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pH/temperature-sensitive 4-arm poly(ethylene glycol)-poly(amino urethane) copolymer hydrogels

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ABSTRACT

A series of novel pH/temperature-sensitive 4-arm poly(ethylene glycol)-poly(amino urethane) copolymers was synthesized via addition polymerization. The resulting copolymers were characterized by ^{1}H, ^{13}C NMR, Fourier transform infrared spectroscopy and gel permeation chromatography. Poly(amino urethane) (PAU) segment acts as a pH/temperature-sensitive block. The copolymer aqueous solutions showed a sol-to-gel-to-aggregation phase transition as a function of pH and temperature when the pH of the copolymer solution is higher than 6.8. The sol–gel phase transition could be controlled by varying the PAU block length and copolymer concentration. The gel window covers the physiological conditions and a white gel was formed rapidly after subcutaneously injecting the copolymer solution (30 wt%) into SD rats. The in vitro release of chlorambucil, an anticancer drug, was sustained over 14 days under physiological conditions.

1. Introduction

Stimuli-sensitive polymeric hydrogels have been used for biomedical applications, such as drug delivery and tissue engineering, because of their hydrophilicity and biocompatibility [1–3]. Thermosensitive polymeric hydrogels comprising of hydrophilic poly (ethylene glycol) (PEG) and various hydrophobic blocks, such as poly (caprolactone) (PCL) [4], poly(lactide-co-glycolide) (PLGA) [5] and poly(phosphazene) [6], are of interest because of their injectability and biocompatibility. Their aqueous solution exists in the sol state at low temperature, but turn into a gel at physiological temperature (37 °C). However, the neutral character of thermosensitive hydrogels limits their applications in the delivery of ionic proteins.

Recently, gelation in response to multiple stimuli, especially pH and temperature, has been an important topic [7–15]. Cationic hydrogels bearing tertiary amine groups were introduced as excellent materials for drug delivery system, because of their ability to bind to anionic drugs/proteins through ionic interactions [10]. Poly(lysine) [16], poly(ethylene imine) [17], poly(amido amine) (PAA) [14,15], poly(amino urethane) (PAU) [7] and poly(β-amino ester) (PAE) [14] are typical examples of cationic polymers that have been used for the delivery of genes, proteins and drug molecules.

The star-shaped structure is known to affect the gelation behavior of block copolymer hydrogels. Choi et al. first reported star-shaped copolymers with 8-arm PEG as the inner block and thermosensitive poly(L-lactide) (PLLA) (PEG(-PLLA)$_8$) and PCL (PEG(-PCL)$_8$) as the outer blocks [18]. Subsequently, PLLA(-PEG)$_3$ with 3-arm PLLA was reported [19]. The gelation of these copolymers is due to the hydrophobic interactions of PLLA and PCL blocks. The gelation in the case of star-shaped PEG(-PLLA)$_8$ and PEG-poly (D-lactide) (PEG(-PDLA)$_8$) was attributed to the formation of a stereocomplex between the enantionmeric PLLA and PDLA segments [20]. Recently, PEG(-PLLA)$_8$ end-capped by cholesterol exhibited a sol-to-gel transition, while PEG(-PLLA)$_8$ itself did not [21]. The gelation was triggered by the hydrophobic interaction of the cholesterol groups. However, no star-shaped polymeric hydrogels which respond to both pH and temperature have so far been reported.

As compared to linear polymers at the same molecular weight, star-shaped polymers have a smaller hydrodynamic radius that allows them to be excreted from the human body via the kidneys [18]. PEG is an important material for biomedical applications, because of its non-toxicity and non-immunogenicity and PAU is a biocompatible polymer [7].

In this study, a series of novel pH/temperature-sensitive hydrogels based on 4-arm poly(ethylene glycol)-poly(amino

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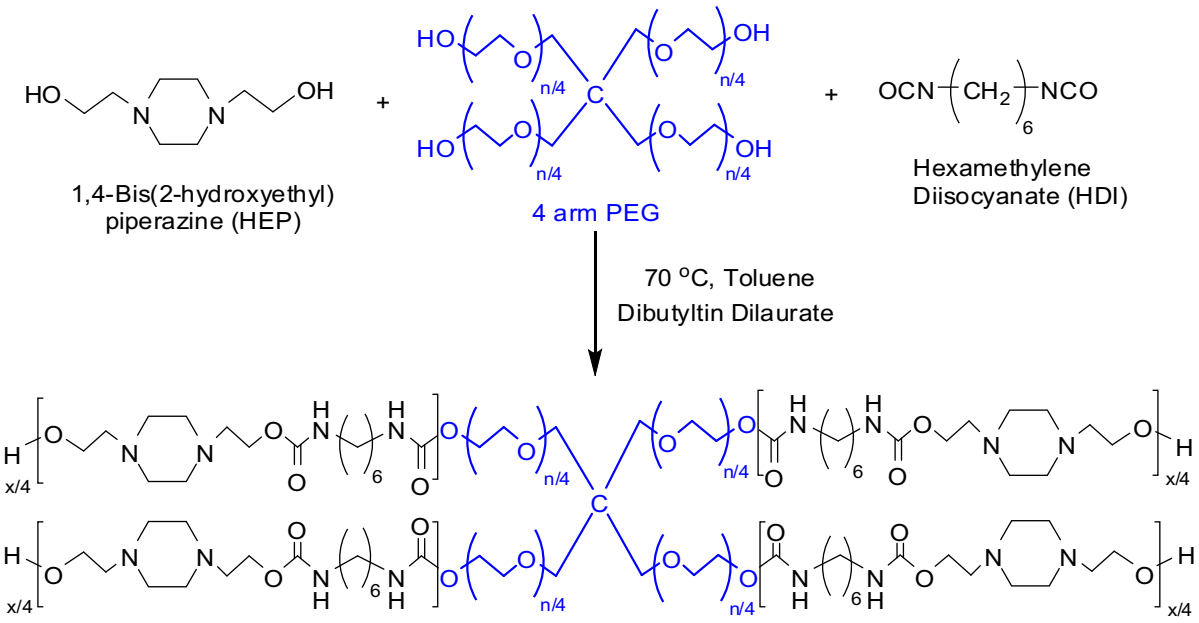

Scheme 1. Synthesis route of PEG(-PAU)$_4$ copolymers.

urethane) (PEG(-PAU)$_4$) copolymers was synthesized. The PEG (-PAU)$_4$ copolymers were obtained by the addition polymerization of the isocyanate groups of 1,6-diisocyanato hexamethylene (HDI) and hydroxyl groups at the end of the 4-arm PEG and 1,4-bis (hydroxyethyl) piperazine (HEP) in toluene in the presence of dibutyltin dilaurate as a catalyst. The resulting copolymers were characterized by ^{1}H, ^{13}C NMR, Fourier transform infrared spectroscopy (FTIR) and gel permeation chromatography (GPC). PAU exhibits hydrophilic properties at relatively low pH, but turns into a hydrophobic block at neutral pH. In contrast to multiblock copolymers with high molecular weights (20,000–30,000) [7], these obtained PEG(-PAU)$_4$ copolymers in aqueous solution exhibited a sol-to-gel-to-aggregation transition upon heating at pH 6.8–7.8 with much lower molecular weights (4000–6000). The hydrogels spanned the physiological conditions (pH 7.4, 37 °C). The in vivo gelation and the sustained release of chlorambucil in vitro under the physiological conditions were investigated.

2. Experimental

2.1. Materials

4-arm PEG (M_n = 2000) was purchased from ID Biochem, Inc. (Seoul, Korea) and used as received. HEP, HDI, dibutyltin dilaurate, phosphate buffer saline (PBS) and anhydrous toluene were obtained from Sigma–Aldrich. Diethyl ether and chloroform were obtained from Samchun Chemical Co. (Korea) and used as received. All other chemicals were of reagent grade and used as received.

2.2. Synthesis of PEG(-PAU)$_4$ block copolymer

The PEG(-PAU)$_4$ block copolymers were synthesized by conjugating PAU blocks to 4-arm PEG by the addition polymerization of the isocyanate groups of HDI and hydroxyl groups at the end of the 4-arm PEG and HEP in toluene in the presence of dibutyltin dilaurate as a catalyst [7]. The feed ratio of the components was calculated so as to obtain hydroxyl groups at the ends of the resulting copolymer. The synthetic process of PEG(-PAU)$_4$ was as follows (P-03): 2.0 g (1.0 mmol) of 4-arm PEG (M_n = 2000) and 0.004 g of dibutyltin dilaurate were added into a 250 mL two-neck round-bottom flask equipped with a magnetic stir-bar. The flask was placed into an oil-bath and dried for 1 h under vacuum at 100 °C and then cooled to 70 °C. After that, 2.06 g (11.7 mmol) of HEP was added into the flask and the mixture was dried under vacuum for 30 min. Then, 60 mL of anhydrous toluene was added. After the reactants were completely dissolved, 1.92 mL (11.7 mmol) of HDI was added to the flask and the reaction was continued for 1 h at 70 °C. Finally, the reaction mixture was evaporated under vacuum, dissolved in chloroform and then precipitated in excess diethyl ether. The precipitated polymer was filtrated and dried under vacuum at room temperature for 48 h. The product yield was over 85%.

2.3. Characterization

^{1}H and ^{13}C NMR was carried out using a 500 MHz spectrometer (Varian Unity Inova 500NB instrument) to examine the structures of the copolymers in $CDCl_3$.

Fourier transform infrared spectroscopy (FTIR) was recorded using an FTIR spectrometer (FT/IR-4100 Type A, TGS, Jasco).

The molecular weights of the copolymers and their distributions were measured by gel permeation chromatography (GPC) using a Waters Model 410 instrument with a refractive index detector (Shodex, RI-101) and three Styragel (KF-803L, KF-802.5 and KF-802) columns in series, at a flow rate of 1.0 mL min^{-1} (eluent: DMF; 40 °C). Poly(ethylene glycol) standards (Waters) were used to determine the molecular weights.

Table 1
Characteristics of PEG(-PAU)$_4$ copolymers.

No.	Feed ratio (mol)[a]			PEG[b]	M_n[c] of PEG(-PAU)$_4$	PDI[c]	M_n[d] of PAU/arm	pK$_a$[e]
	PEG	HEP	HDI					
P-01	1.0	5.85	5.85	2000	4000	1.92	500	6.44
P-02	1.0	8.75	8.75	2000	5000	1.95	750	6.62
P-03	1.0	11.70	11.70	2000	5900	1.98	975	6.75

[a] feed ratio.
[b] provided by ID Biochem, Inc.
[c] measured by GPC.
[d] calculated from the molecular weight of PEG and copolymers.
[e] calculated from the titration curves.

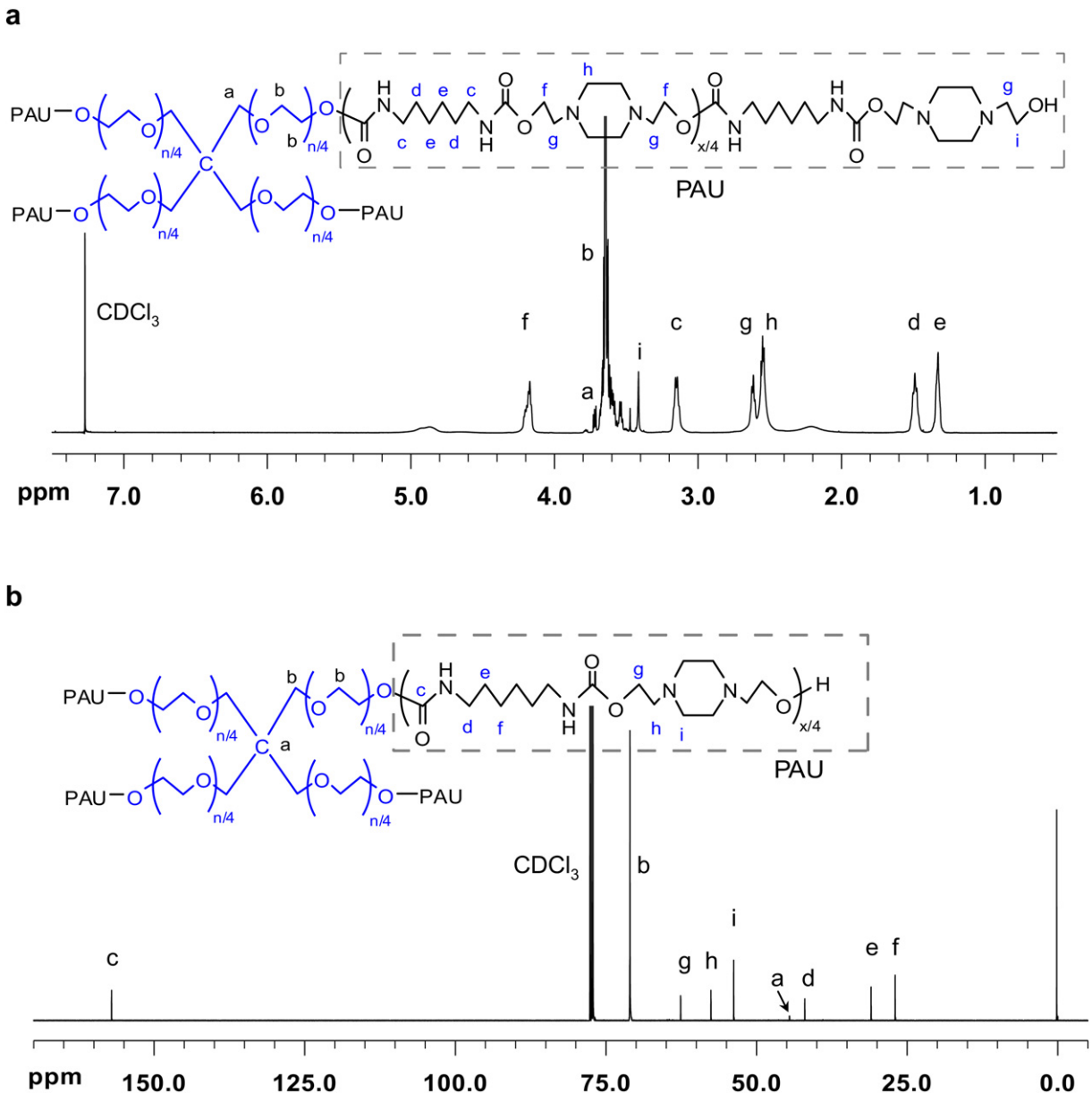

Fig. 1. Proton NMR (a) and ^{13}C NMR (b) of PEG(-PAU)$_4$ copolymer (P-03).

2.4. Acid–base titration

The acid–base titration was conducted to determine the pK_a values of the synthesized copolymers. In brief, 50 mg copolymer was dissolved in 50 mL distilled water and the pH was adjusted to 2.5–3.0 by adding 1 N HCl. Then, the titration profiles were obtained by repeated adding 50 μL of 0.1 N NaOH and recording the pH value. The pK_a values of the copolymers were calculated from the derivative values of the titration curves, which correspond to the inflection point.

2.5. Sol–gel phase transition measurement

The sol (flow) to gel (non-flow) phase transition of the copolymer in aqueous solution was determined by the tube inverting method [10]. In brief, the copolymer was dissolved in phosphate buffered saline (PBS) at pH 1 in a 4 mL vial (10 mm diameter) at a given concentration for 4 h and the pH was adjusted with 5 N NaOH and 5 N HCl and stabilized at 2 °C overnight. Each vial contained around 0.5 mL of the copolymer solution. The sample vials were placed in a water-bath and then slowly heated from 0 to 80 °C. The samples were equilibrated for 20 min at temperature intervals of 2 °C. The sol–gel transition was determined by inverting the vial [10,11].

2.6. Rheology

The viscosity variation of the copolymer aqueous solutions was determined by dynamic mechanical analysis (Bohlin Rotational Rheometer) [11,12]. A polymer solution (30 wt%) in PBS was placed between a 20 mm diameter plate and 100 mm diameter plate with a gap of 250 μm. Oscillation mode with a stress controlled of 0.4 Pa and frequency of 1 rad s^{-1} was performed. The heating rate was 1 °C min^{-1}.

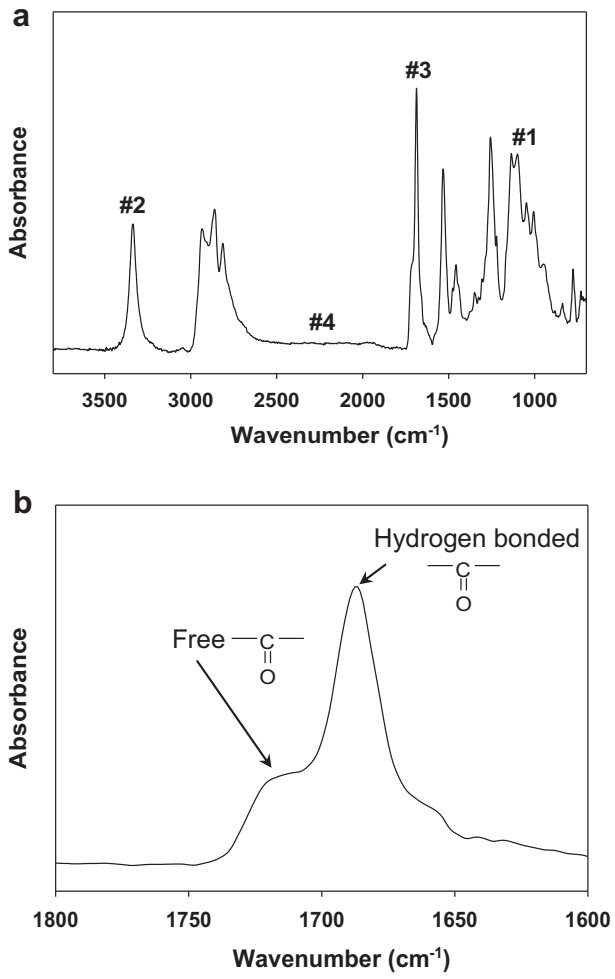

Fig. 2. FTIR spectrum of PEG(-PAU)$_4$ copolymer: a) copolymer P-03 (ether groups of PEG stretching: #1, 1104 cm^{-1}; -NH- of urethane groups stretching: #2, 3334 cm^{-1}; carbonyl of urethane groups stretching: #3, 1720 cm^{-1}; disappear of -NCO stretching: #4, 2267 cm^{-1}) and b) functional carbonyl of urethane groups stretching.

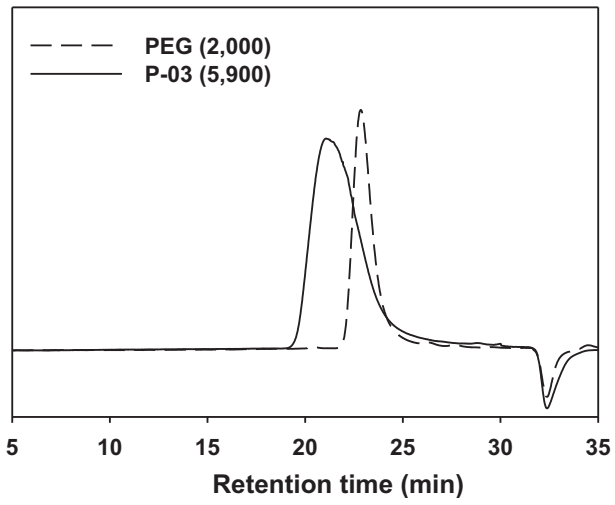

Fig. 3. GPC traces of PEG and PEG(-PAU)$_4$ copolymer (P-03).

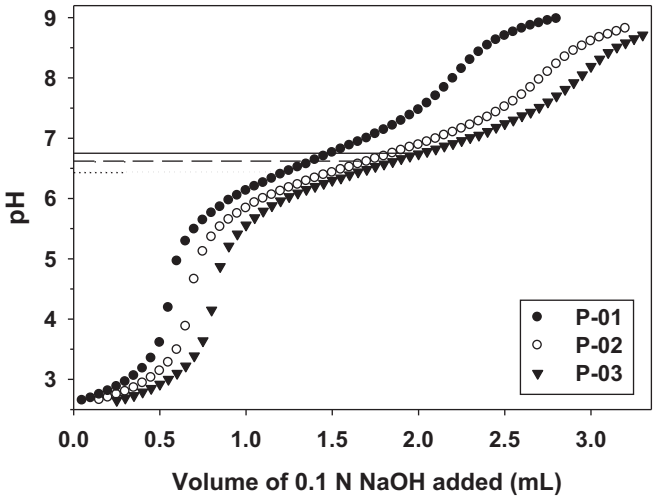

Fig. 4. Acid–base titration profiles of synthesized copolymers.

2.7. In vivo gel formation

To study the gel integrity of the aqueous copolymer solutions in vivo, male Sprague-Dawley (SD) rats (Hanlim Experimental Animal Laboratory, Seoul, Korea) were used. The rats (5–6 weeks old, average body weight 200 g) were handled in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23, revised 1985).

An aqueous solution (200 μL, 30 wt%) of PEG(-PAU)$_4$ (P-03) at pH 6.8 was subcutaneously injected into the back of a male SD rat to investigate the injectability and in vivo gelation of the copolymer solution. 5 min after the injection, the rat was sacrificed and the gel morphology was observed.

2.8. In vitro release of chlorambucil

Chlorambucil was added to the copolymer solution (30 wt%) in PBS buffer at pH 6.0 to obtain final concentrations of 4 mg mL^{-1} and 8 mg mL^{-1}, and stirred at 4 °C for 24 h. After that, the pH was adjusted to 7.4 and stirring was continued for 12 h. Subsequently, 0.5 mL of the drug-loaded polymer solution was placed into a 4 mL vial and then the samples were incubated at 37 °C for 30 min to

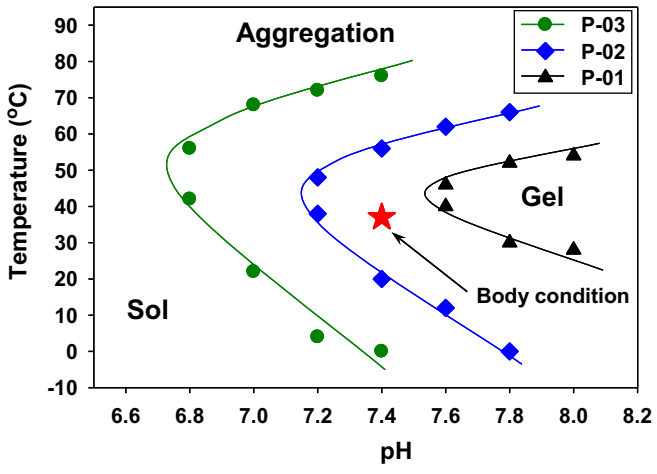

Fig. 5. Sol–gel phase transition diagrams of PEG(-PAU)$_4$ copolymers (30 wt%) with different PAU block lengths.

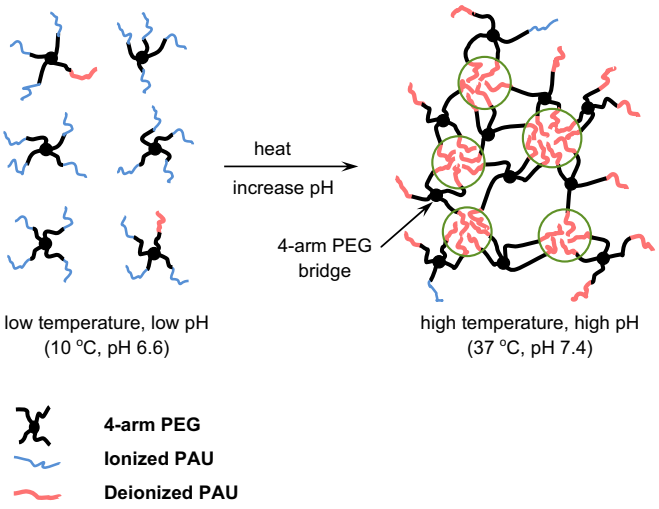

Fig. 6. Schematic showing the sol–gel phase transition of the PEG(-PAU)$_4$ copolymer.

obtain gels. Then, 3 mL of fresh release medium (PBS buffer solution, 37 °C and pH 7.4) was added to each vial. At given time intervals, 1.5 mL of the release medium was withdrawn from the vial and 1.5 mL of fresh release medium was added. The chlorambucil concentration of the release mediums and standard solutions was measured by a UV–Vis spectrophotometer at 254 nm [7]. The comparison of the absorbance was used to calculate the chlorambucil concentration and accumulative release. The standard line was determined by using five different chlorambucil concentrations ranging from 0.2 to 0.00032 mg mL^{-1}.

3. Results and discussion

3.1. Synthesis and characterization of PEG(-PAU)$_4$ copolymer

The PEG(-PAU)$_4$ copolymers were synthesized by the addition polymerization of the isocyanate groups of HDI and hydroxyl groups at the end of the 4-arm PEG and HEP. The synthesis route is shown in Scheme 1. The block length of PAU was controlled by varying the feed ratio of the monomers. The feed ratio of the reactants were calculated to obtain the hydroxyl-terminated copolymers (the number of hydroxyl groups is four equivalents larger than the number of isocyanate groups), as shown in Table 1. The chemical structure of the synthesized copolymers was characterized by ^{1}H and ^{13}C NMR as well as FTIR spectroscopy. Fig. 1a shows the ^{1}H NMR spectrum of the copolymer PEG(-PAU)$_4$ (P-03). The signals at 3.51–3.78 ppm were assigned to the methylene protons of PEG (peak a, b). The signals at 3.12–3.22 ppm were assigned to the first methylene protons of HDI (-OCO-NH-C**H$_2$**-CH$_2$-, c). The signals at 1.43–1.58 ppm and 1.28–1.37 ppm were assigned to the second (-NH-CH$_2$-C**H$_2$**-CH$_2$-, d) and third methylene protons (-NH-CH$_2$-CH$_2$-C**H$_2$**-C**H$_2$**-CH$_2$-, e) of HDI, respectively. The signals at 4.16–4.23 ppm and 2.49–2.71 ppm were assigned to the methylene protons of HEP (peaks f, g, h). The signals at 3.39–3.42 ppm were assigned to the methylene protons near the -OH groups at the end of the copolymer (peak i). The isocyanate peak at 122.9 ppm was not observed in Fig. 1b, indicating that the HDI monomers was completely consumed during the polymerization. The FTIR measurements further confirmed the formation of the copolymers. Fig. 2 shows the IR spectrum of the P-03 copolymer. The peak at 1104 cm^{-1} was attributed to the C-O-C stretching of 4-arm PEG (Fig. 2a). The peak at 3334 cm^{-1} corresponded to the N-H stretching band of the urethane, and the absence of the peaks at 2267 cm^{-1} indicates that the isocyanate groups completely reacted. The peak at 1720 cm^{-1} and the one at a lower frequency in Fig. 2b were attributed to the carbonyl stretching and hydrogen bond carbonyl groups, respectively, further indicating the formation of the functional urethane groups. The NMR and FTIR results confirmed the formation of PEG(-PAU)$_4$ copolymer. Furthermore, the molecular weight of copolymers and their distributions were determined by GPC. Fig. 3 shows the GPC results of 4-arm PEG (M_n = 2000) and PEG(-PAU)$_4$ (P-03) copolymer. The above characterizations clearly indicate the successful synthesis of the PEG(-PAU)$_4$ copolymers. 4-arm PEG with the molecular weight of 2000 (in Scheme 1, n = 45) was used for all experiments and copolymers with different of PAU block length were achieved by changes in the feed ratios of the reactants. The repeat unit in PAU (x value in Scheme 1) of copolymer P-01, P-02 and P-03 are 5.85, 8.75 and 11.70, respectively. The characteristics of the synthesized copolymers are summarized in Table 1.

3.2. Acid–base titration

The pK_a of the synthesized copolymers were studied by the acid–base titration method. Fig. 4 shows the acid–base titration

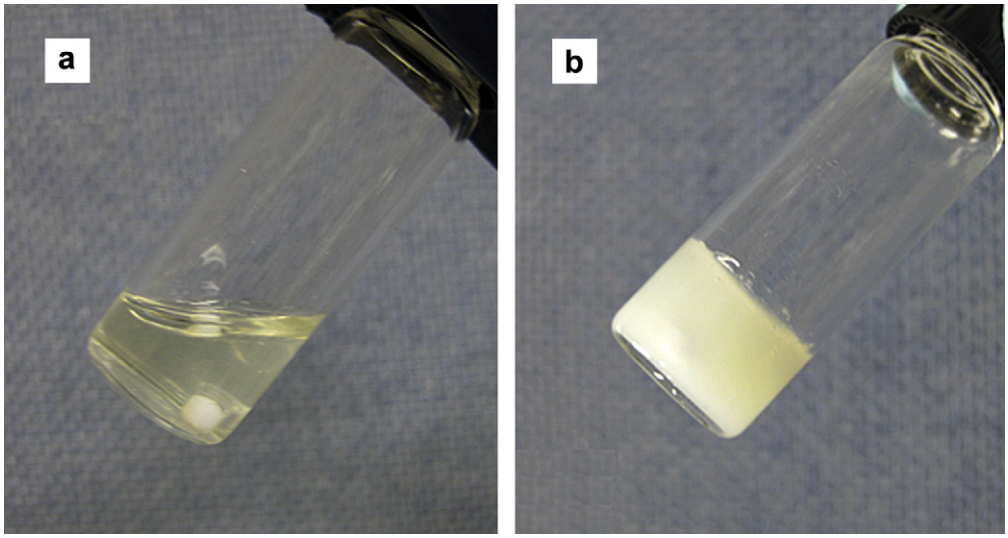

Fig. 7. Photographs of in vitro sol–gel transition of the 20 wt% copolymer solution (P-03) at pH 7.4 upon heating: a) sol at 10 °C and b) gel at 37 °C.

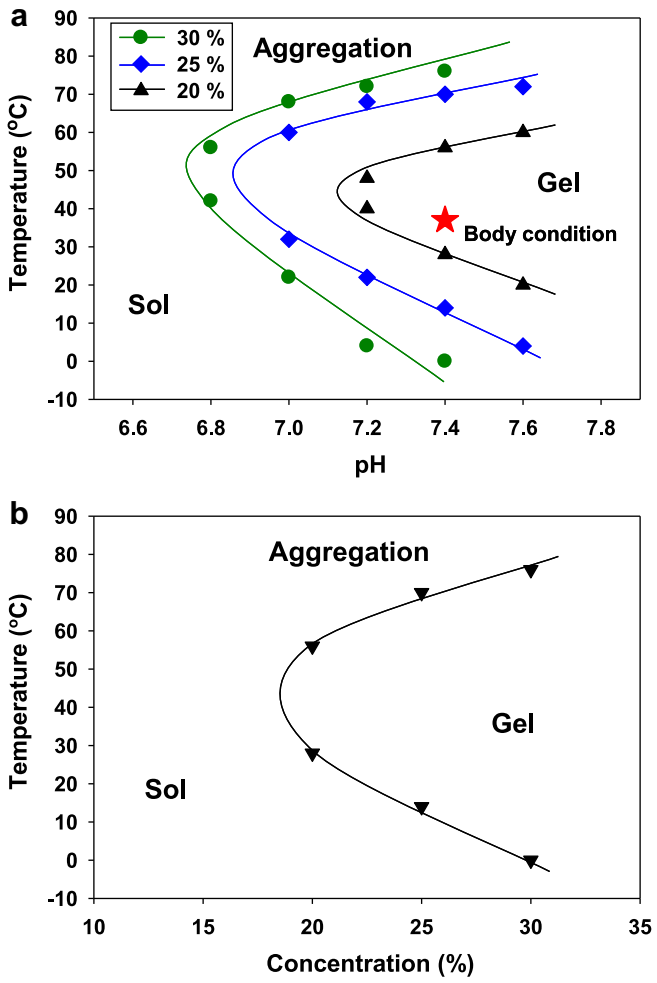

Fig. 8. Sol–gel phase transition diagram of copolymer P-03 with different concentrations. a) Various copolymer concentrations at various pH values, b) Various copolymer concentrations at pH 7.4.

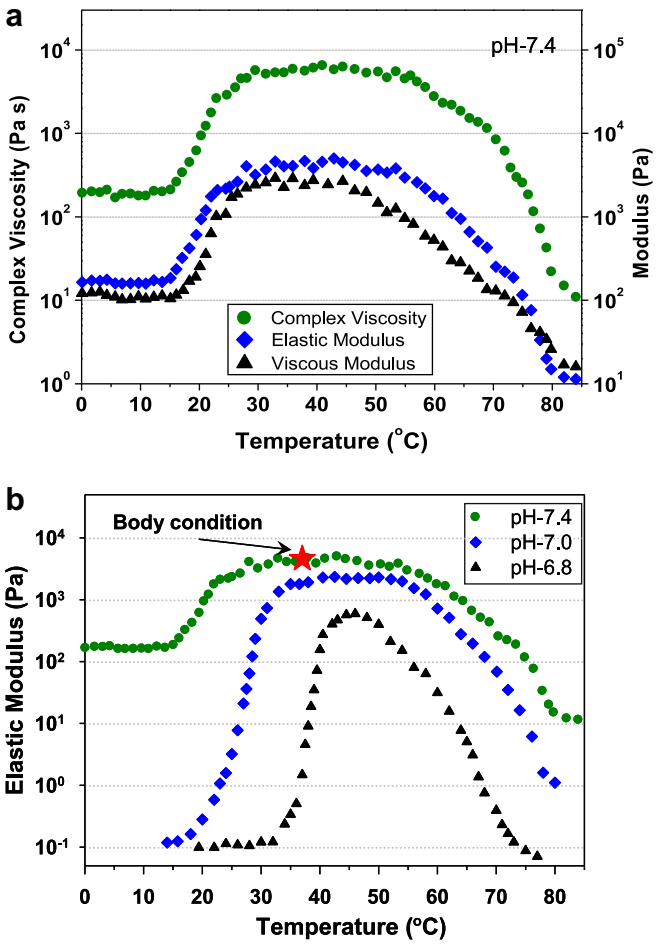

Fig. 9. Rheological properties of 30 wt% copolymer aqueous solution (P-03): a) At pH 7.4, b) At different pH values.

profiles of the copolymers containing different PAU block length. The buffering property of the copolymers was confirmed. The pK_a of copolymers increase from 6.44 to 6.62 and 6.75 with the increase PAU block length of each arm from 500 to 750 and 975 (P-01 to P-02 and P-03, respectively), due to the increase of the functional tertiary amine group of PAU. The pK_a of synthesized copolymers are listed in the Table 1.

3.3. Sol–gel phase transition of copolymers

The sol–gel phase transitions of the PEG(-PAU)$_4$ copolymers in aqueous solution were examined by the tube inverting method under various pH and temperature conditions. The PAU block acts as a pH/temperature-sensitive block [7,12], while the 4-arm PEG acts as a crosslinker between physical crosslinks of PAU blocks [20]. Fig. 5 shows the sol–gel phase transition of the copolymers solution (30 wt%) with different PAU block lengths (P-01, P-02 and P-03). The copolymer solution showed a sol-to-gel-to-aggregation transition as a function of pH and temperature. For the P-02 copolymer, at low pH (such as pH 6.6), the PAU blocks were hydrophilic, because of the ionization of the tertiary amine groups. The electrostatic repulsion between the charged PAU blocks weakened the interactions between the copolymers [7,12]. Thus, the copolymer solutions existed in the sol state in the experiment range of temperature (0–80 °C). At higher pH values (such as pH 7.4) the PAU blocks were deionized. However, at low temperatures (such as 10 °C), the copolymer solutions existed in the sol state because of the weak hydrophobic interactions between the deionized PAU blocks. With increasing temperature, the hydrophobicity of the system increased, and the copolymer solution turned into a gel. This result was supported by our previous study which reported that the PAU block was less hydrophobic at low temperature but the hydrophobicity was increased with increasing temperature [7]. Fig. 5 also shows the influence of the PAU block length on the gel region. With increasing PAU block length of each arm from 500 to 750 and 975 (P-01 to P-02 and P-03, respectively), the gel region became wider and shifted to lower pH values, due to the increase in the interactions between the hydrophobic PAU blocks [13,14].

The PEG(-PAU)$_4$ copolymer in aqueous solution exhibited reversible properties in response to both pH and temperature. At room temperature, the hydrogel precursor existed in the sol state at low pH values (such as pH 6.5) and changed to a gel when the pH was increased (such as to pH 7.4), but the hydrogel rapidly turned into a solution again upon the addition of HCl to decrease pH (such as 6.5). In addition, the copolymer solutions at neutral pH (such as pH 7.0, 10 °C) rapidly turn into a gel when the temperature was increased to 40 °C. However, the switching from a gel to a sol with decreasing temperature to 10 °C would be expected to be slower (1 day), due to the strong hydrogen bonds between the PAU segments.

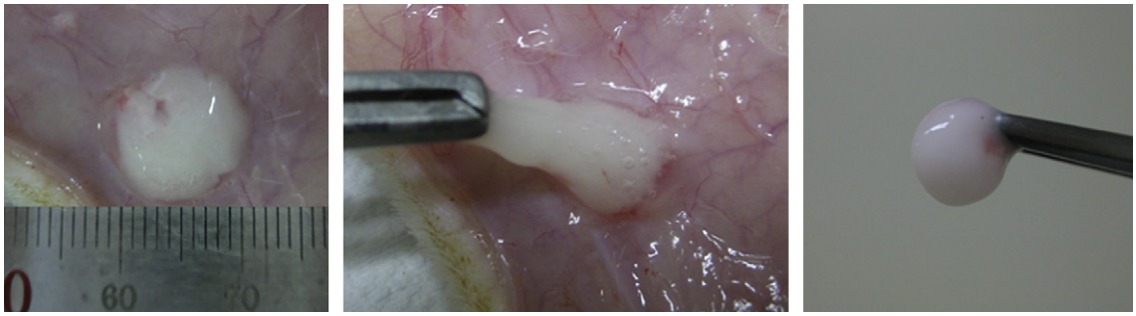

Fig. 10. Photographs of in situ gel formation 5 min after subcutaneously injection of 30 wt% copolymer solution (pH 6.8, P-03) into a rat.

A schematic diagram of the sol–gel mechanism of PEG(-PAU)$_4$ is depicted in Fig. 6. At 10 °C and pH 6.6, the copolymer was a solution, due to the hydrophilic character of the PAU blocks. In contrast, at higher pH and temperature (pH 7.4 and 37 °C), the hydrophobic interactions between the PAU blocks led to the formation of a microscopic domain, resulting in gelation. Fig. 7 shows a photograph of the sol-to-gel transition of the copolymer solutions (20 wt %, P-03) in vitro at pH 7.4 when the temperature was increased from 10 °C (clear sol state) to 37 °C (turbid gel state). The copolymer concentration of 20 wt% was chosen because of the gelation of 30 wt% P-03 at pH 7.4 and 0–78 °C.

Fig. 8 shows the sol–gel transition of the PEG(-PAU)$_4$ copolymer (P-03) solution resulting from the changes in pH and temperature at various concentrations. The gel window became broader and shifted to lower pH values as the polymer concentration was increased from 20 to 30 wt%. This was attributed to the increase in the crosslinker density and the number of interactions between hydrophobic PAU blocks.

3.4. Viscosity

Dynamic rheological analysis was employed to confirm the sol–gel phase transition of the copolymers in solution (Fig. 9). Fig. 9a shows the variation of the complex viscosity, elastic modulus (G′) and viscous modulus (G″) of the copolymer solution (30 wt%, P-03) with temperature at pH 7.4. The copolymer solution exhibited an abrupt increase in viscosity at 16 °C. However, G′ was higher than G″ over the whole range of experiment temperatures (0–78 °C), indicating that the copolymer in solution existed in the gel state at pH 7.4. According to Fig. 9a, at the temperature higher than 50 °C, the G′ and G″ show the fast decrease because of the dehydration of PEG at high temperature [11]. In addition, the moduli of the P-03 solutions (30 wt%) at different pH values were investigated. As shown in Fig. 9b, the temperature at which the viscosity abruptly increased was shifted from 16 to 23 and 37 °C when the pH value was decreased from 7.4 to 7.0 and 6.8, respectively. This indicates that the pH affected the sol–gel transition.

3.5. In vivo gel morphology

To investigate the injectability and in vivo gelation, 200 μL of the copolymer solution (30 wt%, P-03) at pH 6.8 and 20 °C was subcutaneously injected into the back of a male SD rat. 5 min after the injection, the rat was sacrificed and the morphology of the gel was observed. As shown in Fig. 10, a white gel formed in situ in a short time as a result of the pH and temperature changes caused by the rat's body condition. This result indicates that the copolymer solution can be easily injected into the body and rapidly form a gel in situ.

3.6. In vitro release of chlorambucil

Chlorambucil, an anticancer drug, was employed as a model drug to examine the drug release behavior of the PEG(-PAU)$_4$ copolymer hydrogels (P-03, 30 wt%) under physiological conditions (37 °C, pH 7.4). The drug loading concentrations were 4 mg mL^{-1} and 8 mg mL^{-1}. Fig. 11 shows the cumulative release of chlorambucil. 80% of the model drug was released from the hydrogels over 14 days. The in vitro release profiles indicate that the copolymer hydrogels offers sustained release of the hydrophobic drug under physiological conditions.

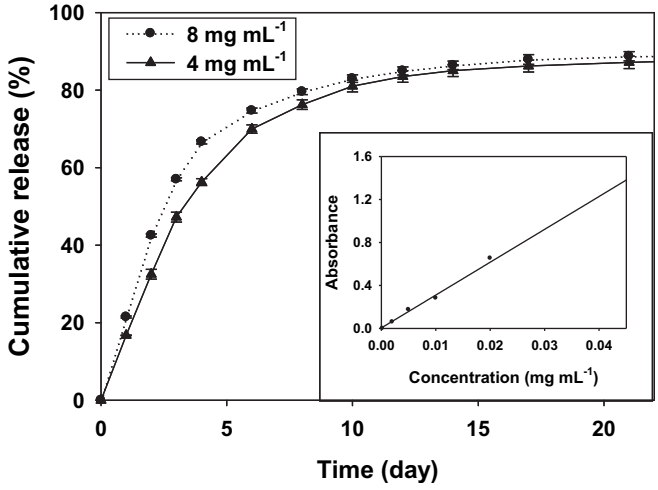

Fig. 11. In vitro release of chlorambucil from the 30 wt% copolymer hydrogels (P-03) at physiological conditions. The standard calibration line is shown in the inset.

4. Conclusion

A series of novel pH/temperature-sensitive PEG(-PAU)$_4$ copolymers was synthesized and characterized. The copolymers in aqueous solution showed a sol-to-gel-to-aggregation phase transition as a function of pH and temperature. The gel can be formed with the short length of PAU block and low molecular weight (4000–6000). The gel window covers the physiological conditions (37 °C, pH 7.4). The sol–gel phase transition could be controlled by varying the PAU block length and copolymer concentration. After injecting the copolymer solution into a rat, a gel formed in situ within a short time. The in vitro release of chlorambucil, an anticancer drug, was sustained over 14 days under physiological conditions. This copolymer hydrogels have the potential to be use as injectable carriers for sustained drug delivery systems.

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