

Synthesis of New pH-Sensitive Amphiphilic Block Copolymers and Study for the Micellization Using a Fluorescence Probe

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Abstract: This paper reports a facile synthesis of new water-soluble poly(ethylene oxide) (PEO)-based amphiphilic block copolymers showing pH sensitive phase transition behaviors. The copolymers were prepared by atom transfer radical polymerization (ATRP) of methacrylamide type of monomers carrying a sulfonamide group using a PEO-based macroinitiator and a Cu(I)Br/Me₆TREN catalytic system in aqueous media. The resulting polymers were characterized by a combination of ¹H-NMR, size exclusion chromatography, and UV/Visible spectrophotometric analysis. The micellization of the block copolymers as a drug-loading mechanism in aqueous media using fluorescein salt was examined as a function of pH. The stable micelle formation and its loading efficacy suggest that the block copolymers can be used as precursors for drug-nanocontainers.

Keywords: pH-sensitive block copolymer, macroinitiator, atom transfer radical polymerization, sulfonamide groups, micellization, fluorescence probe.

Introduction

Stimuli-responsive 'intelligent' materials have received a great attention in the medicinal, biotechnological, or display application fields.¹⁻⁴ Specifically, poly(*N*-isopropylacrylamide-*co*-acrylic acid)s and poly(acrylic acid-*g*-*N*-isopropylacrylamide)s have been found to exhibit double stimuli-responsive phase transitions.^{5,6}

Sulfonamides have been also well-known as an important class of drugs not only retaining a pharmacological efficacy such as antibacterial and/or anti-cancer effect but also exhibiting the different pH values in relation with the *pK_a* values depending on the structures.⁷⁻¹¹ In addition, hydrogel obtained from copolymerization of *N*-isopropylacrylamide (NiPAM) and *N*-[4-((2,6-dimethyl-4-pyrimidinyl)sulfamoyl)phenyl] methacrylamide carrying sulfamethazine group (*pK_a* = 7.4) with *N,N'*-methylenebis(acrylamide) has been found to exhibit the pH-sensitive phase transition behavior.¹²

Many amphiphilic polymeric micelles as colloidal nanoparticles have been used as the nanocontainers carrying drugs for antimicrobial chemotherapy and diagnosis.¹³⁻¹⁹ With this regard, stimuli-responsive water-soluble amphiphilic block copolymers may be used as very useful and bio-related materi-

als to generate drug-loaded polymer micelles drug-loaded in organic or aqueous media. One of the authors has already reported that poly(ethylene glycol) (PEG)-based block copolymers with well-defined architecture exhibiting thermoresponsive phase transition was successfully synthesized through atom transfer radical polymerization (ATRP) of NiPAM using PEG-based macroinitiator and the Cu(I)Br/Me₆TREN catalytic system in aqueous media.²⁰ This informs that relatively polar monomers such as methacrylamide type of vinyl monomers carrying different sulfonamide groups can be polymerized via the ATRP mechanism because the ATRP technique admits a relatively high tolerance to impurity or acidic condition of reagents.^{21,22} New PEO-based amphiphilic block copolymers exhibiting pH-sensitive phase transitions are also expected to be utilized as the efficient drug-carriers via a micellization technique.

Fluorescein has been well-known as a powerful fluorophore for biological experiment because it retains a high fluorescence quantum yield in aqueous media.²³ Furthermore, intracellular pH changes under physiological conditions has been reported to render the fluorescence change in many cells exposed to certain biological stimuli.²⁴ The drug-loading efficacy of the micelles formed by amphiphilic block copolymers is expected to be indirectly assessed through fluorescence probe technique using fluorescein.

Herein we report the results for synthesis of PEO-based

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water-soluble amphiphilic block copolymers in response to the change of pH. The results for the stability and the drug-loading efficacy of micelles generated by pH-sensitive amphiphilic block copolymers are discussed.

Experimental

Materials. Tetrahydrofuran (THF; Oriental Chem. Co., reagent grade), benzene (Dae Jung Chem. Co., reagent grade), and methanol (Oriental Chem. Co., reagent grade) were purified by following the procedures described in the literatures.²⁵ Sulfadimethoxine (Sigma Chem. Co.) and sulfacetamide (Sigma Chem. Co.), tris(2-aminoethyl) amine (Lancaster, 97%), hydrochloric acid (Junsei Chem. Co., 35%), sodium hydroxide (NaOH; Oriental Chem. Co., 97%), formaldehyde (Kanto Chem. Co., 35%), acetone (T. J. Baker, 99.7%, HPLC grade), and copper(I) bromide were used as purchased without further purification. Fluorescein sodium salt (Aldrich Chem. Co.; C. I. 45350; Acid Yellow 73) was used as purchased without further purification. Methacryloyl chloride (Lancaster, 97%) was dried with freshly ground calcium hydride (CaH₂; Aldrich Chem. Co., 95%), followed by distillation to an ampoule cylinder under high vacuum. 2-Bromopropionyl bromide (Aldrich Chem. Co., 97%) and 2-bromoisobutyryl bromide (Aldrich Chem. Co., 98%) were used as purchased without further purification. Ethylene oxide (Aldrich Chem. Co., 99.5%+) was dried with CaH₂ below 5 °C under high vacuum for at least 3 h, followed by distillation of required amount into calibrated cylinder. Potassium *tert*-butoxide (*t*-BuOK; Aldrich Chem. Co., 1.0 M solution in tetrahydrofuran) was also purchased and used without further purification. *n*-Butyllithium (*n*-BuLi; Aldrich Chem. Co., 1.6 M in hexane) was used as an initiator after titration by Gilman's method using 1,2-dibromoethane.²⁶ Tris(2-dimethylaminoethyl)amine (Me₆TREN) as a specific ligand for Cu(I)Br in the atom transfer radical polymerization system was synthesized using tris(2-aminoethyl)amine by following the procedures described in the literature.²⁷

Synthesis of pH Sensitive Block Copolymers. Methacrylamide type of monomers carrying sulfanilamide group such as *N*-[4-(acetyl)sulfamoyl]phenyl] methacrylamide (sulfacetamidyl methacrylamide) and *N*-[4-(2,6-dimethoxy-4-pyrimidinyl)sulfamoyl]phenyl] methacrylamide (sulfadimethoxinyl methacrylamide) were synthesized by the reaction of methacryloyl chloride with the corresponding sulfonamides by following the modified procedures described in the literature.^{7,8,12} The crude monomers were purified by recrystallization in absolute ethanol.

ω -Brominated PEO as a macroinitiator for atom transfer radical polymerization was synthesized by the reaction of 2-bromopropionyl bromide with polymeric alkoxide which synthesized by *n*-BuLi-initiated ring-opening polymerization of ethylene oxide in the presence of *t*-BuOK in the

mixture of benzene and dimethylsulfoxide (DMSO) (6/4~10/1, v/v) by following the procedures described in the literature.²⁸

Atom transfer radical polymerizations (ATRP) of sulfadimethoxinyl methacrylamide and sulfacetamidyl methacrylamide in aqueous media using the PEO-based macroinitiator were carried out as the followings; 2 g of the macroinitiator ($M_n = 5,300$ g/mol) was delivered into a 250 mL of three neck round bottom flask containing the mixture of distilled water and methanol (100/50, mL/mL) deoxygenated by purging N₂ gas for 12 h, followed by adding the Me₆TREN (0.4 mmol) and copper(I) bromide (0.4 mmol) into the solution using a syringe. After that, the degassed aqueous solution consisting of methacrylamide type of monomer carrying sulfonamide group (0.01 mol) and NaOH (0.01 mol) was delivered into the solution of the reaction flask using a cannula at 50 °C with vigorously stirring. The reaction was kept for 24 h, followed by precipitation in HCl aqueous solution (0.01 mol) and filtration. The precipitate was immersed in methanol to extract unreacted monomer and PEO-based macroinitiator for 10 min, followed by filtration and drying in vacuum oven at room temperature.

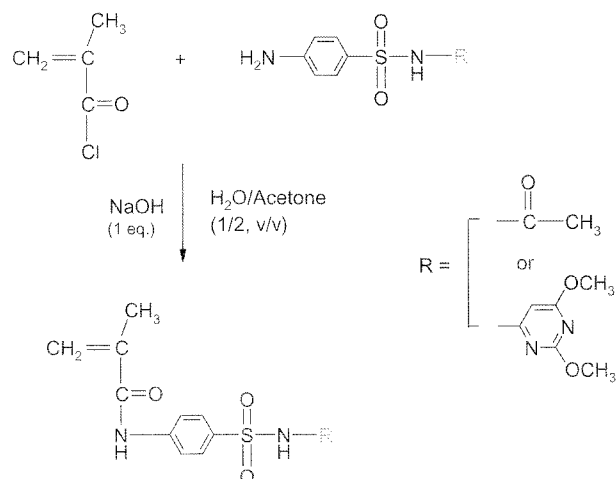
Micellization of Block Copolymers. The micelle formation of PEO-based block copolymer was monitored both by electronic spectral change using a UV/Visible spectrophotometer and by optical chromophoric change using fluorescein as a fluorescence probe with pH. Poly(ethylene oxide-*b*-sulfadimethoxinyl methacrylamide), PEO-*b*-PSDMA, ($M_n = 7,000$) (1 g) and fluorescein salt (0.05 g) were dissolved in aqueous media (pH 10), followed by keeping the solution at room temperature with stirring for at least 6 h. The aqueous HCl (0.1 M) solution was slowly added drop by drop, followed by precipitation in HCl aqueous media and washing with methanol to remove excess fluorescein on the surface of powder. The dried powder was dissolved in sodium phosphate buffer (pH 10) again. The pH-dependent property of powder (micelle containing the fluorescence probe in the core) was observed optically with pH. The photoluminescence property of the powder solution was also monitored with pH using NaOH (aq) and HCl (aq) under 365 nm excitation. In addition, a preliminary study for the drug-loading efficacy (wt%) of the micelle formed by the amphiphilic block copolymers in both aqueous media and dimethyl formamide (DMF) was also performed by using fluorescein as a precursor of water-soluble drug; at first, 0.5 g (0.007 mole) of PEO-*b*-PSDMA ($M_n = 7,000$) was dissolved in 100 mL of both H₂O and DMF, followed by addition of 0.01 g (0.003 mole) of fluorescein into the resulting solutions, respectively. The resulting solutions were kept at 25 °C for 1 h with stirring, followed by precipitation in excess HCl aqueous media and washing several time using methanol. The resulting precipitates were dried at room temperature in vacuum for 48 h prior to characterization. The loading efficacy was determined by the concentration of fluorescein included in the powder using the 'Beer's and Lambert's Law' via UV/Visi-

ble spectroscopic analysis; 0.1 g of the dried powder was dissolved in 10 mL of sodium phosphate buffer (pH 10), followed by controlling the solution to pH = 7.0 after the measurement of the extinction coefficient (ϵ) of fluorescein in aqueous media (pH = 7.0).

Characterization. Size exclusion chromatographic (SEC) analysis was performed at a flow rate of 1.0 mL/min in THF at 30 °C using the Dionex Softron P680 HPLC component system equipped with two Shodex GPC columns (LF-804) and two ultra- μ -Styragel columns (500 Å) with Wyatt Technology Interferometric Refractometer MALLS system after calibration with standard PEO samples (Polymer Laboratories) and SEC analysis of product was also performed using a Water GPC 410 system equipped with three UltrahydrogelTM (120, 150, 500 Å) at a flow rate of 0.6 mL/min in distilled H₂O containing 5% NH₄OH. ¹H-NMR spectroscopic analysis was performed using a Bruker spectrometer (Model; Avance 400 (400 MHz)) in deuterated dimethylsulfoxide (Aldrich Chem. Co.) or CDCl₃ at 25 °C. The %transmittance change in distilled H₂O and buffer solution was monitored using an Agilent 8453 Diode Array spectrophotometer.

Results and Discussion

Synthesis of Methacrylamide Type of Monomers. We synthesized methacrylamide type of vinyl monomers carrying sulfonamide group retaining a different pK_a value as shown



Scheme 1

in Scheme 1.

¹H-NMR spectra of sulfacetylamidyl methacrylamide and sulfadimethoxinyl methacrylamide are shown in Figure 1. The chemical shifts at 6.1 ppm corresponding to the protons on the primary amine group of sulfanilamide disappeared completely and the peaks at 10.2 ppm in both spectra correspond to the proton on the amide group adjacent the vinyl group. In addition, the peaks at 5.6 and 5.85 ppm corresponding to the protons on the vinyl group were also observed

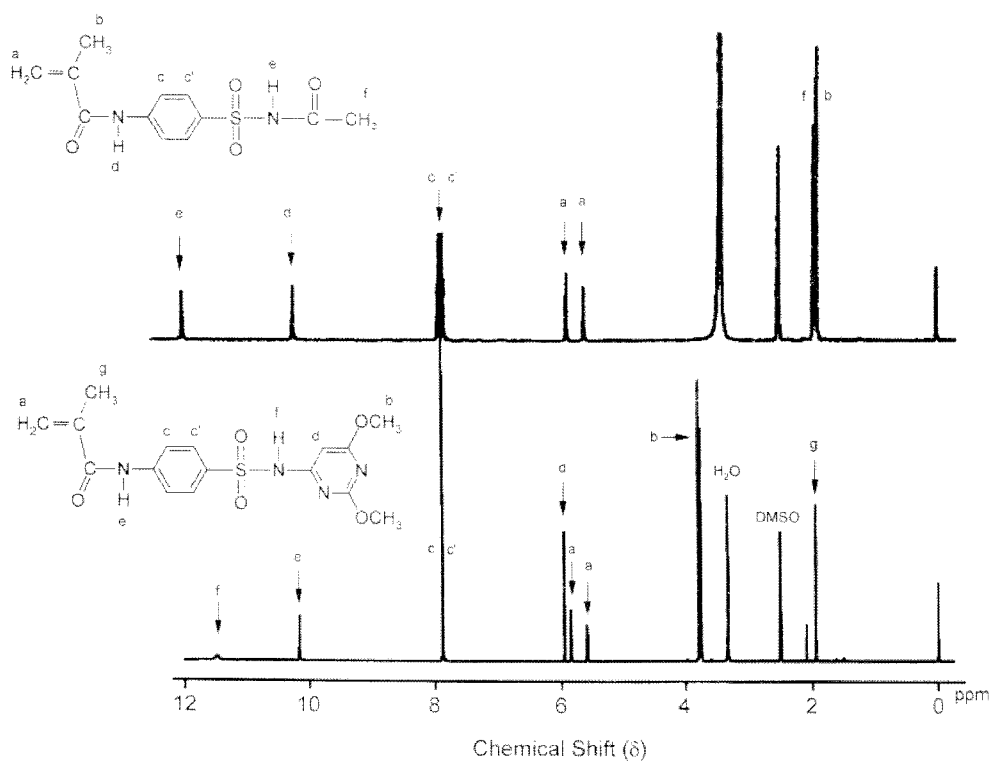
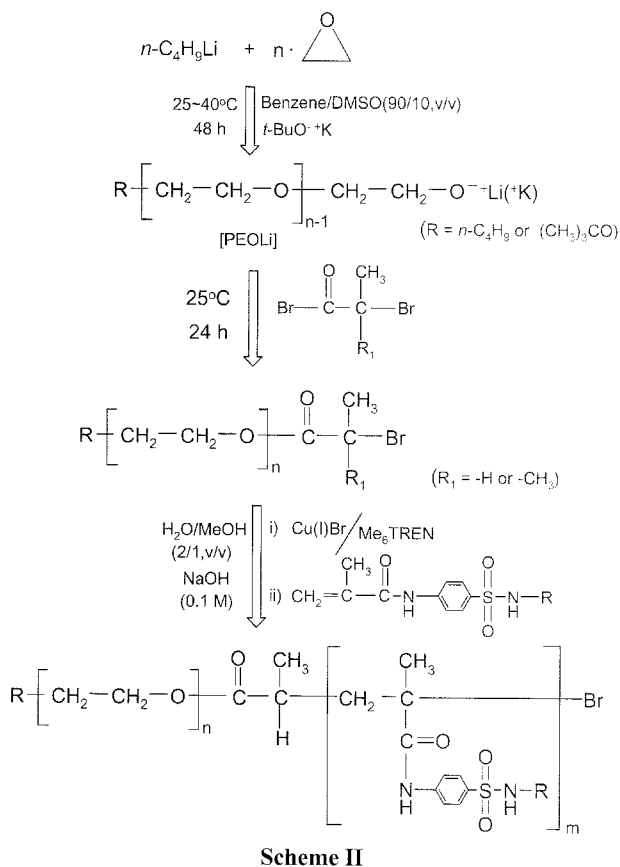


Figure 1. ¹H-NMR spectra of sulfadimethoxine and the corresponding sulfadimethoxinyl methacrylamide in deuterated dimethylsulfoxide at room temperature.



clearly. The final production yields were over 85 mol% based on the incipient moles of methacryloyl chloride used.

Synthesis of Macroinitiator and pH-Sensitive Block Copolymers. We have successfully developed simple and useful method to synthesize ω -functionalized poly(ethylene oxide) (PEO) *via* one pot process as shown in Scheme II.²⁸ Specifically, the molecular weight of PEO can be readily controlled with the ratio of grams of monomer to the moles of $n\text{-BuLi}$ and $t\text{-BuOK}$ taking part in initiation of ethylene oxide partially.²⁸ The active chain-end of the resulting polymeric alkoxide shows the living character as described in the literature.^{28,29} The chain-end functionalization of the living PEO using a proper terminating agent such as 2-bromopropionyl bromide or 2-bromoisobutyryl bromide produces the corresponding ω -brominated PEO which can be utilized as a macromolecular initiator for ATRP. Practically, Figure 2 represents ¹H-NMR spectra of a typical PEO and the corresponding ω -brominated PEO obtained from the reaction with 2-bromopropionyl bromide run in CDCl₃. The chemical shifts at 0.9 and 1.18 ppm in the subset of the upper spectrum must correspond to the protons of the methyl groups on the n -butyl and t -butoxy group on the segment of the initiating agents, respectively. The chemical shift at 1.18 ppm in the subset of the lower spectrum is assigned to the protons on the methyl group of the propionyl group in the chain end group generated as shown in Scheme II. The yield for synthesis of macroinitiator was over 90 mol% on the basis

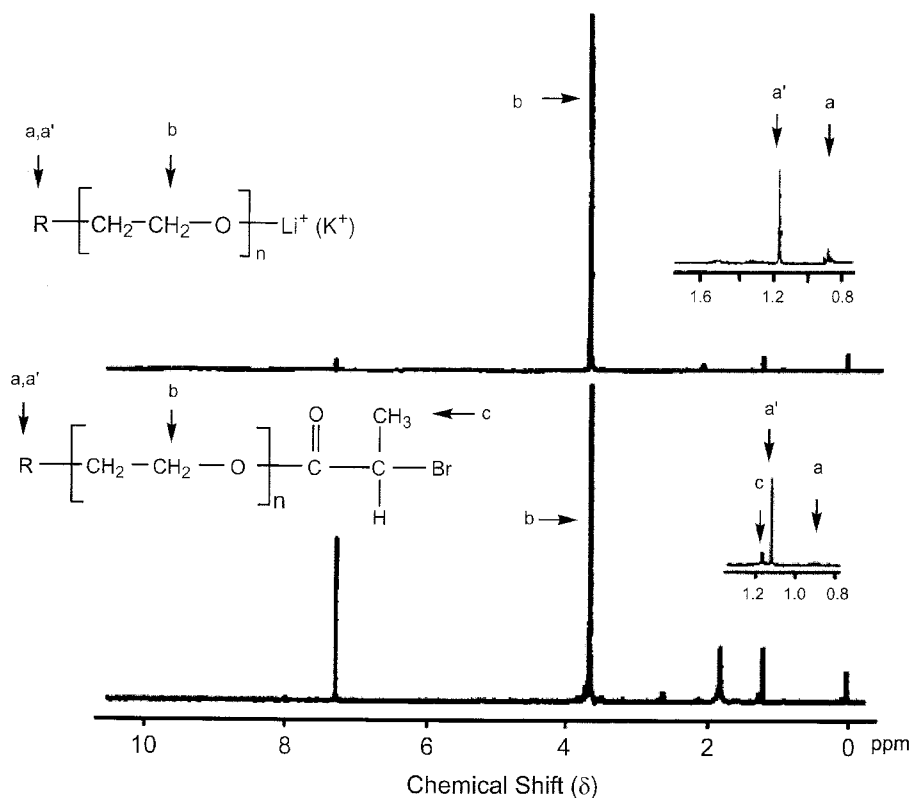


Figure 2. Comparison of ¹H-NMR spectra of poly(ethylene oxide) and the corresponding ω -brominated PEO in CDCl₃.

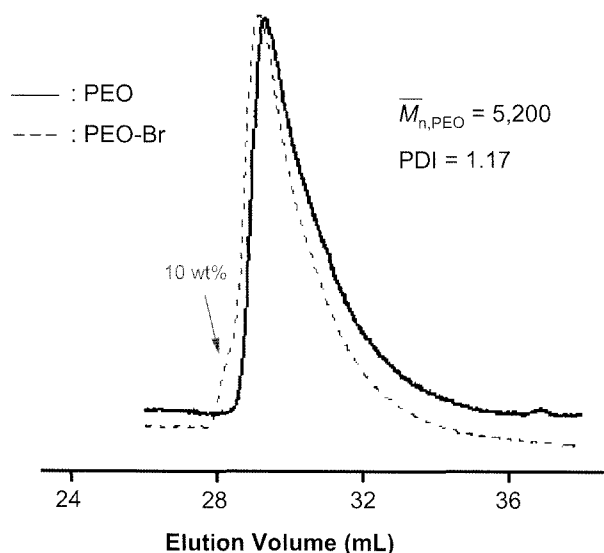


Figure 3. Size exclusion chromatograms of poly(ethylene oxide) and the corresponding ω -brominated poly(ethylene oxide) using THF as solvent at 30 °C with a flow rate of 1.0 mL/min.

of $^1\text{H-NMR}$ spectroscopic analysis. Figure 3 represents size exclusion chromatograms of PEO and the corresponding PEO-based macroinitiator using THF as solvent at 30 °C.

The production yields were found to be almost quantitative except for the formation of a few weight percent of coupled product on the basis of SEC and $^1\text{H-NMR}$ spectroscopic analysis.

Atom transfer radical polymerization (ATRP) of a variety of vinyl monomers using alkyl halide as a true initiator have been well described in the literatures.^{21,30-34} In this experiment, pH-sensitive PEO-*b*-PSDMA and poly(ethylene oxide-*b*-sulfacetamidyl methacrylamide) (PEO-*b*-PSAcMA) were successfully synthesized in aqueous media using ω -brominated PEOs as the macroinitiators for ATRP as shown in Scheme II. All the polymerization conditions and the results for characterization of the corresponding block copolymers are summarized in Table I. The conversion of methacrylamide type of monomers to the corresponding block copoly-

mers in this polymerization system was almost quantitative leading to the control of molecular weights. Unfortunately, as shown in the entry Block Poly 5, no ATRP of sulfadiazinyl methacrylamide occurred in our reaction condition. Up to date, no any detail investigation for no polymerization has been performed. It is simply inferred that the amine group on the dipyridinyl ring of sulfadiazine seemed to affect the ligand conformation between copper atom and amine compound such as Me₆TRENE of ATRP catalytic system leading to the reduction of catalytic activity.

The insolubility in neutral H₂O or THF of the block copolymers exhibiting pH-sensitive phase transition behavior brought no information on the molecular weight by size exclusion chromatographic analysis. Concerning the 'salting-in' effect, the addition of a proper amount of NH₄OH (5 wt%) may swell a proper size of hydrodynamic volume of the resulting block copolymers. All the block copolymers present monomodal distributions in SEC run using the mixture of H₂O/NH₄OH eluent as shown in Figure 4. Although the molecular weight from the SECs was unable to be obtained, the different elution time informed that ATRPs of methacrylamide type of monomers using PEO-based macroinitiator were successful enough to generate the corresponding pH-sensitive block copolymers. Therefore, the molecular weights were calculated on the basis of $^1\text{H-NMR}$ analysis compared with that of PEO obtained by the SEC analysis using THF eluent. In addition, with a synthetic point of view for amphiphilic block copolymers, our reaction process provides a powerful tool to prepare pure pH-sensitive block copolymers because of their simple purification procedures through the simple change of pH of H₂O. For instance, unfunctionalized and/or unreacted PEOs as shown in Figure 3 can be readily extracted out by using H₂O in the wide range of pH.

Figure 5 represents $^1\text{H-NMR}$ spectra of two typical PEO-*b*-PSMA in Table I (Block Poly 1 and Block Poly 3). While the strong peaks at the chemical shift at 5.7 and 5.9 ppm corresponding to the protons on the vinyl group disappear completely, new broad peak at the chemical shift near 1.0 ppm corresponds to the protons on methyl groups at the α -carbon of methacrylamide monomers in both spectra appear,

Table I. Synthesis of PEO-Based Block Copolymers Containing Sulfonamide Group in 70 mL of H₂O or H₂O/CH₃OH at 60 °C for 12 h and the Results for Their Characterizations

Entry	Macroinitiator (MI) ^a (\bar{M}_n) (g/mol)	Monomer (M) (g)	[MI][M] (mol/mol)	PEO- <i>b</i> -PSMA ^b (\bar{M}_n) (g/mol)
Block Poly 1 ^c	5,300	0.8	1/5	1,500 (6,800)
Block Poly 2 ^c	5,300	1.2	1/10	3,000 (8,300)
Block Poly 3 ^d	5,300	1.6	1/5	1,900 (7,200)
Block Poly 4 ^d	5,300	2.3	1/10	3,800 (9,100)
Block Poly 5 ^e	5,300	2.0	1/10	-

^aMolecular weight observed from SEC using THE as eluent at 30 °C. ^bMolecular weight of the sulfonamide group fragment obtained from $^1\text{H-NMR}$ analysis based on that of PEO. ^cBlock copolymer based on sulfadimethoxinyl methacrylamide synthesized in H₂O. ^dBlock copolymer based on sulfacetamidyl methacrylamide in H₂O. ^eBlock copolymer based on sulfadiazinyl methacrylamide synthesized in H₂O.

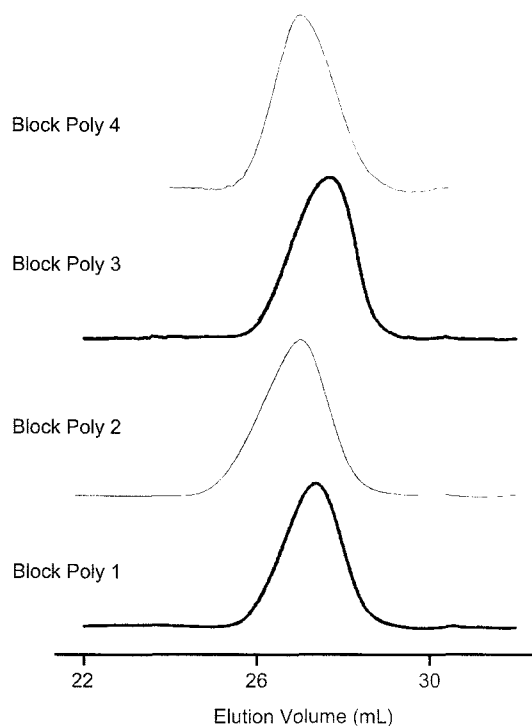


Figure 4. Comparison of SECs of poly(ethylene oxide-*b*-sulfacetamidyl methacrylamide) (PEO-*b*-PSAcMA) (Block Poly 3 & 4) and poly(ethylene oxide-*b*-sulfadimethoxinyl methacrylamide) (PEO-*b*-PSDMA) (Block Poly 1 & 2) in Table I using 5% NH_4OH aqueous media at a flow rate of 0.6 mL/min at room temperature.

which was originally observed at 1.9 ppm as shown in Figure 1. Furthermore, the strong and broad peaks in the range of 7–8 ppm correspond to the protons on the phenyl group of the block copolymers, which originally showed sharp and strong peaks at $\delta = 7.9$ ppm in Figure 1. Considering the results for $^1\text{H-NMR}$ spectroscopic and SEC analyses, the synthesis of PEO-based amphiphilic block copolymers were successful via atom transfer radical polymerization in aqueous media.

To investigate whether the block copolymers retain the pH sensitive properties, their phase transitions with pH was observed by monitoring the % transmittance change with pH using a UV/Visible spectrophotometer. Specifically, the phase transition behaviors of all the block copolymers in Table I are shown in Figure 6. The pK_a values of sulfadimethoxine and sulfacetamide have been reported to be 6.1 and 5.4, respectively.^{8,11} The phase transitions of the block copolymers are expected to be effected by not only the pK_a value but also the molecular weight of the polymeric fragment carrying sulfonamide group. There seems to be a relationship between the pK_a and the corresponding pH. PEO-*b*-PSDMA and PEO-*b*-PSAcMA show the sharp phase transitions in the pH range of 6.0–7.2 and 3.2–4.0, respectively. Both solutions over pH 7.0 exhibit the 100% transmittance arising from complete dissolution. On adding a drop of 0.1 N HCl aqueous solution, the pH value of the polymer solutions decreased rapidly below pH 3.0 within at least 2 min. Although PEO-*b*-PSDMA showed a

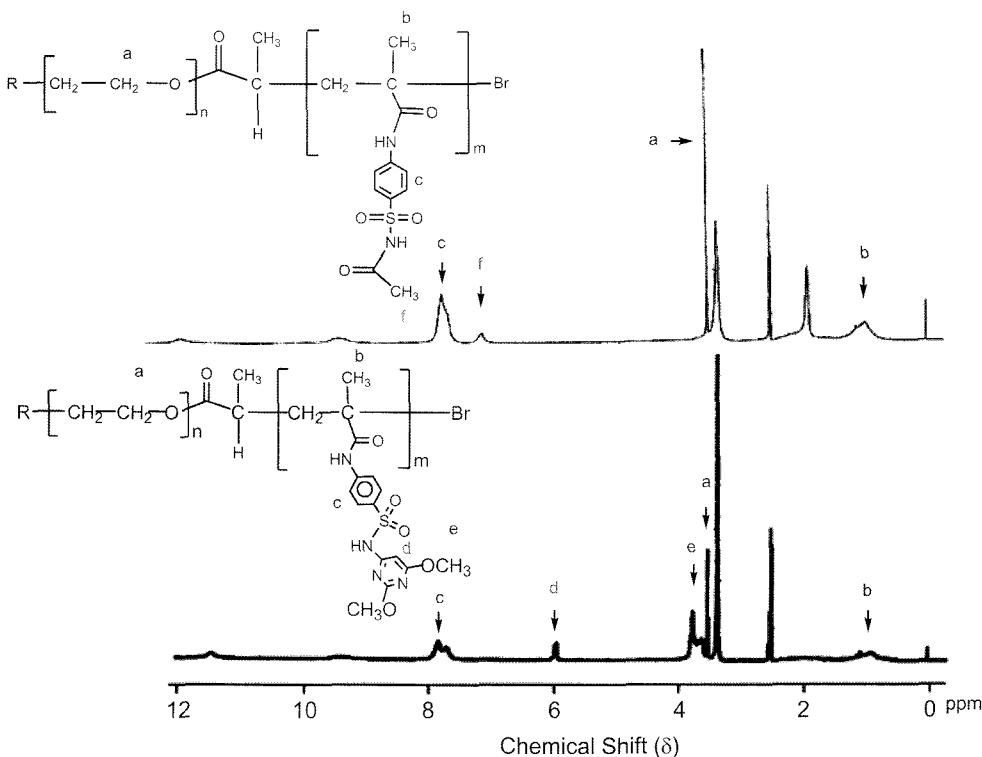


Figure 5. $^1\text{H-NMR}$ spectra of the block copolymers; Block Poly 3 (upper) and Block Poly 1 (lower) in Table I.

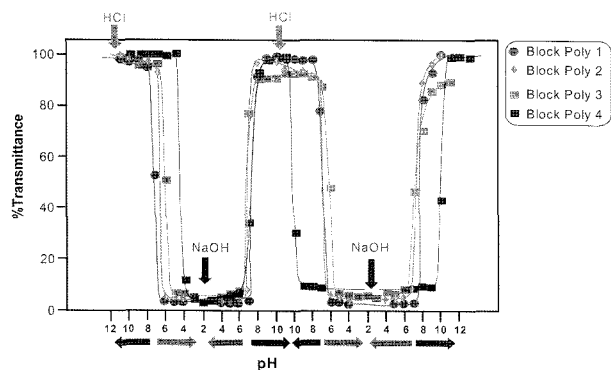


Figure 6. The % transmittance changes of poly(ethylene oxide-*b*-sulfacetamidyl methacrylamide)s and poly(ethylene oxide-*b*-sulfadimethoxinyl methacrylamide)s in Table I with pH change cycle.

sharp pH-sensitive phase transition, however, PEO-*b*-PSAcMA did not bring a rapid dissolution state with the pH change. Presumably, this phenomenon is due to stronger aggregation of the sulfacetamide groups exhibiting stronger acidic property compared with the sulfadimethoxine group. When an aggregation behavior of 0.01 g/10 mL (tetraborated buffer) of PEO-*b*-PSDMA was examined at pH 6.45, it was found that its complete aggregation took over 10 h. With this regard, it is not expected that the water-soluble PEO-based block copolymer exhibiting sharp pH-sensitive phase transition bring faster micelle formation for drug-loading in the drug delivery system. Further study should be performed.

Fluorescence of Block Micelles. A micellization of the pH-sensitive amphiphilic block copolymers seems to play an important role to determine the drug-loading efficacy as well as the micelle stability. In this experiment the fluorescence probe technique using fluorescein was applied for determination of the micelle formation and its stability. As mentioned in the previous section,^{23,24} fluorescein has been used for a biological assessment because of its high fluorescence quantum yield. Specifically, its maximum absorption in pH 4.0 appears at $\lambda_{max} = 440$ nm. The pH increase (basic) of the solution brings new appearance of red-shifted strong absorption band at $\lambda_{max} = 490$ nm, which corresponds to facile electron resonance on the fluorescein structure. In addition, PEO-*b*-PSDMA in pH 6.5 exhibits very weak absorption near 490 nm. The encapsulation was performed by adding slowly 0.1 HCl into the homogeneous mixture solution (pH 10) of PEO-*b*-PSDMA and fluorescein, followed by precipitation in diethyl ether and washing the precipitate with neutral distilled water several times to remove fluorescein molecules adsorbed on the surface of particles. Both visual and optical colors of fluorescein and the Entry Block 1 in the pH range of 6-7 are shown in Figure 7. Clearly, the optical colors of two substances can be clearly distinguished each other. When those were excited by UV irradiation with

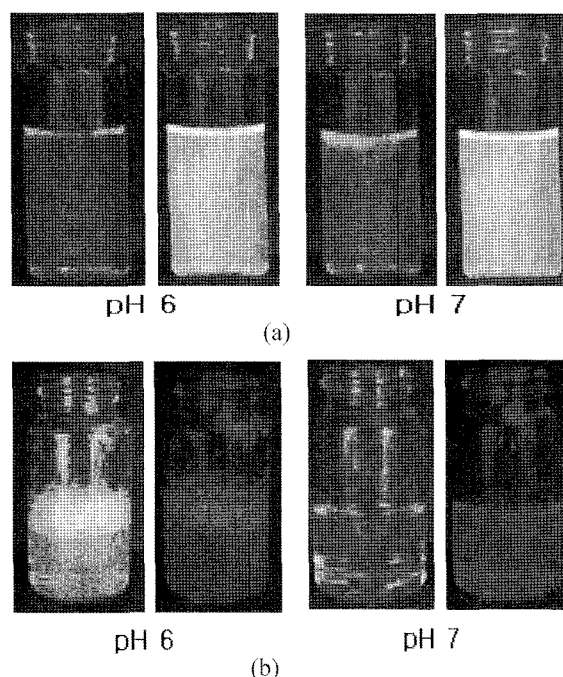


Figure 7. Comparison of optical properties of fluorescein (a) and the Entry Block 1 (b) in different pH; the front image under light and the following image by UV irradiation (365 nm).

365 nm, the color of the absorption maximum of the former looks a light green in the pH range of 6-7, the color of the amphiphilic block copolymer in pH 6-7 seems to be blue as just reflected one. When the dried precipitate is dissolved in buffer solution (pH = 10), the solution looks homogeneous and the visual color is a light green arising from incomplete aggregation of block copolymer containing fluorescein as shown in Figure 8. The decrease of pH brings a turbid solution and the light green color disappears, which indirectly informs a complete encapsulation of fluorescein by the Entry Block 1 in pH 6 via micellization. The color change of the solution is reversible with pH change. Furthermore, the final concentration of fluorescein encapsulated inside the micelle decreases with pH change cycles, which corresponds to the release of fluorescein from the micelle core.

The loading efficacy in this experiment was determined by the concentration of fluorescein in the dried powders. Practically, 0.015 and 0.0073 wt% of the efficacy in aqueous medium and DMF were observed under this experimental condition (see the supporting data). Although these loading efficacies are not confirmative, the water-soluble drug-loading efficacy in aqueous media is twice effective than that in DMF. This result is informative in the drug-loading experiment. Thus, the amphiphilic block copolymers are expected to be used as an effective drug-nanocarrier. Further detail study should be carried out in the future.

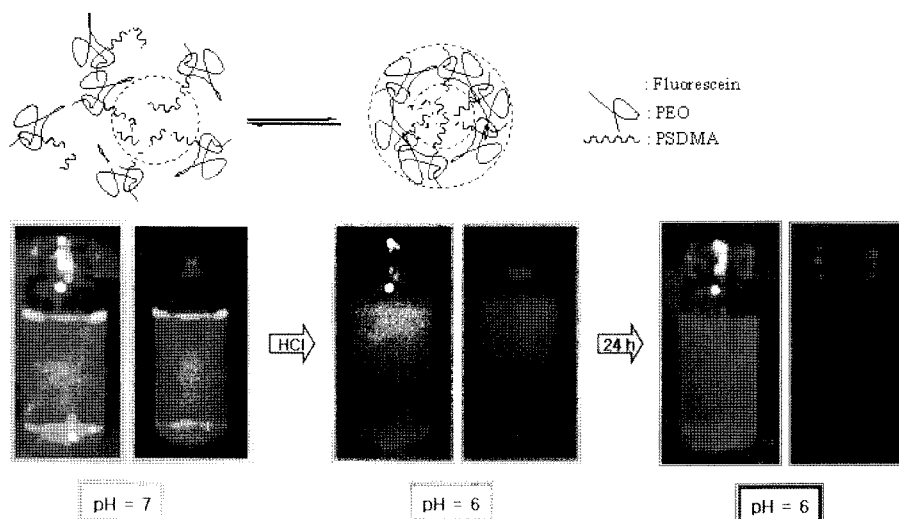


Figure 8. Optical color change of the precipitate consisting of the Entry Block 1 and fluorescein in aqueous media under light and excitation by UV irradiation (365 nm) with pH and time.

Conclusions

We have developed a facile and useful method to prepare PEO-based block copolymers exhibiting water-soluble and pH sensitive phase transitions affecting to drug-loading efficacy as nanocarrier in the drug delivery system. The pH-sensitive block copolymers were successfully prepared by atom transfer radical polymerization of methacrylamide type of monomers carrying sulfonamide group such as sulfadimethoxine and sulfacetamide using the Cu(I)Br/Me₆TREN catalytic system and PEO-based macroinitiator in aqueous media. The molecular weights of the resulting polymers affecting the pH-sensitive property were controlled by the ratio of the weight of monomer to the mole of PEO-based macroinitiator. The study for the micellization via the fluorescent probe technique provided a good evidence for the applicability of the pH-sensitive amphiphilic block copolymers as drug-nanocontainers in a drug delivery system (DDS). The drug-loading was found to be achieved via micellization mechanism. The drug-loading efficacy in the case of a water-soluble drug seems to be more effective in aqueous media than in DMF.

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