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NOTE

Instant preparation of a biodegradable injectable polymer formulation exhibiting a temperature-responsive sol–gel transition

Yasuyuki Yoshida[1], Akihiro Takahashi[2], Akinori Kuzuya[1,2] and Yuichi Ohya[1,2]

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INTRODUCTION

Thermogelling polymers exhibiting a sol–gel transition between room temperature (r.t.) and body temperature can be used as injectable polymers (IPs). An aqueous solution of such polymers is in the sol state at r.t. and becomes a hydrogel *in situ* simply after injection into the body by a syringe. Biodegradable thermogelling polymers are of particular interest because of their potential for use in minimally invasive clinical therapies. Such hydrogels can easily entrap water-soluble bioactive reagents (for example, drugs, proteins, nucleic acids and so on) or living cells simply by mixing them into the polymer solution before administration. Thus, IP systems have been widely investigated for use as sustained drug delivery depots and as scaffolds for tissue regeneration.[1–4] Normally, IPs exhibiting a temperature-responsive sol–gel transition possess amphiphilic structures with a delicate balance of hydrophilic and hydrophobic segments. Poly (ethylene glycol) (PEG) is a water-soluble polymer with excellent biocompatibility that has frequently been used as the hydrophilic segment in IP systems. Diblock and triblock copolymers of PEG and water-insoluble biodegradable aliphatic polyesters, such as poly(L-lactide), poly(D,L-lactide), poly(D,L-lactide-*co*-glycolide) and poly(ε-caprolactone), have been investigated and applied as biodegradable IPs.[5–9] We have reported several biodegradable IP systems exhibiting a temperature-responsive sol–gel transition and relatively high mechanical strength in the gel state using block or graft copolymers of polylactide and PEG having branched strcutures.[10,11] However, almost all of the IPs reported previously are a sticky paste in the dry state at r.t., and require a considerable amount of time (usually more than 1 day) to be dissolved in an aqueous solution. These properties are inconvenient for the preparation of IP formulations in the laboratory and in the clinic, and they represent a significant barrier to the clinical application of IPs. To address this issue, Jeong[12], Ding[13] and Deng[14] reported a method for the solidification of amphiphilic block copolymer-type thermogelling polymers containing PEG and poly(ε-caprolactone) or their copolymers to achieve a powder form in the dry state. However, solubilization in an aqueous solution remained a problem in these systems, and it was necessary to heat the mixture of the polymer and solvent above the melting temperature of the copolymers to achieve dissolution. An additional problem is that aqueous solutions of the copolymer are likely to spontaneously transform into a hydrogel after a certain period at r.t. because of the relatively high crystal-forming tendency of the hydrophobic segments.

Herein, we developed a quick and convenient preparative method for IP formulations. We used a triblock polymer composed of poly(ε-caprolactone-*co*-glycolic acid) (PCGA) and PEG, PCGA-*b*-PEG-*b*-PCGA, and investigated the effects of various additives on the dispersion time (DT) and gelation behavior of the copolymers in phosphate buffer solution (PBS; pH = 7.4, I = 0.14). The results indicated an appropriate combination of copolymer and additive for the quick preparation of an IP formulation exhibiting a temperature-responsive sol–gel transition between r.t. and body temperature. The method developed here should be convenient for use at the clinical scene.

EXPERIMENTAL PROCEDURE

Materials

PEG ($M_n = 1000$ and 1500; PEG_{1000} and PEG_{1500}, polyacrylic acid ($M_n = 5000$), ε-caprolactone (CL), tin-2-ethylhexanoate, maltose monohydrate, sucrose and lactose monohydrate were purchased from Wako Pure Chemical Ind. (Osaka, Japan). PEG ($M_n = 2000$, 4600 and 10 000; PEG_{2000}, PEG_{4600} and $PEG_{10\,000}$) and monomethoxy-PEG ($M_n = 5000$; MeO-PEG_{5000}) were purchased from Sigma-Aldrich (St Louis, MO, USA). Glycolide (GL) was obtained from Polysciences Inc. (Warrington, PA, USA). Polyvinyl alcohol ($M_n = 2000$) was purchased from Kanto Chemical Inc. (Tokyo, Japan). Sodium hyaluronate ($M_n = 90\,000$) was supplied by Kibun Food Chemifa Co., Ltd. (Tokyo, Japan).

PCGA-*b*-PEG-*b*-PCGA triblock copolymers were synthesized by the ring-opening copolymerization of CL and GL in the presence of PEG_{1500} as a macroinitiator and tin-2-ethylhexanoate as a catalyst at 160 °C for 12 h,

[1]Department of Chemistry and Materials Engineering, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Suita, Japan and [2]Organization for Research and Development of Innovative Science and Technology (ORDIST), Kansai University, Suita, Japan
Correspondence: Professor Y Ohya, Department of Chemistry and Materials Engineering, Kansai University, Yamate 3-3-35, Suita, Osaka 564-8680, Japan.
E-mail: yohya@kansai-u.ac.jp
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according to the method reported previously (Supplementary Scheme S1).[13–15] Details of the characterization of the products are provided in Supplementary Information (Supplementary Figure S1 and Supplementary Table S1).

Sample preparation and sol–gel transition

The temperature-responsive sol–gel transition behavior of the copolymer solution or suspension was investigated by the test-tube inversion method.[10] Uniformly dissolved sample solution (named as **S**-sample) was prepared by mixing a predetermined amount of copolymer (solid) with PBS (pH = 7.4, I = 0.14) in a 5 ml vial. The mixture was shaken by a vortex mixer, and then heated at 60 °C, which was above the T_m of the copolymer, for 1 min. During this process, the sample became an opaque hydrogel. The hydrogel obtained in the vial was immersed in ice-cold water for 10 min to recover the sol state. This process was repeated several times. The resulting solution was then incubated at 4 °C for 12 h to remove air bubbles. To determine the sol–gel transition temperature, T_{gel}, the vial of uniformly dissolved sample was immersed in a water bath at the desired temperature for 15 min, removed from the water bath and then inverted repetitively. The temperature was increased in 1 °C increments, and T_{gel} was determined based on the criteria of 'flow' (= sol) and 'no flow' (= gel) over 30 s. Measurements were repeated three times for each temperature to determine the transition temperature for a phase diagram.

Powdery formulations of the copolymer with or without additives were prepared by solvent evaporation and freeze-drying. PCGA-*b*-PEG-*b*-PCGA (100 mg) with or without predetermined amount of additive (5–20 wt% to the copolymer) was put in a sample tube. Acetone or pure water (1 ml) was added to the sample tube, and the mixture was sonicated in a bath-type sonicator for a few minutes to give a clear solution. The obtained solution was added dropwise into 10 ml of pure water and further stirred for 20 min. After removal of acetone by evaporation, the clear aqueous solution of PCGA-*b*-PEG-*b*-PCGA with/without additives was freeze-dried to give a white, powdery mixture.

The apparent minimum DT of the powdery formulation was determined by a nylon mesh permeation test. The powdery polymer sample containing 100 mg of copolymer and a predetermined amount of additive was placed in a vial, and then PBS was added to the vial to achieve a copolymer concentration of 30 wt%. After vigorous mixing using a vortex mixer for a specified period of time at r.t., a sample was taken from the turbid suspension and dropped onto a nylon mesh (mesh size = 150 μm) that was placed on a vacuum filtration apparatus. The DT was defined as the mixing time after which insoluble material was not observed on the nylon mesh. The suspension obtained by this dispersion procedure was named as **D**-sample.

Rheological measurements were performed on a dynamic rheometer (Thermo HAAKE RS600, Thermo Fisher Scientific, Waltham, MA, USA). A solvent trap was used to prevent evaporation of the solvent. Each sample was injected between parallel plates (diameter: 25 mm, gap of 1 mm) using a syringe. The data were collected at a controlled stress (4.0 dyn cm^{-2}) and a frequency of 1.0 rad s^{-1} while heating at 0.5 °C per min from 20 to 50 °C. The gelation temperature (T_{gel}) was defined as the temperature where the storage modulus (G') overtook the loss modulus (G').

RESULTS AND DISCUSSION

Synthesis of PCGA-*b*-PEG-*b*-PCGA triblock copolymer

The synthesis of the PCGA-*b*-PEG-*b*-PCGA triblock copolymer was performed twice, and each run gave slightly different samples (code: **CP4** and **CP5**). The results from the characterization and the proton nuclear magnetic resonance (^{1}H NMR) spectra of the PCGA-*b*-PEG-*b*-PCGA triblock copolymers are shown in Supplementary Table S1 and Supplementary Figure S1, respectively. The molecular weight of each PCGA segment was ~1800 Da, and the total molecular weight of the triblock copolymers was 5000 or 5100 Da. The average degrees of polymerization (DP) for the CL and GA (glycolic acid, not GL) units were calculated from integrations of the *CO*-linked methylene (-CO-CH_2-) peaks of the CL unit at 2.2–2.5 p.p.m. and the methylene (-O-CH_2-CO) peaks of the GA unit at 4.6–4.8 p.p.m. in the ^{1}H NMR spectra. The DP of the CL and GA units for **CP4** were calculated to be 14 and 3.4, respectively, and for **CP5** they were 14 and 1.5, respectively. The CL/GL in the feed was 7.0. If the reactivity of CL and GL was equal, CL/GA should be 3.5 because the GL unit contains two GA units. The CL/GA found in **CP4** and **CP5** was 4.1 and 4.7, respectively. Because these values are >3.5, these results indicate a relatively higher reactivity of CL compared with GL. The average lengths of the caproyl-repeating units and the glycolyl-repeating units, L_c and L_g, were calculated from the 1H NMR spectra[16,17] to be 4.3 and 1.1 for **CP4**, respectively (see Supplementary Information). For **CP5**, these values were 4.9 and 1.1, respectively. The melting point (T_m) of **CP4** was observed to be 33 °C, but the T_m of **CP5** was slightly higher at 37 °C. This difference in T_m reflects the slightly higher crystallinity of **CP5** because of its larger L_c. These copolymers both had a powder morphology at r.t. in the dry state. A photograph of **CP4** after drying is shown in Supplementary Figure S2.

Sol–gel transition behavior of uniformly dissolved samples

The temperature-responsive sol–gel transition behavior for **S**-samples (**CP4-S** or **CP5-S**) in PBS as a function of polymer concentration was investigated by the test-tube inversion method. The results for **CP4-S** are shown in Figure 1 as a phase diagram. The photographs in Figure 1 show the sol and gel states of **CP4-S** in PBS (30 wt%). **CP4-S** exhibited a sol–gel transition in the concentration range of 15–30 wt%. The T_{gel} values of **CP4-S** at 20, 25 and 30 wt% were 36, 35 and 32 °C, respectively. The T_{gel} values of **CP5-S** at 20, 25 and 30 wt% were slightly higher at 38, 36 and 34 °C, respectively. These results, which can be attributed to the slightly shorter hydrophobic segments in **CP5** compared with **CP4**, suggest that the length of hydrophobic segment had greater influence on T_{gel} than the crystallinity. However, the differences were within 2 °C and were not significant. The results from the rheological study of **CP4-S** (30 wt%) are shown in Figure 2 and Table 1. **CP4-S** (30 wt%) showed an increase in storage modulus (G') as temperature increased, and T_{gel} was observed to be 28.3 °C. At 37 °C, G' was 233 Pa. The maximum value of G' was found to be 279 Pa at 40.4 °C.

Effects of additives on DT

The effects of additives on the DT of freeze-dried samples were investigated by a nylon mesh test. The effects of additives on the nanoparticle suspension were also reported previously.[18] Disaccharides and water-soluble polymers were chosen as the additives based on the following expectations: (1) these additives can penetrate into the solid-state PCGA segment in the freeze-dried sample to reduce the size of crystals, and (2) these additives will not disturb the solidification of the polymers. Importantly, none of the

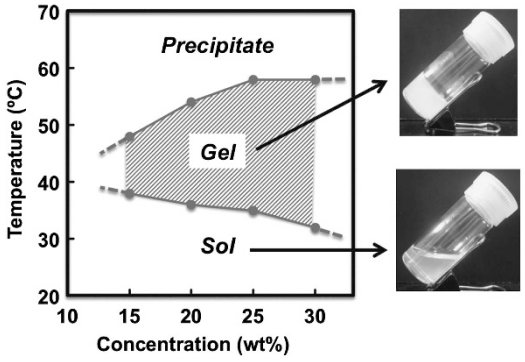

Figure 1 Phase diagram of uniformly dissolved **CP4** in PBS (**CP4-S**) at various concentrations determined by the test-tube inversion. Photographs show **CP4-S** in the sol and gel states. A full color version of this figure is available at *Polymer Journal* online.

additives disturbed solidification, as all of the freeze-dried samples showed a cotton-like morphology in air at r.t. (Figure 3a). With the exception of polyvinyl alcohol and sodium hyaluronate, all of the mixture samples passed the nylon mesh test, meaning that no residual solids were observed on the nylon mesh, and showed a turbid but macroscopically homogeneous distribution after several minutes of vortex mixing (Figure 3b). These samples showed sol–gel transition behavior between 25 and 37 °C (Figure 3c). The effects of the additives on the DT and sol–gel transition are summarized in Table 2. It should be noted that because of limitations in the amount of available sample, **CP5** was used for these experiments rather than **CP4**. However, as described above, **CP5** had T_{gel} values that were comparable to **CP4** in the test-tube inverting test, and the differences were within 2°C. Thus, **CP4** and **CP5** were almost identical and would be expected to give similar results here.

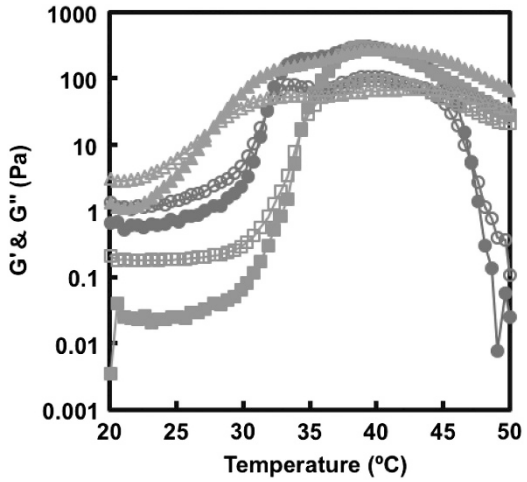

Figure 2 Storage modulus (G') (closed symbol) and loss modulus (G'') (open symbols) as a function of temperature for **CP4-S** (▲, Δ), **CP4-D** (■, □) and **CP4-D-10** (●, ○) in PBS (pH = 7.4, I = 0.14). The concentration of **CP4** was 30 wt%. A full color version of this figure is available at *Polymer Journal* online.

Table 1 Results of rheological studies of the copolymer samples with or without PEG_{2000}

Sample	*Content of PEG_{2000} (wt%)*	T_{gel} (°C)	*G′ at 37°C (Pa)*	*G′ max (Pa) ($T_{G'max}$) (°C)*[a]
CP4-S[b]	0	28.3	233	279 (40.4)
CP4-D[c]	0	34.4	225	308 (38.9)
CP4-D-P10[c]	10	31.9	268	304 (39.4)

Abbreviations: PEG, poly(ethylene glycol); T_{gel}, gelation temperature.
[a]Temperature when the G' is maximum.
[b]**S**-sample, prepared by dissolving in phosphate buffer solution (PBS) with heating process.
[c]**D**-sample, prepared by dispersion of freeze-dried polymer in PBS at room temperature (r.t.).

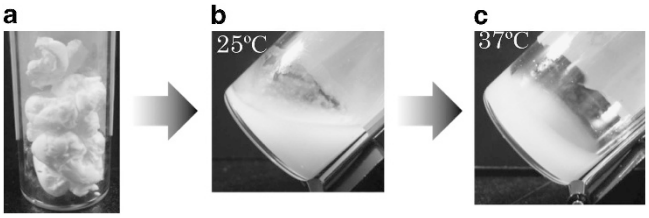

Figure 3 Photographs of **CP4** containing PEG_{2000} (10 wt% of polymer) as an additive. (**a**) After freeze-drying, (**b**) after the addition of PBS and followed by 20 s of vortex mixing and (**c**) after further incubation at 37 °C. The concentration of **CP4** was 30 wt%. A full color version of this figure is available at *Polymer Journal* online.

It took 5 min to obtain a homogeneous suspension of additive-free **CP5**. As a first step, the effects of additives (included at 10 wt% to **CP5**) on shortening DT were investigated. Sucrose, maltose and lactose were used as low-molecular-weight additives. Among these, lactose and maltose showed slight improvements in DT, but the effects were not significant. We then investigated the effects of several water-soluble polymers as additives for shortening DT. Among these, PEG_{2000}, PEG_{4600} and MeO-PEG_{5000} showed a significant shortening effect and reduced DT to <1 min (0.3, 0.5 and 0.5 min, respectively). However, the effect of polyacrylic acid was not significant, and sodium hyaluronate and polyvinyl alcohol did not give a homogeneous suspension. Next, we investigated the effects of molecular weight and concentration of PEG on the DT of the freeze-dried samples using PEG ($M_n = 1000$–$10\,000$). PEGs with molecular weights below 1000 Da were not used as additives because they are a waxy paste or liquid at r.t. and may have negative effects for obtaining a

Table 2 Effects of additives on dispersion time and sol–gel transition of CP5

Additive	*Concentration of additive*[a] *(wt% to polymer)*	*Dispersion by nylon mesh test*[b]	*Dispersion time*[c] *(min)*	*Sol–gel transition at 25–37°C*[d]
None	–	+	5.0	+
PEG_{1000}	5	+	2.0	+
	10	+	3.0	+
	15	+	2.0	+
	20	+	2.0	+
PEG_{1500}	5	+	1.0	+
	10	+	2.5	+
	15	+	2.0	+
	20	+	2.0	+
PEG_{2000}	5	+	3.5	+
	10	+	0.3	+
	15	+	1.0	+
	20	+	4.0	+
PEG_{4600}	5	+	1.5	+
	10	+	0.5	+
	15	+	2.0	+
	20	+	2.0	+
$PEG_{10\,000}$	5	+	1.5	+
	10	+	1.0	+
	15	+	2.0	+
	20	+	4.0	+
MeO-PEG_{5000}	10	+	0.5	+
PAA	10	+	3.5	+
PVA	10	-	ND	ND
HANa	10	-	ND	ND
Sucrose	10	+	4.0	+
Lactose	10	+	2.0	+
Maltose	10	+	2.0	+

Abbreviations: HANa, sodium hyaluronate; MeO-PEG, monomethoxy-PEG; ND, not determined; PAA, polyacrylic acid; PEG, poly(ethylene glycol); PVA, polyvinyl alcohol.
[a]Amount of additives in wt% to the copolymer.
[b]Dispersion state determined by the nylon mesh test. The symbol '+' indicates macroscopically homogeneous distribution (no residue was observed by the nylon mesh test), whereas '–' indicates heterogeneous distribution (residue was observed by the nylon mesh test).
[c]Time to obtain homogeneous distribution state to pass the nylon mesh test.
[d]Determined by test-tube inverting method; '+' indicates that gelation was observed, whereas '–' indicates no gelation was observed.

powder in the dry state. The DTs for PEG_{1000}, PEG_{1500} and $PEG_{10\,000}$ as additives were >1 min at all concentrations tested. The 10 wt% of PEG_{2000} was found to be the best for shortening the DT (0.3 min) of **CP5**. The obtained macroscopically homogeneous suspension exhibited a sol–gel transition between r.t. and body temperature (Figure 3).

Finally, we investigated the effects of the addition of PEG_{2000} on the sol–gel transition temperature and physical properties of the hydrogel. In addition to the results for **CP4-S**, the results of the **D**-sample with PEG_{2000} (10 wt% to **CP4**) (**CP4-D-P10**) and without additives (**CP4-D**) are shown in Figure 2 and Table 1. The T_{gel} of **CP4-D** was 34.4 °C that was higher than that of **CP4-S** (28.3 °C). This value was slightly higher than the T_m of **CP4** (33 °C). This result suggests that the sol–gel transition can occur after melting of the solid-state cores of the micelles for the **D**-samples. On the other hand, the T_{gel} of **CP4-D-P10** was 31.9 °C, and this was higher than the T_{gel} of **CP4-S** but lower than that of **CP4-D**. These results indicate that the addition of PEG_{2000} had a reducing effect on the T_{gel} of the **D**-sample by penetrating into the solid-state cores of the micelles of the **D**-sample. **CP4-D-P10** showed a slightly higher G' than **CP4-S** and **CP4-D**. However, the maximum G' (G'_{max}) and the temperature when G' is maximized ($T_{G'max}$) were similar for these three samples.

We also investigated the effects of the concentration of PEG_{2000} on the T_{gel} and physical properties of **CP4-D** (Supplementary Table S2). The T_{gel} values for **CP4-D-P5** (5%), **CP4-D-P10** (10%) and **CP4-D-P20** (20%) were within 29.9–31.9 °C and were not considerably different. The concentration of the PEG did not have a large effect on T_{gel}. The G'_{max} value of **CP4-D-P20** (151 Pa) was lower than those of **CP4-D-P5** and **CP4-D-P10** (231 and 304 Pa, respectively). This result suggests that a large amount of PEG may have a negative effect on the physical strength of the hydrogel. Moreover, we investigated the evolution of G' and G'' over time for **CP4-D, CP4-D-P10** and **CP4-S** after heating to 37 °C (Supplementary Figure S3 and Supplementary Table S3). The gelation times of these samples were within 1 min. These results indicate that the addition of PEG_{2000} does not have a negative effect on the phase transition behavior of the samples.

For the cases of **D**-samples containing disaccharides as additives, G'_{max} values of the hydrogels were significantly lower (80–110 Pa) than those of **CP4-D** and **CP4-D-P10** (Supplementary Table S4). Interestingly, the addition of an appropriate amount of PEG (10%) slightly improved the physical strength of the hydrogel compared with the low-molecular-weight additives. This result may be explained by the appropriate amount of PEG having a bridging effect during micelle aggregation.[19] These results are consistent with previous reports showing that the aggregation of micelles in block copolymer IP systems was accelerated in the presence of PEG.[20] Overall, the mixture of **CP4** and 10 wt% PEG_{2000} was the best formulation for achieving rapid dispersion and maximizing the physical properties of the hydrogel.

CONCLUSION

We synthesized PCGA-*b*-PEG-*b*-PCGA triblock copolymers that exhibited a sol–gel transition between r.t. and body temperature and had a powder morphology in the dry state at r.t. To prepare a uniformly dissolved solution (**S**-sample) of the copolymer, a heating process (above the T_m) was needed. It took 5 min to obtain a macroscopically homogeneous suspension of the copolymer (**D**-sample) by dispersion of freeze-dried samples in PBS with vortex mixing. However, the DT to prepare **D**-sample could be significantly shortened by the addition of PEG before the freeze-drying process. The shortest DT (0.3 min) was obtained when 10 wt% of PEG_{2000} was used as an additive. The obtained suspension of **CP4** and 10 wt% of PEG_{2000} in PBS (**CP4-D-P10**) exhibited a temperature-responsive sol–gel transition at 31.9 °C as well as a slightly higher physical strength compared with additive-free samples. The formulation developed in this work, which requires a short preparation time from freeze-dried samples (<1 min), should be very convenient for use in clinical applications, as a medical doctor could easily prepare an IP formulation in the clinic.

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Supplementary Information accompanies the paper on Polymer Journal website (http://www.nature.com/pj)