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Thermo-sensitive sol–gel transition of poly(depsipeptide-*co*-lactide)-*g*-PEG copolymers in aqueous solution

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ABSTRACT

A series of biodegradable graft copolymers composed of poly(ethylene glycol) side-chains and a poly-(depsipeptide-*co*-DL-lactide) backbone (PDG-DL-LA-*g*-PEG) were prepared as a novel thermo-gelling system. An aqueous solution of PDG-DL-LA-*g*-PEG (20 wt%) with a certain PEG length and composition showed instantaneous temperature-sensitive gelation at 33 °C. The sol–gel transition temperature (T_{gel}) could be controlled from 33 to 51 °C by varying the PEG length and compositions without a decrease in mechanical strength of the hydrogels. The 20 wt% hydrogel was eroded gradually in PBS at 37 °C for 60 days. This research provides a molecular design approach to create biodegradable thermo-gelling polymers with controllable T_{gel} and mechanical toughness.

1. Introduction

In situ gel-forming polymers have recently drawn attention as promising materials for minimally invasive therapy [1–3]. In particular, thermo-gelling biodegradable polymers with a sol–gel transition point between room temperature and body temperature are expected to be useful for injectable polymer systems in biomedical applications. The polymer solution would be in a sol state in the syringe at room temperature but would then become a hydrogel in situ after injection into the body, without requiring toxic cross-linkers. Such systems would enable pharmaceutical agents or cells to be easily entrapped and form a depot by a simple syringe injection at a target site, where the depot acts as a sustained drug delivery system, or a cell-growing scaffold for tissue engineering [4–10].

To exhibit a temperature-sensitive sol–gel transition in water, such polymers should possess amphiphilicity with a delicate balance of hydrophilicity and hydrophobicity [11]. Poly(ethylene glycol) (PEG) has typically been used as the hydrophilic unit of thermo-gelling polymers due to its biocompatibility. Poly(L-lactide) (PLLA), poly(D-lactide) (PDLA), poly(DL-lactide-*co*-glycolide) (PLGA), poly(ε-caprolactone), poly(β-hydroxybutyrate) and their modified polymers have been primarily used as the biodegradable hydrophobic unit [11–17]. Most of these thermo-gelling polymers adopt a linear-type di- or triblock architecture. However, the mechanical strength of the hydrogels prepared from these copolymer solutions was not sufficiently high for their clinical application as implant materials. It is generally considered that thermo-gelling polymers with higher total molecular weights should be able to produce hydrogels with higher mechanical strengths [18]. However, there is a molecular weight limitation for such thermo-gelling polymers as injectable polymer systems due to their linear-type di- or triblock architecture, because the sol–gel transition temperature strongly depends on the PEG length. For instance, the total molecular weight of PEG-*b*-PLGA-*b*-PEG triblock copolymers should be 4000–5000 Da to adjust the gel formation temperature between room temperature and body temperature [13]. Thus, it should be possible to develop a thermo-gelling polymer which possesses an appropriate gel formation temperature and can form a mechanically strong hydrogel for achieving ideal injectable polymer systems.

Jeong et al. have overcome the problem concerning the total molecular weight of thermo-gelling polymers composed of PEG and PLGA or PLLA by employing graft or multiblock architectures [18–21]. The corresponding PLGA-*g*-PEG graft copolymer (M_n: 9300 Da) and PEG–PLLA multiblock copolymer (M_n: 6700 Da) in aqueous solutions (25 wt%) showed storage modulus of ca. 10 Pa and 280 Pa at 37 °C, which are not high enough. Kim et al. designed a star-shaped (4-arm) PLGA–PEG block copolymer (M_n: 8600 Da) as an injectable polymer to overcome this problem [21], but they did not report on the mechanical strength of the resulting hydrogel.

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We have previously reported the synthesis of biodegradable copolymers of depsipeptide and PLLA, poly(depsipeptide-*co*-L-lactide), having reactive side-chain groups, such as COOH, NH_2, and SH, by varying the amino acid units of the depsipeptide [22,23]. Moreover, it was possible to impart many functionalities to these copolymers via chemical modification of various functional groups.

In this paper, thermo-gelling aqueous solutions of biodegradable graft copolymers with comb-like architectures, poly(depsipeptide-*co*-DL-lactide) grafted with PEGs (PDG-DL-LA-*g*-PEG), are reported. The molecular weight limitation can be improved greatly by comb-like graft copolymer systems. Moreover, we also investigated the structural dependence on the transition temperature and mechanical strength of the hydrogel derived from these copolymers.

2. Experimental section

2.1. Materials

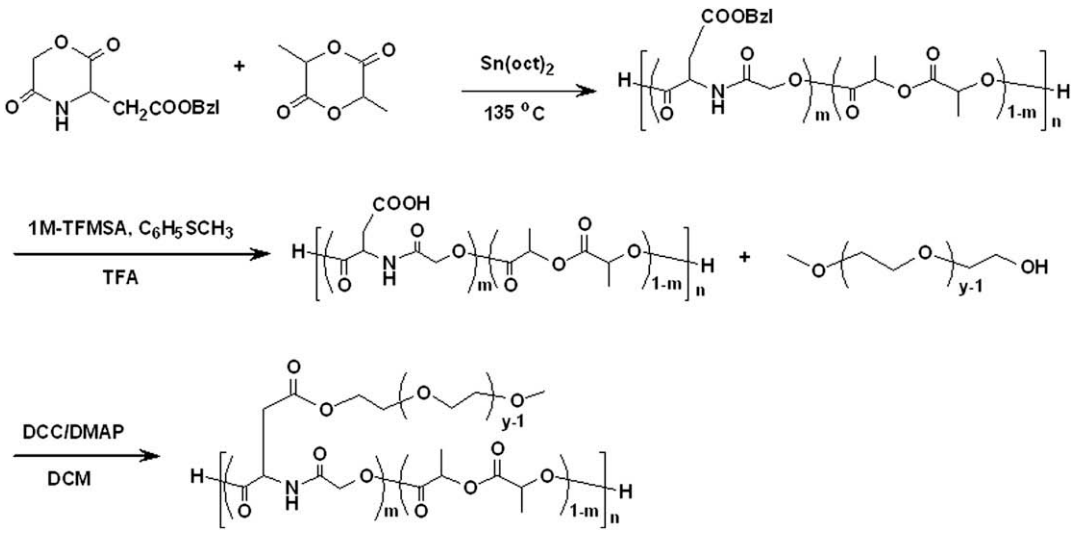
DL-Lactide (DL-LA) was purchased from PURAC. Monomethoxypoly(ethylene glycol) (MeO-PEG350 and MeO-PEG550) (M_n: 350, 550 Da, respectively) were purchased from Aldrich. DL-LA and MeO-PEG were dried under vacuum prior to use. Dicyclohexyl carbodiimide (DCC, Aldrich) and *N*-dimethylaminopyridine (DMAP, Aldrich) were reagent grade and used as-received without further purification. Dry methylene chloride (DCM) was purchased from Wako Pure Chemical Industries, Ltd., and used as a solvent for coupling reactions without purification. All other organic solvents were purified by normal distillation methods. Tin 2-ethylhexanoate [$Sn(oct)_2$] was also purchased from Wako Pure Chemical Industries, Ltd. All other reagents were of commercial grade and used without further purification.

2.2. Synthesis of poly[(Glc-Asp)-co-DL-LA] copolymers

The synthesis of the cyclodepsipeptide consisting of glycolic acid (Glc) and Asp, cyclo(Glc-Asp), was performed using similar methods as reported previously [24]. A series of poly[(Glc-Asp)-*co*-(DL-lactide)] (PGD-DL-LA) copolymers was synthesized through bulk ring-opening copolymerization of DL-LA and cyclo[Glc-Asp(OBzl)] using tin 2-ethylhexanoate as a catalyst according to the same method reported previously, as shown in Scheme 1 [25]. Briefly, under a nitrogen atmosphere, DL-LA (1.1 g, 7.64 mmol) and cyclo[Glc-Asp(OBzl)] (470 mg, 1.89 mmol) were placed into a glass tube, followed by the addition of a freshly prepared solution of tin 2-ethylhexanoate (3.76 mg, 9.4 μmol) in anhydrous THF (3.7×10^{-2} M) in a glove box. The solvent was removed under vacuum overnight. The tube was then purged with argon and sealed in vacuo. The sealed tube was placed in an oil bath at 160 °C for 2 min, then at 135 °C for 24 h. Deprotection of the poly{[Glc-Asp(OBzl)]-*co*-(DL-lactide)} was carried out by acid treatment with a mixture of trifluoromethane sulfonic acid, thioanisole and trifluoroacetic acid. The purification of the reaction mixture was performed three times by the reprecipitation method using chloroform as a solvent and diethyl ether as a non-solvent, and dried under vacuum overnight to give PGD-DL-LA copolymer (yield: 71%). The averaged number of molecular weight (M_n) and the polydispersity indexes (M_w/M_n) of these copolymers were determined by size exclusion chromatography (SEC) (column: TSKgel Multipore $H_{xL}M \times 2$; detector: RI; standard: PEG; eluent: DMF). The molecular composition of DL-LA and cyclo(Glc-Asp) in the obtained copolymers was estimated from ^{1}H NMR measurements (JEOL GSX-400, DMSO-d_6).

2.3. Synthesis of PGD-DL-LA-graft-PEG copolymers

A series of PGD-DL-LA-*g*-PEG copolymers was synthesized through coupling reactions between the side carboxyl groups of PGD-DL-LA and the end hydroxyl group of MeO-PEG. In brief, *N*,*N*-dicyclohexyl carbodiimide (DCC) (156 mg, 0.76 mmol) was added to an ice-cooled solution of PGD-DL-LA (580 mg, COOH: 0.51 mmol), MeO-PEG350 (268 mg, 0.77 mmol) and 4-dimethylaminopyridine (DMAP) (23 mg, 0.19 mmol) in anhydrous DCM (3 ml). The reaction mixture was stirred at 0 °C for 4 h and then at room temperature for 24 h. Precipitated dicyclohexylurea was removed by filtration, and the filtrate was poured into a mixture of *n*-hexane/ethanol (8/2) to remove the excess MeO-PEG350 giving a white precipitate of PGD-DL-LA-*g*-PEG350 copolymer (yield: 82%). The degree of substitution of PEG chains onto the PGD-DL-LA backbone, total M_n, and molecular composition of PGD-DL-LA-*g*-PEG were estimated from ^{1}H NMR measurements ($CDCl_3$). The M_w/M_n of these copolymers was determined by SEC (standard: PEG; eluent: DMF).



Scheme 1. Synthesis of PGD-DL-LA-*g*-PEG copolymers.

2.4. Sol–gel transition

The temperature-responsive sol–gel transition of these copolymers in water or PBS (pH 7.4, ionic strength = 0.14) was investigated by the test tube inversion method with a temperature increment of 1 °C. Each sample of a given concentration was prepared by dissolving the copolymer in PBS in a 5-ml vial at 4 °C and incubating for 12 h at 4 °C. The vial was then immersed in a water bath at the desired temperature for 20 min. The sol–gel transition temperature was determined based on the criteria of "flow" (=sol) and "no flow" (=gel) 2 min after the vial was inverted, with a temperature increment of 1 °C per step. Measurements were repeated three times at each temperature to determine the transition temperature in the phase diagram. Water soluble poly(DL-lactide-*co*-glycolide)-*b*-PEG-*b*-poly(DL-lactide-*co*-glycolide) triblock copolymer (PLGA-*b*-PEG-*b*-PLGA) (M_n of one PLGA segment = 980 Da, M_n of PEG = 1000 Da, total M_n = 2960 Da, M_w/M_n = 1.05, PEG content = 34.5 wt%) was synthesized as a typical thermo-gelling biodegradable polymer [26], and its properties were compared with a series of PGD-DL-LA-*g*-PEG copolymers.

2.5. Fluorescent probe measurements

The critical aggregation concentration (CAC) values of the copolymers in water or PBS were determined using pyrene as a fluorescence probe [27–32]. Pyrene was first dissolved in acetone and then the resulting solution was added to water and adjusted to a concentration of 1.8×10^{-5} mg/ml. Acetone was subsequently removed by reducing the pressure and stirring for more than 5 h at 20 °C. The concentration of the polymer in water or PBS was varied from 1×10^{-6} to 1.0 mg/ml. The pyrene and polymer aqueous solutions were mixed and equilibrated at room temperature for 1 day before measurement. The fluorescence spectra were obtained at 20 °C using a fluorescence spectrophotometer (Hitachi, F-2500). The emission wavelength was set to 375 nm, which was chosen according to the maximum intensity obtained in the excitation spectra. The excitation spectra exhibit a shift from 335 to 338 nm, indicating the partition of pyrene into the hydrophobic core of the micelles. The intensity ratios I_{338}/I_{335} were monitored.

2.6. Dynamic light scattering measurements

The average micelle diameter of these copolymers in water or PBS was measured by dynamic light scattering (DLS) measurements on a DLS-7000 apparatus (Otsuka Electronics Co.) with vertically polarized incident light at a wavelength of 488 nm supplied by an argon laser operated at 15 mW. Each copolymer (3 mg) was dissolved in 3.0 ml of water, and then the solutions were filtered through a Durapore (Millipore) 0.2 μm membrane prior to measurement. The experimental temperature range was from 10 to 50 °C, and the sample solution was equilibrated for 60 min at each temperature before measurement. The micelle diameter is an average of three measurements per point.

2.7. Rheological measurements

Aqueous solutions of PGD-DL-LA-*g*-PEG copolymers with a certain concentration (20 wt%) were prepared by dissolving the copolymer in water or PBS at 4 °C for 5 h. Temperature dependences of the storage modulus (G') and the loss modulus (G'') of these copolymer solutions were studied by dynamic rheometry (HAAKE, Thermo HAAKE RS600). A solvent trap was used to prevent evaporation of the solvent. Each copolymer solution was applied between parallel plates of 25 mm diameter and a gap of 1.0 mm using a syringe, and the plates were heated at rates of 0.5 °C/min. The data were collected under controlled stress (4.0 dyn/cm^2) and a frequency of 1.0 rad/s.

2.8. Swelling and degradation test

The swelling and in vitro degradation behavior of copolymer hydrogels in PBS at 37 °C was investigated. Copolymer hydrogels (20 wt%) prepared in a 5-ml vial were soaked in PBS and incubated at 37 °C. After predetermined periods, the excess amount of PBS was fully removed from the swollen hydrogels and the resulting hydrogels were weighed. The PBS was freshly replaced after every weight measurement. The percentage of weight change of these hydrogels was calculated using the following equation, where W_0 is the initial weight just after preparation of the hydrogel and W_t is the weight after incubation in PBS:

$$\text{Hydrogel weight change } (\%) = (W_t/W_0) \times 100$$

3. Results and discussion

3.1. Synthesis and characterization of PGD-DL-LA-g-PEG copolymers

The PGD-DL-LA copolymers were synthesized by ring-opening polymerization of cyclo(Glc-Asp) and DL-lactide in the presence of tin 2-ethylhexanoate as a catalyst. Results of copolymerization of cyclo(Glc-Asp) and DL-lactide are summarized in Table 1. The molar ratio of (Glc-Asp) units in the PGD-DL-LA copolymer was determined by the NMR integration ratio of peak **c** attributed to methylene protons of the (Glc-Asp) units at δ 4.5–4.7 ppm and peak **a** attributed to methine protons of the DL-lactide units at δ 5.1–5.2 ppm, as shown in Fig. S1 (see Supporting Information). As seen from the GPC elution in Fig. 1B, the PGD-DL-LA copolymer (P2) showed a narrow symmetric distribution, indicating that the copolymer was uncontaminated with unreacted DL-lactide and cyclo(Glc-Asp). The molar fractions of (Glc-Asp) unit in the copolymers [x (mol.%) values in Table 1] were 10.7, 16.2, 14.7, and 12.3, and the M_n values were 18,200, 16,400, 11,700, and 11,300 Da for P1, P2, P3, and P4, respectively.

Amphiphilic PGD-DL-LA-*g*-PEG copolymers were synthesized through coupling reactions of PGD-DL-LA (P1, P2, or P3) and MeO-PEG350 or MeO-PEG550 as shown in Scheme 1. Fig. 1A shows a typical ^{1}H NMR spectrum of the resulting graft copolymer. The successful coupling of PGD-DL-LA and MeO-PEG was proved by the complete shift of peak **f** attributed to the methylene protons of (Glc-Asp) from δ 2.7–2.9 to δ 2.9–3.1 ppm as well as the appearance of peak **b** attributed to the methylene protons of PEG at δ 4.1–4.2 ppm nearest the newly formed ester bond. The average number of PEG chains introduced into a PGD-DL-LA backbone was estimated by the

Table 1
Results of copolymerization of DL-lactide and cyclo(Glc-Asp).

Sample	X^a (mol.%)	x^b (mol.%)	$[m,n]^c$	$M_n \times 10^{-3d}$ (Da)	$M_w/M_n{}^d$
P1	13.0	10.7	[14,122]	18.2	1.8
P2	19.0	16.2	[18,108]	16.4	1.6
P3	17.0	14.7	[11,71]	11.7	1.5
P4	15.0	12.3	[9,75]	11.3	1.7

[a] Molar fraction of cyclo(Glc-Asp) in feed.
[b] Molar fraction of (Glc-Asp) units in PGD–DL–LA estimated by ^{1}H NMR in DMSO-d_6.
[c] m,n: numbers of (Glc-Asp) units and lactide units in one polymer chain, respectively.
[d] Determined by GPC.

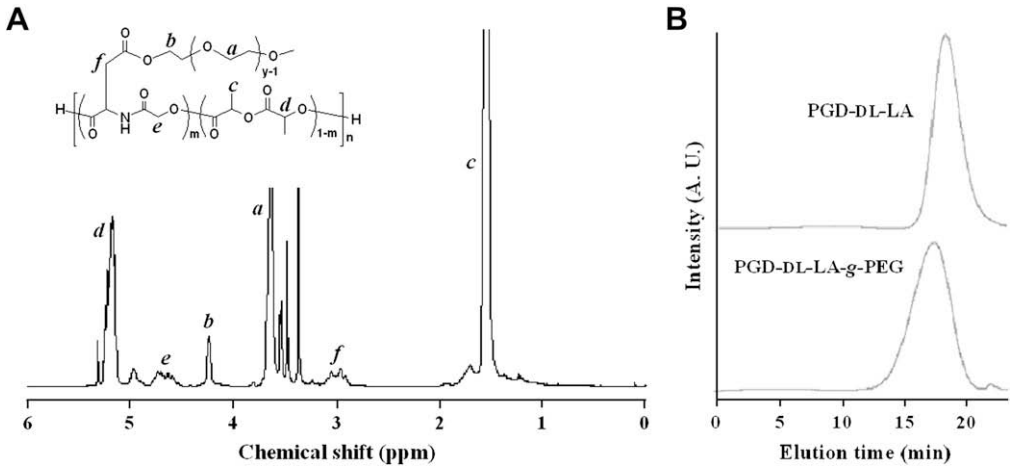


Fig. 1. (A) ^{1}H NMR spectrum of PGD-DL-LA-*g*-PEG(11K-350-26) copolymer in $CDCl_3$. (B) GPC elution curves of PGD-DL-LA (P2) and PGD-DL-LA-*g*-PEG(16K-350-28).

integral ratio of peak **a** attributed to methylene protons of PEG at δ 3.6–3.7 ppm and peak **f** attributed to methylene protons of (Glc-Asp) at δ 2.9–3.1 ppm. The degree of substitution of PEG chains per carboxyl group of the corresponding PGD-DL-LA was calculated as over 95% in all PGD-DL-LA-*g*-PEG copolymers studied here. The characteristics of these copolymers are listed in Table 2. Five kinds of PGD-DL-LA-*g*-PEG copolymers with different PEG content were synthesized. The M_n of these graft copolymers studied here ranged between 15,100 and 26,050 Da. The code used in Table 2 can best be described by the formula PGD-DL-LA-*g*-PEG(*x*–*y*–*z*), in which *x* is the M_n of PGD-DL-LA, *y* is the M_n of PEG, and *z* is the PEG content in the PGD-DL-LA-*g*-PEG copolymer. Fig. 1B shows a typical result of GPC analysis of PGD-DL-LA-*g*-PEG (16K-350-28). The PGD-DL-LA-*g*-PEG showed a narrow symmetric distribution with a higher molecular weight than the corresponding PGD-DL-LA, indicating that the PGD-DL-LA-*g*-PEG was uncontaminated with unreacted PEG. All the obtained copolymers readily dissolved in water at 20 °C (concentration was up to 5 wt%). The copolymers were difficult to dissolve in water spontaneously over 5 wt%, but a homogeneous suspension could be obtained by sonication in an ultrasound bath for 1 h at 10 °C.

3.2. Sol–gel transition behavior

In concentrated PGD-DL-LA-*g*-PEG copolymer suspensions except for PGD-DL-LA-*g*-PEG(16K-550-38), a temperature-responsive transition between a sol and a macroscopic gel was observed. Although PGD-DL-LA-*g*-PEG(16K-550-38) dissolved well in water at room temperature and the concentrated aqueous solution also showed a temperature-responsive phase transition, it was a sol-to-precipitate transition. Thus, the total M_n of PGD-DL-LA-*g*-PEG graft copolymers that showed a temperature-induced sol–gel transition ranged between 15,100 and 25,500 Da. The state diagrams of these copolymer suspensions determined by the test tube inversion method are shown in Fig. 2. Photographs of PGD-DL-LA-*g*-PEG(11K-350-26) copolymer suspension (15 wt%) at 25 °C and 37 °C are also shown. Above the critical gelation concentration (CGC), the copolymer suspensions could spontaneously form a physical gel depending on temperature. The lower boundary curves give the sol-to-gel transition temperature, T_{gel}. As the polymer concentration increased, T_{gel} decreased, probably due to an increase in the availability of physical cross-linking points. The higher boundary curves give the gel-to-precipitate transition temperature. This temperature-responsive phase transition was found to be reversible. When the temperature was lowered below T_{gel}, the solution once again changed into a transparent sol state. The T_{gel} of 20 wt% copolymer suspensions were 33, 39, and 51 °C for PGD-DL-LA-*g*-PEG(11K-350-26), PGD-DL-LA-*g*-PEG(16K-350-28), and PGD-DL-LA-*g*-PEG(18K-550-29), respectively (Table 3). The T_{gel} depended on PEG content as well as the M_n of PEG chains of these PGD-DL-LA-*g*-PEG copolymers. The T_{gel} increased with increasing PEG content and M_n of PEG chains. Although PGD-DL-LA-*g*-PEG(18K-550-29) and PGD-DL-LA-*g*-PEG(16K-350-28) copolymers possessed similar PEG content, PGD-DL-LA-*g*-PEG(18K-550-29) showed a quite higher T_{gel} than PGD-DL-LA-*g*-PEG(16K-350-28). Thus, it was found that PGD-DL-LA-*g*-PEG copolymer with a lot of short PEG chains could lead to a lower T_{gel} when comparing between PGD-DL-LA-*g*-PEG copolymers that have the same PEG content. According to the state diagram, the T_{gel} of PGD-DL-LA-*g*-PEG(11K-350-26) suspensions (13–20 wt%) is between room temperature and body temperature (37 °C), which is very suitable for injectable biomaterials.

Table 2
List of PGD-DL-LA-*g*-PEG copolymers studied.

Code	$M_n \times 10^{-2}$ (Da)			M_w/M_n[c]	# of PEG[d]	PEG content[b] (%)	Thermo-gelation[e]
	MeO-PEG	PGD-DL-LA[a]	PGD-DL-LA-*g*-PEG[b]				
18K-550-29	5.5	182.4	254.5	1.9	13.5	29.1	Yes
16K-550-38	5.5	164.2	260.5	1.8	17.8	37.6	No
11K-550-35	5.5	113.5	173.5	1.9	10.9	35.4	Yes
16K-350-28	3.5	164.2	224.4	1.8	17.2	27.8	Yes
11K-350-26	3.5	113.5	150.6	1.7	10.6	26.3	Yes

[a] Estimated by ^{1}H NMR in DMSO-d_6.
[b] Estimated by ^{1}H NMR in $CDCl_3$.
[c] Determined by GPC.
[d] Averaged number of grafted PEG chains per PGD-DL-LA backbone.
[e] Thermo-gelation of PGD-DL-LA-*g*-PEG copolymer aqueous solution (20 wt% was determined by the test tube inverted method).

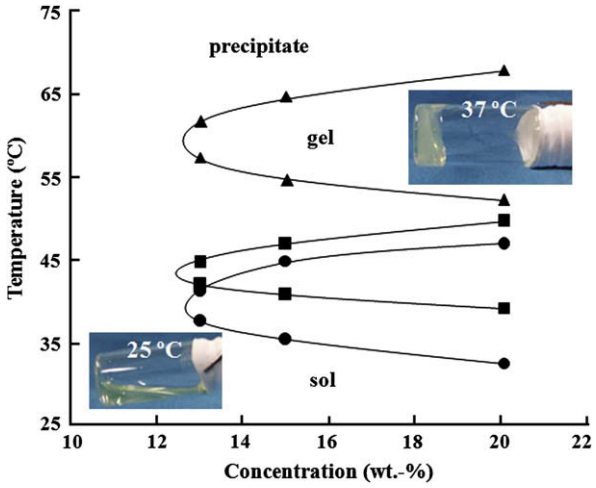


Fig. 2. The state diagrams of PGD-DL-LA-*g*-PEG and PLGA-*b*-PEG-*b*-PLGA copolymers in water at various concentrations and temperatures. Insets are optical images of a sol and a gel of PGD-DL-LA-g-PEG(11K-350-26) in a vial. PGD-DL-LA-*g*-PEG(11K-350-26) (●), PGD-DL-LA-*g*-PEG(16K-350-28) (■), and PGD-DL-LA-*g*-PEG(18K-550-29) (▲).

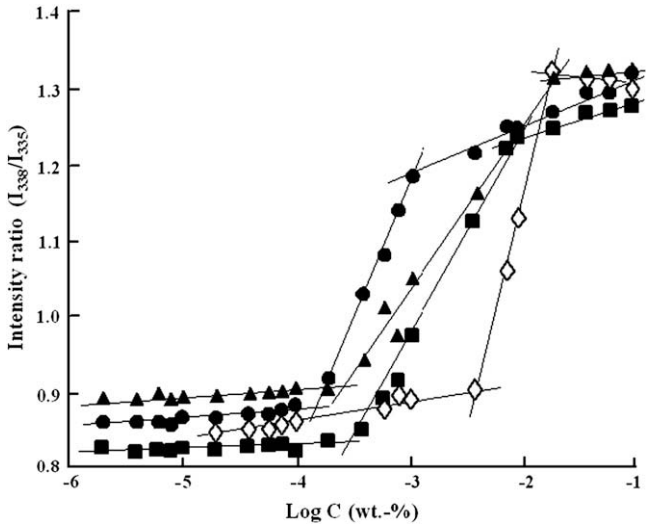


Fig. 3. Plots of fluorescence intensity ratio I_{338}/I_{335} of pyrene versus the logarithm of polymer concentration. PGD-DL-LA-*g*-PEG(11K-350-26) (●), PGD-DL-LA-*g*-PEG(16K-350-28) (■), PGD-DL-LA-*g*-PEG(18K-550-29) (▲), and PLGA-PEG-PLGA (◇).

3.3. Micelle formation

The temperature-responsive phase transition of amphiphilic copolymers composed of hydrophilic PEG and hydrophobic polyesters such as PLA and PLGA was expected to show a strong correlation with aggregation (micelle formation) ability and intermicelle aggregation in aqueous solution. Therefore, the CAC and micelle formation of PGD-DL-LA-*g*-PEG copolymers in dilute aqueous media were investigated using pyrene as a fluorescence probe. The excited spectra of pyrene exhibit a shift from 334.8 nm to 337.7 nm, indicating the partition of pyrene into the hydrophobic domains of the aggregates (data not shown) [27–32]. The shift is utilized to determine the critical aggregation behavior of such amphiphilic molecules in aqueous media. Fig. 3 shows the plots of the fluorescence intensity ratios (I_{338}/I_{335}) obtained from the fluorescence excitation spectra versus copolymer concentrations. These copolymer samples showed a crossover point, indicating the formation of aggregates by self-assembly. The critical aggregation concentration (CAC) was determined from each crossover point. The CAC values of these copolymers are listed in Table 3. The 11K-350-26 copolymer with the shortest PEG side-chain and smallest PEG content studied here showed the lowest CAC values. The lower CAC value indicates a strong tendency toward formation of aggregates. Previously, Jeong et al. reported that PLGA-*g*-PEG copolymers with a certain balance of hydrophilicity and hydrophobicity could form micelles with a core–shell structure [19]. We do not have concrete evidence concerning the structure of the aggregates, but considering the results of this fluorescence study and reports from Jeong et al., it is thought that the PGD-DL-LA-*g*-PEG copolymers could also form polymeric micelles composed of a hydrophobic PGD-DL-LA core and a hydrophilic PEG shell extended outside.

The hydrodynamic diameter (R_h) of these micelles was estimated by DLS measurement at 20 °C (Table 3). The micelles generated from PGD-DL-LA-*g*-PEG or PLGA-*b*-PEG-*b*-PLGA copolymers exhibited nanometric sizes with R_h approximately 10–25 nm. There was no difference in R_h among the three PGD-DL-LA-*g*-PEG micelles, while PLGA-*b*-PEG-*b*-PLGA copolymers could form micelles about two times larger than PGD-DL-LA-*g*-PEG micelles. The PLGA-*b*-PEG-*b*-PLGA copolymer studied here is known to form flower-like micelles in aqueous media [26]. Considering the M_n of the PEG chain (1000) and the flower-like structure of PLGA-*b*-PEG-*b*-PLGA micelles, it is easily estimated that both the PGD-DL-LA-*g*-PEG(18K-550-29) and PLGA-*b*-PEG-*b*-PLGA micelles possess a PEG layer with similar thickness. In other words, the difference in whole micelle size between PGD-DL-LA-*g*-PEG(18K-550-29) and PLGA-*b*-PEG-*b*-PLGA can be mainly due to the size of the micelle core which is composed of hydrophobic PGD-DL-LA or PLGA. On the other hand, PGD-DL-LA-*g*-PEG(18K-550-29) could form a smaller micelle core, while PGD-DL-LA-*g*-PEG(18K-550-29) has approximately a six times longer hydrophobic chain length than PLGA-*b*-PEG-*b*-PLGA. This obviously means that the PGD-DL-LA backbones cannot maintain an extended conformation in the core. Thus, inter-chain overlapping and entangling, as well as inter-chain crumpling of the PGD-DL-LA backbones, would occur during the self-assembly process which can result in the core–shell micelle, as shown in Fig. 4.

Table 3
Characteristics of PGD-DL-LA-*g*-PEG and PLGA-*b*-PEG-*b*-PLGA copolymer aqueous solutions.

Sample	Gelation temp.[a] (°C)	CAC 10^{-4} wt%	R_h[b] (nm)				Max. G'[c] (Pa/°C)
			20 °C	30 °C	40 °C	50 °C	
18K-550-29	51	2.62	13	15	15	17	461/52
16K-350-28	39	3.54	10	12	17	18	492/41
11K-350-26	33	1.60	17	18	24	42	339/38
PLGA–PEG–PLGA	15	37.2	25	28	43	40	79/18

[a] Gelation temperature of copolymer aqueous solutions at 20 wt% was determined from the test tube inverted method.
[b] Determined by DLS measurements of copolymer aqueous solutions at 0.3 wt%.
[c] Maximum G' values and the temperature were determined from rheological data of copolymer aqueous solutions at 20 wt% by changing the measurement temperature.

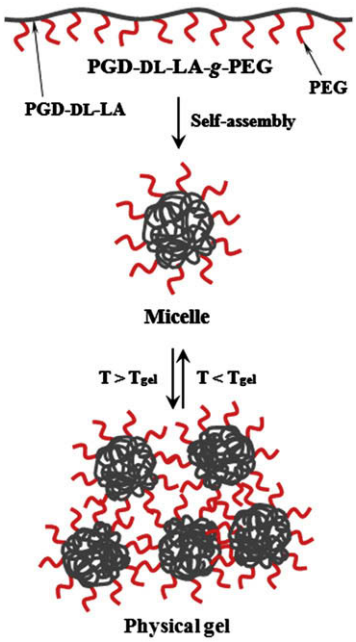


Fig. 4. Schematic representation of the aqueous solution behavior of PGD-DL-LA-*g*-PEG copolymers. Amphiphilic graft copolymers were self-assembled into micelles in aqueous media, and the micellar gel network was formed above T_{gel}.

3.4. Thermo-responsive micellar aggregation

In order to obtain additional information concerning the temperature-responsive behavior, the micelle size and its distributions for PGD-DL-LA-*g*-PEG and PLGA-*b*-PEG-*b*-PLGA copolymers were measured by DLS at various temperatures. The R_h values are listed in Table 3, and the size distributions of these micelles at different temperatures are shown in Fig. 5. All of the micelles showed unimodal distributions at 20 °C, which indicates well-defined micelles. As the temperature increases, the micelle size also increases gradually. These temperature-responsive phenomena agreed well with a previous report concerning temperature-responsive gelation of PLGA-*b*-PEG-*b*-PLGA copolymer aqueous solutions by Kim et al. [26]. They have proposed a micellar-bridging mechanism for the gelation of PLGA-*b*-PEG-*b*-PLGA copolymers. This mechanism could also be adopted for temperature-induced gelation of aqueous PGD-DL-LA-*g*-PEG copolymer solutions. On the other hand, the responsiveness to temperature changes was quite different between PGD-DL-LA-*g*-PEG graft copolymers with comb-like architectures and PLGA-*b*-PEG-*b*-PLGA triblock copolymers. Indeed, PGD-DL-LA-*g*-PEG(18K-550-29) and PGD-DL-LA-*g*-PEG(16K-350-28) micelles showed a slight temperature dependency in the range from 20 to 50 °C, while a relatively higher dependency was observed for PGD-DL-LA-*g*-PEG(11K-350-26) and PLGA-*b*-PEG-*b*-PLGA micelles. Considering these results, it can be proposed that the individual primary micelles tend to combine together (intermicellar aggregation) gradually with increasing solution temperature, and the tendency

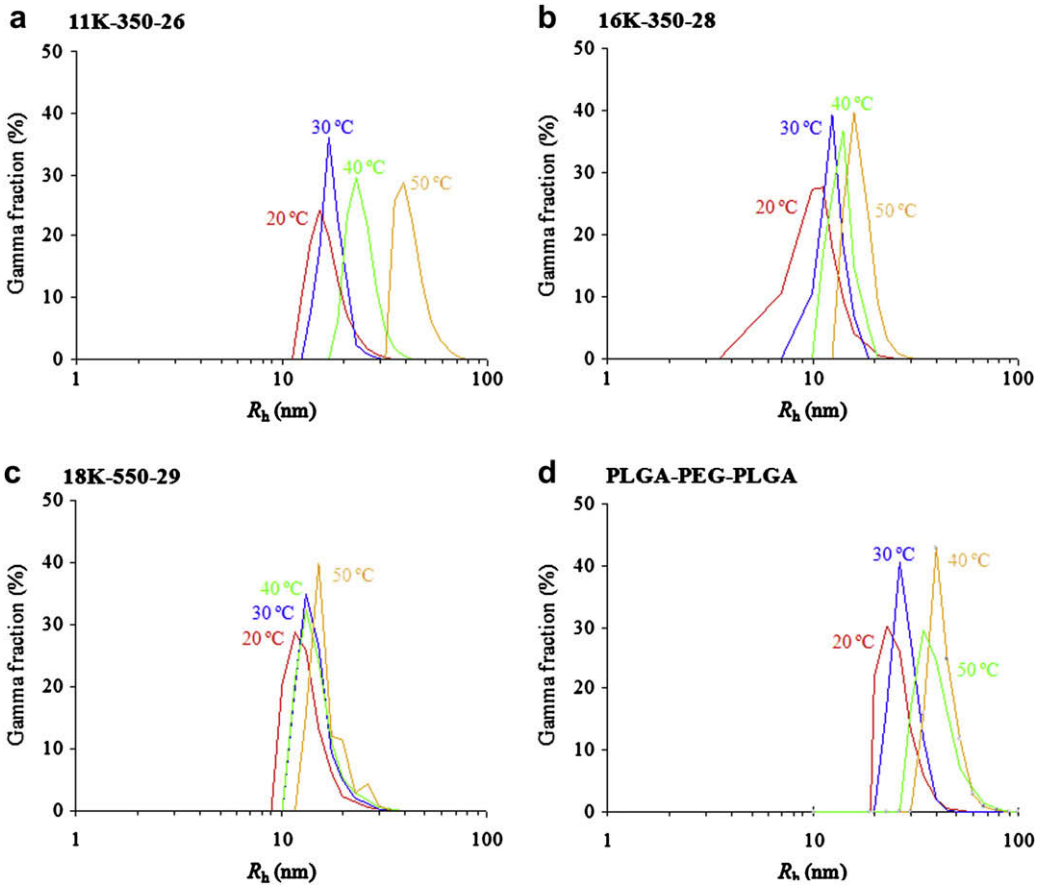


Fig. 5. Size of aggregates and their distribution in aqueous solution of PGD-DL-LA-*g*-PEG copolymers (0.3 wt%) as a function of temperature. (a) PGD-DL-LA-*g*-PEG(18K-550-29), (b) PGD-DL-LA-*g*-PEG(16K-350-28), (c) PGD-DL-LA-*g*-PEG(11K-350-26), and (d) PLGA–PEG–PLGA.

to form combined micelles is stronger for PGD-DL-LA-*g*-PEG(11K-350-26) and PLGA-*b*-PEG-*b*-PLGA primary micelles than PGD-DL-LA-*g*-PEG(18K-550-29) and PGD-DL-LA-*g*-PEG(16K-350-28) primary micelles. These results suggest that the thermodynamic stability of PGD-DL-LA-*g*-PEG(18K-550-29) and PGD-DL-LA-*g*-PEG(16K-350-28) primary micelles is significantly higher than those of PGD-DL-LA-*g*-PEG(11K-350-26) and PLGA-*b*-PEG-*b*-PLGA primary micelles due to the formation of a phase-separated core–shell structure with a tightly packed hydrophobic core. The temperature-induced intermicellar aggregation may be derived from the instability of the hydrophobic core in these micelles. In other words, the hydrophobic core becomes softer and looser with increasing temperature, leading to collapse of the phase-separated core–shell structure of the micelles and subsequent intermicellar aggregation. The lower thermodynamic stability of micelle cores is one of the reasons for a relatively lower T_{gel}. Previously, Tew et al. have reported that the stability of a hydrophobic core prepared from semicrystalline PLLA-*b*-PEG-*b*-PLLA copolymers is higher than that from amorphous PDLLA-*b*-PEG-*b*-PDLLA copolymers [33]. However, since both the PGD-DL-LA and PLGA used here as hydrophobic segments are amorphous copolymers, the crystallinity should not be a key to influence the thermodynamic stability in these cases. Thus, these results strongly support the structure of a hydrophobic core in PGD-DL-LA-*g*-PEG micelles. We can propose that a key factor in the thermodynamic stability of these micelles is the structure of the hydrophobic cores. Inter-chain overlapping, entangling, and inter-chain crumpling among PGD-DL-LA segments based on the comb-like architecture with amphiphilicity would result in higher thermodynamic stability. These physical interactions could not work effectively for PGD-DL-LA-*g*-PEG(11K-350-26) probably because of its shorter hydrophobic segment. Therefore, in addition to the structure of the hydrophobic cores, the molecular weight of the hydrophobic PGD-DL-LA backbone may also be an important factor.

3.5. Rheological properties of hydrogels

Dynamic mechanical analysis was carried out to determine the storage modulus (G') and loss modulus (G'') in the hydrogel formation process. Fig. 6 shows the temperature dependence of G' and G'' for PGD-DL-LA-*g*-PEG and PLGA-*b*-PEG-*b*-PLGA copolymer aqueous solutions (20 wt%). The maximum G' values and the corresponding temperature are listed in Table 3. The G' value of the PLGA-*b*-PEG-*b*-PLGA suspension increased gradually with increasing temperature and equaled the G'' value at 14 °C (gel point), subsequently reaching maximum values (ca. 79 Pa) at around 18 °C. By contrast, whereas PGD-DL-LA-*g*-PEG suspensions did not show a gradual increase of G' values at low temperatures (below each gel point), these G' values increased drastically above each gel point. These maximum G' values were 461, 492, and 339 Pa for PGD-DL-LA-*g*-PEG(18K-550-29), PGD-DL-LA-*g*-PEG(16K-350-28), and PGD-DL-LA-*g*-PEG(11K-350-26) hydrogels, respectively, in the temperature range from 38 to 52 °C. These maximum G' values were ca. 4 times larger than that of the PLGA-*b*-PEG-*b*-PLGA hydrogel, and were higher than the PLGA-*g*-PEG hydrogel reported by Jeong [20]. When comparing the maximum G' values of PGD-DL-LA-*g*-PEG copolymers, it was found that PGD-DL-LA-*g*-PEG hydrogel, which has a total high molecular weight of the hydrophobic PGD-DL-LA backbone, tends to show a higher maximum G' value. The order of maximum G' values of these thermogels agree with the

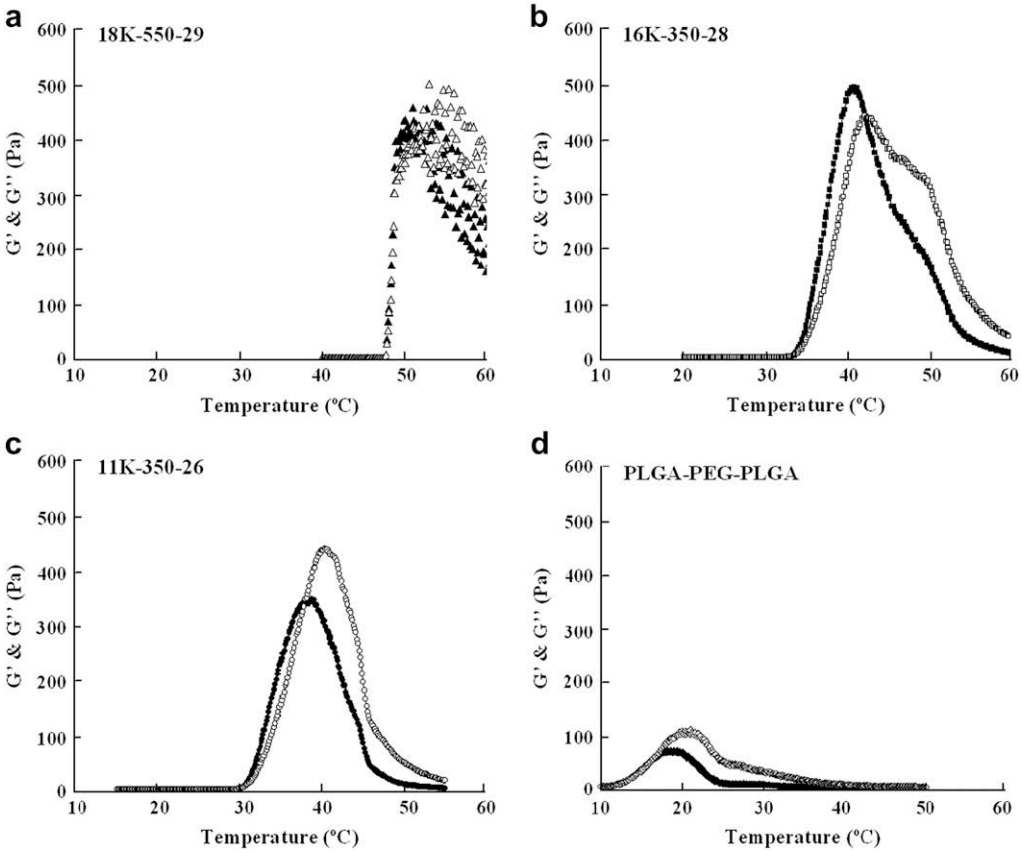


Fig. 6. Storage modulus G' (solid symbols) and loss modulus G'' (open symbols) as a function of temperature for PGD-DL-LA-*g*-PEG copolymer aqueous solutions (20 wt%). (a) PGD-DL-LA-*g*-PEG(18K-550-29), (b) PGD-DL-LA-*g*-PEG(16K-350-28), (c) PGD-DL-LA-*g*-PEG(11K-350-26), and (d) PLGA–PEG–PLGA.

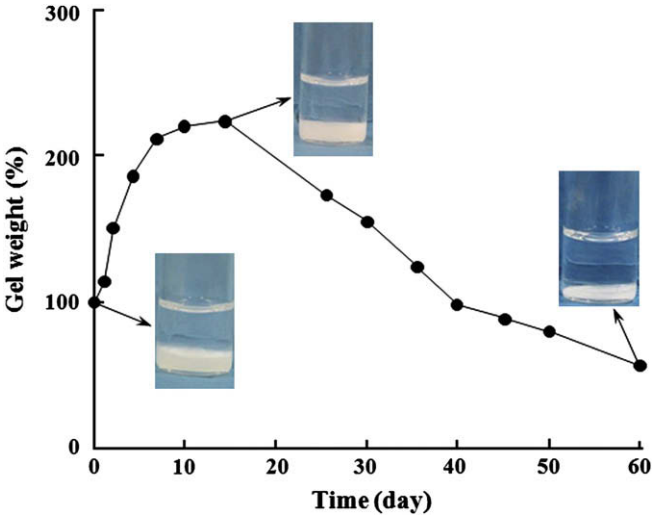


Fig. 7. Swelling and degradation profiles of 20 wt% PGD-DL-LA-*g*-PEG(16K-350-28) hydrogel in PBS at 37 °C.

thermodynamic stability results of the micelle cores, indicating that the higher thermodynamic stability affords the higher mechanical strength. Thus, in these core–shell type polymer micelles, high thermodynamic stability due to entangling and crumpling between the hydrophobic segments is important in terms of allowing formation of a mechanically strong hydrogel above the transition temperature.

To the best of our knowledge, there have been no reports concerning the temperature-responsive sol–gel transition of amphiphilic PEG–polyester copolymers in which the molecular weight of each hydrophobic polyester segment is over 10,000 Da, since such longer hydrophobic segments generally lead to insolubility in aqueous media. The comb-like graft copolymer systems have three parameters, length of main-chain, length of graft-chain and number of graft-chain, which could provide a wide range of hydrophobic hydrophilic balance and total molecular weight. We used DL-lactide as a monomer in this research. Other cyclic monomers, such as glycolide, ε-caprolactone, trimethylene carbonate, can be used in these graft copolymer systems. Such variation may afford further improvement to the mechanical strength at 37 °C.

3.6. Swelling and degradation of hydrogels

In order to study the hydrolytic profiles of the thermogels, PGD-DL-LA-*g*-PEG(16K-350-28) copolymer was dissolved in water (20 wt%), followed by incubation at 37 °C for 60 days. Fig. 7 shows the time course of weight change of the hydrogels. Photographs of the hydrogels just prepared, after 15, and 60 days are also shown. PGD-DL-LA-*g*-PEG(16K-350-28) hydrogels displayed gradual swelling for up to 15 days. This swelling behavior can be interpreted as a decrease in physical cross-linking points due to partial hydrolysis of the polymer. Swelling and erosion of the hydrogel occurred gradually over at least 60 days. The molecular weight of the PGD-DL-LA-*g*-PEG(16K-350-28) copolymer determined by GPC decreased to 1970 Da from the initial value (26,050 Da) after 60 days. The slow erosion profile and relatively long degradation time of the hydrogels render them suitable for use as injectable biomaterials such as drug delivery depots, as they are expected to allow sustained release of encapsulated pharmaceutical agents or temporary scaffolds for tissue regeneration.

4. Conclusion

Aqueous PGD-DL-LA-*g*-PEG copolymer solutions showing a sol–gel transition via a small temperature increase were developed. These comb-like graft copolymer systems could impart relatively high total molecular weight and mechanical strength to thermo-gelling polymers. The sol–gel transition temperature could be controlled from 33 to 51 °C by varying PEG length and compositions without a decrease in mechanical strength of the hydrogels. Since the comb-like graft copolymer systems have three parameters, length of main-chain, length of graft-chain and number of graft-chain, a wide variation of hydrophobic hydrophilic balance and total molecular weight can be easily obtained to improve the character of the thermo-gelling system. Thus, this research provides novel insights into molecular design for thermo-gelling biodegradable polymer systems. The hydrogels underwent gradual erosion for 60 days in PBS at 37 °C. This slow erosion profile and relatively long degradation time of the hydrogels render them suitable for use as injectable biomaterials such as drug delivery depots, as they are expected to allow sustained release of encapsulated pharmaceutical agents or temporary scaffolds for tissue regeneration.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.polymer.2009.05.045.

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