Poly(γ-glutamic acid) Hydrogel Prepared from Microbial Poly(γ-glutamic acid) and Alkanediamine with Water-Soluble Carbodiimide

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ABSTRACT: Poly(γ-glutamic acid)(PGA) hydrogels have been prepared from microbial PGA produced by *Bacillus subtilis* F-02-1, water-soluble carbodiimide (WSC), and alkanediamines such as 1,3-propanediamine (1,3-PD), 1,4-butanediamine (1,4-BD), and 1,6-hexanediamine (1,6-HD) in aqueous medium. The carboxyl groups of PGA were activated by the addition of WSC in deionized water, and the PGA-WSC adduct was produced. PGA hydrogels could be produced after the mixing of PGA-WSC and alkanediamine in deionized water. This alkanediamine to which both amino groups reacted with the carboxyl groups of PGA plays the role of a crosslinking point. When the amount of PGA was 100 mg, WSC was 50 mg or more, and 1,3-PD was 25 μL or more in 2 mL of deionized water, PGA hydrogels could be produced. Specific water contents (weight of absorbed water/weight of dry gel) ranged from 300 to 1,993 g/g in the case of 1,3-PD. If the PGA-WSC adduct was freeze-dried, the yield of the PGA hydrogel became higher than that when PGA-WSC was not freeze-dried. The highest yield of the PGA hydrogel from 100 mg of PGA, 100 mg of WSC, and 100 μL of 1,3-PD in 2 mL of deionized water using the freeze-dry method was 39.9 mg of dry PGA hydrogel with a 650 g/g specific water content. The order of yield was 1,6-HD > 1,4-BD > 1,3-PD from 100 mg of PGA−100 mg of WSC in 2 mL of deionized water. The order of the specific water content was 1,3-PD (462 g/g) > 1,4-BD (234 g/g) > 1,6-HD (199 g/g). This order may be due to the higher reaction probability between the activated carboxyl groups in the PGA-WSC and both amino groups in the alkanediamine with longer methylene chains, indicating that the crosslinking density of the PGA hydrogel is higher and the specific water content is lower. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **65:** 1889–1896, 1997

Key words: poly(γ-glutamic acid); hydrogel; water-soluble carbodiimide; 1,3-propanediamine; alkanediamine

INTRODUCTION

Hydrogels are soft solid polymer structures which contain a significant volume fraction of water (often over 90%). The three-dimensional polymer structure in the hydrogel is usually held together by crosslinking not only by physical interactions such as van der Waals or hydrogen bonds, but also by covalent bonds formed by crosslinking reagents or γ-irradiation. Some biomedical applications which use hydrogels include soft tissue augmentation, controlled drug release, separations, and biosensors.

In recent years, hydrogels prepared from natural polymers have received attention for environmental preservation. Hydrogels originating from microbial poly(γ-glutamic acid) (PGA)[1,2] and poly(ε-lysine)(PL)[3,4] can be prepared by γ-irradi-

ation. These microbial poly(amino acid)s are water soluble, hydrodegradable, and biodegradable. Until now, another type of poly(amino acid) other than these two poly(amino acid)s produced by microorganisms has not been found.

Several bacteria produce PGA (**1** in Scheme 1) outside of the cells.[5-12] PGA is water soluble and biodegradable with a high relative molecular mass (M_r 100,000–1,000,000). PGA can be used as a thickener, humectant, sustained release material, or drug carrier with biodegradability in the fields of food, cosmetics, or medicine. PGA was discovered as a capsule of *Bacillus anthracis* in 1937.[13,14] Since it was shown that PGA accumulated in the culture broth of *Bacillus subtilis* as a product of fermentation,[15] much research has been done on PGA. Some researchers have also been interested in a viscous material called *natto,* a traditional food in Japan. In 1905, Sawamura[16] isolated a bacterium from *natto* and named it *B. natto* sawamura. It was then shown that *natto* contained PGA and a mucin and that the mucin from *natto* consisted of a polysaccharide (levanform fructan).[17] The gene for PGA production in *B. subtilis* was then reported.[18,19] It was suggested that the function of the plasmid in *B. subtilis* was involved in PGA production. Recently, the PGA degradation enzyme was purified from the culture filtrate of a filamentous fungus.[20] It was shown that PGA was degraded with *endo*-type specificity by this enzyme.

As a modification of microbial PGA, much research has been done on the various reactions of PGA. For example, the esterification of carboxyl groups of PGA was studied,[21-23] indicating that esterified PGAs are thermoplastics. Poly(γ-glutamic acid α-benzyl ester) can form fibers and membranes.[24] In addition, PGA hydrogels were prepared by adding crosslinking agents such as hexamethylene diisocyanate in organic solvents such as dimethyl formamide or dimethyl sulfoxide.[25,26] We recently described a convenient methodology for the hydrogel formation of PGA[1,2] and PL[3,4] by γ-irradiation. These hydrogels can be expected to be used as water-sorption materials. The preparation of a hydrogel from a naturally occurring polymer is important with respect to an environmental friendly material which is both biodegradable and independent of oil resources. In this article, we report the preparation and swellability of the PGA hydrogel from water-soluble carbodiimide (WSC) and alkanediamines such as 1,3-propanediamine (1,3-PD), 1,4-butanedia-
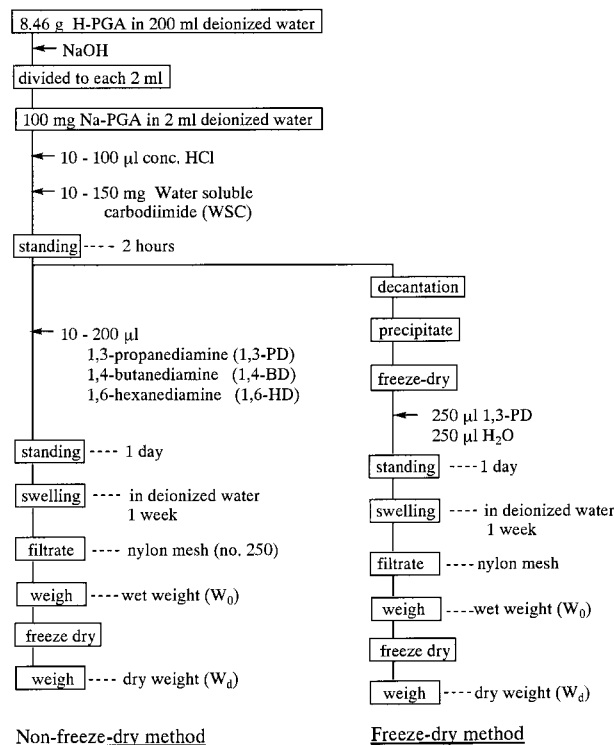

Figure 1 Preparation methods of PGA hydrogel from microbial PGA, WSC, and alkanediamine in aqueous medium.

mine (1,4-BD), or 1,6-hexanediamine (1,6-HD) in aqueous medium.

EXPERIMENTAL

Materials

PGA fermented by *B. subtilis* F-02-1 was obtained from Meiji Seika Kaisha, Ltd. (Tokyo). The number-average molecular weight, M_n, of PGA was 5.5×10^5 as analyzed by gel permeation chromatography [poly(styrene sulfonic acid, sodium salt) standard used]. WSC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride, obtained from Dojin (Tokyo), and 1,3-PD, 1,4-BD, and 1,6-HD, obtained from Wako Pure Chemical Co. (Tokyo), were used without further purification.

Preparation of PGA Hydrogel from PGA and Alkanediamine with WSC

The preparation methods of the PGA hydrogel are indicated in Figure 1. H-PGA (8.54 g) (corresponding to 10 g of Na-PGA) was put into 200 mL of deionized water. Sodium hydroxide was added

to this solution to neutralize it. Na-PGA, obtained from the water-insoluble H-PGA, was water soluble. This solution was divided into 2 mL each after the pH was checked. Concentrated HCl (36 wt % HCl concentration, $10-100$ μL) was added to 2 mL of the Na-PGA solution. WSC ($10-150$ mg) was added immediately to this solution. This solution was shaken vigorously using a touch mixer. PGA-WSC was obtained as a precipitate. This solution was left standing for 2 h. In the case of the non-freeze-dry method, 1,3-PD, 1,4-BD, or 1,6-HD ($10-200$ μL) was directly added to this solution. 1,4-BD and 1,6-HD have melting points higher than room temperature, so these alkanediamines were added to the reaction mixture after heating. The resultant PGA hydrogels were swollen to equilibrium in deionized water for 1 week. During this time, the uncrosslinked PGA and reacted WSC were removed by daily changing of the swelling media. After equilibration of the swelling, water was removed by filtration through a 250 mesh nylon net. In the case of the freeze-dry method, the resultant precipitate was separated by decantation after the addition of WSC to PGA solutions and 2 h of standing. This precipitate was freeze-dried. The solution mixture (500 μL), of 1,3-PD and deionized water, was added to the freeze-dried PGA-WSC. Other steps were the same as for the non-freeze-dry method.

Swelling of PGA Hydrogel

The weights of the wet PGA hydrogels (W_0) were measured after equilibration in deionized water at 4°C. The PGA hydrogels were then lyophilized, and the weights of the dry PGA hydrogels (W_d) were measured. The specific water content was calculated from the following expression:

Specific water content (g/g)

$$= (W_0 - W_d)/W_d \quad (1)$$

Characterization of PGA Hydrogel

The gel composition of the hydrolyzed PGA hydrogel was measured by ^{1}H nuclear magnetic resonance (NMR) analysis. One hundred microliters of $1M$ NaOH was added to $2-3$ mg of the freeze-dried PGA hydrogel in a 1.5-mL microtest tube. This solution was heated at 60°C for 12 h. This solution was then neutralized by 100 μL of $1M$ HCl and freeze-dried. This sample was dissolved in D_2O with 0.05% sodium 2,2,3,3-d$_4$-3-(trimeth-

ylsilyl)propionate as the standard. The ^{1}HNMR spectra of the hydrolyzed PGA hydrogels were recorded on a JEOL (Tokyo) GX-270 spectrometer at room temperature with 4-s pulse repetition, 32 accumulations, and 16×10^3 datum points.

RESULTS AND DISCUSSION

Investigation of WSC and 1,3-PD Concentrations for PGA Hydrogel Formation

As a modification of PGA, the chemical preparation of a PGA hydrogel by a crosslinking reaction was studied, and indicated that a transparent PGA hydrogel with a high capability for water sorption could be produced using the non-freeze-dry method (Fig. 1). This crosslinking reaction system involves a carboxyl group in the PGA polymer chain being activated by WSC, and then, 1,3-PD is added to the activated carboxyl group. The WSC and 1,3-PD concentrations for the crosslinking reaction were investigated next. Table I shows the concentrations of WSC and 1,3-PD, dry gel weights, specific water contents (weight of absorbed water/weight of dry gel), and gel compositions of the PGA hydrogels. When the amount of PGA was 100 mg, WSC was 50 mg or more, and 1,3-PD was 25 μL or more in 2 mL of deionized water, the PGA hydrogel could be produced. In the case of 100 mg of PGA (0.66 mmol of glutamic acid residue) and 100 μL of 1,3-PD (1.18 mmol), about 2 equiv of 1,3-PD was added per glutamic acid residue in PGA. The dry gel weight of the PGA hydrogel was increased from 2.0 to 19.8 mg, and the specific water content of the PGA hydrogel was decreased from 646 to 300 g/g, with an increasing amount of WSC. The changing of the 1,3-PD amount showed a tendency similar to that of WSC. To clarify the amount of 1,3-PD incorporated in the PGA, a gel composition was determined by ^{1}H-NMR measurement of a hydrolyzed PGA hydrogel. The hydrolyzed PGA was obtained using $1M$ NaOH at 60°C for 12 h (see Experimental section). The gel composition of glutamic acid residue (glu) in PGA and 1,3-PD in the PGA hydrogel, as indicated in Table I, was calculated from the ratio of the (a) proton in the glutamic acid residue of PGA and the (e) proton in 1,3-PD, as shown in Figure 2. From this NMR spectrum, N-acyl urea remained in the PGA hydrogel after swelling for 1 week. The amount of 1,3-PD incorporated into PGA was independent of the amount of WSC and was increased from 17

Table I   Preparation Conditions and Swellabilities of PGA Hydrogel Prepared from PGA, WSC, and 1,3-PD

WSC (mg)	1,3-PD (μL)	Dry Gel Weight (mg)	Specific Water Content[a] (g/g)	Gel Composition[b]	
				glu[c] (mol %)	1,3-PD (mol %)
10	100	0	—	—	—
25	100	0	—	—	—
50	100	2.0	646	n.d.	n.d.
100	100	6.5	462	69	31
150	100	19.8	300	66	34
100	10	0	—	—	—
100	25	2.5	834	83	17
100	50	3.9	700	74	26
100	200	4.7	662	65	35

One hundred milligrams of PGA in 2 mL of deionized water was used for a crosslinking reaction. Concentrated HCl (60 μL) was added to Na-PGA solution. WSC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride; n.d., not determined.

[a] Specific water content is weight of absorbed water/weight of dry hydrogel.
[b] Gel composition was determined by ^{1}H-NMR measurement of a hydrolyzed PGA hydrogel.
[c] Glutamic acid residue in PGA polymer chain.

to 35 mol % with an increase from 25 to 200 μL in the amount of 1,3-PD added to the reaction mixture.

The proposed reaction pathway from H-PGA to the PGA hydrogel is indicated in Scheme 1. PGA obtained from Meiji Seika Kaisha (Tokyo) was the H-form **1** (free acid type of carboxylic acid at side chain of PGA) and was water insoluble. It
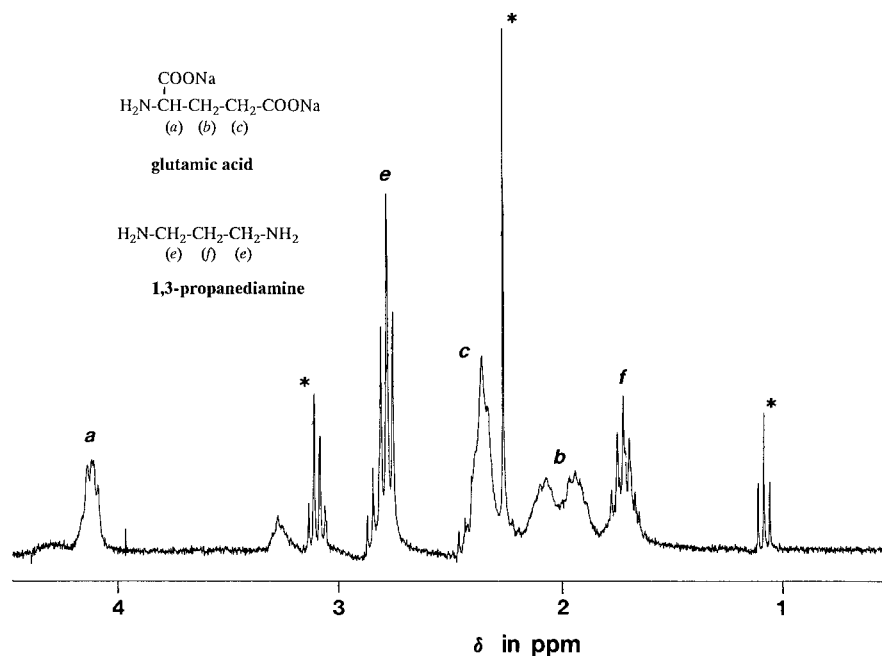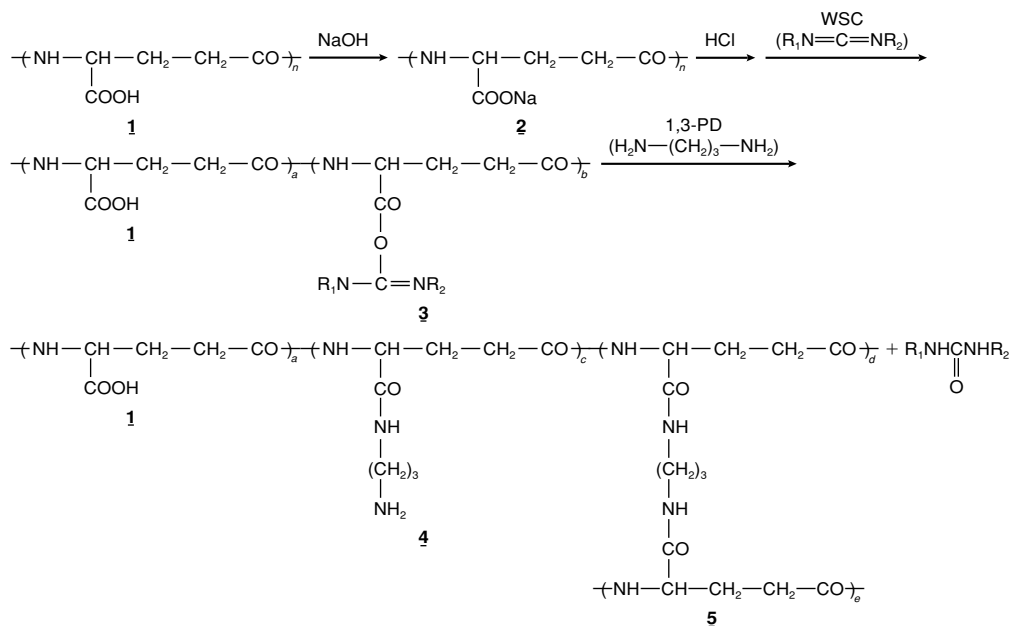

Figure 2 ^{1}H-NMR spectrum (270 MHz) of hydrolyzed PGA hydrogel in D$_2$O. The sample was prepared from 100 mg of WSC and 100 μL of 1,3-PD in 2 mL of deionized water using the non-freeze-dry method. Chemical shifts are in ppm from sodium 2,2,3,3-d$_4$-3-(trimethylsilyl)propionate. *N-Acyl urea is eliminated from the PGA-WSC adduct after the addition of 1,3-PD.

$$\text{+NH-CH-CH}_2\text{-CH}_2\text{-CO+}_n \xrightarrow{\text{NaOH}} \text{+NH-CH-CH}_2\text{-CH}_2\text{-CO+}_n \xrightarrow[\text{(R}_1\text{N=C=NR}_2\text{)}]{\text{WSC}} \xrightarrow{\text{HCl}}$$

Scheme 1

was impossible to form a hydrogel from this H-PGA, so this PGA was neutralized to Na-PGA **2** by sodium hydroxide, as shown in the scheme. WSC would not react with Na-PGA; therefore, concentrated HCl was added to this Na-PGA solution, and then the WSC powder was added immediately to this solution. This order of addition of HCl and WSC is important. In this way, the water-insoluble adduct **3** (PGA-WSC) was produced. With the addition of 1,3-PD to this solution, modified PGAs (**4** and **5**) are formed. The unit **5** plays a role as the crosslinking point of the polymer network, so that the PGA hydrogel is formed. However, 1,3-PD incorporated into a hydrogel was about 30 mol % in the case of 100 μL of 1,3-PD (Table I), indicating that a large amount of unit **4** may be produced. If all of the 1,3-PD molecule becomes the crosslinking point, like unit **5** between the PGA molecules, this material cannot absorb much water, such as over 300 times its dry weight.

The yield of PGA hydrogel (1–10% per PGA in the reaction solution) was very low. This may be due to the low reactivities of the reaction from PGA and WSC to PGA-WSC and the reaction from PGA-WSC and 1,3-PD to PGA hydrogel. In addition, another reason for the low yield may be that microgels can pass through the nylon mesh. On the other hand, the amount of 1,3-PD incorporated into the hydrogel was increased from 17 to

35 mol %; however, the specific water content was not significantly decreased (from 834 to 662 g/g) when the amount of 1,3-PD was increased from 25 to 200 μL. This indicates that unit **4** was mainly produced in the case of the addition of a large amount of 1,3-PD. In this way, it was found that highly water-absorbant hydrogel could be produced from PGA with WSC and 1,3-PD.

Investigation of Freeze-Dry Method on PGA Hydrogel Formation

To enhance the yield of the PGA hydrogel, a freeze-dry method for the PGA hydrogel formation, as shown in Figure 1, was studied. We tried to increase the reactivity between PGA-WSC (compound **3** in Scheme 1) and 1,3-PD by the freeze drying of the precipitated PGA-WSC. By using this method, the concentration of 1,3-PD can be increased up to 100% 1,3-PD solution. In the case of the non-freeze-dry method, as explained before, there was 2 mL of deionized water in the reaction mixture. The amount of freeze-dried PGA-WSC was approximately 100 mg from 100 mg of PGA and 100 mg of WSC in 2 mL of deionized water. The dry gel weight and "swellability" of the PGA hydrogels using the non-freeze-dry method and the freeze-dry method are shown in Table II. In the case of the non-freeze-dry method (last column in Table

Table II Preparation Conditions and Swellabilities of PGA Hydrogel Prepared from Freeze-Dried Samples of PGA and WSC with Various 1,3-PD Concentrations

1,3-PD (μL)	H_2O (μL)	Dry Gel Weight (mg)	Specific Water Content[a] (g/g)
500	0	8.3	488
250	250	6.9	906
100	400	2.9	1,993
50	450	0.5	n.d.
25	475	1.3	n.d.
100	2000[b]	2.6	563

Concentrated HCl (60 μL) was added to Na-PGA solution. Freeze-dried precipitate after the addition of 100 mg of WSC to 100 mg of PGA in 2 mL of deionized water was used for the crosslinking reaction with 1,3-PD and H_2O. n.d., not determined.

[a] Specific water content is weight of absorbed water/weight of dry hydrogel.

[b] PGA hydrogel was prepared from WSC and 1,3-PD using the non-freeze-dry method.

II), 2.6 mg of PGA hydrogel was formed. When 500 μL of 1,3-PD or 250/250 μL of (1,3-PD/H_2O) was added to the freeze-dried PGA-WSC, 8.3 or 6.9 mg of the PGA hydrogel was produced. The specific water contents of 250/250 μL of (1,3-PD/H_2O) using the freeze-dry method and 100/2,000 μL of (1,3-PD/H_2O) using the non-freeze-dry method were 906 and 563 g/g, respectively. The crosslinking density of a hydrogel from the 250/250 μL of (1,3-PD/H_2O) was lower compared with the hydrogel from the non-freeze-dry method, in spite of the higher concentration of 1,3-PD as the crosslinker. This may be due to the alkaline hydrolysis of the PGA polymer main chain so that the molecular weight of PGA decreases and the specific water content is increased. In this way, the PGA hydrogel with a higher yield and higher swellability can be produced by the freeze-dry method compared with the non-freeze-dry method.

Investigation of Concentrated HCl Amount for PGA Hydrogel Formation

The reaction from Na-PGA to H-PGA for this reaction system is very important. This reaction rate is thought to be dependent on the concentrated HCl amount added to the Na-PGA solution. There was an optimal pH for the reaction between PGA and WSC. Therefore, the amount of concentrated

HCl added to the Na-PGA solution before the addition of WSC using the non-freeze-dry method and freeze-dry method was investigated, as indicated in Table III. The optimal yield (39.9 mg) was obtained using 100 mg of PGA, 100 mg of WSC, and 250/250 μL of (1,3-PD/H_2O) with 50 μL of concentrated HCl by the freeze-dry method. The amount of the incorporated 1,3-PD in this hydrogel was 43 mol %, based on NMR analysis. The yield of the PGA hydrogel using the freeze-dry method was higher than that for non-freeze-dry method for any amount of concentrated HCl. The reaction solution with the addition of 50 μL of concentrated HCl had a pH 2 using pH test paper. The addition of 30 μL or less indicated a pH 3–4. It was found that the optimal pH for the reaction between PGA and WSC was pH 2. PGA was precipitated by the addition of 50 μL of concentrated HCl to 100 mg of PGA in 2 mL of deionized water for 1 day; however, no precipitate appeared during standing for 2 h because the PGA precipitation reaction had a slow reaction rate. The precipitate appeared with the addition of WSC. The specific water content decreased from 650 to 346 when increasing the amount of concentrated HCl from 50 to 80 μL. This may indicate that the reactivity between PGA-WSC and 1,3-PD is higher under acidic conditions. Thus, the optimal amount of concentrated HCl on PGA hydrogel formation was 50 μL per 100 mg of PGA in 2 mL of deionized water using the freeze-dry method, which changed the pH of the reaction mixture to 2. The highest yield of the PGA hydrogel from 100 mg of PGA, 100 mg of WSC, and 250/250 μL of 1,3-PD/H_2O using the freeze-dry method was 39.9 mg of dry PGA hydrogel with a 650 g/g specific water content.

PGA Hydrogel with 1,4-BD or 1,6-HD

Other alkanediamines as crosslinkers were investigated using the non-freeze-dry method, as shown in Table IV. A mole of 1,4-BD or 1,6-HD is equal to 100 μL of 1,3-PD (1.18 mmol). In the case of 1,4-BD, the dry gel weight of the PGA hydrogel increased and the specific water content decreased with increasing WSC or 1,4-BD, as was observed in 1,3-PD (Table I). A similar tendency was observed for the PGA hydrogels from 1,6-HD. The order of the yield was 1.6-HD (13.0 mg) > 1,4-BD (9.8 mg) > 1,3-PD (6.5 mg) in the case of 100 mg of PGA and 100 mg of WSC. The order of the specific water content was 1,3-PD (462 g/g) > 1,4-BD (234 g/g) > 1,6-HD (199 g/g). These

Table III   Preparation Conditions and Swellabilities of PGA Hydrogel Prepared from PGA and WSC with Various Amounts of Concentrated HCl

	Non-Freeze-Dry Method		Freeze-Dry Method		
Conc. HCl (μL)	Gel Dry Weight (mg)	Specific Water Content (g/g)	PGA-WSC Weight (mg)	Gel Dry Weight (mg)	Specific Water Content (g/g)
10	0	—	0	0	—
20	0	—	0	0	—
30	0	—	0	0	—
40	0	—	79.4	3.9	420
50	3.3	626	113.3	39.9	650
60	7.3	639	72.1	25.7	508
70	1.7	n.d.	38.8	15.8	402
80	2.5	639	37.8	10.2	346

In the case of the non-freeze-dry method, PGA hydrogel was prepared from 100 mg of PGA, 100 mg of WSC, and 100 μL of 1,3-PD in 2 mL of deionized water. In the case of the freeze-dry method, freeze-dried PGA-WSC after the addition of 100 mg of WSC to 100 mg of PGA in 2 mL of deionized water with various amounts of concentrated HCl was used for the crosslinking reaction with 250 μL of 1,3-PD and 250 μL of H_2O.

results may be due to the length of the methylene of alkanediamine: a longer methylene chain in the alkanediamine may cause a higher possibility that both amino groups in the alkanediamine react with the activated carboxyl group in PGA-WSC. The crosslinking density may increase and

Table IV   Preparation Conditions and Swellabilities of PGA Hydrogel Prepared from PGA, WSC, 1,4-BD, and 1,6-HD

WSC (mg)	1,4-BD (μL)	1,6-HD (μL)	Dry Gel Weight (mg)	Specific Water Content[a] (g/g)
10	119	0	0.6	n.d.
25	119	0	1.6	n.d.
50	119	0	5.9	376
100	119	0	9.8	234
150	119	0	21.4	229
100	11.9	0	0	—
100	29.8	0	7.9	411
100	59.5	0	8.5	221
100	238	0	15.4	248
10	0	157	1.2	n.d.
25	0	157	3.3	303
50	0	157	8.2	203
100	0	157	13.0	199
150	0	157	27.9	177
100	0	15.7	0	—
100	0	39.3	4.3	233
100	0	78.5	6.2	303
100	0	314	17.4	159

One hundred milligrams of PGA in 2 mL of deionized water was used for the crosslinking reaction. Concentrated HCl (60 μL) was added to the Na-PGA solution using the non-freeze-dry method. n.d., not determined.

[a] Specific water content is weight of absorbed water/weight of dry hydrogel.

the specific water content decrease for the alkanediamine with a longer methylene chain.

CONCLUSION

Water-absorbed hydrogel could be produced from a naturally occurring polymer and alkanediamine with WSC in aqueous medium. The specific water contents of these hydrogels ranged from 159 to 1,933 (g of water/g of dry gel). However, the yield of the PGA hydrogel was very low from PGA and an alkanediamine with WSC, so research is continuing to improve this reaction system. In addition, this reaction system can be applied to the immobilization of a compound as an enzyme or a drug with amino functional groups to the PGA polymer chain.

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