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Synthesis and evaluation of biotin-conjugated pH-responsive polymeric micelles as drug carriers

Ji Hyun Kim[a,1], Yi Li[a,1], Min Sang Kim[a], Seong Woo Kang[a], Ji Hoon Jeong[b], Doo Sung Lee[a,*]

[a] Department of Polymer Science and Engineering, Sungkyunkwan University, Suwon 440-746, South Korea
[b] College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea

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ABSTRACT

pH-Responsive polymeric micelles have been investigated as drug carriers for chemotherapy. Ligand-mediated polymeric micelles, which can penetrate the target tumors due to their high binding affinity to a specific receptor on the surface of tumors, were developed to achieve targeted drug delivery. In this study, biotin-conjugated methoxypoly(ethylene glycol)-grafted-poly(β-amino ester) was prepared for active and pH-sensitive tumor targeting. These polymers were modified by cholesteryl chloroformate to improve the hydrophobicity of the micelle core. The structure of the biotin-conjugated polymer was confirmed by ^{1}H NMR spectroscopy, and the existence of biotin at the surface of the polymeric micelles was evaluated by an 4′-hydroxyazobenzene-2-carboxylic acid/avidin (HABA/avidin) binding assay at different pHs. The micelle properties were determined by dynamic light scattering and the result showed that the mean size of the polymeric micelles was approximately 20 nm. For cancer therapy, doxorubicin (DOX) was loaded into the polymeric micelles with a high loading efficiency. From the *in vitro* cellular uptake results, the biotin-conjugated polymeric micelles can effectively release doxorubicin at acidic tumor cells compared to the micelles without biotin. Overall, biotin-conjugated pH-responsive polymeric micelles have great potential to be used as drug carriers.

1. Introduction

Polymeric micelles formed by self-assembly of amphiphilic copolymers under aqueous conditions have been investigated in the field of drug delivery systems owing to their biodegradability, high water-solubility, high drug loading capacity and low toxicity, which are induced by the prolonged circulation in the blood and enhanced accumulation in tumor tissue *via* enhanced permeability and retention (EPR) effect (Rösler et al., 2001; Maeda, 2001). Specific stimulus (temperature, pH, enzymes, *etc.*) sensitive polymers are useful candidates for cancer therapy to achieve a specific drug accumulation at the tumor site. Polymeric micelles that exhibit a transformation of the structure by pH change are used to selectively deliver various anti-cancer drugs to tumor site by passive targeting because the tumor extracellular environment is more acidic than normal tissues and the blood stream due to the high rate of aerobic and anaerobic glycolysis (proton production by lactate formation and ATP lysis) in cancer cells (Wang et al., 2009; Ganta et al., 2008; Oishi et al., 2006; Gillies and Frechet, 2005; Mahoney et al., 2003). However, a more effective and active targeting system was needed to enhance the intracellular uptake of drug carriers within cancer cells at the tumor site. To increase the delivery of a given drug to a specific target site, polymeric micelles can be surface conjugated using various targeting moieties or ligands, such as folate, saccharide, biotin and peptides (Weitman et al., 1992; Lee et al., 2005; Joralemon et al., 2004; Yoo and Park, 2004; Wu et al., 2010). Among them, biotin is used widely as a tumor targeting ligand for various anti-cancer drugs. The avidin–biotin interaction is the strongest known non-covalent biological interaction. The bond between biotin and avidin is stable and unaffected by a wide range of pH values, temperatures, organic solvents or other denaturing agents (Wilchek et al., 2006; Ben-Shabat et al., 2006; Green, 1975).

This study focused on a tumor-targeted drug delivery system based on biotin-conjugated polymeric micelles. Biocompatible and amphiphilic graft copolymers composed of methoxypoly(ethylene glycol) and pH-responsive poly(β-amino ester) (PAE-g-PEG) were prepared. These polymers can self-assemble to form nanoparticles with a core–shell structure and is capable of loading hydrophobic drugs. In addition, PAE-g-PEG was modified with cholesteryl chloroformate (PAE-g-PEG-ch) to improve the hydrophobicity of the micelle core. Biotin moieties were conjugated to the copolymers (Biotin-PAE-PEG-ch) for active tumor targeting. The structures of the biotin-conjugated copolymers were confirmed by ^{1}H NMR spectroscopy, and the existence of biotin at the surface of the

* Corresponding author. Tel.: +82 31 290 7267; fax: +82 31 292 8790.
E-mail address: dslee@skku.edu (D.S. Lee).
[1] These authors contributed equally to this work.

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biotin-conjugated polymeric micelles was evaluated using a HABA/avidin binding assay. The size of the polymeric micelles was determined by dynamic light scattering (DLS) and the critical micelle concentration (CMC) of the polymer in PBS solution was determined by fluorescence measurements. DLS showed that the copolymers could form micelles in aqueous media with mean sizes of 13–25 nm. The Biotin-PAE-g-PEG exhibited pH-responsive micellization behavior, whereas Biotin-PAE-g-PEG-ch could maintain micelle formation, even at low pH due to the hydrophobic interaction between cholesterol groups. In the case of Biotin-PAE-g-PEG, HABA/avidin binding assay proved that biotin was exposed to the micelle surface at any pH. Micelles composed of cholesterol-conjugated polymers showed that the degree of biotin exposure was dependent on pH. These results can be helpful for selective tumor-target drug delivery systems.

2. Materials and methods

2.1. Materials

Methoxypoly(ethylene glycol) (MPEG, Mn = 2000), succinic anhydride, N,N′-dicyclohexyl carbodiimide (DCC), 4-(dimethyl amino)pyridine (DMAP), anhydrous dioxane, anhydrous dichloromethane (DCM), anhydrous tetrahydrofuran (THF), 3-amino-1-propanol (AP), 1,4-butanediol diacrylate (BD), biotin, cholesteryl chloroformate, trimethylamine (TEA), phosphate buffer saline (PBS) tablets, HABA/avidin reagent and doxorubicin (DOX) were purchased from Sigma–Aldrich. Diethyl ether was supplied by Samchun Chemicals (Korea). Carboxylic acid-modified MPEG (MPEG-COOH) was prepared according to the procedure reported elsewhere (Zalipsky et al., 1983). 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). CCK-8 solution was obtained from Promega Corporation (Madison, WI, USA).

2.2. Synthesis of biotin-conjugated PAE-g-PEG-ch (Biotin-PAE-g-PEG-ch)

Methoxypoly(ethylene glycol) grafted poly(β-amino ester) (PAE-g-PEG) was synthesized as described previously (Kim and Lee, 2010). Briefly, AP (3.8 g, 50 mmol) was charged into round bottom flask and BD (10 g, 50 mmol) was weighed directly into liquid AP. The mixture was kept at 100 °C for 5 h. After the reaction, the flask was allowed to cool to room temperature, the viscous product was dissolved in DCM and the solution was precipitated in diethyl ether. The final product (PAE) was obtained after drying at 30 °C for 2 days. MPEG-COOH (3.84 g, 1.83 mmol) together with PAE (5 g, 18.3 mmol), DCC (0.75 g, 3.6 mmol) and DMAP (0.45 g, 3.6 mmol) were dissolved at room temperature in anhydrous DCM and the mixture was stirred for 24 hrs. After filtering the mixture to remove dicyclohexyl urea (DCU), the filtrate was precipitated in diethyl ether. PAE-g-PEG was obtained by subsequent drying (yield: 83%).

To improve the hydrophobicity of PAE-g-PEG, cholesteryl chloroformate was conjugated as follows. PAE-g-PEG (4 g, 8 mmol), cholesteryl chloroformate (0.36 g, 0.8 mmol) and triethylamine (0.2 ml, 1.6 mmol) were dissolved in anhydrous THF at room temperature for 24 hrs. After removing the triethylammonium chloride by filtering, the reactant solution was precipitated in diethyl ether. The resulting cholesterol-conjugated PAE-g-PEG (PAE-g-PEG-ch) was dried under vacuum at 30 °C for 2 days (yield: 85%).

The biotin-conjugated PAE-g-PEG-ch was prepared as follows. PAE-g-PEG-ch (2.0 g, 4 mmol) together with biotin (0.10 g, 0.4 mmol), DCC (0.17 g, 0.8 mmol) and DMAP (0.10 g, 0.08 mmol) were dissolved at room temperature in anhydrous DCM and the mixture was stirred for 2 days. After removing the DCU by filtering, the final Biotin-PAE-g-PEG-ch product was obtained by precipitation in diethyl ether and dried in vacuum oven (yield: around 50%).

2.3. Characterization of Biotin-PAE-g-PEG-ch

The chemical structure of Biotin-PAE-g-PEG-ch was characterized by ^{1}H NMR spectroscopy recorded on a Varian-Unity Inova 500NB spectrometer operated at 500 MHz. Dimethyl-d_6 sulfoxide (DMSO-d_6) was used as the solvent and substitution ratio of PEG, cholesteryl chloroformate and biotin was calculated by comparing the proton peaks. The molecular weight (Mn) and polydispersity index (PDI) were confirmed by gel permeation chromatography (GPC). The molecular weight was calibrated with poly(methyl methacrylate) standards.

2.4. Fluorescence measurement

The critical micelle concentration (CMC) of Biotin-PEA-g-PEG-ch in PBS buffer solution was determined using a fluorescence probe technique with pyrene. The fluorescence spectra were recorded using an AMNCO-BOWMAN®Serise 2 luminescence spectrometer (SLM-AMINCO, USA) at room temperature. The pyrene solution in THF (1 ml) was dispersed in the PBS buffer solution (1 L), and the THF was eliminated using a rotary evaporator at 60 °C for 1 hr. The pyrene dissolved buffer solution was prepared at a concentration of 1.0×10^{-6} M. The excitation spectra were recorded from 310 to 350 nm with the condition of an emission wavelength at 392 nm.

2.5. Dynamic light scattering

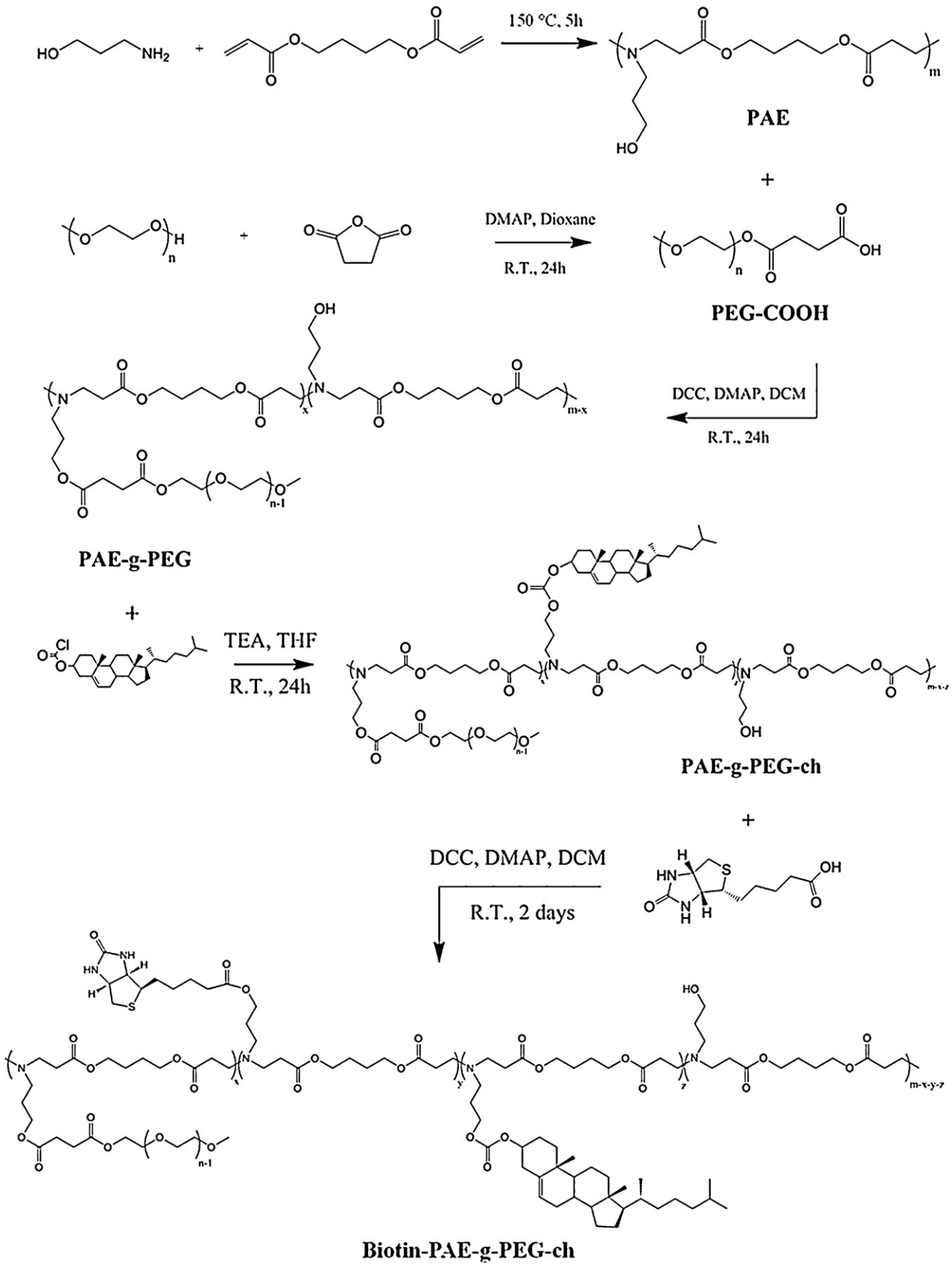
The size of the polymeric micelles was determined by dynamic light scattering (DLS) using a Malvern PCS100 spectrogoniometer and Brookhaven BI-9000AT digital autocorrelator with a helium laser at 633 nm. The scattering angle was kept at 90 °C and the temperature was adjusted to 37 °C. For the DLS measurements, the concentration of polymer in a PBS buffer solution was kept at 5 mg/ml and the micelle size was measured at different pH values. Before being transferred into the light scattering cuvettes, the polymer solution was filtered through a syringe filter. The mean diameter was evaluated using the Stokes–Einstein equation.

2.6. Biotin binding assay

The exist of available biotin on micelles at different pH was confirmed using a HABA/avidin reagent (Sigma–Aldrich). The HABA/avidin reagent was reconstituted with 10 mL of deionized water and added dropwise to the polymer in a PBS buffer solution (5 mg/mL) at various pH values. The absorbance was measured by UV–visible spectroscopy (ELISA, SpectraMaz M5, Molecular Devices, USA) at 500 nm.

2.7. Preparation of DOX-loaded polymeric micelles

Doxorubicin (DOX) was loaded into the pH-sensitive micelles using a film hydration method (Ko et al., 2007). Briefly, DOX·HCl (1 mg) and polymers (10 mg) were dissolved in 10 mL of chloroform/methanol (1:1, v/v) with 1.5 equiv. of TEA. The co-solvent was evaporated using a rotary evaporator, and the polymer/drug thin film was dispersed by adding 10 ml of distilled water (pH 7.4) under gentle shaking for 5 min. The dispersed DOX-loaded polymeric micelles were passed through syringe filters (0.45 μm, Millipore) to remove the unloaded doxorubicin. The drug loading efficiency and drug content of the DOX-loaded polymeric micelles



Scheme 1. Schematic illustration of synthesis for biotin-conjugated pH-responsive amphiphilic copolymer.

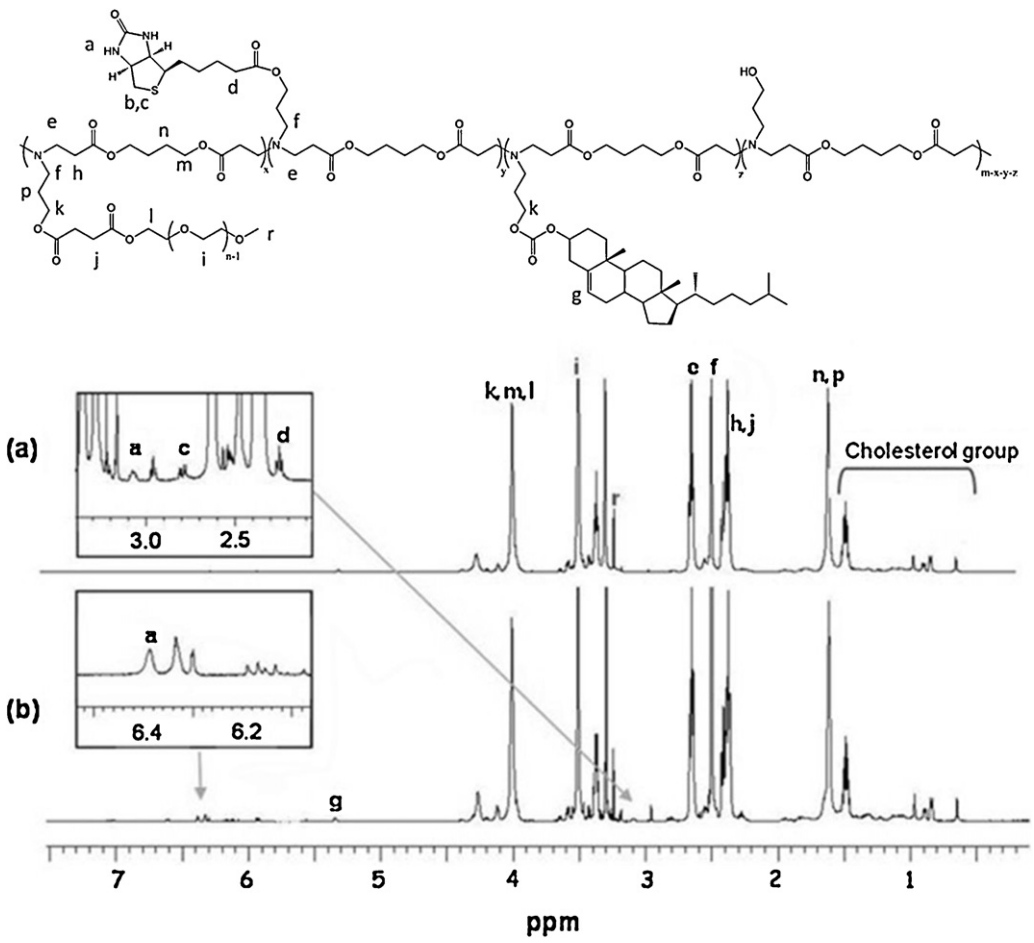


Fig. 1. ^{1}H NMR spectra of PAE-g-PEG-ch (a) and Biotin-PAE-g-PEG-ch (b).

were quantified by UV–visible spectroscopy at 479 nm. The particle sizes of the DOX-loaded and unloaded polymeric micelles were measured by dynamic light scattering.

2.8. In vitro cytotoxicity study

An adenocarcinoma breast cancer cell line of MCF-7, which was obtained from the Korea Cell Line Bank, was cultured in RPMI medium (Gibco, Grand Island, NY) containing 10% FBS (Gibco) and 1% penicillin–streptomycin (Gibco) at 37 °C in a humidified 5% CO_2–95% air atmosphere. The MCF-7 cells were seeded in a 96-well flat-bottom microplate at density of 5×10^3 cells per well and incubated for 2 days. Various concentrations of DOX-loaded polymeric micelles were prepared with a PBS buffer solution (pH 6.4 and pH 7.4). To evaluate their cell viability, each different polymer formulation and culture medium were added to the cells. After incubation for 24 h, a CCK-8 solution was added to each well and the cells were incubated again for 4 h. Finally, cell survival was assessed by CCK-8 assay.

2.9. In vitro cellular uptake

For fluorescence cellular uptake studies, MCF-7 cells (1.0×10^5 cells) in culture media were grown in a 35 mm coverglass-bottom dish. After 3 days, the medium was replaced by 1 mL of fresh medium at pH 6.4 or 7.4 and DOX-loaded polymeric micelles with PBS buffer solution (20 μg/ml DOX equiv.) were added. After 1, 5, 10 and 30 min, the culture dishes were washed 3 times with PBS (pH 7.4), fixed with 4% (wt./vol.) formaldehyde in PBS (pH 7.4) for 30 min at 4 °C, and the cellular uptake of DOX in polymeric micelles at pH 6.4 or 7.4 was visualized by confocal laser scanning microscopy (Carl Zeiss LSM510) with excitation and emission wavelength of 488 and 560–615 nm, respectively.

3. Results and discussion

3.1. Synthesis and characterization

The synthesis route of Biotin-PAE-g-PEG-ch is illustrated in Scheme 1. PAE-g-PEG was modified by cholesteryl chloroformate to make the PAE chain more hydrophobic. Cholesteryl chloroformate was coupled to the hydroxyl group of PAE-g-PEG using coupling agent. Fig. 1 shows the ^{1}H NMR spectrum of PAE-g-PEG-ch, which confirms the successful conjugation of biotin to PAE-g-PEG-ch. The substitution ratio of cholesterol was calculated from integration values of the PAE peaks at 2.65 ppm (—CH_2—, e) and the cholesterol peaks at 5.37 ppm (—CH—, g). Similarly, the substitution ratio of biotin was calculated from the integration values of the PAE peaks at 2.65 ppm (—CH_2—, e) and the biotin peaks at 6.39 ppm (—CH—, a). The substitution ratios of cholesterol and biotin were both 5 mol%. The Mn of Biotin-PAE-g-PEG-ch determined by GPC was 11,800 g/mol with a PDI of 1.60.

3.2. pH-Sensitive micelle formation

The micelle formation of Biotin-PAE-g-PEG-ch in PBS buffer solution (pH 7.4) was assessed by fluorescence technique using pyrene as the hydrophobic fluorescence probe, as described

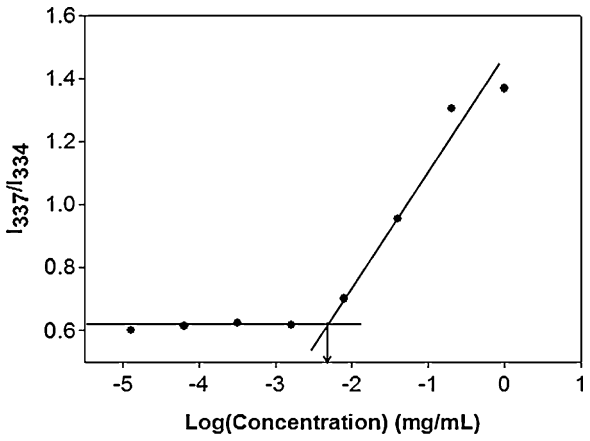


Fig. 2. The critical micelle concentration (CMC) of Biotin-PAE-g-PEG-ch polymer. Intensity ratio (I_{337}/I_{334}) from pyrene excitation spectra as a function of polymer concentration in PBS.

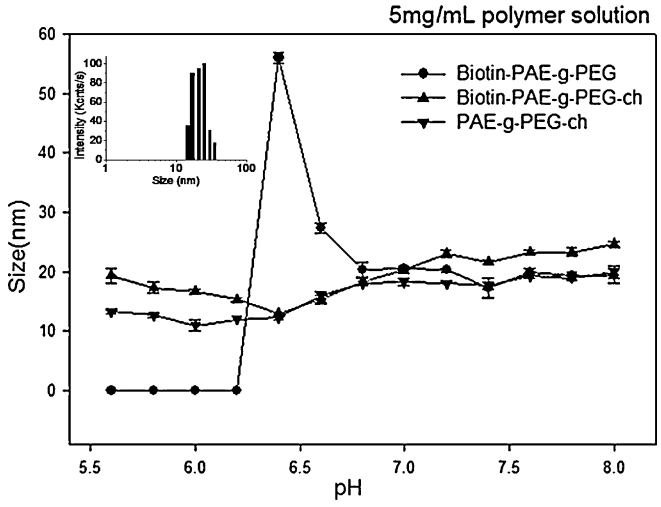


Fig. 3. Micelle size of polymeric micelles as a function of pH at 25 °C. (The inset shows the size distribution of Biotin-PAE-g-PEG-ch micelles at pH 7.4.)

previously (Nishyama et al., 2005). The change of the I_{337}/I_{334} ratio as a function of concentration indicated the formation of micelles. The CMC was determined by the interception of the extrapolated line (Fig. 2). The as-calculated CMC of Biotin-PAE-g-PEG-ch was 0.0039 mg/mL at pH 7.4.

The pH sensitivity of the polymers was examined by DLS as a function of pH. As shown in Fig. 3, Biotin-PAE-g-PEG could form micelles above pH 6.6 and the size of micelles was around 23 nm. PAE is fully ionized below pH 6.4 and the partial ionization of Biotin-PAE-g-PAE is sufficient to disrupt (the remarkable increase of size at pH 6.4 was attributed to the loose of the micelle core). Whereas, the size of cholesterol grafted PAE-g-PEG and cholesterol grafted Biotin-PAE-g-PEG ranged from 15 nm to 25 nm at different pHs. In case of the PAE-g-PEG-ch series, the hydrophobic core of the micelles was strengthened by modification with cholesterol, so that

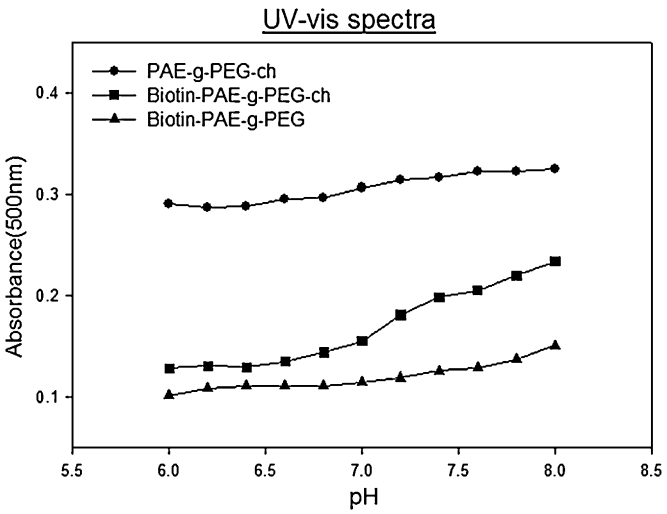


Fig. 4. UV–vis spectra of HABA/avidin complex after the addition of polymeric micelles.

a higher degree of ionization is needed to destabilize the micelle. These results confirmed that PAE-g-PEG modification with cholesterol not only increases the colloidal stability but also changes the pH-sensitive range of the micelles.

3.3. Biotin binding assay

The presence of functional biotin on the surface of polymeric micelle was confirmed by the developed biotin-affinity assay. Fig. 4 presents the UV–visible spectra of the avidin/HABA complex before and after the addition of biotin-conjugated micelles. The absorbance of the HABA/avidin complex at 500 nm decreased upon the addition of the biotin-conjugated polymeric micelles, suggesting that biotin on the micelles displaced HABA from the HABA/avidin complex as a result of its higher affinity for avidin.

In the case of biotin-conjugated PAE-g-PEG, the absorbance of polymeric micelles decreased the entire range of pH compared to PAE-g-PEG. But, the absorbance of biotin-conjugated PAE-g-PEG-ch decreased sharply from pH 7.4 to pH 6.5, which means that the hydrophobicity of the micelle core by the modification with cholesterol is strong enough to maintain the micelle particle and biotin exists on the micelle core above pH 7.4 *via* a pH-sensitive molecular chain (PAE) that is shielded by the PEG shell of the micelle. At pH < 7.4, partial ionization of PAE is sufficient for the micelles to swell and then biotin is exposed on the micelle surface. Subsequently, exposed biotin can interact with the cells, which facilitates biotin receptor-mediated endocytosis. When the pH was decreased further, most of polymeric micelles destabilize, resulting in enhanced drug release and disruption of the cell membranes.

3.4. Characterization of the DOX-loaded polymeric micelles

DOX-loaded polymeric micelles were prepared using a solvent casting method, as mentioned above. Table 1 lists the results of the drug contents, loading efficiency and sizes of the DOX-loaded

Table 1
Characterization of DOX-loaded polymeric micelles.

	Polymer/DOX weight ratio (mg/mg)	Drug content (%, w/w)	Loading efficiency (%, w/w)	Size (nm)	
				Polymer	DOX–polymer
Biotin-PAE-g-PEG-ch	100/10	5.5	61	34.7	58.0
Biotin-PAE-g-PEG	100/10	5.9	65	73.0	73.6
PAE-g-PEG-ch	100/10	4.2	47	19.5	32.4

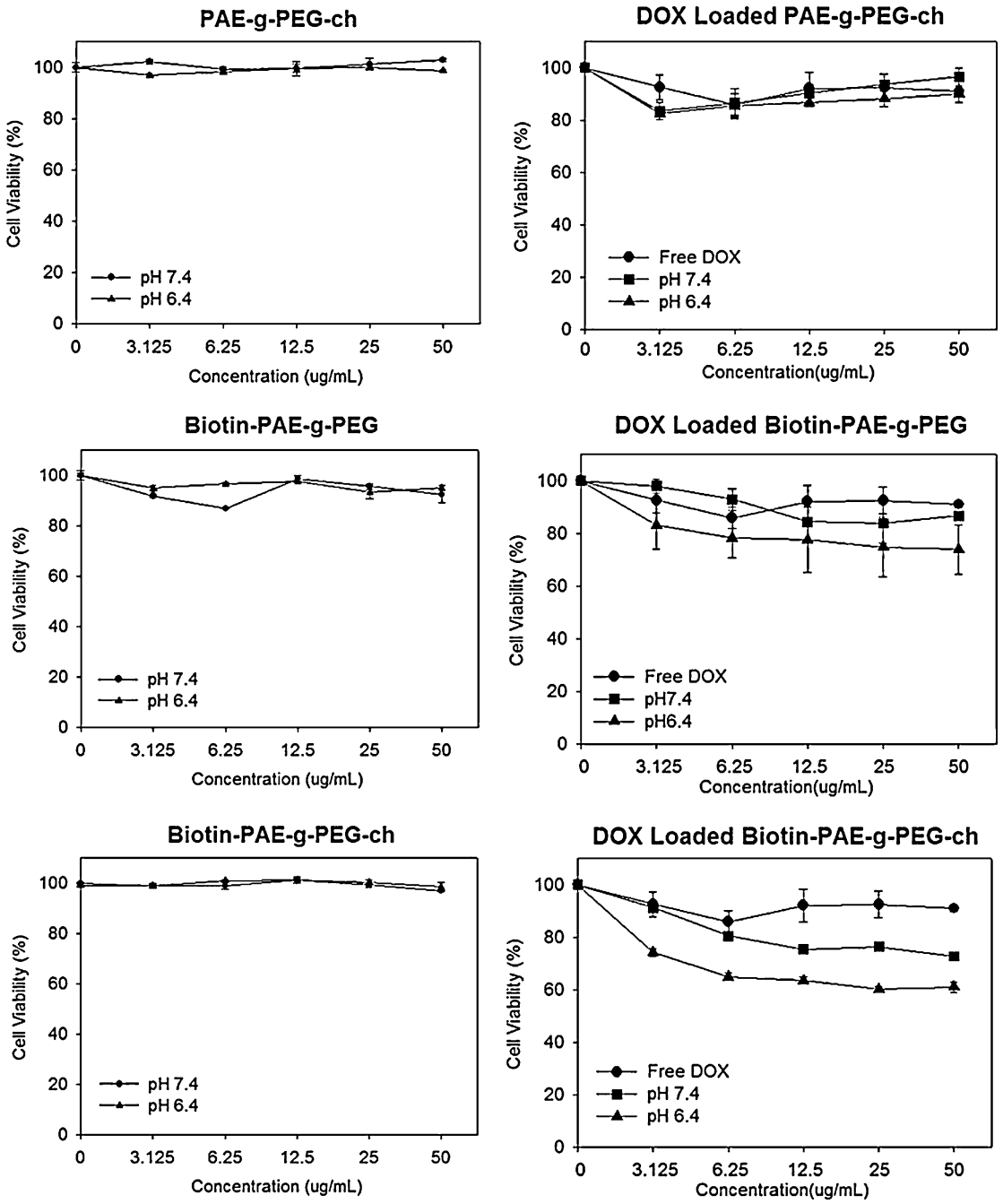


Fig. 5. Effect of concentration on *in vitro* cytotoxicity of polymeric micelles and DOX-loaded polymeric micelles at pH 6.4 and pH 7.4.

polymeric micelles. After incorporating DOX into the pH-sensitive micelles, the micelle size increased slightly, regardless of the identity of the polymer, indicating that the DOX molecules were trapped in the hydrophobic inner cores, which increased the size of the pH-sensitive micelles. The size of DOX loaded PAE-g-PEG-ch micelles is 32.4 nm which is the smallest compared to other drug loaded micelles. After the conjugation of biotin, the size of drug loaded Biotin-PAE-g-PEG-ch increased to 58.0 nm. This can be explained by the conjugation of a hydrophilic moiety, which make the micelle core less condensed. Similarly, DOX loaded Biotin-PAE-g-PEG has the biggest particle size due to the absence of the hydrophobic cholesteryl group. The drug content and loading efficiency were calculated using the following equations:

$$\text{Drug contents} = \frac{\text{Amount of DOX in polymeric micelles}}{\text{Weight of polymeric micelles}} \times 100\%$$

$$\text{Loading efficiency} = \frac{\text{Residual amount of DOX in polymeric micelles}}{\text{Feeding amount of DOX}} \times 100\%$$

3.5. *In vitro cell cytotoxicity*

The cell viability of DOX-loaded polymeric micelles was evaluated *in vitro* by cytotoxicity tests using CCK-8 assay with MCF-7 cell line at various polymer concentrations. For comparison, the cytotoxicity of the empty micelles was also evaluated. As shown in

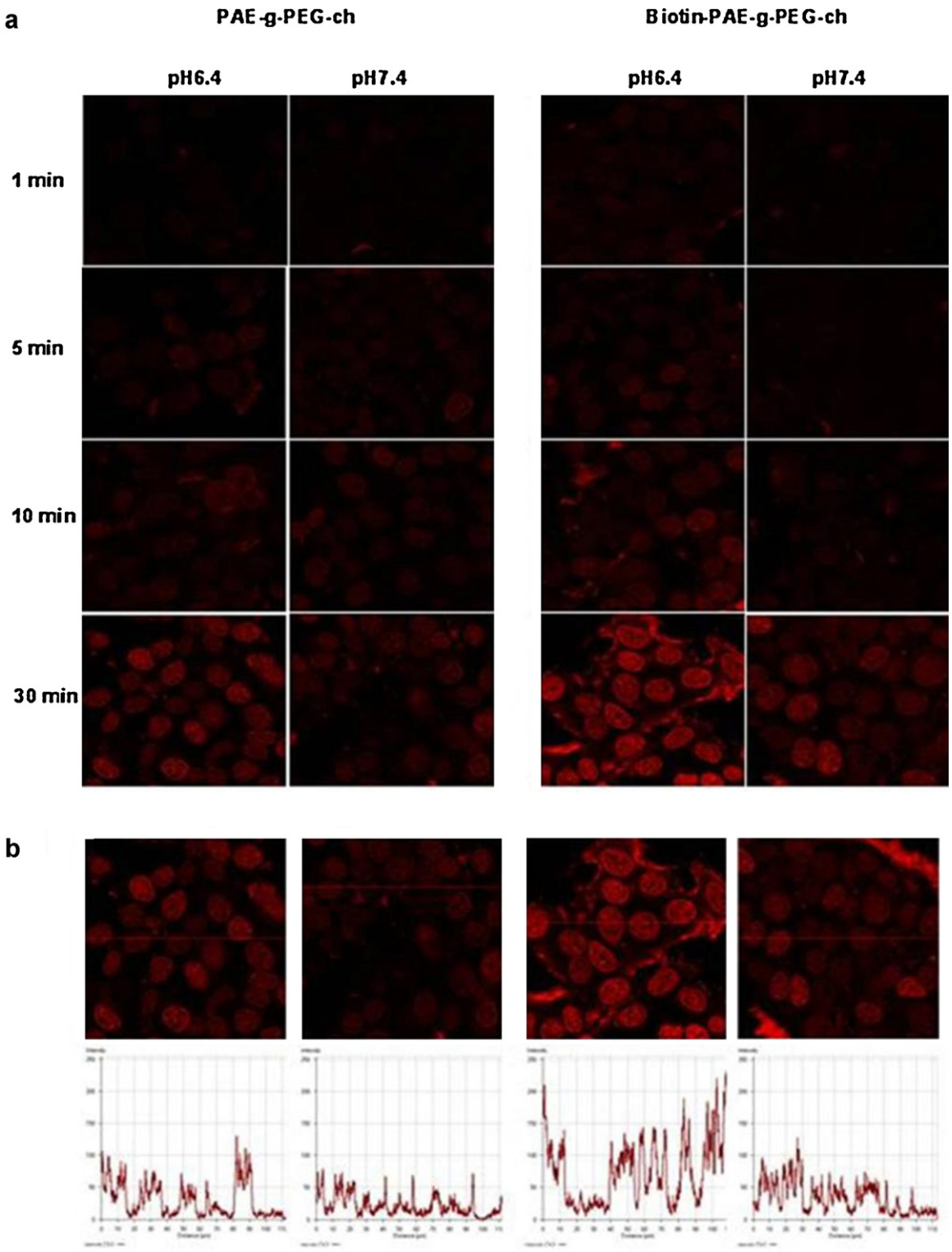


Fig. 6. (a) Confocal laser scanning microscopy of PEA-g-PEG-ch and Biotin-PEA-g-PEG-ch after incubation for 1 min, 5 min, 10 min and 30 min and (b) fluorescence intensity trace of the whole scale at different pH after 30 min.

Fig. 5, empty micelles did not exhibit toxicity, even at high concentration. At all concentrations, the cell viability of the MCP-7 cell was >85%. On the other hand, DOX-loaded biotin-conjugated polymeric micelles showed low viability at high concentrations at pH 6.4. In addition, the uptake of DOX was enhanced for DOX loaded Biotin-PAE-g-PEG-ch micelles compared to DOX loaded Biotin-PAE-g-PEG micelles, which means biotin has the ability to adhere to the cells and increases the uptake of the micelles.

3.6. *In vitro* cellular uptake

The *in vitro* cellular uptake of DOX-loaded polymeric micelle was evaluated by confocal laser scanning microscopy, which emits red fluorescence. From Fig. 6, the enhancement of cellular uptake with Biotin-PAE-g-PEG-ch over PAE-g-PEG-ch after treatment for 1, 5, 10 and 30 min at different pH conditions, such as pH 6.4 and pH 7.4, showed more micelles had been taken into the MCF-7 cells.

In case of PAE-g-PEG-ch, DOX was released slowly by the tertiary amine ionization of PAE at pH 6.4 and pH 7.4. The highest intensity occurred for biotin-conjugated PAE-g-PEG-ch at pH 6.4 after 30 min. In addition the fluorescence intensity trace of the entire scale of cells was also highest under such conditions. This makes it possible for the biotin-conjugated PAE-g-PEG-ch to penetrate into the tumors due to the high binding affinity between biotin and the specific receptor on the surface of tumors. These findings suggest that biotin-conjugated polymeric micelles might be suitable for active targeting to the acidic extracellular environments of the tumors.

4. Conclusion

From these *in vitro* studies, our pH-sensitive anticancer drug carrier system made of biotin conjugated PAE-g-PEG-ch can effectively release doxorubicin under acidic conditions. The size and presence of biotin on the surface are both pH-sensitive. Therefore, biotin conjugated polymeric micelles might increase the target drug accumulation at the tumor site or in tumor cells through ligand receptor mediated endocytosis with less drug distribution to the normal tissues. Overall, these Biotin-PAE-g-PEG pH-sensitive polymeric micelles have potential in clinical treatments.

Acknowledgments

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