# Temperature-Sensitive Poly(caprolactone-co-trimethylene carbonate)–Poly(ethylene glycol)–Poly(caprolactone-co-trimethylene carbonate) as in Situ Gel-Forming Biomaterial

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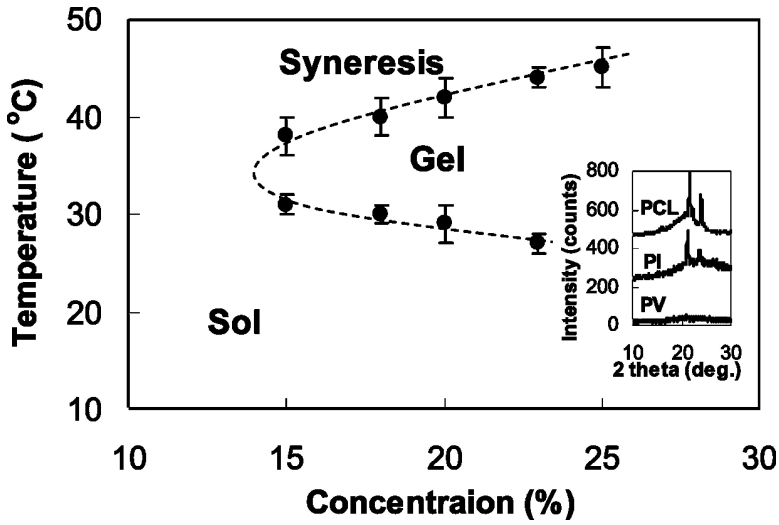


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# Temperature-Sensitive Poly(caprolactone-*co*-trimethylene carbonate)−Poly(ethylene glycol)−Poly(caprolactone-*co*-trimethylene carbonate) as in Situ Gel-Forming Biomaterial

**So Hyun Park,[†] Bo Gyu Choi,[†] Min Kyung Joo,[†] Dong Keun Han,[‡] Youn Soo Sohn,[†] and Byeongmoon Jeong*,[†]**

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ABSTRACT: We are reporting a new thermogelling poly(caprolactone-*co*-trimethylene carbonate)−poly(ethylene glycol)−poly(caprolactone-*co*-trimethylene carbonate) (PCTC−PEG−PCTC) triblock copolymer. By incorporating the comonomer (trimethylene carbonate) up to 25−40 wt % in the polycaprolactone block, the resulting PCTC−PEG−PCTC triblock copolymer achieved sol stability while keeping the thermogelling property in a physiologically important temperature range of 10−50 °C. The precipitation of the polymer in water at 20 °C was a concern for a poly(caprolactone)−poly(ethylene glycol)−poly(caprolactone) (PCL−PEG−PCL) system for practical applications (*Macromolecules* 2006, *39*, 4873). The PCTC−PEG−PCTC triblock copolymer thermogel did not show the characteristic peaks of the polycaprolactone crystalline domain at $2\theta = 21.5°$ and 23.5° in the X-ray diffraction spectra. The sol-to-gel transition temperature of PCTC−PEG−PCTC aqueous solution could be controlled in a range of 15−30 °C by varying the composition and molecular weight of PCTC. The PCTC−PEG−PCTC was not degraded in a phosphate buffer saline for 50 days, whereas significant changes in molecular weight and composition of the polymer were observed for the polymer implanted in the subcutaneous layer of rats over the same period of time. The in situ formed hydrogel of PCTC−PEG−PCTC was investigated as a three-dimensional cell culture medium. Compared with the traditional culture on a plate (two-dimensional culture), the chondrocytes cultured in the in situ formed PCTC−PEG−PCTC hydrogel (three-dimensional culture) showed a higher expression of collagen type II and aggrecan and suppression of collagen type I (fibroblastic gene), suggesting the excellent differentiation of the cell in the PCTC−PEG−PCTC hydrogel.

## Introduction

Thermogelling aqueous solutions of biodegradable polymers have recently been drawing attention as an implantable depot for sustained drug release and tissue engineering applications.[1,2] The aqueous polymer solution is a low viscous sol at room temperature (20 °C) or lower and forms a gel at body temperature (37 °C). Poly(ethylene glycol)/poly(lactic acid-*co*-glycolic acid) triblock and graft copolymers,[3,4] poly(ethylene glycol)/poly(propylene fumarate),[5] chitosan/glycerol phosphate,[6] polyphosphazene,[7] multiblock copolymers of poly(ethylene glycol) with poly(propylene glycol), poly(L-lactic acid), or poly(3-hydroxybutyrate),[8−10] and poly(ethylene glycol)/poly(trimethylene carbonate)[11] are examples of thermogelling biodegradable polymers. The control of critical gel concentration, phase diagram, mechanism of sol−gel transition, and their biomedical applications were extensively investigated. Especially, the fine-tuning of the sol−gel transition temperature by end-group modifications and a polymer with a very low critical gel concentration also have been recently reported.[12−14] We also reported poly(caprolactone-*b*-ethylene glycol-*b*-caprolactone) (PCL−PEG−PCL) as a biodegradable thermogelling polymer.[15] The triblock copolymer has a powder morphology at room temperature and is thus not only simple to transfer or weigh but also easily dissolved in water. However, the fact that the polymer aqueous solution becomes an opaque gel in 30 min at 20 °C was a concern unless the injection is performed quickly after the redissolution of the polymer in water. Multiblock copolymers that were synthesized by coupling the PCL−PEG−PCL triblock copolymers showed partially improved sol stability over the PCL−PEG−PCL triblock copolymers.[16] However, the precise control of molecular weight of the multiblock copolymer is not easy because a small difference in the progress of the coupling reaction can lead to a large difference in the molecular weight of the multiblock copolymer.

In this research, we assumed that the copolymerization of the trimethylene carbonate and caprolactone would increase the amorphous character of the material and thus avoided the precipitation problem shown in the PCL−PEG−PCL triblock copolymer aqueous solution. We designed a poly(caprolactone-*co*-trimethylene carbonate)−poly(ethylene glycol)−poly(caprolactone-*co*-trimethylene carbonate) (PCTC−PEG−PCTC) triblock copolymer. Even though both poly(glycolic acid) and poly(L-lactic acid) are crystalline polymers, their copolymers are amorphous when the L-lactic acid composition is in a range of 25−75 wt %.[17] The PEG molecular weight was fixed at 1000 Da considering the previous thermogelling PCL−PEG−PCL (1000−1000−1000) triblock copolymer.[15]

The objectives of this study are to (1) find an composition of caprolactone and trimethylene carbonate to give the PCTC−PEG−PCTC triblock copolymer sol stability as well as the thermogelling property, (2) to confirm that the crystallinity of the polycaprolactone is related to the sol stability of the polymer, (3) to study the structure−property relationship of the sol−gel transition by varying composition and block length of PCTC, (4) to check degradability of the PCTC−PEG−PCTC triblock copolymer in phosphate buffer saline as well as in the subcutaneous layer of a rat, and (5) to investigate the feasibility of the in situ formed PCTC−PEG−PCTC triblock copolymer hydrogel as a three-dimensional cell culture system.

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**Table 1. Sequences of PCR Primers**

| target genes[a] | forward primer (5′-3′)reverse primer (5′-3′) | size (bp) |
|---|---|---|
| collagen type I | GGACCTCAAGATGTGCCACT<br>CTTGGGGTTCTTGCTGATGT | 178 |
| collagen type II | GCACCCATGGACATTGGAGGG<br>GACACGGAGTAGCACCATCG | 366 |
| aggrecan | GAGGTCGTGGTGAAAGGTGT<br>GTGTGGATGGGGTACCTGAC | 206 |
| SOX-9 | TTCATGAAGATGACCGACGA<br>GTCCAGTCGTAGCCCTTGAG | 203 |
| GAPDH | AGGTCATCCACGACCACTTC<br>GTGAGTTTCCCGTTCAGCTC | 202 |

[a] GAPDH: D-glycerol aldehyde-3-phosphate dehydrogenase. SOX-9: sex-determining region box-9.

**Table 2. List of the Polymers**

| | structure[a] | $M_n$[b] | $M_n$[c] | $M_w/M_n$[c] |
|---|---|---|---|---|
| PI | $(CL)_{8.8}-(EG)_{22.7}-(CL)_{8.8}$ | 1000−1000−1000 | 2900 | 1.3 |
| PII | $(CL)_{5.9}(TMC)_{2.4}-(EG)_{22.7}-(CL)_{5.9}(TMC)_{2.4}$ | 910−1000−910 | 3200 | 1.3 |
| PIII | $(CL)_{7.7}(TMC)_{2.7}-(EG)_{-}(CL)_{7.7}(TMC)_{2.7}$ | 1160−1000−1160 | 3400 | 1.2 |
| PIV | $(CL)_{8.4}(TMC)_{3.8}-(EG)_{22.7}-(CL)_{8.4}(TMC)_{3.8}$ | 1350−1000−1350 | 3300 | 1.4 |
| PV | $(CL)_{7.5}(TMC)_{4.8}-(EG)_{22.7}-(CL)_{7.5}(TMC)_{4.8}$ | 1350−1000−1350 | 3600 | 1.3 |
| PVI | $(TMC)_{9.8}-(EG)_{22.7}-(TMC)_{9.8}$ | 1000−1000−1000 | 3400 | 1.3 |

[a] CL, TMC, and EG indicate the caprolactone, trimethylene carbonate, and ethylene glycol repeating units in the PCTC−PEG−PCTC triblock copolymers. [b] Determined by $^1$H NMR ($CDCl_3$). [c] Determined by gel permeation chromatography relative to poly(ethylene glycol) standards.

We investigated the phase behavior of the polymer solution using various instrumental methods such as rheometry, UV−vis spectroscopy, X-ray diffraction, and $^{13}$C NMR spectroscopy, and the degradation of the polymer was studied by gel permeation chromatography and $^1$H NMR spectroscopy. The gene expression of chondrocytes cultured in the PCTC−PEG−PCTC triblock copolymer hydrogel (three-dimensional culture) was compared with that cultured on the traditional cell culture plate (two-dimensional culture).

## Materials and Experimental Methods

**Materials.** $\epsilon$-Caprolactone, trimethylene carbonate, stannous octoate, poly(ethylene glycol) (PEG) (MW = 1000 Da), and 1,6-diphenyl-1,3,5-hexatriene were used as received from Aldrich. Toluene (Aldrich) was distilled over sodium before use. Cell culture medium (Dulbecco's modified Eagle's medium) and heat-inactivated fetal bovine serum were purchased from Gibco (Germany).

**Triblock Copolymer Synthesis.** The PCTC−PEG−PCTC triblock copolymer was prepared by ring-opening polymerization of caprolactone and trimethylene carbonate in the presence of PEG as an initiator and stannous octoate as a catalyst. For example, to synthesize the PCTC−PEG−PCTC triblock copolymer (PV in Table 2), the PEG (MW = 1000) (8.5 g, 8.5 mmol) was dissolved in anhydrous toluene (100 mL), and the solvent was distilled off to a final volume of 50 mL. $\epsilon$-Caprolactone (16.9 g, 148.1 mmol) and trimethylene carbonate (7.6 g, 74.5 mmol) were added, and then 20 mL of toluene was distilled off to remove the residual water in the reaction system. Then, stannous octoate (32 $\mu$L, 0.08 mmol) were added to the reaction mixtures and stirred at 120 °C for 20 h. The product was precipitated into diethyl ether. The polymer was redissolved in methylene chloride and then precipitated by slowly adding diethyl ether. The residual solvent was removed under vacuum. The final yield was about 75%. FTIR ($cm^{-1}$): 2943, 2868, 1737, 1461, 1402, 1356, 1259, 1164, 1105, 1038, 953. $^1$H NMR ($CDCl_3$) of PCTC−PEG−PCTC triblock copolymer: $\delta$ 1.1−1.5 ($-OCH_2CH_2\mathit{CH_2}CH_2CH_2CO-$); $\delta$ 1.6−1.7 ($-OCH_2\mathit{CH_2}CH_2$-$\mathit{CH_2}CH_2CO-$) and ($OCH_2\mathit{CH_2}CH_2COO$); $\delta$ 1.7−2.2 ($OCH_2CH_2$-$\mathit{CH_2}COO$); $\delta$ 2.2−2.5 ($OCH_2CH_2CH_2CH_2\mathit{CH_2}CO$); $\delta$ 3.5−3.8 ($-O\mathit{CH_2CH_2}-$); $\delta$ 4.0−4.4 ($-O\mathit{CH_2}CH_2CH_2CH_2CH_2CO-$), ($O\mathit{CH_2}CH_2CH_2COO$), and ($-CH_2\mathit{CH_2}O$-connected to PCTC).

Other polymers were similarly prepared, and the peaks at 1.7−2.2 ppm (trimethylene carbonate), 2.2−2.5 ppm (caprolactone), and 3.5−3.8 ppm (PEG) were used to calculate the number-average molecular weight and composition of the PCTC−PEG−PCTC triblock copolymers.

**Gel Permeation Chromatography.** The gel permeation chromatography system (Waters 515) with a refractive index detector (Waters 410) was used to obtain the molecular weights and molecular weight distributions of the PCTC−PEG−PCTC triblock copolymers. *N,N*-Dimethylformamide was used as an eluting solvent. The poly(ethylene glycol)s in a molecular weight range of 400−20 000 Da were used as the molecular weight standards. An OHPAK SB-803QH column (Shodex) was used.

**NMR Study.** A 250 MHz NMR spectrometer (9503DPX; Bruker) was used for $^1$H NMR (in $CDCl_3$) to study composition of the polymers, and a 500 MHz NMR spectrometer (Unity-inova; Varian) was used for $^{13}$C NMR to see spectral changes of the PCTC−PEG−PCTC triblock copolymers (in $D_2O$) as a function of temperature.

**Phase Diagram.** The sol−gel transition of the PCTC−PEG−PCTC triblock copolymer aqueous solution was investigated by the falling ball method.[18,19] The aqueous polymer solution and a steel ball (diameter ($D$) = 4.0 mm, density ($\rho_s$) = 6.4 g/cm$^3$) were put in the NMR tube with an inner diameter of 4.2 mm. The time for the steel ball to fall 5.0 cm was measured with a temperature increment of 2 °C per step. The temperature at which the elapsed time of the steel ball abruptly increased was selected as the sol−gel transition temperature. The syneresis temperature was determined by visual observation that macroscopic phase separation occurred between water and polymer. The sol−gel transition temperature and syneresis temperature were statistically treated by the three measurements.

**Dynamic Mechanical Analysis.** The sol−gel transition of the polymer aqueous solution was also investigated by dynamic mechanical analysis (Rheometer RS 1; Thermo Haake).[18,20] The aqueous polymer solution (25 wt %) was placed between parallel plates of 25 mm diameter and a gap of 0.5 mm at 10 °C for 20 min. The data were collected under a controlled stress (4.0 dyn/cm$^2$) at a frequency of 1.0 rad/s. The heating rate was 0.5 °C/min.

**UV−vis Spectroscopy.** PCL−PEG−PCL (PI) and PCTC−PEG−PCTC (PII to PV) triblock copolymer aqueous solutions (25 wt %) were prepared, and the absorbance at 500 nm was monitored at 20 °C as a function of time using UV−vis spectrophotometer (SCINCO: S-3130). Absorbance was an average of the three measurements.

**X-ray Diffraction Spectra.** The PCL−PEG−PCL and PCTC−PEG−PCTC triblock copolymer aqueous solutions (25 wt %) were kept at room temperature for 60 min. The X-ray diffraction data of the polymer solution or gel were recorded with a Rigaku SWXD diffractometer using Cu K$\alpha$ radiation at a scanning rate of 1°/min at room temperature (20 °C). The X-ray diffraction spectra of polycaprolactone (MW = 1250) and poly(ethylene glycol) (MW = 1000) were also studied for comparison.

**Dye Solubilization.** 1,6-Diphenyl-1,3,5-hexatriene (DPH) solution in methanol (10 $\mu$L at 0.4 mM) was injected into an PCTC−PEG−PCTC triblock copolymer (PV) aqueous solution (1.0 mL) in a polymer concentration range of 0.000 05−1.0 wt %. Using a UV−vis spectrophotometer (SCINCO: S-3130), the absorption spectra of the solutions were recorded from 300 to 400 nm to see the solubilization of the dye. Increases in the absorbance at 340, 356, and 378 nm are typical phenomena of the solubilization of the dye in the micelle.[21,22] The critical micelle concentration was determined by absorbance at 378 nm relative to 400 nm.

**Dynamic Light Scattering.** The apparent size of PCTC−PEG−PCTC triblock copolymer micelles was studied by a dynamic light scattering instrument (Zetasizer nano ZS; Malvern) as a function of temperature at a concentration of 0.03 wt % that was above the critical micelle concentration of the polymer in water. A He−Ne laser operating at 633 nm was used as a light source. Measurements of scattered light were made at an angle of 90° to the incident beam.

The results of dynamic light scattering were analyzed by the regularized CONTIN method. The decay rate distributions were transformed to an apparent diffusion coefficient. From the apparent diffusion coefficient, the apparent hydrodynamic radius of the polymer micelle was obtained by the Stokes−Einstein equation.

**In Situ Gel Formation and Degradation.** Aqueous solution (25 wt %) of the PCTC−PEG−PCTC triblock copolymer (PV) was prepared by heating the polymer in water to 60 °C in a bath for 30 s followed by quenching in an ice bath for 30 s. The polymer aqueous solution (0.5 mL) was injected into a 4.0 mL vial (inner diameter = 11 mm), which was thermostated at 37 °C to form a gel. After 2 min, 3.0 mL of the phosphate buffer saline (150 mM, pH = 7.4) containing $NaN_3$ (0.02 wt %) at 37 °C was added to the preformed gel, and the vial was shaken at 100 strokes/min. The buffer at 37 °C was replaced daily by a fresh one (3.0 mL). To confirm the injectability in a gel state, the polymer aqueous solution (PV; 0.5 mL, 25 wt %) was heated to 37 °C in the bath for 2 min, and the gel was injected through a syringe with a 21-gauge needle.

For in vivo study, the PCTC−PEG−PCTC triblock copolymer (PV) aqueous solution (25.0 wt %, 0.5 mL) was heated to 37 °C in a syringe with a 21-gauge needle for 2 min to form a gel and then subcutaneously injected the preformed gel into healthy rats of body weight of about 200 g. After taking the sample at a given time interval, each rat was sacrificed. The samples were freeze-dried, and the polymer fraction was extracted with chloroform (5.0 mL). Chloroform was evaporated under vacuum for further analyses.

**Chondrocyte Isolation and Three-Dimensional Cell Culture.** Chondrocytes were isolated from the knee articular cartilage of 4-week-old New Zealand white rabbits by collagenase digestion.[23,24] Isolated chondrocytes were monolayer cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin under 5% $CO_2$ at 37 °C atmosphere and then subcultured to passage 1. Harvested cell (passage 1) suspension ($1 \times 10^6$ cells) was mixed with PCTC−PEG−PCTC aqueous solution (0.25 mL) to a final polymer concentration of 25 wt % and incubated in 24-well culture plate at 37 °C/5 min to derive sol-to-gel transition. Dulbecco's Modified Eagle Medium (0.5 mL) containing 10% fetal bovine serum and 1% penicillin/streptomycin was added on the cell-encapsulated PCTC−PEG−PCTC hydrogel under 5% $CO_2$ at 37 °C atmosphere and replaced every 3 days. Chondrocytes were also monolayer cultured in the plate without PCTC−PEG−PCTC for comparison.

**RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR).[24,25]** After 1, 14, 21, and 28 days of culture, the total RNA was extracted from the cell encapsulated PCTC−PEG−PCTC hydrogel using the $TRI_{ZOL}$ reagent (Invitrogen), according to the manufacturer's instructions. The extracted RNA pellet was dissolved in nuclease-free water, and the RNA concentration was determined using a UV−vis spectrophotometer (SCINCO: S-3130) at 260 nm. For the expression of cartilage-specific genes, total RNA (0.2 $\mu$g) was amplified from the chromosomal DNA by the Maxime PCR PreMix (INTRON, Korea). The PCR amplifications were carried out for 35 cycles under the following conditions: 30 s at 95 °C for denaturation, 45 s at 57 °C for annealing, and 30 s at 72 °C for extension. The PCR products were electrophoresed in a 1.2 wt % agarose gel and visualized by SYBR green. PCR primers are summarized in Table 1.

## Results and Discussion

The PCTC−PEG−PCTC triblock copolymer was prepared by ring-opening polymerization of $\epsilon$-caprolactone and trimethylene carbonate on the PEG in the presence of stannous octoate as a catalyst. The peaks at 3.5−3.8 ppm (ethylene of PEG), 2.2−2.5 ppm (methylene of the caprolactone next to carbonyl), and 1.7−2.2 ppm (methylene of the trimethylene carbonate next to carbonyl) in the $^1$H NMR spectra were well separated and used to calculate the composition of the triblock copolymer (Supporting Information, Figure S1-a). The IR spectra show the ether peak at 1000−1300 $cm^{-1}$ and the carbonyl peak at 1730−1740 $cm^{-1}$. The ester of the caprolactone and carbonate

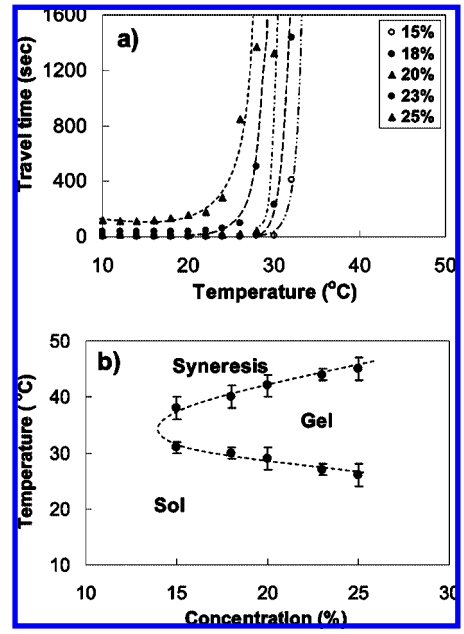


**Figure 1.** (a) Travel time of a steel ball through a sol or gel phase as a function of temperature and concentration. (b) Phase diagram of the PCTC−PEG−PCTC (PV) aqueous solution determined by the falling ball method. Each data point is an average of the three measurements.

of the trimethylene carbonate were not separated in the IR spectra (Supporting Information, Figure S1-b). On the basis of the principle of equal reactivity, the block length and composition of both sides of the PEG were assumed to be identical.[26] The list of polymers studied in this research is summarized in Table 2. The crystallization during the mixing of the polymer aqueous solution with cells is inconvenient and may be harmful to the cells. The goal of this study was to find out a PCTC−PEG−PCTC triblock copolymer that has not only a thermogelling property but also sol stability, which was a concern for the PCL−PEG−PCL (1000−1000−1000) triblock copolymer system, and investigate its application as a three-dimensional cell culture system.[15] First, the PEG molecular weight was fixed at 1000 Da, and the composition of caprolactone/trimethylene carbonate was varied at a fixed total molecular weight of the PCTC−PEG−PCTC triblock copolymer (PIV and PV in Table 2). Second, the composition of caprolactone/trimethylene carbonate was fixed at 75/25 (by wt %), and the total molecular weight of hydrophobic block (PCTC) was varied (PII and PIII in Table 2). In addition, the poly(trimethylene carbonate)−poly(ethylene glycol)−poly(trimethylene carbonate) (1000−1000−1000) triblock copolymer (PVI in Table 2) was synthesized for comparison.

The aqueous solution of the PCTC−PEG−PCTC underwent clear sol-to-turbid gel-to-syneresis transitions as the temperature increased. The sol-to-gel transition accompanied an abrupt change in the viscosity. Therefore, the sol-to-gel transition temperature was determined by the falling ball method which measured the travel time of a steel ball through a finite distance (5 cm) of a sol or gel phase (Figure 1a).[18,19] The syneresis temperature was determined by visual observation of macroscopic phase separation between water and polymer. After heating the polymer aqueous solution over the syneresis temperature, it could be returned to a sol phase again by cooling it to a low temperature by putting the vial in an ice bath. The same transition temperatures were observed when the sol was reheated in the same manner as the first heating cycle, indicating the reversibility of the phase transition. A typical phase diagram

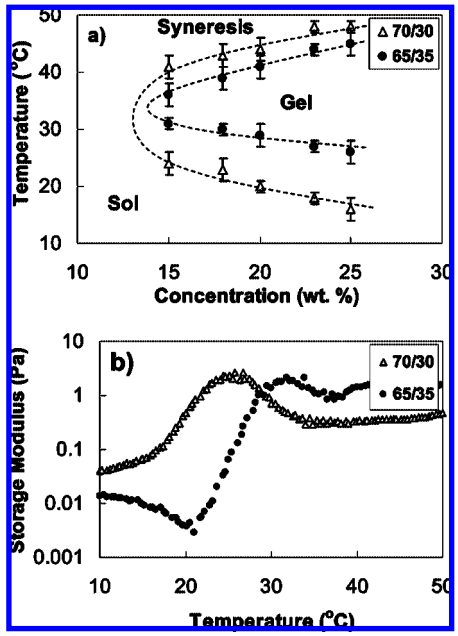


**Figure 2.** (a) Effect of the composition of the hydrophobic block on the phase diagram of the PCTC−PEG−PCTC (1350−1000−1350). The ratio of caprolactone/trimethylene carbonate (by weight) is shown as the legends. 70/30 and 65/35 are PIV and PV in Table 2, respectively. Each data point is an average of the three measurements. (b) The storage modulus of the PCTC−PEG−PCTC aqueous solution (25 wt %) as a function of temperature.

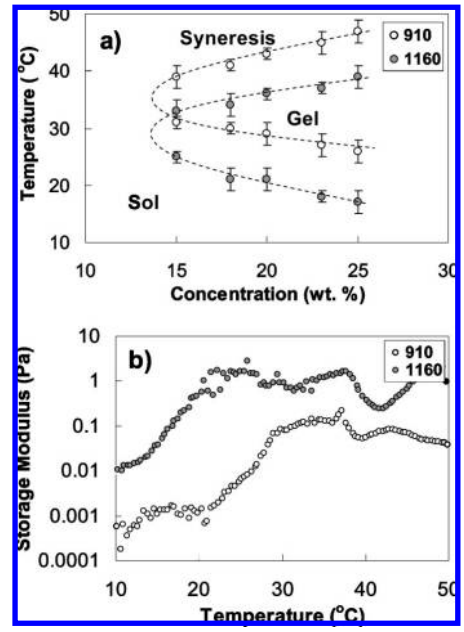


**Figure 3.** (a) Effect of the molecular weight of the PCTC block on the phase diagram of the PCTC−PEG−PCTC. The molecular weight of PCTC is shown as the legends (910 (PII) and 1160 (PIII)), respectively. The molecular weight of PEG is 1000, and the ratio of caprolactone/trimethylene carbonate is 75/25. Each data point is an average of the three measurements. (b) Storage modulus of the PCTC−PEG−PCTC aqueous solution (25 wt %) as a function of temperature.

of the PCTC−PEG−PCTC (PV) aqueous solution is shown in Figure 1b.

To investigate the effect of polymer composition on the phase diagram, the molecular weight of the PCTC−PEG−PCTC was fixed at 1350−1000−1350 and the caprolactone/trimethylene carbonate composition was varied from 70/30 (PIV) to 65/35 (PV). The sol-to-gel transition temperature increased and the gel window decreased as the trimethylene carbonate composition increased, suggesting that the trimethylene carbonate is less hydrophobic than caprolactone (Figure 2a). The poly(trimethylene carbonate)−poly(ethylene glycol)−poly(trimethylene carbonate) (1000−1000−1000: PVI) triblock copolymer is soluble in water and did not undergo a sol-to-gel transition as the temperature increased, whereas the PCL−PEG−PCL (1000−1000−1000) underwent a sol-to-gel transition in the physiologically important temperature range of 10−50 °C. This fact also suggests that the poly(trimethylene carbonate) is intrinsically less hydrophobic than the polycaprolactone. The maximal storage modulus was observed at 22−28 °C for the PCTC−PEG−PCTC thermogel with a 70/30 (caprolactone/trimethylene carbonate) composition and 31−36 °C for the PCTC−PEG−PCTC thermogel with a 65/35 (caprolactone/trimethylene carbonate) composition (Figure 2b). Hydrogel can be defined as the polymer that is not dissolved and keeps its integrity in the excess amount of water. We proved the PCTC−PEG−PCTC (65/35: 25 wt % in water) as an in situ gelling material for chondrocyte 3D culture as would be discussed in next section. The gel was stable and kept its integrity for more than 50 days, even though the excess amount of phosphate buffer saline was replaced daily. Therefore, the in situ formed PCTC−PEG−PCTC gel can be defined as a hydrogel even though the gel has rather a low storage modulus.

When the molecular weight of hydrophobic block (PCTC) increased at the fixed composition of caprolactone/trimethylene carbonate (75/25 by wt %), the sol-to-gel transition temperature decreased and the storage modulus of the gel increased (Figure 3a,b). For example, the transition temperature of the polymer aqueous solution (25 wt %) decreased from 26 to 17 °C by increasing the molecular weight of the hydrophobic block (PCTC) from 910 to 1160.

The increase in the hydrophobicity of the PCTC−PEG−PCTC, which was induced by increasing PCTC block length or by increasing caprolactone/trimethylene carbonate ratio, lowered the sol-to-gel transition temperature and increased the maximal storage modulus of the gel. Such trends suggest that the thermal gelation of the PCTC−PEG−PCTC triblock copolymer is driven by hydrophobic interactions.

The stability as a sol phase at room temperature (20 °C) was measured by absorbance at 500 nm (Figure 4a). The initially clear sol of PCL−PEG−PCL turned into a turbid sol and then a turbid gel at 20 °C over 30 min. As the composition of trimethylene carbonate in the PCTC increased, the sol stability of the PCTC−PEG−PCTC triblock copolymer aqueous solution was significantly improved. The triblock copolymers of PCTC−PEG−PCTC (PII, PIV, and PV) aqueous solutions formed a clear sol even after several days. The X-ray diffraction spectra showed that the crystallinity of the polycaprolactone ($2\theta$ = 21.5 and 23.5) is responsible for the sol instability of PI and PIII (Figure 4b). As the trimethylene carbonate composition of the PCTC−PEG−PCTC triblock copolymer increased, the characteristic crystalline peaks of the polycaprolactone ($2\theta$ = 21.5 and 23.5) decreased. From this result, the sol stability was proven to be related to the crystallinity of the polycaprolactone, and the sol stability problem could be solved by copolymerizing the caprolactone and trimethylene carbonate to make PCTC−PEG−PCTC instead of PCL−PEG−PCL. However, the PCTC−PEG−PCTC lost the powder morphology of PCL−PEG−PCL and showed a paste morphology as a neat polymer.

On the basis of the sol−gel transition temperature (25−30 °C), sol stability at room temperature (20 °C), and a maximal storage modulus of the gel at 30−40 °C, PCTC−PEG−PCTC (1350−1000−1350; CL/TMC = 65/35 by weight; PV) might be very promising as an in situ gelling biomaterial.

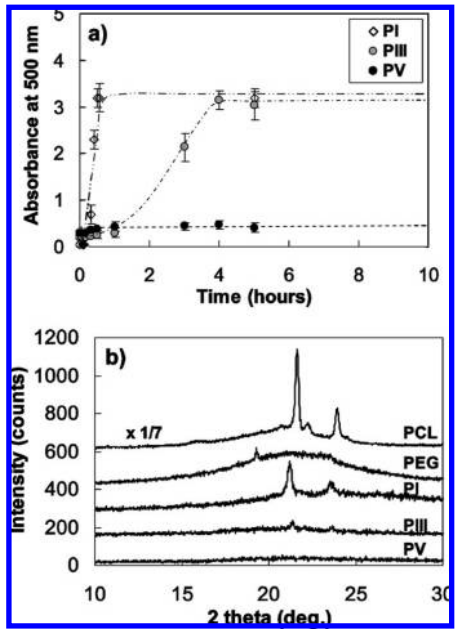


**Figure 4.** (a) Absorbance of the PCTC−PEG−PCTC aqueous solution (25 wt %) at 500 nm measured at 20 °C. The absorbance of PCL−PEG−PCL (PI) aqueous solution (25 wt %) is shown as a reference. The PII, PIV, and PVI aqueous solutions were transparent similar to PV. Each data point is an average of the three measurements. (b) X-ray diffraction spectra of the PCTC−PEG−PCTC aqueous system (25 wt %) at 20 °C. The neat polycaprolactone (MW = 1250 Da) and poly(ethylene glycol) (MW = 1000 Da) spectra are shown for comparison. The characteristic crystalline peaks of the polycaprolactone ($2\theta$ = 21.5 and 23.5) shown for PI and PIII are disappeared for PV.

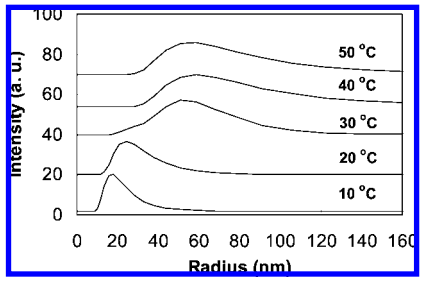


**Figure 5.** Micelle size of the PCTC−PEG−PCTC (PV) in water (0.03 wt %) determined by dynamic light scattering as a function of temperature.

The polymers consisting of hydrophilic PEG block and hydrophobic PCTC blocks self-assembled to micelles in water. As the block copolymers form micelles in water, the absorbance at 340, 356, and 378 nm increases.[21,22] The critical micelle concentration determined by hydrophobic dye (1,6-diphenyl-1,3,5-hexatriene) solubilization was about $8 \times 10^{-3}$ wt % (Supporting Information, Figure S2). The micelle size was studied as a function of temperature at 0.03 wt %, which is above the critical micelle concentration. The micelle size abruptly increased from 25 to 55 nm when the temperature increased from 20 to 30 °C, suggesting that micelle aggregation might be involved in the sol-to-gel transition (Figure 5).[15,20]

The $^{13}$C NMR spectral change of the PCTC−PEG−PCTC triblock copolymer aqueous solution (25 wt % in $D_2O$) as a function of temperature gave information on the phase transition at the molecular level. The carbonyl peak of the PCTC block at 172−174 ppm increased in intensity and sharpness, whereas the ethylene peak of the PEG block at 69−71 ppm was broadened and shifted to downfield (Figure 6). Dehydration of the hydrophilic PEG block and an increase in the molecular motion of the hydrophobic PCTC block are responsible for such

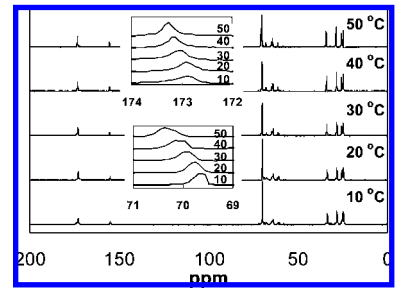


**Figure 6.** $^{13}$C NMR spectra of PCTC−PEG−PCTC (PV) (25 wt % in $D_2O$) as a function of temperature. Change in the ester carbonyl peak at 172−174 ppm and the ethylene (PEG) peak at 69−71 ppm are enlarged. The temperature unit (°C) is omitted in the enlarged spectra to save space.

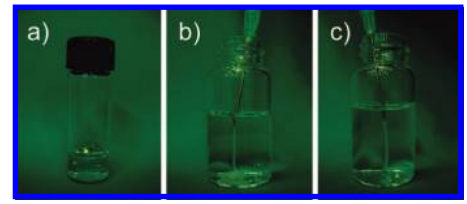


**Figure 7.** In situ gel formation from PCTC−PEG−PCTC aqueous solution (PV; 25 wt %): (a) polymer solution at 20 °C, (b) in situ gel formation by injection of the polymer aqueous solution (20 °C) into water (37 °C) through a 21-gauge needle, and (c) gel injection into water (37 °C) through a 21-gauge needle.

changes as the temperature increases.[4,15,20] The storage modulus showed a maximal value at 31−36 °C in Figure 2b, which might be a balanced state as a hydrogel between the increased molecular motion of the PCTC and dehydration of PEG. The decrease in gel modulus at high temperature is caused by the increased molecular motion of PCTC and the shrinkage of gel volume by the dehydration of the PEG.

To see the potential applicability as an injectable biomaterial, the polymer aqueous solution was injected in a gel as well as sol states. Figure 7a shows the clear sol phase of the PCTC−PEG−PCTC triblock copolymer (PV) aqueous solution (25 wt %) at 20 °C. When the clear sol at 20 °C was injected into water at 37 °C, it instantaneously turned into a gel (Figure 7b). In addition, the gel formed by putting the syringe containing the polymer aqueous solution in a 37 °C bath was soft enough to be injected through the 21-gauge needle (Figure 7c). Gel injection reduces an initial burst for a drug when the sol−gel transition polymer was used as a drug delivery depot.[27] On the basis of the above characteristics of the PCTC−PEG−PCTC triblock copolymer, the following procedure can be suggested to make a depot system. First, a polymer aqueous solution is prepared. Second, the solution can be mixed with a drug. The polymer/drug formulation can be heated to 30−37 °C in a syringe to form a gel. Then, an implant can be prepared in an animal by a subcutaneous injection of the preformed gel formulation.

The transparent PCTC−PEG−PCTC triblock copolymer (PV) aqueous solution (0.5 mL; 25 wt %) was heated to 37 °C in a syringe with a 21-gauge needle and then subcutaneously injected the preformed gel into rats to see the duration and degradation in in vivo conditions. The transparent gel turned into opaque gel and stayed for more than a month (see Supporting Information, Figure S3, for the photos), suggesting the PCTC−PEG−PCTC as a promising in situ gelling polymer. The polymer in the remaining gel was extracted by chloroform after freeze-drying the sample. The polymer recovered from the subcutaneous layer of the rat (in vivo; solid lines in Figure 8a) showed a decrease in molecular weight and the formation of the lower molecular

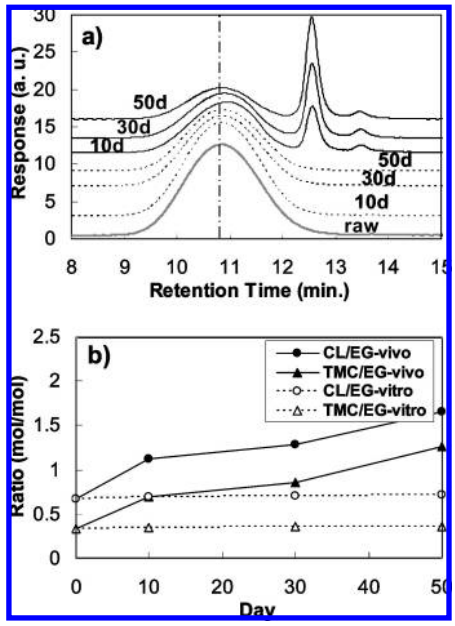


**Figure 8.** (a) Changes in the gel permeation chromatograms of the PCTC−PEG−PCTC (PV) during degradation in vitro (phosphate buffer saline) and in vivo (rat). Raw polymer (raw), 10 days (10 d), 30 days (30 d), and 50 days (50 d) after degradation study started were compared for in vivo (solid lines) and in vitro (dotted lines) studies. (b) Compositional changes of the remaining gel for in vivo (vivo) and in vitro (vitro) studies. CL/EG and TMC/EG indicate the mole ratio of the caprolactone unit (2.2−2.4 ppm) to the ethylene glycol unit (3.5−3.7 ppm) and the trimethylene carbonate unit (1.9−2.1 ppm) to the ethylene glycol unit of the polymer, respectively, which were determined by $^1$H NMR spectra (in $CDCl_3$) by the following equations of CL/EG = $2A_{2.2-2.4}/A_{3.5-3.7}$ and TMC/EG = $2A_{1.9-2.1}/A_{3.5-3.7}$.

weight fractions at 12.6 and 13.4 min, corresponding to 1050 and 400 Da relative to PEG standards, respectively. No decrease in molecular weight of the PCTC−PEG−PCTC triblock copolymer was observed for the polymer incubated in the phosphate buffer saline (in vitro; dotted lines in Figure 8a) at 37 °C, over the same period. The composition of the remaining gel determined by $^1$H NMR ($CDCl_3$) showed that the PCTC fraction relative to PEG almost doubled (Figure 8b) in 50 days of incubation in the rats. This might be due to the preferrential elimination of PEG-rich segments during the degradation. On the other hand, the composition of the polymer recovered from the phosphate buffer saline (in vitro) after 50 days incubation at 37 °C did not change. In addition, the sol-to-gel transition temperatures were not changed by the incubation in phosphate buffer saline at 37 °C for 50 days, suggesting that the stability of the PCTC−PEG−PCTC under the in vitro conditions. These results suggest that new peaks at 12.6 and 13.4 min are degraded PCTC fragments that were not dissolved away in the subcutaneous layer of the rats. Polycaprolactone and poly(trimethylene carbonate) homopolymers were reported to be degraded faster in in vivo after implantation in the subcutaneous layer of the rat or rabbit than in in vitro conditions.[28,29] The involvement of enzymes such as lipases and the reactive oxygen species generated by inflammatory cells was suggested for such a behavior.[28,30−32]Polycaprolactone is a slowly degrading polymer in the phosphate buffer saline, and the in vitro degradation rate even more decreases by the copolymerization with trimethylene carbonate.[33] Current PCTC−PEG−PCTC was significantly degraded in the rat, whereas it was quite stable against hydrolysis in the phosphate buffer saline over 50 days, suggesting that similar process including the involvement of enzymes (lipases) and reactive oxygen species might be involved in the degradation of the polymer under the in vivo conditions. The detailed degradation mechanism of the polymer is under investigation.

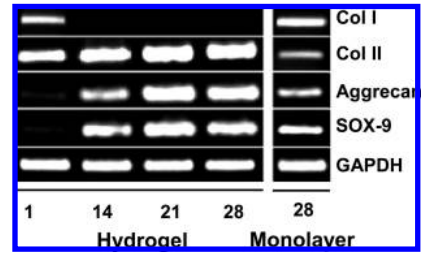


**Figure 9.** Gene expression of chondrocytes cultured in in situ formed PCTC−PEG−PCTC gel (hydrogel) for 1, 14, 21, and 28 days, are compared with that cultured on the two-dimensional tissue culture plate (monolayer) for 28 days. Col I (collagen type I), Col II (collagen type II), aggrecan, SOX-9 (sex-determining region box-9), and GAPDH (D-glycerol aldehyde-3-phosphate dehydrogenase) expression level were compared.

Articular chondrocytes undergo rapid changes in phenotype and gene expression when isolated from cartilage tissue and cultured on the culture plate.[24,34−36] To investigate the feasibility of current thermogelling PCTC−PEG−PCTC as a three-dimensional cell culture system, chondrocytes were cultured in the in situ formed PCTC−PEG−PCTC hydrogel. The chondrocytes cultured on the regular culture plate were compared as a control. The RT-PCR showed that collagen type II and aggrecan were strongly expressed when the chondrocytes were cultured in an in situ formed three-dimensional PCTC−PEG−PCTC hydrogel, compared with the traditional two-dimensional culture system (Figure 9). In addition, collagen type I (fibroblastic gene) disappeared in the three-dimensional culture system of PCTC−PEG−PCTC hydrogel. In osteoarthritic cartilage, chondrocytes were reported to be dedifferentiated to a fibroblast-like phenotype and express collagen type I, III, or V instead of collagen type II and aggrecan.[25,37] The present results indicate that chondrocytes in PCTC−PEG−PCTC hydrogel can enhance the gene expression of chondrocytic differentiation, suggesting that PCTC−PEG−PCTC hydrogel might have a great potential as a three-dimensional matrix for cartilage engineering.

## Conclusions

The sol stability issue of PCL−PEG−PCL triblock aqueous solution was solved by preventing the crystallization of the PCL through the copolymerization of caprolactone with trimethylene carbonate. X-ray diffraction spectra confirmed that the crystallization of PCL block was responsible for the sol instability. The PCTC−PEG−PCTC aqueous solution showed sol-to-gel-to-syneresis transitions as the temperature increased. The PCTC−PEG−PCTC triblock copolymer gel was stable in phosphate buffer at 37 °C for 50 days whereas the gel significantly (>90%) disappeared in the subcutaneous layer in the rat. The feasibility of current PCTC−PEG−PCTC as an in situ thermogelling biomaterial was tested for a three-dimensional cell culture. Compared with two dimensionally cultured chondrocytes, the three dimensionally cultured chondrocytes in the PCTC−PEG−PCTC hydrogel strongly expressed the collagen type II and aggrecan, whereas suppressed collagen type I, suggesting the potential of the material as a three-dimensional cell culture matrix.

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**Supporting Information Available:** $^1$H NMR spectra of the PCTC−PEG−PCTC (in $CDCl_3$), determination of the critical

micelle concentration by the dye solubilization method, and the photos taken during the in vivo gel duration experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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