerization with monomers and chemical conjugation.[21–23] However, the bioconjugation technique, using an appropriate conjugation agent (such as *p*-nitrophenyl chloroformate), could obtain the narrow distribution of polymers.[24] In addition, the previously studied mPEG-PCL-FA system showed low binding efficiency with the cell because FA was conjugated with the terminal hydrophobic chain.[25] To increase efficiency of uptake, FA should conjugate with the terminal of the hydrophilic part. Cell binding and uptake efficiency can be controlled by modulating the PEG chain length.

We synthesized the tri-block copolymer (PEG-PCL-PEG, PECE) using various molecular weights of PEG by the bioconjugation technique. FA conjugated with hydrophilic chains of tri-block copolymeric nanoparticles containing anticancer drug was prepared for targeted drug delivery (Fig. 1). Chemical structure of synthetic copolymers was analyzed by ^{1}H-NMR, molecular weight and distribution were analyzed by gel permeation chromatography, and size and morphology of prepared nanoparticles were evaluated by laser scattering and electron microscopy. As an anticancer agent, doxorubicin (DOX) was loaded in prepared nanoparticles, and drug release tests were performed to examine their release characteristics. The drug loading contents and encapsulation efficiency were calculated. Cellular uptake efficiencies of FA conjugated block copolymer nanoparticles were evaluated by fluorescence-activated cell sorting (FACS) and epi-fluorescence microscopy. The MTT assay for three types of nanoparticles was performed for evaluation of cytotoxicity.

MATERIALS AND METHODS

Materials

Poly ε-caprolactone diol (PCL-diol, M_W: 2000) was purchased from Aldrich (San Diego, CA). *p*-nitrophenyl chloroformate (pNC), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimde (DCC), 3-(4,5-dimethylthiaol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), folate (FA), and doxorubicin (DOX) were obtained from Sigma (St. Louis, MO). Diamine polyethylene glycol (DA-PEG) (M_W: 2000, 3400, and 5000) were purchased from NOF corporation (Japan). All other chemicals were of analytical grade.

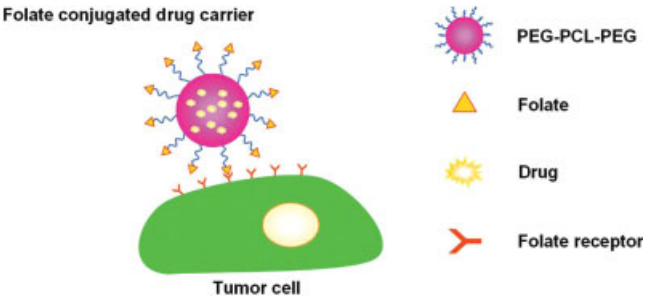

Figure 1. Conceptual scheme of FA receptor mediated drug carrier. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Synthesis and characterization of FA conjugated tri-block copolymers

PCL-diol (1.0 mmol) dissolved in methylene chloride was activated by adding 4.0 mmol of pNC and 4.0 mmol of pyridine at 0°C. The reaction was carried out for 3 h at room temperature under nitrogen atmosphere. The resultant was filtered and precipitated by dropping into cold diethyl ether and dried under vacuum until the next procedure.

To synthesize PECE tri-block copolymer, activated PCL-diol (0.1 mmol) dissolved in dimethyl formamide (DMF) was added in a dropwise manner to DA-PEG (0.25 mmol) dissolved in DMF at 4°C to avoid the unwanted multi-block copolymer. This reaction was carried out under nitrogen atmosphere for 6 h. The purification process and storage method were as previously mentioned.

Folate conjugated tri-block copolymers (FA-PECEs) were synthesized by mixing 0.02 mmol of PECE tri-block copolymer and 0.04 mmol of FA mixed with 0.16 mmol of DCC and NHS in 10 mL dimethyl sulfoxide at room temperature for 10 h. After the reaction, the filtrated products were dialyzed in distilled water. A synthetic scheme of FA-PECE is shown in Figure 2(a).

To increase drug loading contents, DOX was conjugated with PCL-diol using the above-mentioned method [Fig. 2(b)]. Activated PCL-diol (0.02 mmol) dissolved in DMF was added to DOX (0.05 mmol) dissolved in DMF at 4°C. This reaction was carried out under nitrogen atmosphere for 6 h. The products (DOX-PCL-DOX) were purified in the same manner as mentioned above.

The average molecular weight and polydispersity index (PDI) of prepared PECEs were obtained using gel permeation chromatography (GPC, Yonglin Autochro-GPC, Yonglin, Korea). The GPC columns were series of μStyeagel columns (HR-1, HR-2, HR-3, and HR-4), and tetrahydrofuran was used as a solvent. The yield of polymerization was calculated as mass percentage (see Table I), and tri-block copolymers were synthesized with relatively high yield.

Critical micelle concentration (CMC) of synthetic tri-block copolymer was measured using a surface tension meter (Fisher Scientific, USA) at 25°C.[26]

Structural characterization of tri-block copolymer was performed by ^{1}H-NMR (500 MHz NMR spectrometer, AVANCE 500, Bruker, Germany) for observation of the

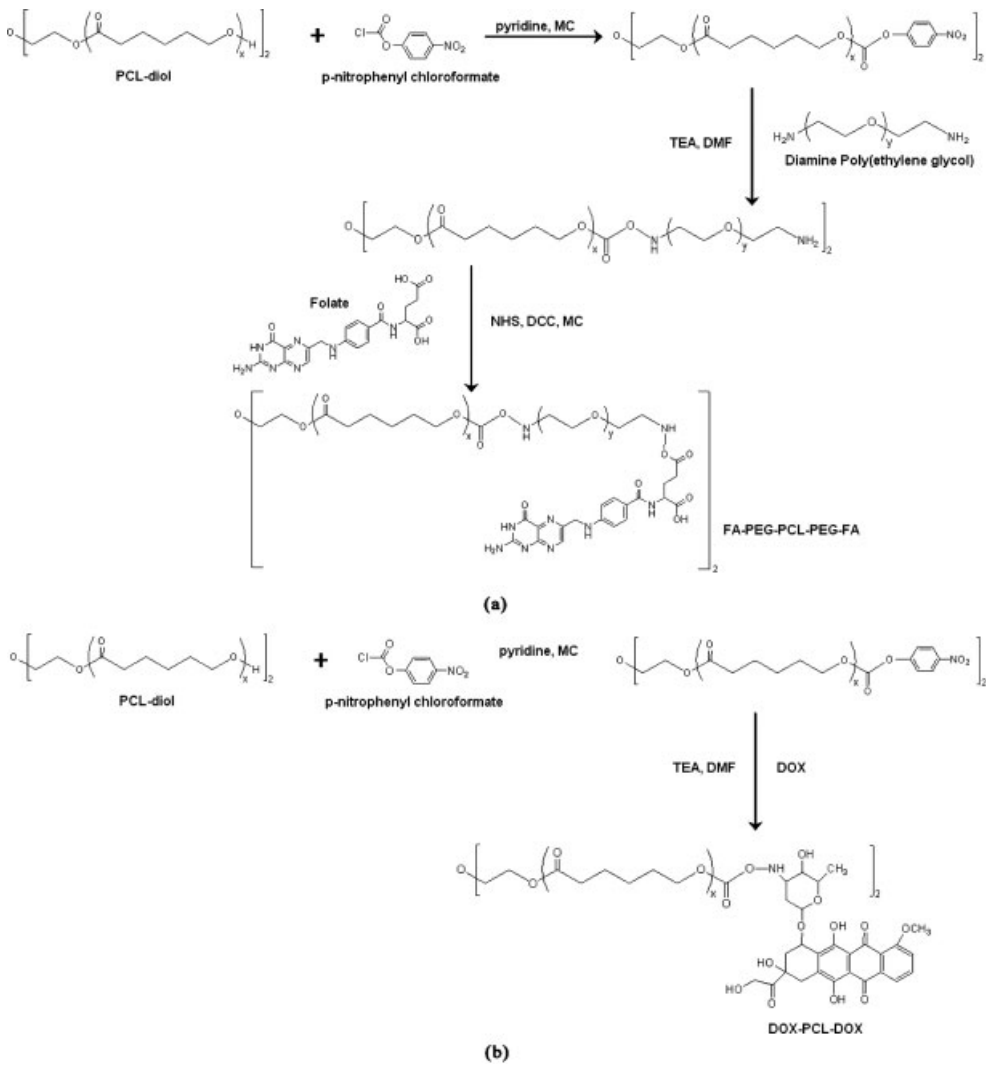

Figure 2. Synthetic scheme of FA conjugated PEG-PCL-PEG tri-block copolymers (a) and DOX conjugated with PCL-diol (b).

functional groups. Chloroform-*d* and dimethyl sulfoxide-d_6 were used as solvents.

Preparation of FA-PECE nanoparticles loaded with DOX

FA-PECE nanoparticles loaded with DOX (DOX-FA-PECE) were prepared by the nanoprecipitation method.[25] Seven milligrams of FA-PECE, 3 mg of DOX-PCL-DOX, and 2 mg of DOX were dissolved in 2 mL acetone in the presence of 2.5 mg of triethylamine as a catalyst.[13] The solution was added dropwise to 50 mL distilled water under moderate stirring at 10°C. The acetone was removed under reduced pressure. The nanoparticles were purified and stored after freeze-drying. Fluorescein isothiocyanate (FITC) was conjugated with the amine group of FA for

TABLE I
Molecular Weight, Polydispersity Index, Yield, and CMC of Synthetic PECE Tri-Block Copolymers

Copolymer Name	M_n of DA-PEG	M_w[a]	PDI[a]	Yield of Polymerization (%)[b]	CMC (μg/mL)
PECE-1	2,000	6,738	1.21	78.5	0.08
PECE-2	3,400	9,614	1.14	83.1	0.15
PECE-3	5,000	13,012	1.37	81.4	0.32

[a]Determined by gel permeation chromatography, PDI (polydispersity index) $= M_w/M_n$.

[b]Evaluated after purification process; Yield of polymerization $(\%) = \dfrac{\text{mass of synthesized tri-block copolymer}}{\text{mass of introduced polymers}}$

FACS analysis and epi-fluorescent microscopic study during the nanoparticle preparation process.

The morphology of prepared nanoparticles was observed by transmission electron microscopy (TEM) (JSM5410LV, JEOL, Nikon, Japan). A drop of polymeric nanoparticle suspension was placed on a copper grid coated with formvar film, and dried at room temperature. The sample was negatively stained with 0.3% phosphotungstic acid.

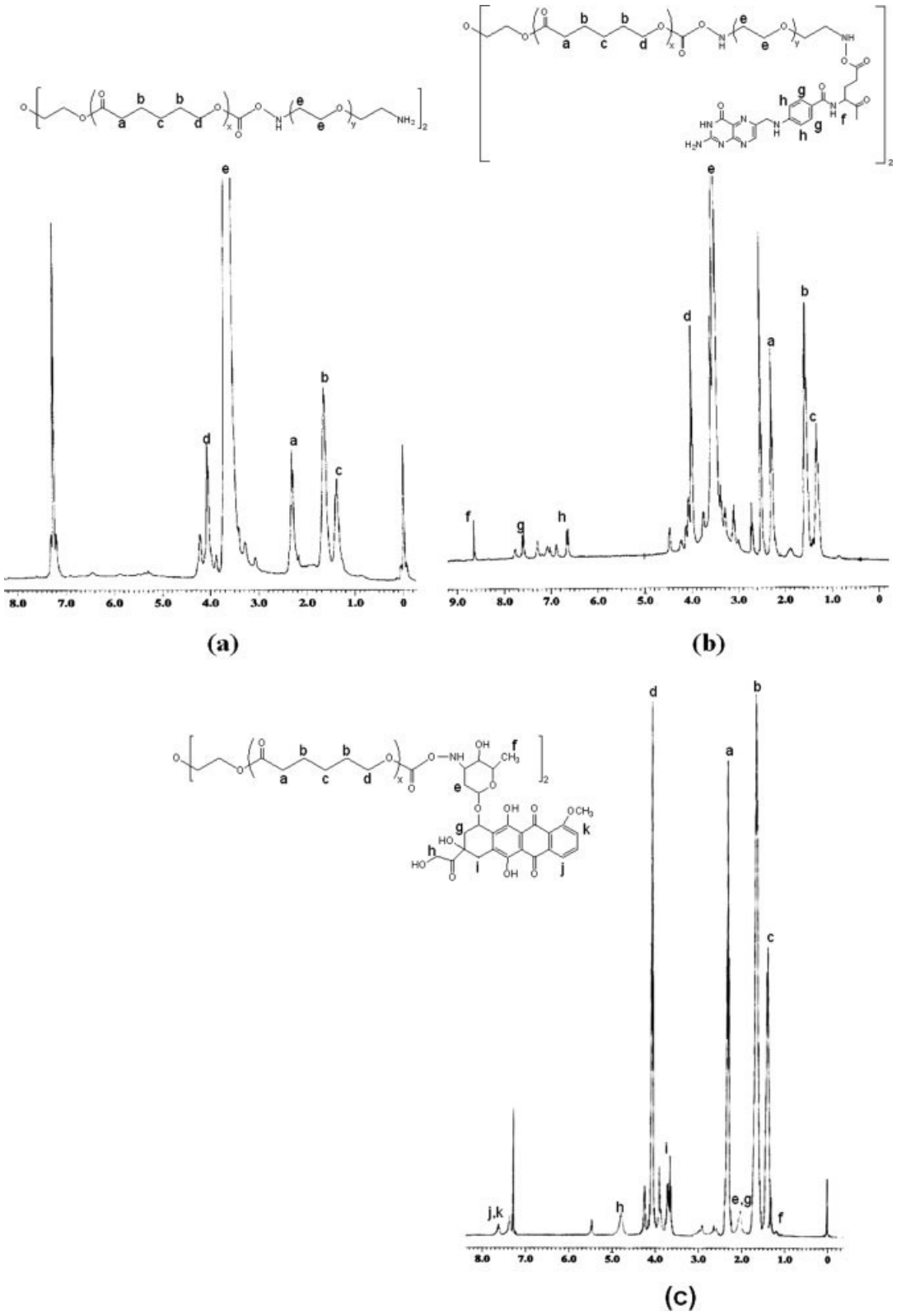

Figure 3. ^{1}H-NMR spectra of PEG-PCL-PEG, tri-block copolymers (PECE-2) (a), FA conjugated tri-block copolymers (FA-PECE-2) (b), and DOX conjugated PCL (c).

TABLE II
Physicochemical Characteristics of the Prepared PECE Nanoparticles and Nanoparticle Yields

Copolymer Name	Size (nm)[a]	Polydispersity Index[a]	Zeta Potential (mV)	Nanoparticle Yield (%)[b]
PECE-1	93.3 ± 10.1	0.317	−4.7	91.5
PECE-2	82.2 ± 7.3	0.289	−3.8	89.4
PECE-3	57.7 ± 8.7	0.216	−6.3	91.2

[a]Mean ± standard deviation ($n = 3$)

[b]$\text{Nanoparticle yield } (\%) = \dfrac{\text{weight of prepared nanoparticles}}{\text{weight of injected polymer and drug}} \times 100$

Dynamic laser scattering (DLS) (Zetasizer Nano ZS, Malvern, UK) analysis was performed to determine the size, size distribution, and zeta potential.

The colloidal stability of the prepared nanoparticles was evaluated from their resistance to sodium sulfate induced nanoparticle aggregation. One hundred microliter samples of nanoparticle suspensions (20 mg/mL) were added to 5 mL of sodium sulfate solutions of varying concentrations (0–1.0*M*) at 37°C. After 10 min, the turbidity of the suspensions was measured at 560 nm using a UV spectrophotometer.

Drug release studies

To obtain the drug release profile, 10 mg of prepared DOX-FA-PECE nanoparticles was suspended in 2 mL of phosphate buffer solution (pH 7.4) and sealed in a dialysis tube that was immersed in 25 mL buffer solution at 37.5°C. The system was shaken at moderate speed. At regular time intervals, the amount of released drug was monitored by measuring the absorbance (480 nm) using a UV spectrophotometer (Optizen 2120UV, MECASYS, Korea). Drug loading contents and entrapment efficiency were measured in the same manner using a calibration curve.

Cell uptake efficiency of nanoparticles

The OVCAR3 cell is a human ovarian carcinoma cell line that expresses FA receptor α-isoform (FR-α) at the surface, and the A549 cell is a human lung carcinoma cell line with defective FA receptors, purchased from American Type Culture Collection (ATCC, Manassas, VA). These cells were maintained in a 5% CO_2 humidified atmosphere at 37°C in an FA-free RPMI 1640 medium (Gibco, Carlsbad, USA), containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

1×10^6 cells incubated with FITC labeled nanoparticles in previously described media at 37°C for 4 h and then washed twice with PBS were collected by trypsinization. The resultant was washed three times with 0.2% FBS and 0.02% NaN_3 in PBS. These samples were resuspended in 400 μL, 4% paraformaldehyde, and scanning of the cell-associated fluorescence was performed using a FACScalibur (Beckton-Dickinson, Mansfield, MA) at the wavelength of $\lambda = 488$ nm. Specific binding and cellular uptake of prepared nanoparticles were visualized using an epi-fluorescence microscope (Olympus BX51; Olympus Optical, Tokyo, Japan).

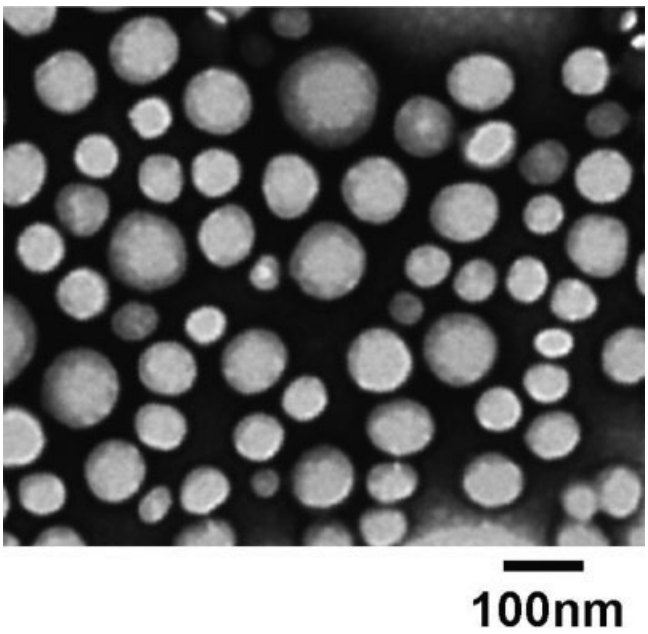

Figure 4. TEM image of FA-PECE-2 nanoparticles loaded with DOX.

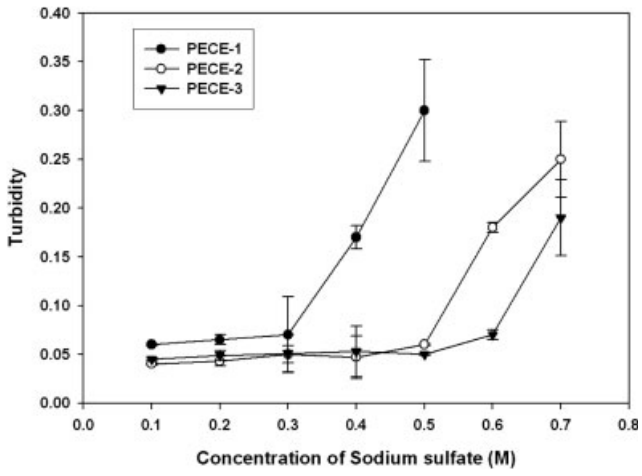

Figure 5. Turbidity evolution of prepared PECE nanoparticle suspensions with injected sodium sulfate.

TABLE III
Drug Loading Contents and Entrapment Efficiency of the PECE Nanoparticles ($n = 3$)

Copolymer Name	Drug Loading Contents (%)[a]	Entrapment Efficiency (%)[b]
PECE-1	2.41 ± 0.54	60.3 ± 7.26
PECE-2	2.94 ± 0.97	62.9 ± 10.74
PECE-3	3.26 ± 0.73	58.4 ± 8.19

[a]$\text{Drug loading contents } (\%) = \dfrac{\text{weight of drug in nanoparticles}}{\text{weight of prepared nanoparticles}} \times 100$

[b]$\text{Entrapment efficiency } (\%) = \dfrac{\text{weight of drug in nanoparticles}}{\text{weight of injected drug}} \times 100$

Cytotoxicity test

Cytotoxicity of free DOX and DOX-FA-PECE nanoparticles was evaluated by measuring the inhibition of cell growth using a MTT assay.[27] Cell growth medium was Dulbecco's Modified Eagle's Medium (DMEM), and a 5% CO_2 atmosphere was maintained. The target cells (a cellular density of 4×10^3 cells/mL) were cultured on 96 well plates. The cells were incubated with various concentrations of free DOX and prepared nanoparticles for 72 h, the MTT assay was performed, and the cell viability was determined.

RESULTS AND DISCUSSION

The high yield of block copolymers was synthesized by chemical conjugation. The chemical structure of the prepared PECEs was confirmed by ^{1}H-NMR [Fig. 3(a)]. The PCL peak was observed at 1.4, 1.7, 2.4, and 4.1 ppm (PCL backbone), and the characteristic band of PEG was observed at 3.65 ppm in $CDCl_3$. After FA was conjugated, its characteristic peak was observed at 6.65, 7.6, and 8.7 ppm in DMSO-d_6 [Fig. 3(b)]. The chemical shifts of DOX conjugated PCL-diol were observed at 1.35 (—C—CH_3), 3.9 (—O—CH_3), and 4.85 (—C=O—CH_2—OH) ppm [Fig. 3(c)].

The molecular weight and distribution of synthetic copolymers were measured by GPC (Table I). The tri-block copolymers were synthesized in a narrow molecular distribution as various lengths of PEG chain. For the same PCL chain length, the CMC of tri-block copolymer increases as the PEG chain length increases (CMC of PECE-1, 16.7 μmol/L; CMC of PECE-2, 11.3 μmol/L; and CMC of PECE-3, 6.2 μmol/L). These results came from the hydrophobic chain (PCL) worked with driving force for micellization in a solution.

The spherical and moderately uniformly sized nanoparticles were prepared by the nanoprecipitation method. The mean size and the size distribution of prepared particles were measured by laser scattering (Table II). The size of the nanoparticles decreased as the PEG/PCL ratio increased. Zeta potential values were evaluated to verify colloidal stability. Although the values were relatively lower than the zeta potential of naked polyester nanoparticles,[28] the nanoparticles were dispersed by steric hindrance.[29] In addition, zeta potential was an important factor in determining the interaction of nanoparticles with the cell membrane, which is usually negatively charged. TEM photography of prepared nanoparticles is shown in Figure 4. The sample was negatively stained with 0.3% phosphotungstic acid, and the nanoparticles are shown as white circles.

The turbidity of PECE nanoparticle suspension in the presence of sodium sulfate is shown in Figure 5. The turbidity increased as the concentration of sodium sulfate increased and the relatively long PEG chain could prevent the aggregation of nanoparticles.

The amount of DOX in the nanoparticles and the entrapment efficiency are shown in Table III. The conjugation of DOX with PCL-diol and injection of triethylamine during the preparation procedure could increase the loading amount and entrapment

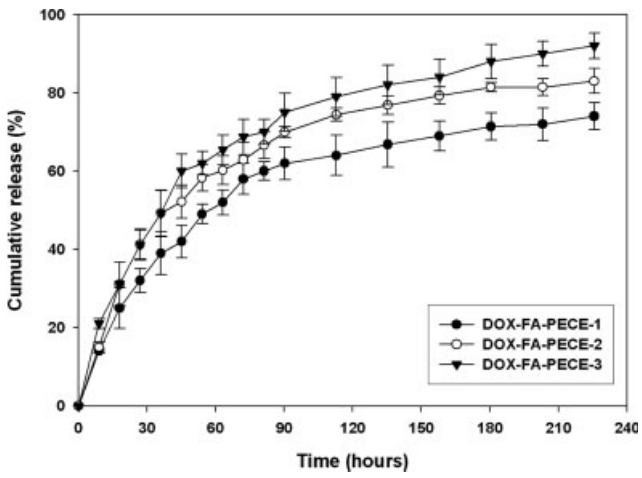

Figure 6. Drug release profile of FA-PECE nanoparticles loaded with DOX ($n = 3$).

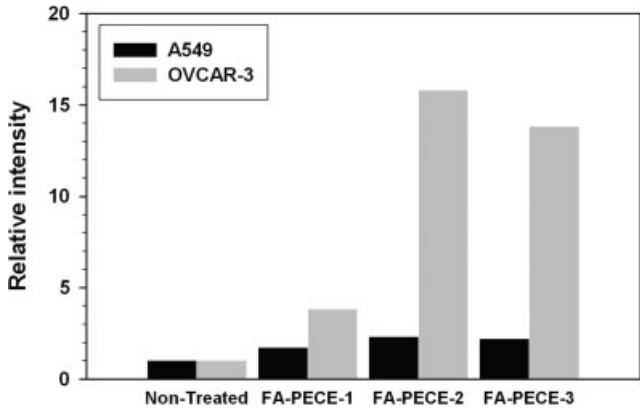

Figure 7. Relative fluorescence intensity by FACS analysis of A549 and OVCAR3 cells incubated with FA-PECE nanoparticles.

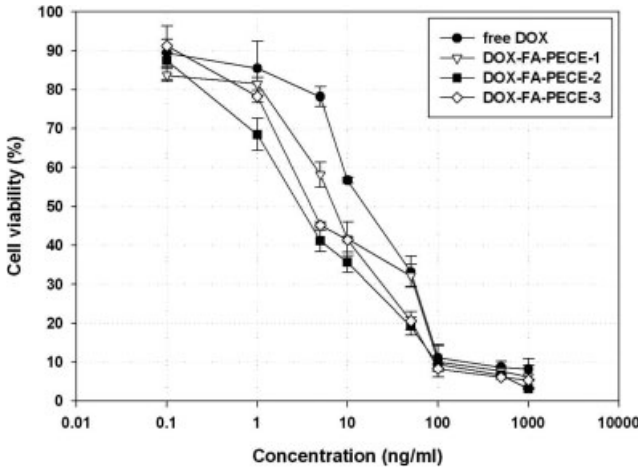

Figure 9. The cell viability of free DOX and DOX-FA-PECE nanoparticles in OVCAR3 cells by MTT assay.

efficiency.[13,28] Drug release from the prepared DOX-FA-PECE nanoparticles is shown in Figure 6. The release test was performed three times and the obtained values were used for calculation of mean value and standard deviation. The length of the PEG chain affected the release characteristics. The drug release rate accelerated as the PEG/PCL ratio increased. The results demonstrated that the long length of PEG chain promotes the uptake of water that induces degradation of the PCL core of nanoparticles.

FACS analysis provided the specific binding and cellular uptake efficiency of nanoparticles. As shown in Figure 7, OVCAR3 cells showed seven times higher mean fluorescence intensity than A549 cells according to the order of the expression level of FA receptors for FA-PECE-2. The FA-PECE-3 nanoparticles were evaluated with relatively lower intensity than FA-PECE-2 nanoparticles because of the long length of the PEG chain, which hides the FA in the PEG matrix.

The cellular uptake efficiencies of FA conjugated nanoparticles for both A549 and OVCAR3 cells were determined using epi-fluorescence microscope. FA receptor expressing OVCAR3 cells showed higher specific binding and cellular uptake efficiency than FA receptor defective A549 cells (Fig. 8). Vivid green fluorescence from FITC was clearly observed for FA conjugate nanoparticle treated OVCAR cells, whereas faint green fluorescence was noted for FA conjugate nanoparticle treated A549 cells. These FACS analysis and epi-fluorescence microscopy results indicate that FA conjugated PECE nanoparticles could successfully bind specifically and then internalize the cells through a receptor mediated endocytosis pathway.

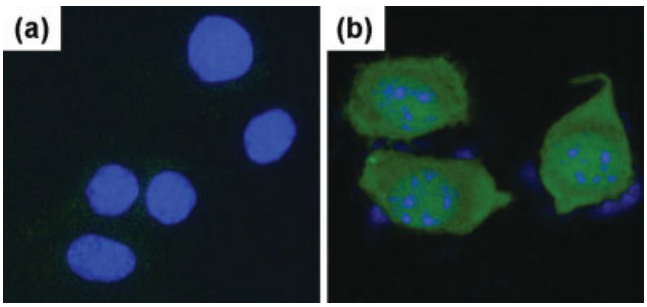

Figure 8. Fluorescence microscopic images of A549 (a) and OVCAR3 (b) cells incubated with FA-PECE-2 nanoparticles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The cytotoxicity was measured after 72 h of incubation of cells treated with free DOX and DOX-FA-PECEs using MTT solution (Fig. 9). The DOX-FA-PECEs showed higher cell toxicity than free DOX, which means the prepared nanoparticles were more bound with cancer cells than free DOX and uptake followed an endocytosis pathway. Then, released DOX and DOX from polymer degradation acted on cancer cells, and the cytotoxic effects were demonstrated. The presence of the PEG chain of prepared polymers reduced the negative charge of PCL, and the FA conjugated with PECE nanoparticles increased binding efficiency and cellular uptake. In addition, DOX-FA-PECE-2 nanoparticles were more toxic than other PECE nanoparticles due to greater binding efficiency, as shown in Figure 7.

CONCLUSIONS

FA-PECE nanoparticles containing an anticancer drug were prepared by the nanoprecipitation method for targeted drug delivery. The chemical structures of synthesized tri-block copolymer against various molecular weights of DA-PEG and physicochemical properties of PECE nanoparticles were ana-

lyzed. Because of the conjugation of DOX with PCL, the high amounts of drug loading and sustained release of DOX from PEGylated nanoparticles were possible. In addition, the drug release rate could be controlled with various chain lengths of PEG. The colloidal stability was enhanced as PEG chain length increased. However, too long a PEG chain prevents the binding affinity with target cancer cells because of steric hindrance. In the case of medium PEG chain length, excellent cellular uptake efficiency was presented via FACS analysis and epi-microscopy, and seven times higher, mean fluorescence intensity against FA receptor expressing OVCAR3 cells was evaluated according to the order of the expression level of FA receptors. At these cellular binding efficiency levels, DOX-FA-PECE nanoparticles demonstrated the different cytotoxicities. These results establish a potential anticancer drug carrier for a receptor mediated targeted drug delivery system.

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