# *In Vitro* Degradability and Stability of Hydrophobically Modified pH-Sensitive Micelles Using MPEG-Grafted Poly(β-amino ester) for Efficient Encapsulation of Paclitaxel

**Min Sang Kim, Doo Sung Lee**

*Department of Polymer Science and Engineering, Sungkyunkwan University, Suwon, Gyeonggi 440-746, Korea*

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**ABSTRACT:** Methoxypoly(ethylene glycol)-grafted poly(β-amino ester) was synthesized for the fabrication of pH-sensitive micelles, and these micelles were modified with deoxycholic acid to facilitate the hydrophobic interaction between the micellar core and paclitaxel. The micelle properties were studied by dynamic light scattering and fluorescence spectrometry. An *in vitro* degradation study showed that the synthesized polymers degraded hydrolytically within 24 h under physiological conditions. The stability of paclitaxel-loaded pH-sensitive micelles was evaluated *in vitro*. The introduced deoxycholic acid more stabilized the micelles at pH 7.4 compared to the micelles without modification. But the pH-sensitive region of the micelles was lowered from pH 6.8 to pH 5.8. These results indicate that pH-sensitive micelles with improved stability have great potential as hydrophobic drug carriers for tumor targeting. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 3431–3438, 2010

**Key words:** micelles; degradation; graft copolymers; self-assembly

## INTRODUCTION

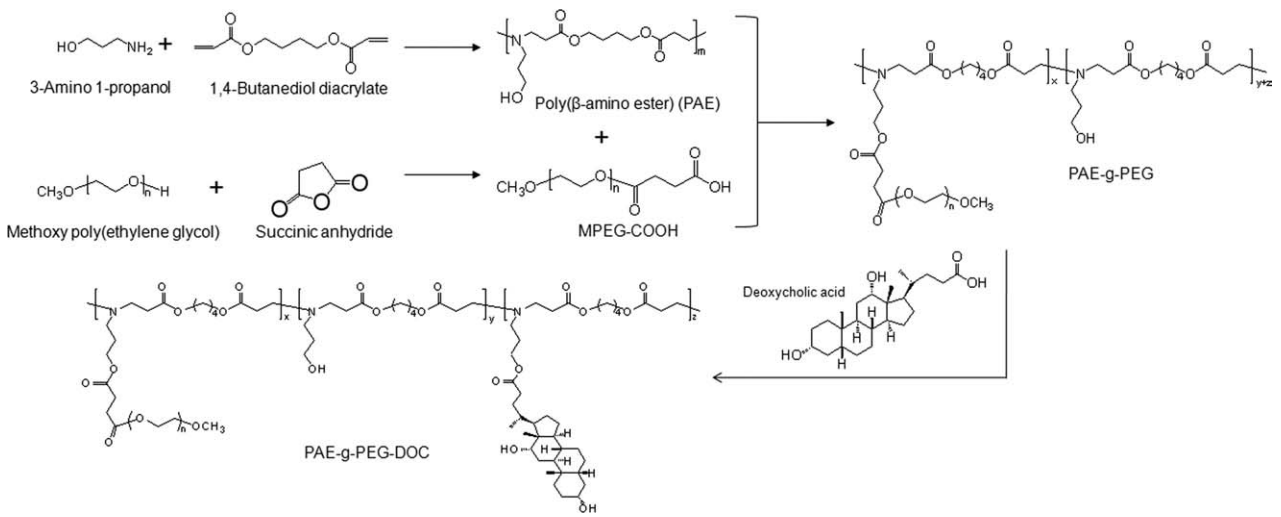
Stimulus-sensitive polymeric micelles have attracted a great deal of attention as drug delivery systems for cancer therapy because they can deliver therapeutic drugs to cancer tissues following micelle degradation triggered by a specific stimulus, such as temperature, pH, or enzymes.[1–5] pH-sensitive polymeric micelles are particularly useful systems for cancer therapy,[6–12] because pH changes occur naturally within cancer tissue (pH 7.2–6.0) due to the high glycolysis rate,[13–15] or endosome(<pH 6.0) which is acidified by proton-translocating ATPases[8,9] without the need for artificial alterations to the environmental conditions. If a polymeric micelle could release drug only in acidic tumor tissues or endosome, the therapeutic efficacy of the delivered drug would be enhanced both by increasing the amount of drug accumulated at the tumor site and by decreasing the side effects caused by delivering the drug to unwanted sites.

Many kinds of degradable polymers, by hydrolysis or enzymatic decomposition, have been applied to drug delivery systems.[16,17] These degradable properties prevent the accumulation of the polymer within the body system after the completion of the delivery mission. However, destabilization of the micelle structure during polymer degradation is inevitable. To deliver a drug to the target site, such as tumor tissue, after long circulation time and enhanced permeation and retention (EPR) effect in the body, the micelle must maintain its stability before reaching the tumor tissue or escaping from the body by renal filtration.

pH-sensitive micelles composed of degradable polymers, which could be used for selective drug release in acidic tumor tissues or endosome, have similar limitations. When applied to cancer therapy, pH-sensitive micelles should release drug only in target site.[6–12] However, slow release of the drug or instability of the micelle inevitably occurs under normal blood stream or tissue conditions (pH 7.4) due to the degradation of the polymer. Therefore, the drug can accumulate in normal tissue or escape from the body, which causes side effects or reduces the therapeutic efficacy, respectively. To overcome this problem, pH-sensitive micelles are required to maintain stability at pH 7.4 and decompose at pH 6.5, thereby releasing the drug.

In this study, graft copolymer composed of biocompatible methoxypoly(ethylene glycol) and pH-sensitive poly(β-amino ester) (PAE-*g*-PEG), which forms micelles at pH values above 7.4 but is destabilized at pH values below 7.0, was designed and characterized. In addition, PAE-*g*-PEG was modified

*Correspondence to:* D. S. Lee (dslee@skku.edu).




**Scheme 1** Schematic illustration of synthesis for pH-sensitive amphiphilic copolymer.

with deoxycholic acid (PAE-*g*-PEG-DOC), frequently used hydrophobic moiety to modify the hydrophilic polymer,[18,19] to induce a hydrophobic interaction with paclitaxel, a representative chemotherapeutic drug. We hypothesized that a hydrophobic interaction between the micellar core and hydrophobic drug increases the stability of the micelles, regardless of the degradation of the polymer. After confirming a correlation between introduced hydrophobic moiety and pH sensitivity of micelles, the *in vitro* degradation of the copolymers and the stability of the paclitaxel-loaded micelles, as a function of time and pH, was evaluated.

## EXPERIMENTAL

### Materials

Methoxypoly(ethylene glycol) (MPEG, $M_n$ provided by manufacturer was 2,000), succinic anhydride (purity = 99 %), *N,N′*-dicyclohexyl carbodiimide (DCC, purity = 99%), 4-(dimethyl amino)pyridine (DMAP, purity = 99%), anhydrous dioxane, anhydrous dichloromethane (DCM, purity = 99.9%), anhydrous tetrahydrofuran (THF, purity = 99.9%), 3-amino 1-propanol (AP, purity = 99%), 1,4-butanediol diacrylate (BD, purity = 90%), and deoxycholic acid (purity = 99%) were used as received (from Aldrich). Carboxylic acid-modified MPEG (MPEG—COOH, $M_n$, calculated by the $^1$H NMR result was 2100) was prepared according to the procedure reported elsewhere.[20] Diethyl ether was supplied by Samchun chemicals (Korea, purity = 90%). Paclitaxel (Genexol®; Samyang Genex) was used as received and its purity was > 99%.

### Synthesis of pH-sensitive polymers

PAE was synthesized by Michael addition polymerization,[10] and MPEG was grafted to PAE (PAE-*g*-PEG). Briefly, a mixture of BD (18 g, 90 mmol) and AP (6.9 g, 90 mmol) was reacted at 100°C for 5 h. After dissolving the product (PAE) in DCM, a viscous liquid was precipitated in diethyl ether. The product was dried at 30°C for 2 days. MPEG—COOH (7.7 g, 3.7 mmol) and PAE (10 g, 36.6 mmol) were coupled using DCC (1.5 g, 7.4 mmol) and DMAP (0.9 g, 7.4 mmol) at room temperature for 24 h. To determine the added amount of PAE, the molecular weight of repeat unit in PAE was used for the calculation. After filtering to remove dicyclohexyl urea (DCU), PAE-*g*-PEG was obtained by precipitation in diethyl ether and subsequent drying. The yield was 82%.

To improve hydrophobicity, deoxycholic acid was conjugated to PAE-*g*-PEG using DCC and DMAP. PAE-*g*-PEG (2 g, 4.1 mmol) and a predetermined amount of deoxycholic acid were placed in a two-neck round-bottom flask and dissolved in THF (10 mL). DCC and DMAP were added to the solution and reacted at room temperature for 24 h. After removing the DCU by filtering, the reactant solution was precipitated in diethyl ether. The resulting deoxycholic acid-conjugated PAE-*g*-PEG (PAE-*g*-PEG-DOC) was dried under vacuum at 30°C for 2 days (Scheme 1). The yield of polymers was more than 70%.

### Characterization

The molecular structure and composition of the copolymer were determined from the $^1$H NMR spectra recorded on a Varian-Unity Inova 500NB spectrometer operated at 500 MHz. Chloroform ($CDCl_3$) was used as the solvent with 0.03 v/v% tetramethylsilane (TMS) as a reference. The substitution ratio of MPEG and deoxycholic acid was calculated by comparing the proton-peak integrations from the $^1$H NMR

**TABLE I**
**Synthesized Polymers**

| Sample | $M_n$[a] (g/mol) | PDI[a] | Average value (feed) | | | Average value (calculated)[b] | | |
|---|---|---|---|---|---|---|---|---|
| | | | x | y | z | x | y | z |
| PAE | $7.6 \times 10^3$ | 2.5 | – | | – | – | – | – |
| PAE-*g*-PEG | $1.3 \times 10^4$ | 2.1 | 1.4 | – | – | 0.56 | – | – |
| PAE-*g*-PEG-DOC3 | $1.7 \times 10^4$ | 1.6 | 1.4 | 11.2 | 1.4 | 0.56 | 12.92 | 0.52 |
| PAE-*g*-PEG-DOC9 | $1.7 \times 10^4$ | 1.6 | 1.4 | 7.4 | 5.2 | 0.56 | 12.18 | 1.26 |

[a] Obtained from GPC measurements.
[b] Calculated by integration of $^1$H NMR peaks.

spectra. Gel permeation chromatography (GPC) experiments, with a series of styragel columns (Shodex-KF 801, 802.5, and KF 804L) and a refractive-index detector (Shodex, RI-101) were performed to measure the molecular weight and molecular weight distribution of copolymers. THF was used as the solvent at a flow rate of 1 mL/min. Calibration was carried out using poly(methyl methacrylate) standards (Shodex) over the molecular weight range of 2000–49,600.

### Fluorescence spectrometry

The critical micelle concentration (CMC) of the PAE-*g*-PEG and PAE-*g*-PEG-DOC series was determined using a fluorescence probe technique with pyrene.[21] A stock solution of pyrene in THF (1 mL) was added to a phosphate buffered saline (PBS) solution (1 L), and the THF was eliminated using a rotary evaporator at 60°C for 1 h. The final concentration of pyrene was $1 \times 10^{-6}$ M. Polymer solutions at various concentrations were prepared by dissolution and dilution in the pyrene-solubilized PBS solution (pH 7.4). The excitation spectra of pyrene were measured using a fluorescence spectrometer (AMINCO• BOWMAN® Series2) at a fixed emission wavelength of 392 nm.

### Dynamic light scattering

The dynamic light scattering (DLS) data were collected on a Malvern PCS100 spectrogoniometer and Brookhaven BI-9000AT digital autocorrelator equipped with a He–Ne laser (633 nm) (light collected at 90°). The CONTIN algorithms were used to calculate the Laplace inversion of the autocorrelation function to obtain the size distribution of the micelles. The mean hydrodynamic diameter was evaluated using the Stokes–Einstein equation. The concentration of the sample solution was kept at 5 mg/mL, and the micelle size was measured three times at several pH values. Polymer degradation possibly occurs during experimental time scale. So as to exclude degradation effect, all samples were measured within 15 min after preparation.

### Degradation study

The hydrolytic degradation of the PAE-*g*-PEG and PAE-*g*-PEG-DOC series was conducted in PBS solution at pH 7.4 and 6.5. After dissolving all polymers at a concentration of 5 mg/mL, the solution pH was adjusted to each pH. Each sample was incubated at 37°C and removed from the incubator at predetermined intervals. After freeze drying, the samples were analyzed by GPC.

### Drug loading method

Paclitaxel-loaded pH-sensitive micelles were prepared by the film hydration method.[11] Briefly, Paclitaxel (1 mg) and the polymers (10 mg) were dissolved in 2 mL of DCM. After evaporating the organic solvent using a rotary evaporator, the polymer/paclitaxel thin film was dispersed by adding 5 mL of pH 7.4 PBS with gentle shaking for 5 min. The dispersed paclitaxel-loaded pH-sensitive micelles were passed through 0.2 μm syringe filters to remove the unloaded paclitaxel. The drug loading efficiency was analyzed by HPLC.

### Micelle stability

The dependence of the stability of the paclitaxel-loaded pH-sensitive micelles on pH was evaluated at 37°C. After incubation of the paclitaxel-loaded micelle solution for predetermined time periods, the solution was filtered through a 0.2 μm syringe filter to remove the paclitaxel that had aggregated due to its low solubility in water. The filtered micelle solution was analyzed by HPLC to determine the paclitaxel concentration.

### HPLC analysis

Reverse-phase HPLC was performed on an NS-2004 series HPLC composed of a quaternary gradient pump, variable programmable UV/Vis detector, and data management software (Multichro 2000). UV/Vis detection at 204 nm, using a C-18 column (Zorbax C18, Agilent) was used for the analysis of paclitaxel.

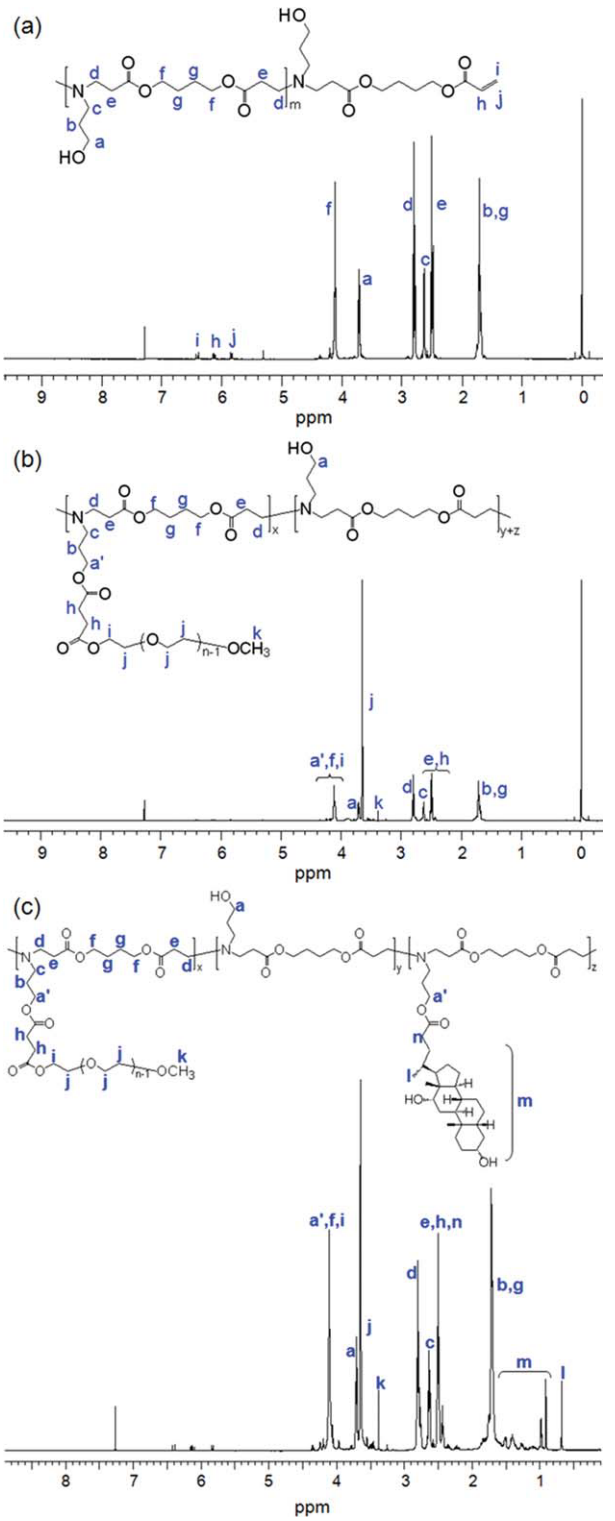



**Figure 1** 1H NMR spectra of PAE (a), PAE-*g*-PEG (b), and PAE-*g*-PEG-DOC3 (c). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

A mixture of acetonitrile and water (50 : 50 v/v) was used as the mobile phase at a flow rate of 0.5 mL/min. The paclitaxel concentrations were obtained using a calibration curve prepared from paclitaxel dissolved in acetonitrile–water (50 : 50 v/v).

## RESULTS AND DISCUSSION

### Synthesis and characterization

PAE-*g*-PEG was synthesized from MPEG grafting to hydroxyl group containing PAE and PAE-*g*-PEG-DOC series were obtained by PAE-*g*-PEG modification with deoxycholic acid. The $M_n$ of PAE, which was prepared by means of Michael addition polymerization, was $7.6 \times 10^3$ g/mol, and that of PAE-*g*-PEG was $1.3 \times 10^4$ g/mol (determined from the GPC analysis, Table I). For the coupling between MPEG and PAE, an MPEG—COOH solution (10 mol % of hydroxyl groups on PAE) was reacted with PAE. Figure 1(b) shows the $^1$H NMR spectrum of PAE-*g*-PEG. Among the proton peaks, the $CH_3$-proton signal of MPEG (3H, k) and the —$CH_2$— proton signal of PAE (6H, b+g) were used to calculate the degree of substitution. Calculated integration ratio indicated that 4 mol % MPEG, indicating the average value for $x$ is 0.56, was substituted to PAE.

The substitution ratio of deoxycholic acid was calculated from the $^1$H NMR results. As shown in Figure 1(c), the substitution ratio of deoxycholic acid was calculated from the integration values of the PAE peaks at 2.80 ppm (—$CH_2$—, d) and the ($CH_3$—, l) peaks at 0.68 ppm, which corresponds to the methyl proton of deoxycholic acid. The increase in molecular weight was confirmed by GPC (Table I), indicating the successful conjugation of deoxycholic acid. When a 10 mol % deoxycholic acid, indicating 1.4 units of hydroxyl groups on PAE, was reacted to PAE-*g*-PEG, the resultant substitution ratio was 3 mol %, which means the substitution of 0.52 units. Resulting polymer denoted as PAE-*g*-PEG-DOC3. When the amount of deoxycholic acid was increased to 30 mol % (5.2 units of hydroxyl groups on PAE), a substitution ratio of 9 mol % (1.26 units) was obtained (PAE-*g*-PEG-DOC9).

### Physical properties of pH-sensitive micelles

The micelle formation of the polymers at pH 7.4 PBS was monitored by fluorescence spectrometry in the presence of pyrene. The change of the $I_{337}/I_{334}$ ratio as a function of concentration indicated the formation of the micelles. Figure 2(a) shows that the maximum excitation wavelength shifts to higher wavelength with increasing polymer concentration and Figure 2(b) indicates the change in the intensity ratio of the peaks at 337 and 334 nm in the excitation spectra plotted against the polymer concentration. The CMC of PAE-*g*-PEG was 0.061 mg/mL, while that of the PAE-*g*-PEG-DOC3 decreased to

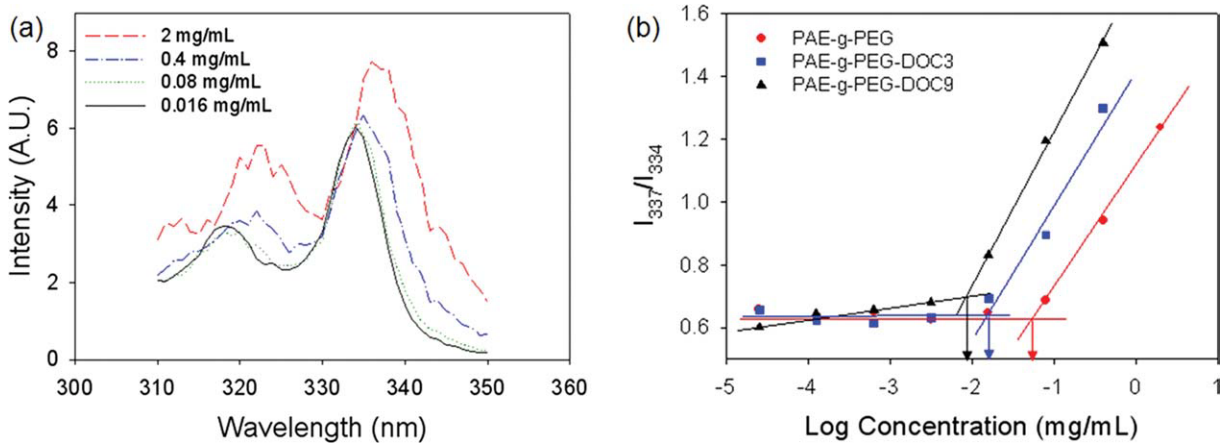



**Figure 2** Representative fluorescence excitation spectra as a function of concentration (PAE-*g*-PEG solution) taken at a fixed emission wavelength of 392 nm (a), and determination of the CMC in pH 7.4 PBS from the fluorescence intensity ratio (I337/I334) (b). The concentration of pyrene was $1 \times 10^{-6}$ M. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

0.013 mg/mL and that of PAE-*g*-PEG-DOC9 was further decreased to 0.008 mg/mL. The introduction of deoxycholic acid appeared to stabilize the micellar core due to the increased hydrophobic interaction.

The pH sensitivity of the polymers was examined by DLS measurements as a function of the pH. As shown in Figure 3(a), no signal was detected by DLS for the PAE-*g*-PEG copolymer solution at pH values below 6.8, whereas the average diameter of micelles was $\sim$ 28 nm at pH values above 6.8. This observation indicated that the protonation of PAE (protonated at pH values below 6.8 but deprotonated at pH values above 6.8) affected micelle behavior. In contrast, the scattering intensity, indicating the relative micelle concentration [Fig. 3(b)], confirmed that the disruption of micelles began when the pH dropped below 7.2, due to the protonation of the PAE component. Therefore, at pH values below 7.2, PAE was protonated while the micelles formed. The hydrophobic interaction of the PAE chains in PAE-*g*-PEG is weak; thus, the micelle can easily dissociate under partial protonation of PAE.

When the PAE-*g*-PEG contains deoxycholic acid, destabilization of pH-sensitive micelles occurs at lower pH. PAE-*g*-PEG-DOC3 and PAE-*g*-PEG-DOC9 are fully destabilized at pH 6.2 and pH 5.8, respectively. Different with PAE-*g*-PEG, it seems that hydrophobic interaction of deoxycholic acid dominates the micelle formation of PAE-*g*-PEG-DOC series at below pH 6.6 in spite of PAE protonation. The slight increase in the micelle size was observed at pH 6.4 and pH 6.0 in case of PAE-*g*-PEG-DOC3 and PAE-*g*-PEG-DOC9, respectively. It seems that the ionization of PAE reduces the hydrophobic interaction in micellar core and expands the micelle size prior to full disruption of micelles. As the increase of the substitution ratio of deoxycholic acid, the higher degree of protonation of PAE is needed to fully destabilize the pH-sensitive micelles. Scheme 2

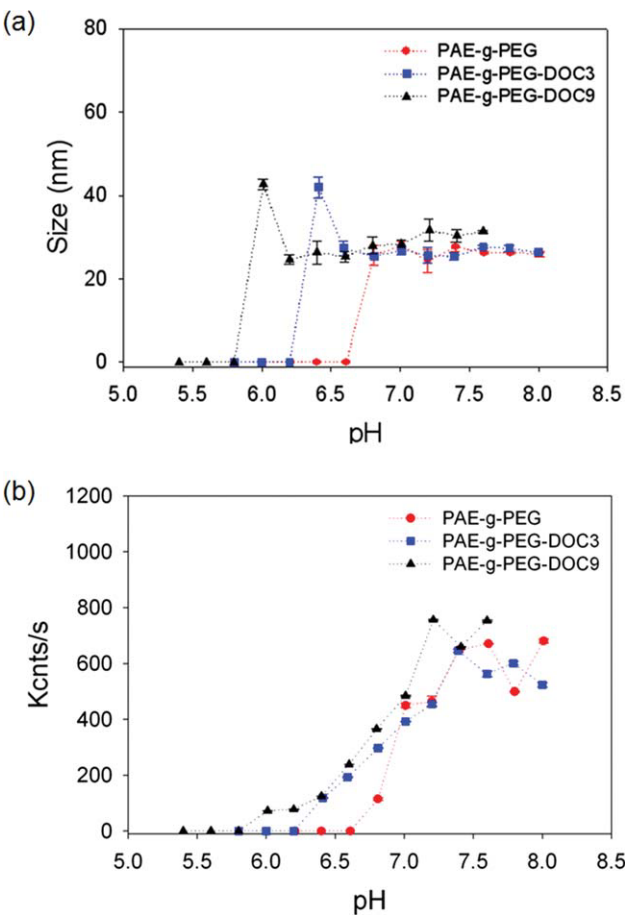
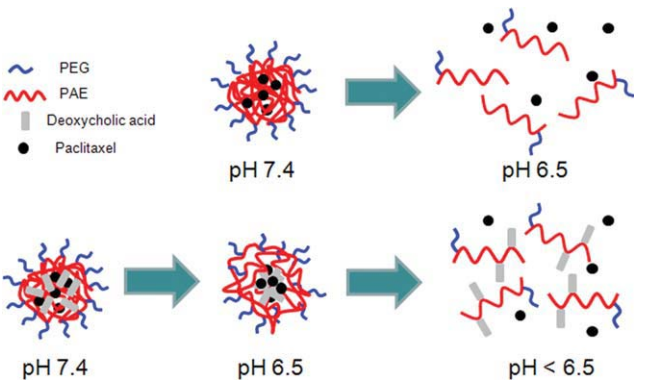



**Figure 3** Micelle size (a), and scattering intensity (b) as a function of the pH for the 5 mg/mL solution. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]




**Scheme 2** Schematic illustration of pH-sensitive micellar behavior of PAE-*g*-PEG and PAE-*g*-PEG-DOC series. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

summarizes the pH-sensitive properties of PAE-*g*-PEG with or without deoxycholic acid.

### Degradation study

Degradability of PAE by hydrolysis has been previously reported.[22] The hydrolytic degradation of micelles in PBS at 37°C showed that the molecular weights of the polymers decreased to one fifth of their initial values. Degradation occurred via the hydrolysis of the amino ester bond. As shown in Figure 4(a), MPEG remained after degradation. All polymers showed similar degradation patterns and degraded faster at pH 6.5 than at pH 7.4 [Fig. 4(b)] due to the incomplete solubility of PAE, possibly caused by micelle formation, inhibited access of the water molecules at pH 7.4. These results indicated that the hydrophobicity of the polymers did not affect their degradation.

### Paclitaxel loading and *in vitro* stability

Paclitaxel was loaded into the pH-sensitive micelles by the film hydration method, and the loading efficiency was evaluated by HPLC analysis. After incorporating paclitaxel into the pH-sensitive micelles, the average micelle diameter slightly increased, regardless of the identity of the polymer, indicating that the paclitaxel molecules were trapped in the hydrophobic inner cores and increased the size of the pH-sensitive micelles. Figure 5 shows narrow distribution of micelles. As shown in Table II, the loading efficiency was higher than 90%, implying that almost all paclitaxel molecules were solubilized by the pH-sensitive micelles.

The physical stability of the paclitaxel-loaded micelles was studied at 37°C by observing the residual amount of solubilized paclitaxel after removing the precipitated paclitaxel (Fig. 6). If removed from the micellar core, paclitaxel precipitated due to its low solubility in water[23] thus precipitates were observed during incubation at 37°C. Paclitaxel was assumed to be released from the micelle due to the degradation of the polymer or pH-sensitive micelle stability. In the case of PAE-*g*-PEG without deoxycholic acid, a different precipitation time was observed depending on pH even though initial precipitation was observed at pH 7.4. At pH 7.4, paclitaxel precipitated within 20 h due to the degradation of the polymer and insufficient hydrophobic interaction. After adjusting the solution pH to 6.5, most paclitaxel precipitated instantaneously and could be removed by filtration within 15 min. This indicated that the stability of the micelles was dependent on the degradation of the polymer and pH.

When deoxycholic acid was conjugated to PAE-*g*-PEG, the paclitaxel-loaded micelle became more stable. For the PAE-*g*-PEG-DOC3 micelle, the

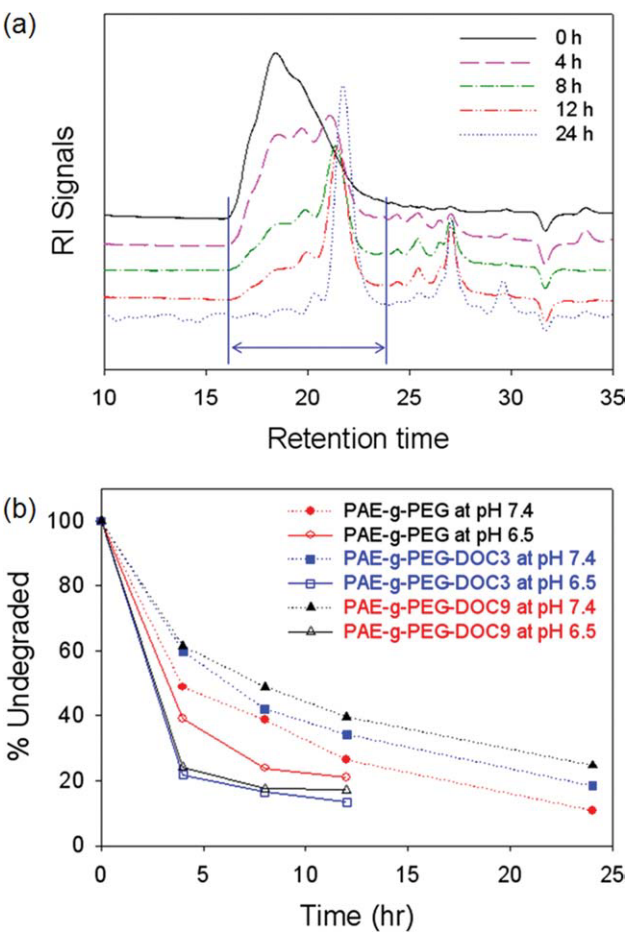



**Figure 4** Representative GPC traces of PAE-*g*-PEG hydrolyzed in pH 7.4 PBS (arrow indicates residual polymer) (a), and hydrolytic degradation profiles as a fuction of time (b). The % undegraded was calculated from the ratio of the $M_n$ value of the degraded polymer to that of the initial polymer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

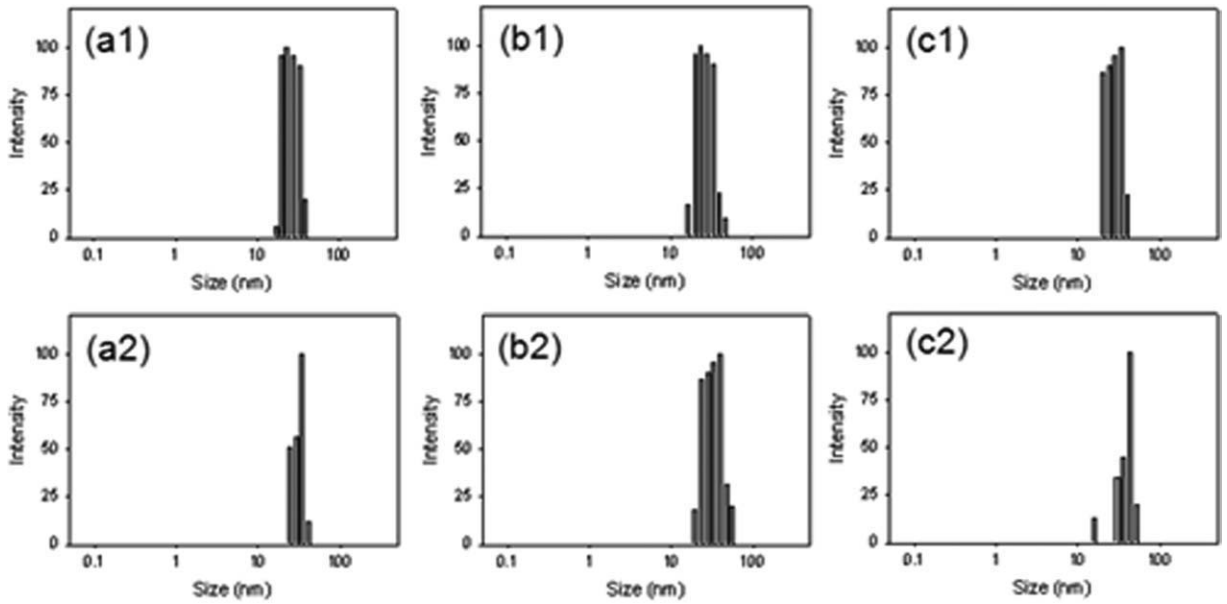



**Figure 5** Size distribution of micelles; PAE-*g*-PEG (a1), PAE-*g*-PEG-DOC3 (b1), and PAE-*g*-PEG-DOC9 (c1) and paclitaxel-loaded micelles; PAE-*g*-PEG (a2), PAE-*g*-PEG-DOC3 (b2), and PAE-*g*-PEG-DOC9 (c2) at pH 7.4 PBS.

precipitation time of paclitaxel was 40 h at pH 7.4, 10 h at pH 6.5, and 15 min at pH 6.0 as shown in Figure 6. For the PAE-*g*-PEG-DOC9 micelle, the precipitation time of paclitaxel was 80 h at pH 7.4, 50 h at pH 6.5, and 8 h at pH 5.5. These results showed that the introduction of deoxycholic acid stabilized the micelle, due to the hydrophobic interaction between deoxycholic acid and paclitaxel, in spite of the instability caused by the degradation of polymers and the disruption of micelles as the pH was lowered. All polymers degraded within 24 hrs, but the micelles showed different levels of stability, which corresponded to the hydrophobic interaction with paclitaxel. From these results, it can be concluded that degradation of the polymer destabilized the micelles, but that this instability could be overcome through hydrophobic modification.

In our previous articles,[10–12] we have prepared PEG-*b*-PAE copolymers composed of 4,4′-trimethylene dipiperidine and diacrylate monomers, and the feasibility for drug delivery carriers has been evaluated. When we intend to give other functionality, such as hydrophobicity control and targeting ability, PEG-*b*-PAE has limitation on modification because it does not have reaction site. On the other hand, hydroxyl group of the developed PAE may provide the reaction site for modification. Indeed, we are preparing target molecule conjugated PAE-*g*-PEG.

## CONCLUSIONS

In summary, the novel pH-sensitive copolymer, forming micelles, was synthesized via coupling reaction between MPEG—COOH and hydroxyl group containing PAE, thus, this copolymer was possible to be modified with deoxycholic acid to improve the micellar stability. Deoxycholic acid conjugation significantly improved the stability of paclitaxel-loaded micelle due to the increased hydrophobic interaction. Incorporating a deoxycholic acid shifted the transition of micelle to low pH. They exhibited pH-sensitive properties that can release the drug at cancer tissue (<pH 6.5) or endosome (<pH 6.0). Prior to an *in vivo* study, the results concerning the

**TABLE II**
**Micelle Size and Loading Efficiency**

| Sample | Empty micelle size[a] (nm) | PTX loaded micelle size[a] (nm) | Loading efficiency[b] (%) |
|---|---|---|---|
| PAE-*g*-PEG | 27.7 ± 1.2 | 29.0 ± 1.2 | 93.7 ± 3.2 |
| PAE-*g*-PEG-DOC3 | 25.3 ± 0.9 | 33.7 ± 1.8 | 92.0 ± 12.1 |
| PAE-*g*-PEG-DOC9 | 30.3 ± 1.5 | 37.3 ± 3.9 | 97.3 ± 1.8 |

[a] Obtained from DLS measurements (at pH 7.4, $n = 3$).
[b] Analyzed by HPLC ($n = 3$).

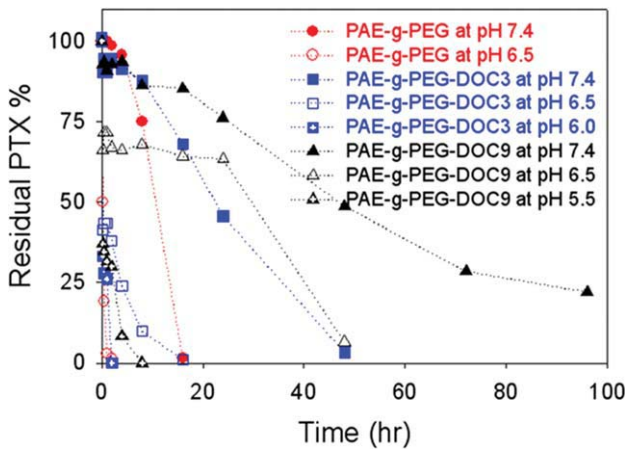



**Figure 6** Residual paclitaxel percentage inside the micelle. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degradability and pH-sensitive stability may provide useful information.

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