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In situ Gel Forming Stereocomplex Composed of Four-Arm PEG-PDLA and PEG-PLLA Block Copolymers

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Abstract: Injectable hydrogels are quite promising materials due to their potential to minimize invasive implantation and this provides versatile fitness irrespective of the damaged regions and facilitates the incorporation of bioactive agents or cells. *In situ* gel formation through stereocomplex formation is a promising candidate for injectable hydrogels. In this paper, a new series of enantiomeric, four-arm, PEG-PLA block copolymers and their stereocomplexed hydrogels were prepared by bulk ring-opening polymerization of D-lactide and L-lactide, respectively, with stannous octoate as a catalyst. The prepared polymers were characterized by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, Fourier-transform infrared (FT IR) spectroscopy, gel permeation chromatography (GPC) and thermal gravitational analysis (TGA), confirming the tailored structure and chain lengths. The swelling and degradation behavior of the hydrogels formed from a selected copolymer series were observed in different concentrations. The degradation rate decreased with increasing polymer content in the solution. The rheological behavior indicated that the prepared hydrogel underwent *in situ* gelation and had favorable mechanical strength. In addition, its feasibility as an injectable scaffold was evaluated using a media dependence test for cell culture. A Tris solution was more favorable for *in situ* gel formation than PBS and DMEM solutions were. These results demonstrated the *in situ* formation of hydrogel through the construction of a stereocomplex with enantiomeric, 4-arm, PEG-PLA copolymers. Overall, enantiomeric, 4-arm, PEG-PLA copolymers are a new species of stereocomplexed hydrogels that are suitable for further research into injectable hydrogels.

Keywords: injectable hydrogel, *in situ* hydrogelation, stereocomplex formation, 4-arm PEG-PLA enantiomers.

Introduction

Hydrogels are considered important biomaterials in various biomedical fields on account of their properties, such as highly hydrophilic nature, flexible structure and good biocompatibility.[1-6] After applying an injectable functionality to hydrogels, there have been many advances in hydrogels in areas of drug delivery and tissue engineering.[7-15] Injectable hydrogels are quite promising materials because they can enable minimized invasive implantation, which have versatile fitness irrelevant to damaged regions and facilitate the incorporation of bioactive agents or cells.[7,8] Injectable hydrogels are based on the *in situ* hydrogel formation triggered by external stimuli, mild chemical reactions and stereocomplexation, etc. The *in situ* forming hydrogels are advantageous for biomedical applications because the hydrogel formation do not require toxic crosslinking agents or organic solvents. External stimuli such as temperature[16-19] and pH[20-22] sensitive reactions permit to form a physical hydrogel of which the sol-gel transition is reversible and has the weak mechanical properties. On the other hands, light stimulus[23-26] and mild chemical reactions such as Michael addition[27-29] and enzymatic reaction[30,31] permit to form a chemical hydrogel of which the sol-gel transition is irreversible and has the good mechanical properties. Exceptionally, stereocomplex formation[32] allows forming a physical hydrogel, but the sol-gel transition and physical properties are similar to chemical hydrogels.

In situ hydrogel formation through stereocomplexation has been highlighted as one such attractive hydrogel. For biodegradable hydrogels, enantiomeric block copolymers of poly(lactic acid) (PLA) have been investigated for potential *in situ* hydrogel formation. The copolymers contained enantiomeric PEG-PLA block copolymers with triblock[33-37] and

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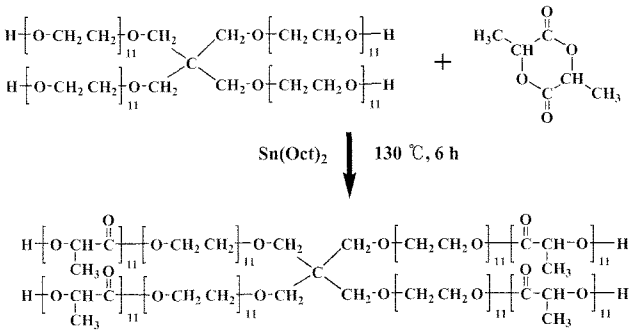
8-arm (star-shape).[38-40] In their studies, hydrogelation through stereocomplex formation was confirmed, and the gelation behaviors and rheological properties were investigated. In addition, de Jong and coworkers prepared and characterized PLA-dextran graft copolymers and their hydrogels.[41-43] This effort lead to carry out more studies on applications for protein delivery because potential growth factors play important roles in tissue repair and regeneration. Recent studies reported that potential proteins could be released from stereocomplexed hydrogels in a controlled manner and their conformational state was stable.[44-46] However, such outcomes were not extended to further studies on cellular responses to the hydrogels, such as the cell viability, adhesion, proliferation, migration, and differentiation, which are considered to be essential for applications to injectable scaffold.

This paper reports one series of PLA stereocomplexes, enantiomeric four-arm PEG-PLA block copolymers, and their hydrogels. The synthesis and hydrogel studies were carried out in a similar manner to other stereocomplexes and their hydrogels. Furthermore, feasibility as an injectable scaffold for tissue repair and regeneration was also evaluated. These results demonstrate the production of a hydrogel through stereocomplex formation from enantiomeric 4-arm PEG-PLA copolymers was accomplished. This hydrogel has potential applications as a cell-supporting scaffold.

Experimental

Materials. 4-Arm poly(ethylene glycol) (4-arm PEG, M_w =2,000 g/mol) was obtained from NOF Co. (Tokyo, Japan) and the D-lactide and L-lactide were supplied by Purac (Gorinchem, The Netherlands). Stannous octoate (Sn-Oct) and Trizma hydrochloride buffer solution (1 M) were purchased from Sigma (St. Louis, MO, USA). Phosphate buffered saline (PBS) and Dulbecco's modified eagle medium (DMEM) were supplied by Life Technology (Carlsbad, CA, USA). All chemicals and solvents were used without further purification.



Scheme I. Synthesis of 4-arm PEG-PLLA and PEG-PDLA copolymers.

Synthesis of 4-Arm PEG-PLA Block Copolymers. 4-Arm poly(ethylene glycol)-poly(D-lactide) (4-arm PEG-PDLA) and 4-arm poly(ethylene glycol)-poly(L-lactide) (4-arm PEG-PLLA) block copolymers were prepared by bulk ring-opening polymerization of D-lactide and L-lactide, respectively, using 4-arm PEG as a tetra hydroxyl group initiator and stannous octoate as a catalyst, as shown in Scheme I. As a typical procedure, D-lactide or L-lactide (5.31 g, 37 mmol), 4-arm PEG (6 g, 3 mmol), and St-Oct (0.03 g, 0.074 mmol) were inserted into an ampoule and maintained at 130 °C for 6 h under nitrogen. The feed amounts of 4-arm PEG were varied to provide a variety of chain lengths and block ratios of block copolymers (Table I). After the reaction was complete, the mixture was dissolved in methylene chloride and precipitated twice in cold diethyl ether. The residual solvent was eliminated by a vacuum oven in 3 days, and a semi-transparent viscous solution of the resulting polymers was obtained in high yield (9.6 g, 85%).

Characterization. The structure and composition of 4-arm PEG-PDLA and 4-arm PEG-PLLA was determined by ^{1}H NMR and FT IR. For the ^{1}H NMR measurements, the copoly-

Table I. Synthesis of 4-Arm PEG-PDLA and 4-Arm PEG-PLLA Block Copolymers

No	PLA	Feed Amount (mmol)			GPC Results			
		4-Arm PEG	Lactide	Sn-Oct	DP_{PEG}	DP_{PLA}	M_n	M_w/M_n
1	PDLA	23	37	0.074	45	48	5,473	1.09
2		25				47	5,360	1.09
3		27				44	5,170	1.07
4		30				43	5,080	1.08
5		32				41	4,990	1.16
6	PLLA	23	37	0.074	45	51	5,670	1.10
7		25				50	5,620	1.10
8		27				45	5,270	1.08
9		30				43	5,150	1.08
10		32				39	4,840	1.16

mer solutions dissolved in $CDCl_3$ were measured using a NMR-400 (Varian, 400 MHz, USA) at 37 °C. The sample for FT IR was prepared by adding the polymer solutions dissolved in methylene chloride into two NaCl disks. The resulting samples were examined using a Nicolet Magma-IR 550 (USA). The molecular weight and polydispersity of the copolymers were determined by chromatography (GPC), M616LC System (Waters, USA) using tetrahydrofuran (THF) as the eluent and polystyrene as the standard polymer. The thermal properties of the copolymers and their stereocomplex were analyzed by thermogravimetric analysis (TGA), TGA Q50 (TA Instrument, USA). The stereocomplex sample was prepared by mixing each polymer dissolved in methylene chloride. The mixture was left to stand for several hours at room temperature to allow solvent evaporation.

***In situ* Hydrogelation.** The prepared copolymers were dissolved separately in Tris buffer (0.5 mL) to a concentration of 20 wt%. The solutions were then mixed and gently agitated at 25 °C to allow *in situ* hydrogel formation. PBS and DMEM were also used as solvents in the same manner to observe any media-dependent hydrogelation. Various solutions were mixed with the enantiomeric copolymers with different chain lengths to obtain hydrogels. The time in which a gel was formed, which is denoted by the gelation time, was determined using vial tilting method. The state in which no flow was observed within 10 min after inverting the vial was regarded as the gel state.

Gel Swelling Ratio. The hydrogel samples (1 mL) were prepared at 18, 20 and 22 wt% concentrations, and weighed accurately (W_i). The samples were then incubated in 1 mL of PBS at 37 °C. At each time interval, the PBS solution was removed from the samples and the weight of the hydrogels was determined (W_t) in order to calculate the swelling ratio. After weighing, fresh PBS solutions were added to the hydrogels. The experiments were carried out in triplicate.

The swelling ratios were determined using the following equation:

$$\text{Swelling ratio} = (W_t - W_i) / W_i$$

where, W_t and W_i are the weights of the water-swollen samples and the initial hydrogel samples, respectively.

Rheological Analysis. The rheological experiment was performed using a Reologica rheometer (Reologica instruments AB®) equipped with a flat plate measuring geometry (25 mm diameter, 0.35 mm gap between plates) at a frequency of 0.1 Hz. Twenty 20 wt% solutions of the copolymers in the Tris buffer were mixed and placed quickly on the plate of the rheometer using a double injection syringe. The gelation of the copolymer solutions was monitored by measuring the storage and loss moduli at 37 °C for 1 h in 30 sec intervals.

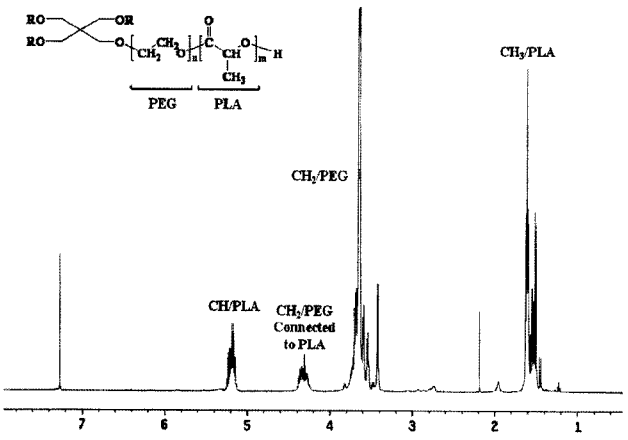


Figure 1. ^{1}H NMR spectrum of a 4-arm PEG-PDLA block copolymer in $CDCl_3$.

Results and Discussion

Synthesis of 4-Arm PEG-PDLA and PEG-PLLA Block Copolymers. Figure 1 shows the ^{1}H NMR spectrum of a 4-arm PEG-PLA block copolymer. The signals at 5.20 ppm and 1.55 ppm were assigned to a methine proton and methyl protons of the PLA block, respectively, indicating successful ring-opening polymerization of the lactide. A signal at 3.65 ppm was derived from the methylene protons of oxyethylene, indicating the presence of a PEG chain. The FT IR spectrum of a 4-arm PEG-PLA block copolymer showed absorption peaks at 2888 (νC-H), 1755 (νC=O) and 1109 (νC-O) cm^{-1}, which confirmed the presence of lactate and oxyethylene groups on the chain of the block copolymer (data not shown). The GPC results revealed that the molecular weights ranged from 5,000-5,600, and the degree of polymerization (DP) of the lactate units were 10-13 per arm of 4-arm PEG-PLA. Table I summarizes the feed amounts and synthetic results of the enantiomeric copolymers. The chain lengths of the PLA block were tailored by varying the feed amounts in accordance with the feed ratios of the 4-arm PEG with lactides. However, the resulting DP values did not exactly coincide with the feed ratios. This is possibly due to the incomplete polymerization of PLA. The polydispersity indices (PDI, M_w/M_n) of all copolymers were also maintained at approximately 1.1, indicating the monodisperse properties of the synthesized copolymer chains.

Table II. Gelation Time of the 4-Arm PEG-PDLA + 4-Arm PEG PLLA Hydrogel

No	Mixed Solutions	Gelation Time (min)
A	1 + 6	insoluble
B	2 + 7	insoluble
C	3 + 8	1
D	4 + 9	5
E	5 + 10	10

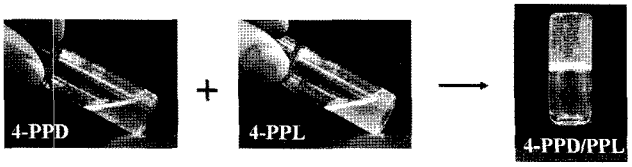


Figure 2. Photographs showing *in situ* hydrogel formation by mixing 20 wt% solutions of 4-arm PEG-PDLA and 4-arm PEG-PLLA at room temperature.

***In situ* Hydrogelation by Stereocomplex Formation.** The enantiomeric copolymers with similar chain lengths were mixed with each other, as shown in Table II. Solutions A and B were insoluble in aqueous buffers and could not form a hydrogel. This is because the chain lengths of a hydrophobic PLA block were slightly too long to be dissolved in aqueous solution. Solutions C, D and E were soluble in aqueous media and formed hydrogels but the gelation times were different. de Jong *et al.* reported that stereocomplex formation occurred in a blend of the enantiomeric PLAs when the DP of PLA was 7-11.[43] In the present case, the PDLA or PLLA with more than 12 lactate units was not soluble in water. Solutions C, D and E, which formed a hydrogel, were made by copolymers with the PLA DPs of 10-11. These results correspond to those reported by de Jong *et al.*. In addition, the gelation time increased with decreasing PLA chain length. This was attributed to decreases in the capacity of the PLA blocks for stereocomplex formation. Figure 2 shows images of the *in situ* hydrogel formation caused by mixing the two solutions of enantiomeric 4-arm PEG-PLA copolymers. Although the solutions of the copolymers were slightly translucent, the resulting hydrogel was almost transparent. This observation suggests indirectly that the hydrogel network was crosslinked by stereocomplexes between the PDLA and PLLA blocks. In many cases of physical gels, the hydrophobic interaction of the amphiphilic copolymers results in opaque hydrogels. However, in this case, it is believed that stereocomplex formation minimized the extensive crystalline phases that are observed in common physical hydrogels. Thermal analysis of the copolymers also shows that the block copolymers form a stereocomplex. Figure 3 shows the TGA thermograms of the stereocomplex along with 4-arm PEG and 4-arm PEG-PDLA. The thermal degradation rate of 4-arm PEG was accelerated at approximately 400 °C. The thermal degradation behavior of 4-arm PEG-PDLA showed two phases consisting of degradation around 250 and 400 °C. This demonstrates that a PDLA chain exists along with PDLA in the copolymer chain. Interestingly, the stereocomplex of 4-arm PEG-PDLA with 4-arm PEG-PLLA shows slightly different degradation behavior from 4-arm PEG-PDLA. The thermal behavior was the same at 400 °C, but was slightly different around 250 °C. This suggests that the structure or any other parameters of stereocomplexes are affected and are different from the un-complexed form (4-arm PEG-PDLA).

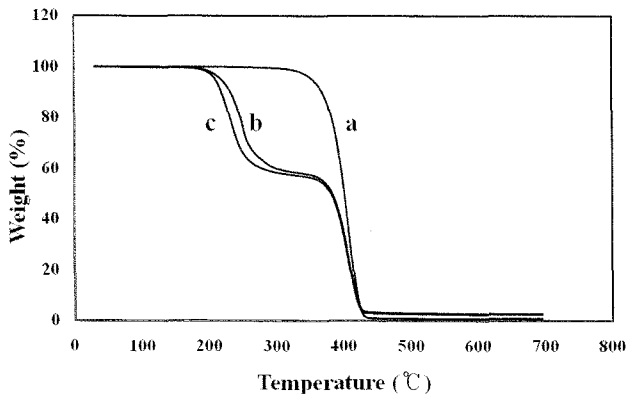


Figure 3. TGA thermograms of 4-arm PEG (a), 4-arm PEG-PDLA (b) and the stereocomplex (c).

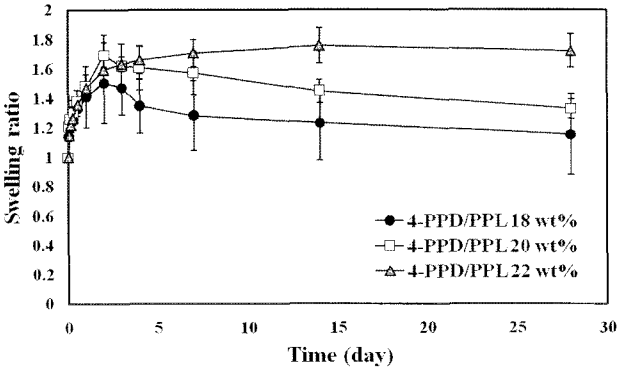


Figure 4. Swelling ratio of the stereocomplexed hydrogels with different concentrations as a function of time.

Swelling and Degradation Behavior of Hydrogels. Figure 4 shows the gel swelling and degradation profiles of stereocomplexed hydrogels at concentrations of 18, 20 and 22 wt%. The swelling ratio of the hydrogels increased with solution concentration over a period of 2~3 days. After that period, the hydrogels began to show slow degradation behavior. After 4 weeks, in the cases of the 18 and 20 wt% hydrogels, the swelling ratio of the maximum swelled hydrogel was 70-80%, and the swelled hydrogels were too fragile compared with the initial hydrogel strength. However, in case of the 22 wt% hydrogel, the swelling ratio was retained at a similar level to the initial value. The overall slow degradation behavior of the hydrogels could be attributed to the stereocomplexes formed by blending the block copolymers with PDLA and PLLA.[47] In the 22 wt% hydrogel, a relatively wider range of the stereocomplex region could endure the degradation of the PLA chains caused by hydrolysis, whereas the 18 and 20 wt% hydrogels were unlikely to have sufficient stereocomplex regions to endure the hydrolysis of the PLA chain like the 22 wt% hydrogel. The biodegradable property of a hydrogel plays an impor-

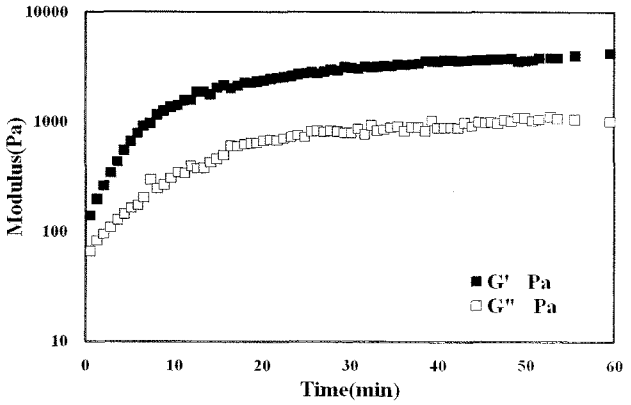


Figure 5. Storage and loss moduli of the stereocomplexed hydrogel (20 wt%) as a function of time.

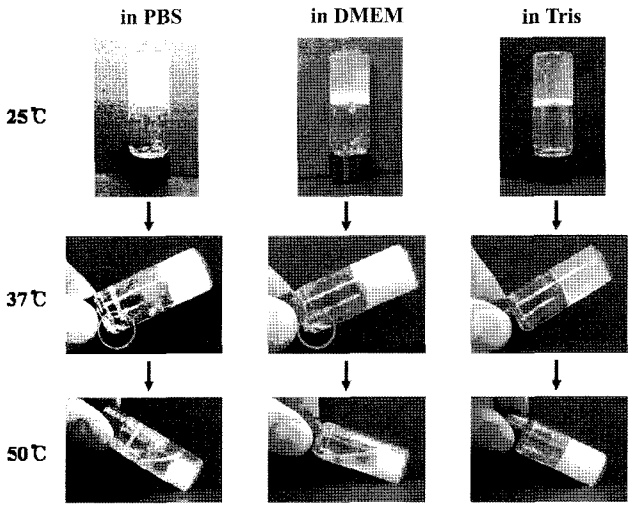


Figure 6. Photographs showing the solutions mixed with the 4-arm PEG-PLA copolymers dissolved in different aqueous media at 25, 37 and 50 °C.

tant role in performing the functions as a scaffold for tissue repair or regeneration.[3,7] Based on this result, the degradation time of stereocomplexed hydrogel can be modulated by varying the polymer concentration, and also related with the other parameters that affect the stereocomplex. For example, the chain length or composition of copolymers affects the solubility as well as the strength or capacity of the stereocomplex, which can be considered another parameter that can be used to modulate the degradation time of the hydrogel.[41]

Rheological Properties of Hydrogels. Rheological tests were carried out to confirm the gelation time and mechanical strength of a stereocomplexed hydrogel. Figure 5 shows the variations in the storage (G') and loss (G'') moduli of 20 wt% hydrogel measured for 60 min. Increases in both moduli with time means an association of polymeric chains, resulting in a polymeric network through chemical and/or physical crosslinking. Both moduli profiles shown in Figure 5 indicate that two types of polymers were mixed and associated to form a polymeric network, which was probably caused by stereocomplex formation. Some researchers examined the rheological properties of stereocomplexes from PLAs and their derivatives under a variety of conditions, such as polymer concentration, blending ratios and chain length.[33-43] The effects of those parameters on the mechanical and rheological properties of hydrogels have already been reported. The present results also show that the enantiomeric polymers form a hydrogel with a polymeric network through stereocomplex formation. This rheological result makes it possible to estimate the mechanical strength of a stereocomplexed hydrogel, which makes suitable as an injectable biomaterial enabling it to retain its structure in a physiological environment.

Feasibility as Injectable Scaffolds. The stereocomplexed hydrogel from the 4-arm PEG-PLA enantiomers were characterized from a few fundamental properties including gel swelling and degradation, rheological property, and gelation time. The results showed that the *in situ* stereocomplexed hydrogel has properties suitable for use as an injectable scaffold for tissue repair and regeneration. These properties were sufficient to allow further investigations to be carried out for biomedical applications. First, the influence of a few media for cell culture on hydrogel properties was investigated. Figure 6 shows images of the hydrogels prepared in different media. The solutions in all media formed a hydrogel at 25 °C, even though each sample showed a different color and transparency. However, at 37 °C, water flowing out from the hydrogel was observed in the cases of the PBS and DMEM media, and all hydrogels were opaque or translucent. This was attributed to the hydrophobic interaction between the PLA chains that did not form a stereocomplex. Generally, opacity is observed in physical hydrogels from thermosensitive amphiphilic copolymers because extended crystalline phases are formed in a hydrogel matrix. The water leakage from hydrogels also is likely to be derived from increases in the region of the crystalline phase due to a hydrophobic interaction. As a noteworthy phenomenon, the solution in Tris medium did not show water leakage. Increasing the temperature to 50 °C caused more severe water leakage as well as shrinkage of the gel matrices in the PBS and DMEM solutions due to the more extended crystalline phases, whereas no change was observed in the Tris buffer solution. These indicate that hydrogelation through stereocomplex formation might be dependent on the media species, probably derived from the buffering capacity or composition of media. This demonstrates that the Tris buffer is suitable as a solvent of injectable hydrogels for biomedical applications.

In addition, a cell viability test was also performed to evaluate the cell compatibility of the stereocomplexed hydrogels. However, the results from the *in vitro* cell culture revealed the hydrogels to be highly cytotoxic due to acidifi-

cation of the hydrogel matrices. This was attributed to the generation of lactic acid caused by the degradation of PLA chains. It is known that PLA ultimately degrades to H_2O and CO_2 in the body. Nevertheless, local acidification in a hydrogel matrix is likely to be an unavoidable problem under *in vitro* conditions.

Conclusions

A novel series of enantiomeric 4-arm PEG-PLA block copolymers were prepared via the bulk ring-opening polymerization of lactides, showing a tailored structure and chain lengths. The swelling/degradation and rheological results revealed that *in situ* hydrogelation and mechanical strength are favorable for injectable hydrogels. In addition, evaluations of the feasibility as an injectable scaffold concluded that the Tris buffer solution is favorable for *in situ* hydrogel formation but acidification of the hydrogel matrix can limit *in vitro* evaluations. Recently, there have been attempts to overcome such local acidification due to PLA degradation using various methods such as the conjugation of basic molecules.[48] We also keep on trying to find a solution using basic molecules, additives, buffer media, and so on. If the acidification problem is solved, the stereocomplexed hydrogel may be applied to injectable scaffolds for tissue regeneration.

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