Journal of Environmental Polymer Degradation, Vol. 4, No. 2, 1996

Preparation Conditions and Swelling Equilibria of Biodegradable Hydrogels Prepared from Microbial Poly(γ-glutamic Acid) and Poly(ε-lysine)[1]

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Biodegradable hydrogels prepared by γ-irradiation from microbial poly(amino acid)s are reviewed. pH-sensitive hydrogels were prepared by means of γ-irradiation of poly(γ-glutamic acid) (PGA) produced by *Bacillus subtilis* IFO3335 and poly(ε-lysine) (PL) produced by *Streptomyces albulus* in aqueous solutions. The preparation conditions, swelling equilibria, hydrolytic degradation, and enzymatic degradation of these hydrogels were studied. A hydrogel with a wide variety of swelling behaviors has been produced by γ-irradiation from a mixture solution of PGA and PL.

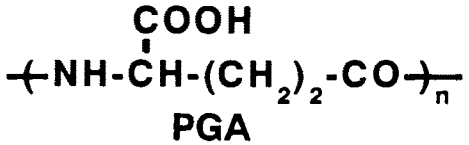
KEY WORDS: Poly(γ-glutamic acid); poly(ε-lysine); hydrogel; biodegradation; enzymatic degradation.

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 cross-linking not only by physical interactions such as Van der Waals or hydrogen bonds, but also by covalent bonds formed by cross-linking reagents or γ-irradiation. Some biomedical applications which employ 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-irradiation. These microbial poly(amino acid)s are water soluble, hydrodegradable, and biodegradable. Until now a type of poly(amino acid) besides these two poly(amino acid)s produced by microorganisms has not been found.

Several bacteria produce PGA (Scheme 1) outside of the cells [5–10]. 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, and medicine. PGA was discovered as a capsule of *Bacillus anthracis* in 1937 [11, 12]. Since it was shown that PGA accumulated in the culture broth of *B. subtilis* as a product of fermentation [13], 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 [14] isolated a bacterium from *natto* and named it *B. natto* sawamura. It was then shown that *natto* contained PGA and a mucin



PGA

Scheme 1

[1] Paper presented at the 4th International Workshop on Biodegradable Plastics and Polymers, October 11–14, 1995, Durham, New Hampshire, USA.

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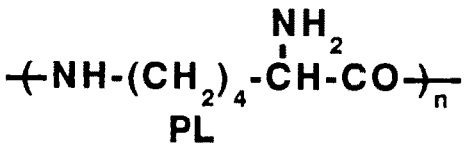

Scheme 2

and that the mucin from *natto* consisted of a polysaccharide (levan-form fructan) [15]. The gene for PGA production in *B. subtilis* was reported [16, 17]. It was suggested that the plasmid in *B. subtilis* was involved in PGA production. Recently PGA degradation enzyme was purified from the culture filtrate of a filamentous fungus [18]. It was shown that PGA was degraded with endo-type specificity by this enzyme.

Streptomyces albulus, an actinomycete, produces PL (Scheme 2) outside of the cell [19, 20]. PL is water soluble and biodegradable, and the molecular weight is approximately 4000. PL is an L-lysine homopolymer (25–30 residues) with a linkage between the carboxyl group and the ε-amino group. PL was discovered as a result of screening for a Dragendorff-positive (alkaloid screening method) substance [19]. The production conditions have been investigated [20]. The decline of pH during the fermentation process was an essential condition for the accumulation of PL. To enhance the productivity, a two-step cultivation was investigated and 4–5 g/L PL could be produced in 8–9 days [21]. PL showed antimicrobial activity against Gram-positive and -negative bacteria at concentrations of 1–8 μg/ml [22]. The activity of the antiphage of PL was also reported.

As a modification of microbial poly(amino acid), much research has been done on the various reactions of PGA. For example, esterifications of carboxyl groups of PGA were studied [23, 24], indicating that esterified PGAs were thermoplastics. Poly(γ-glutamic acid α-benzyl ester) could form fibers and membranes [25]. In addition, PGA hydrogels were prepared by adding cross-linking agents such as hexamethylene diisocyanate [26, 27]. 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 naturally occurring polymer is important with respect to an environmentally friendly material which is both biodegradable and independent of oil resources.

In this article, the next (second) section deals with the cross-linking reaction of PGA by γ-irradiation as a modification of PGA, the swelling properties of PGA hydrogels in various solutions, and the hydrolytic degradation of PGA hydrogels. The third section deals with PL hydrogels including enzymatic degradation. The fourth section is concerned with amphoteric PGA/PL hydrogels with a wide variety of swelling behaviors.

HYDROGELS PREPARED FROM MICROBIAL POLY(γ-GLUTAMIC ACID)

Effect of γ-Irradiation Dose on Preparation of PGA Hydrogels

As a modification of PGA, the preparation of a PGA hydrogel by γ-irradiation was studied, indicating that a transparent PGA hydrogel with a high capability for water sorption could be produced [1, 2]. PGA prepared from *Bacillus subtilis* IFO3335 (IFO; Institute of Fermentation Osaka, Japan) was used in this study. The irradiation conditions for the PGA solution were investigated. Figure 1 shows the specific water content (weight of absorbed water/weight of dry hydrogel) of a PGA hydrogel prepared by γ-irradiation (1.6 kGy/h) of a PGA aqueous solution (5 wt%). When the γ-irradiation dosage was 20 kGy or more, a transparent hydrogel could be produced. In the case of 20 kGy, this PGA hydrogel was very weak, but the specific water content of this gel was approximately 3500. It was found that the PGA hydrogel prepared by γ-irradiation had a high

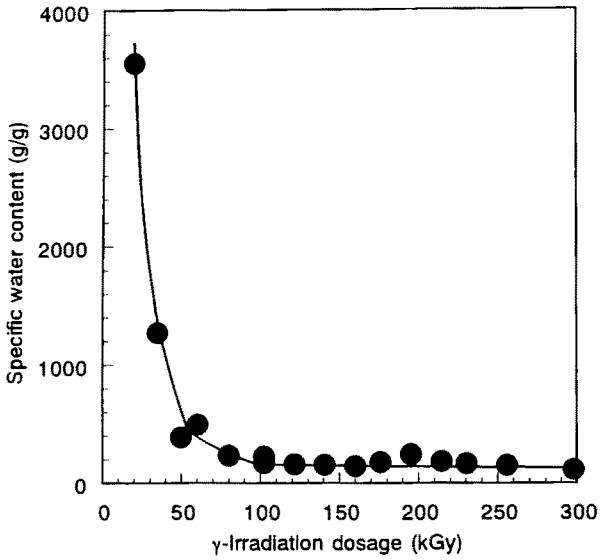

Fig. 1. Change in specific water content (weight of absorbed water/weight of dry gel) of poly(γ-glutanic acid) (PGA) hydrogels during γ-irradiation. The PGA concentration of irradiated aqueous solution was 5 wt%.

water-sorption capability. The specific water content of the PGA hydrogel decreased markedly with increasing dosage of γ-irradiation. This may be due to the increase in the cross-link density of the PGA hydrogel. The specific water content was kept almost-constant at about 200 when over 100 kGy of γ-irradiation was used.

Gamma-ray-induced cleavage of C—H bonds may generate free radicals at the methyne carbons of PGA. The subsequent intermolecular radical combination may lead to cross-linking. The detailed cross-linking mechanism of PGA hydrogels will be investigated in the future.

Effect of pH on PGA Hydrogel Swelling

The influence of various pH's and the presence of salts in the swelling medium of a hydrogel is of importance in agricultural and biomedical applications such as diapers, water reservoirs in agriculture, and hydrogels as implants for drug release applications. Figure 2 shows the specific water content of PGA hydrogels in 25 m*M* McIlvain buffers of various pH's. The specific water content of PGA hydrogels over pH 6 were decreased compared to 200 in Fig. 1. This is due to the effect of the buffer ions. It can be seen that the degree of swelling of PGA hydrogels is strongly dependent on the pH. It was reported that PGA was precipitated at a low pH due to the aggregation of carboxyl groups [24, 28]. Similarly, negatively charged carboxyl groups are incorporated into the polymer network, therefore, the PGA hydrogels swell in the high-pH region (>3) due to the ionic repulsion of the dissociated carboxyl groups and collapse in the low-pH region (<3) because of the aggregation of undissociated carboxyl groups.

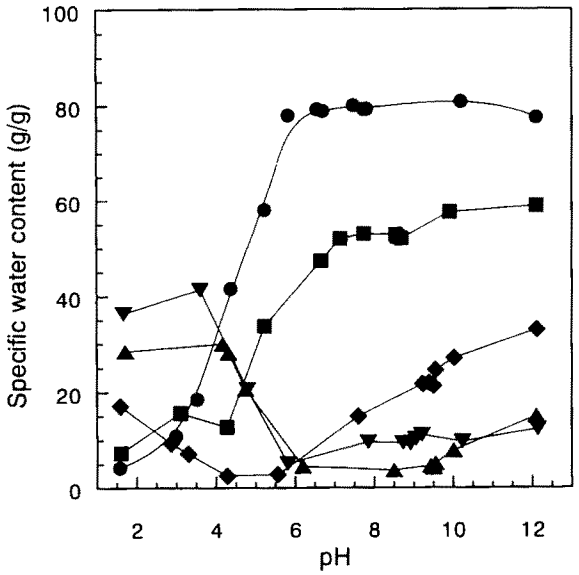

Fig. 2. Swelling of PGA/poly(ε-lysine) (PL) mixed hydrogels (90 kGy) in aqueous solutions of various pH's. PGA/PL wt%: (●) 100/0; (■) 80/20; (♦) 50/50; (▲) 20/80; (▼) 0/100. McIlvaine buffer, 25 m*M*, was used.

Effect of NaCl or $CaCl_2$ Concentration on PGA Hydrogel Swelling

Figure 3 shows the degree of swelling (weight of the wet hydrogel at equilibrium in solution/weight of the wet hydrogel in deionized water) of a PGA hydrogel (100 kGy, 5 wt% PGA concentration) in aqueous solutions of various NaCl or $CaCl_2$ concentrations. The degree of swelling decreased with an increase in the NaCl or $CaCl_2$ concentration. In the case of $CaCl_2$, which is a divalent electrolyte, the degree of swelling decreased markedly, 0.07, at 0.1 wt% $CaCl_2$ concentration. This property is similar to that of the poly(acrylic acid) hydrogel. Thus, the swelling of the PGA hydrogel may be due to the ionic osmotic pressure generated from mobile counter ions to charged ions in the network, similar to the swelling mechanism of the poly(acrylic acid) hydrogel [29].

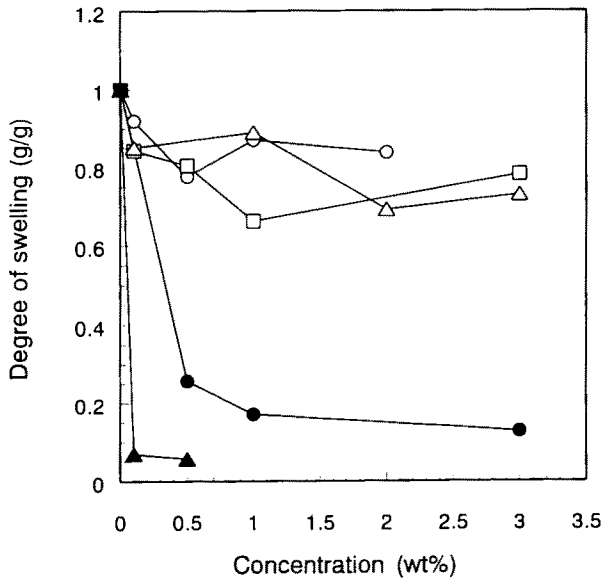

Fig. 3. Swelling of PGA hydrogels in aqueous solutions of various NaCl (●) and $CaCl_2$ (▲) concentrations and PL hydrogels in aqueous solutions of various NaCl ( ), Na_2SO_4 ( ), and $CaCl_2$ (△) concentrations. PGA hydrogels were prepared with 100-kGy γ-irradiation at a 5 wt% PGA concentration. PL hydrogels were prepared with 90-kGy γ-irradiation at a 5 wt% PL concentration.

Hydrolytic Degradation of PGA Hydrogels

Because it was reported that non-cross-linked PGA was hydrodegradable [30], PGA hydrogels may be thought to be hydrodegradable. Hydrolytic degradation studies of PGA hydrogels were performed at 40 and 100°C. PGA hydrogel samples (15 g) were placed in a 50-ml glass bottle with 20 ml deionized water. After heating and glass filtration, the filtrated amount of degraded PGA gel was measured with a total organic carbon amount (TOC) analyzer. The degraded amount of PGA gel and specific water content of the heat-treated residual hydrogels at particular temperatures are shown in Table I. PGA hydrogels were not degraded when held at 40°C for 3 h. When the medium temperature was raised to 100°C, however, the gel was degraded to 76% in 45 min. In this case, the specific water content of the residual hydrogel was increased from 200 to 530. This means that the ionic repulsion due to the ionic carboxyl group is increased. It can be seen that partial hydrolytic degradation of the gels resulted in an increase in the anionic carboxyl group concentration inside the gel. The osmotic pressure was thus enhanced, and elastic refractive forces were decreased because the cross-link density of the gels may be decreased due to scission of the amide connection. Thus, it was found that PGA hydrogels were stable under 40°C but were almost completely degraded at 100°C in deionized water after about 1 h. Thermal hydrolytic degradation may be extended to the biodegradability of PGA hydrogels, but appropriate biodegradation tests, e.g., composting and wastewater treatment environments, must be evaluated for confirmation.

Table I. Hydrolytic Degradation of Poly(γ-Glutamic Acid) (PGA) Hydrogels[a]

Heating		Degradation ratio	Specific water
Temp (°C)	Time (min)	(%)	content
—	0	—	200
40	180	0	196
100	15	4	274
100	30	22	653
100	45	76	530
100	60	92	—[b]

[a] These PGA hydrogels were prepared with a 87-kGy γ-irradiation dose and 5 wt% PGA concentration.

[b] Specific water content could not be measured because the structure of the PGA hydrogel was not maintained.

HYDROGELS PREPARED FROM MICROBIAL POLY(ε-LYSINE)

Effect of γ-Irradiation Dose on Preparation of PL Hydrogels

PL is a water-soluble and biodegradable polymer produced by a microorganism. Many water-soluble polymer solutions have been known to convert to a hydrogel with a high water-sorption ability by means of γ-irradiation. Thus the preparation of a PL hydrogel by γ-irradiation was studied as a modification of PL, indicating that a transparent PL hydrogel could be produced [26, 27]. The irradiation conditions for the PL solution were investigated. PL fermented by *Streptomyces albulus* used in this study was obtained from Chisso Corp. (Japan). Figure 4 shows the specific water content of a PL hydrogel prepared by γ-irradiation of a PL aqueous solution (5 wt%). After the induction period (70-kGy γ-irradiation), a transparent hydrogel could be produced. In the case of 75 kGy, the specific water content of this gel was 160. The specific water content of the PL hydrogel decreased markedly with increasing dose of γ-irradiation. This may be due to the increase in cross-link density of the PL hydrogel. The specific water content was kept almost-constant at about 10 at over 100 kGy. In this region, the cross-link density may be saturated.

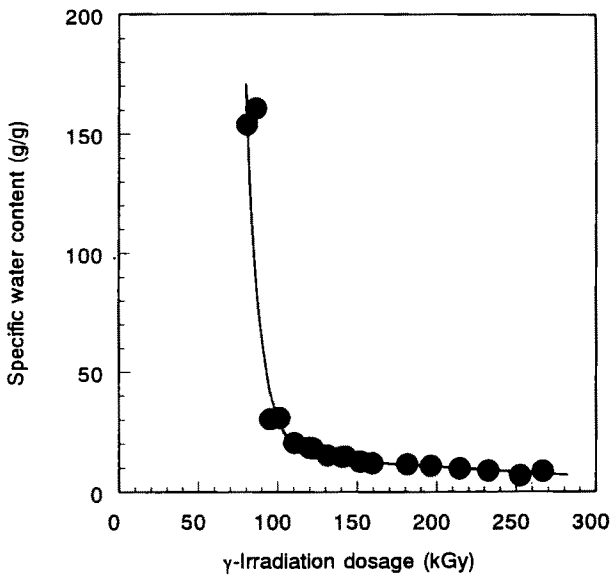

Fig. 4. Change in specific water content of PL hydrogels during γ-irradiation. The PL concentration of irradiated aqueous solution was 5 wt%.

Effect of pH on PL Hydrogel Swelling

Swelling equilibria of PL hydrogels prepared with 90-kGy γ-irradiation and 5 wt% PL concentration were measured in aqueous solutions of various pHs. Figure 2 shows the specific water content of PL hydrogels in aqueous solutions of various pH's. It can be seen that the degree of swelling of PL hydrogel was strongly dependent on the pH. The PL hydrogel swelled in the low-pH region (<4.0). Because of their positive charge, the amino groups ($-NH_3^+$) are incorporated into the polymer network; the gel swells in the low-pH region due to the ionic repulsion of the protonated amino groups and collapses at high pH values because of elastic force without ionic repulsion. Thus, it was found that the PL hydrogel was a pH-sensitive hydrogel and it is expected to be used as an artificial muscle or a drug carrier in response to an input signal.

Enzymatic Degradation of PL Hydrogels

A PL hydrogel from a naturally occurring polymer may be used in an environment or a human body. In these cases, the biodegradability of the gel is an important property. It was known that non-cross-linked PL can be degraded by a neutral protease, *protease A* (Amano Pharmacy Ltd., Japan), produced from a microorganism (*Aspergillus oryzae*). Therefore, biodegradation of the PL hydrogel by this enzyme was investigated. The enzymatic degradation was carried out at 40°C in a 25 m*M* phosphate buffer (pH 7.0). PL hydrogels (ca. 0.5 g) were placed in small bottles containing 20 ml of buffer with *protease A* (6 mg). The reaction solution was incubated at 40°C with shaking. The total organic carbon (TOC) amounts in the filtered supernatant were periodically measured by a TOC analyzer. Figure 5 shows the enzymatic degradation profiles of PL hydrogels (101, 147, and 203 kGy and 5 wt% PL concentration) as a function of degradation time. The degradation reaction of the PL hydrogel (ca. 0.5 g, wet) was carried out at 40°C and 3 mg/10 ml enzyme concentration in 20 ml phosphate buffer. The degradation ratio using 25 mg/10 ml enzyme concentration was almost same as that using 3 mg/10 ml enzyme. The degradation ratio was calculated from the TOC amount in filtered supernatant of the reaction mixture (gel, enzyme, and buffer). The PL hydrogel was not degraded in 20 h without enzymes (Fig. 5) at 40°C. The rate of PL hydrogel degradation by *protease A* is strongly dependent upon the γ-irradiation dose during the cross-linking reaction and decreases with an increase in the

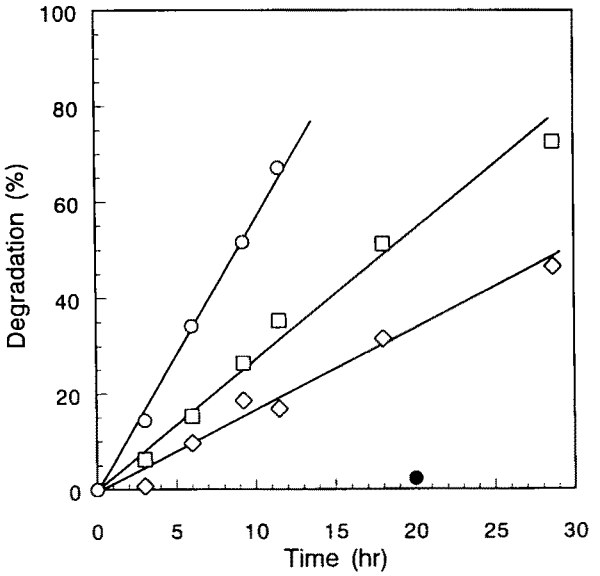

Fig. 5. Enzymatic degradation profiles on PL hydrogels in an aqueous solution (20 ml) of *protease A* (6 mg) at 40°C and pH 7.0. PL hydrogels were prepared with 101 kGy (○), 147 kGy (□), and 203 kGy (◇) of γ-irradiation at a 5 wt% PL concentration. (●) PL hydrogel prepared with 101 kGy immersed in the aqueous solution without enzyme at 40°C and pH 7.0 for 20 h.

γ-irradiation dose. This may be due to the increase in the cross-link density of the PL hydrogel. A PL hydrogel with a higher cross-link density needs more chain scission at the amide bonds by the protease for degradation. The increase in cross-link density can be presumed because the specific water content decreases with an increase in the γ-irradiation dose (21.9 for 101 kGy, 11.5 for 147 kGy, and 6.9 for 203 kGy). Thus, the PL hydrogel is biodegradable and the biodegradation rate of the PL hydrogel can be controlled by the γ-irradiation dose during the cross-linking reaction.

AMPHOTERIC HYDROGEL PREPARED FROM A MICROBIAL POLY(AMINO ACID) MIXTURE

Preparation of Hydrogels from a PGA/PL Polymer Mixture

Aqueous PGA/PL polymer mixed solutions of 5 wt% concentration were cross-linked when exposed to γ-radiation under a N_2 atmosphere [31]. PGA and PL are polyanion and polycation, respectively, but were not precipitated under these preparation conditions. Upon

irradiation with 90 kGy, the water-soluble microbial polymer mixture was converted into a series of water-swellable hydrogels with various specific water contents. The composition of hydrogels produced as a result of γ-irradiation reflected the composition of the starting materials. Preferential cross-linking of PGA or PL was not observed.

Figure 6 depicts the effect of the composition ratio on the specific water content of hydrogels of a 5 wt% aqueous PGA and PL mixed solution and 90 kGy γ-irradiation dose. As shown in Fig. 6, PGA and PL exhibited specific water contents of 90 and 26, respectively. The specific water content of PGA/PL hydrogel increased upon increasing the ratio of PGA in the PGA/PL polymer mixture. This was caused by the difference in ionic group composition in the gel network, i.e., carboxyl and amino groups. In deionized water (pH 7.0), the PGA molecule has an anionic carboxyl group ($-COO^-$) but an almost-nonionic amino group ($-NH_2$) is observed in the PL molecule. The overall equilibrium swelling is thus dependent mainly on the PGA ionic group content in gels because the Donnan effect can be considered as the main driving force for the swelling of these ionizable PGA/PL mixed gels. Upon increasing the PGA content, the charge density in the PGA/PL hydrogel is increased. This leads to an increase in the extent of swelling due to weaker ionic swelling pressure.

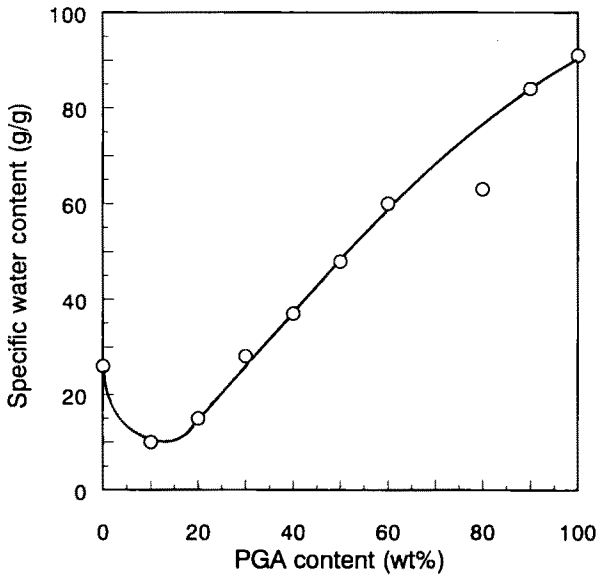

Fig. 6. Specific water contents of PGA and PL mixed hydrogels as a function of PGA content. These hydrogels of 5 wt% aqueous PGA and PL mixed solutions were irradiated with 90-kGy γ-irradiation.

Swelling Equilibrium in Solutions of Various pH's

The effect of the ratio of PGA/PL on the swelling behavior of PGA/PL mixed gel prepared with a 90-kGy irradiation dose is shown in Fig. 2. The specific water contents for gels with ratios of 100/0, 80/20, 50/50, 20/80, and 0/100 wt% (PGA/PL) were measured as a function of pH. In the case of 50/50 wt% (PGA/PL) for-

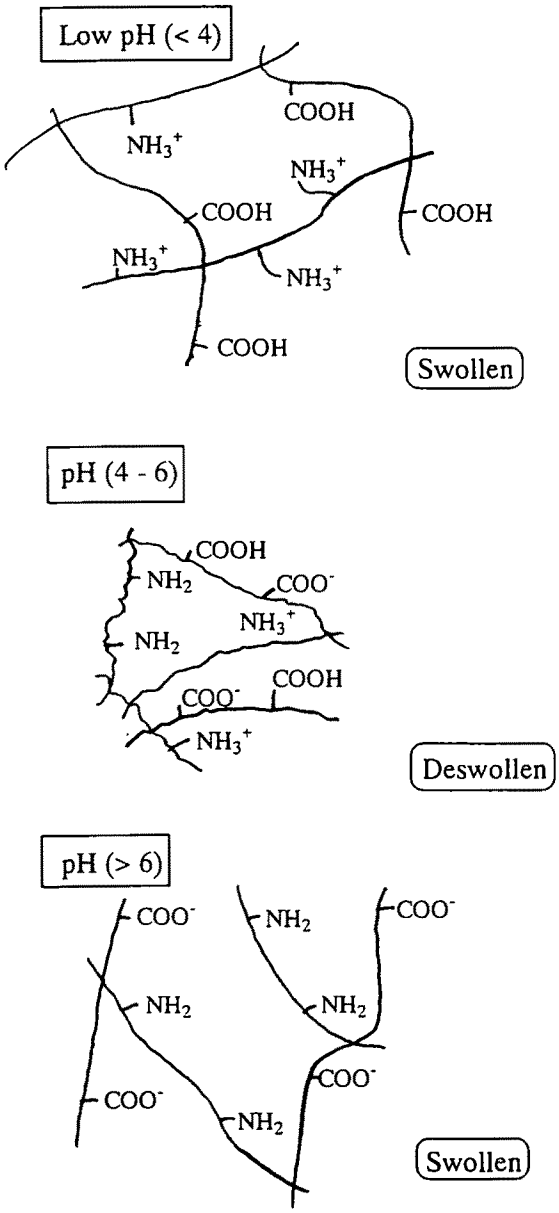

Fig. 7. Hypothetical sketch of the swelling-deswelling molecular structure of 50/50 wt% (PGA/PL) hydrogels in aqueous solutions in the <4.0, 4.0–6.0, and >6.0 pH regions.

mulation gel, the swelling response to pH was characteristic (Fig. 2). This hydrogel swelled at low pH (<4.0) and high pH (>6.0). On the other hand, this gel deswelled at pH from 4.0 to 6.0. From these results, it can be presumed that 50/50 wt% (PGA/PL) gel contains both carboxyl and amino groups and shows characteristic swelling behavior described above. This is to be expected from the variation of ionic composition with pH as shown in Fig. 7.

Gels with networks containing both carboxyl and amino groups can be prepared by γ-irradiation as a result of mixing the two polymers with oppositely charged structures as shown in Fig. 7. Since the charge state of the ionic groups varies with pH, the dominant charges in the PGA/PL mixed gel are the protonated amino group (Fig. 7, top) and the unprotonated carboxyl group (Fig. 7, bottom) at pH <4.0 and pH >6.0, respectively. In this pH region, the PGA/PL gel is swelled due to the increase in ionic swelling pressure. In contrast, at pH 4.0–6.0, most of the ionic groups are absent due to protonation of the carboxyl group and deprotonation of the amino group as shown in Fig. 7 (middle). The gel is thus deswelled in this region. Thus, PGA/PL mixed gels show characteristic pH sensitivity.

CONCLUSION

The water absorbance of a hydrogel produced from a naturally occurring polymer is at best about several hundred times its own weight. Since these hydrogels are biodegradable, they are gentle to the environment, and they may be used for the manufacture of diapers and the greening of deserts. Chemically synthesized water-absorbent polymers capable of absorbing water to 10,000 times their own weight are available but are not degraded in the soil and may have a deleterious effect on the environment. The hydrogels described here are biodegradable and exert no adverse influence on the environment. However, when using water containing salt, the water absorbency is lowered, so research is continuing to improvement this property.

REFERENCES

1. M. Kunioka (1993) *Kobunshi Ronbunshu* **50**, 755-760 (in Japanese).
2. H. J. Choi and M. Kunioka (1995) *Radiat. Phys. Chem.* **46**, 175-179.
3. M. Kunioka and H. J. Choi (1995) *J. Appl. Polym. Sci.* **58**, 801-806.
4. M. Kunioka (1995) *Sen-i Gakkaisi* **51**, 137-142 (in Japanese).
5. S. Murao (1969) *Kobunshi* **16**, 1204-1212 (in Japanese).
6. F. A. Troy (1973) *J. Biol. Chem.* **248**, 305-315.
7. C. Cheng, Y. Asada, and T. Aida (1989) *Agr. Biol. Chem.* **53**, 2369-2375.
8. H. Kubota, T. Matsunobu, K. Uotani, H. Takebe, A. Satoh, T. Tanaka, and M. Taniguchi (1993) *Biosci. Biotech. Biochem.* **57**, 1212-1213.
9. M. Kunioka and A. Goto (1994) *Appl. Microbiol. Biotechnol.* **40**, 867-872.
10. G. A. Birrer, A.-M. Cromwick, and R. A. Gross (1994) *Int. J. Biol. Macromol.* **16**, 265-275.
11. G. Ivánovics and L. Erdös (1937) *Z. Immunitätsforsch* **90**, 5-19.
12. G. Ivánovics and V. Bruckner (1937) *Z. Immunitätsforsch* **90**, 304-318.
13. M. Bovarnick (1942) *J. Biol. Chem.* **145**, 415-424.
14. S. Sawamura (1905) *J. Coll. Agr. Tokyo* **5**, 189-191.
15. H. Fujii (1963) *Nippon Nogeikagaku Kaishi* **37**, 407-411 (in Japanese).
16. T. Hara and S. Ueda (1982) *Agr. Biol. Chem.* **46**, 2275-2281.
17. T. Hara, A. Aumayr, Y. Fujio, and S. Ueda (1982) *Appl. Environ. Microbiol.* **44**, 1456-1458.
18. T. Tanaka, O. Hiruta, T. Futamura, K. Uotani, A. Satoh, M. Taniguchi, and S. Oi (1993) *Biosci. Biotech. Biochem.* **57**, 2148-2153.
19. S. Shima and H. Sakai (1977) *Agr. Biol. Chem.* **41**, 1807-1809.
20. S. Shima and H. Sakai (1981) *Agr. Biol. Chem.* **45**, 2497-2502.
21. S. Shima, S. Ohshima, and H. Sakai (1983) *Nippon Nogeikagaku Kaishi* **57**, 221-226 (in Japanese).
22. S. Shima, H. Matsuoka, T. Iwamoto, and H. Sakai (1984) *J. Antibiot.* **37**, 1449-1455.
23. H. Kubota, Y. Nambu, and T. Endo (1993) *J. Polym. Sci. A Polym. Chem.* **31**, 2877-2879.
24. S. A. Giannos, D. Shah, R. A. Gross, D. L. Kaplan, and J. M. Mayer (1990) *ACS Polym. Prep.* **31**, 209-210.
25. K. Yahata, J. Sadanobu, and T. Endo (1992) *Polym. Prepr. Japan* **41**, 1077 (in Japanese).
26. M. Inagaki, N. Tubokawa, and T. Endo (1991) *Polym. Prepr. Japan* **40**, 411 (in Japanese).
27. K. Kakinoki, A. Kishida, M. Akashi, and T. Endo (1993) *Chem. Soc. Japan Prepr.* **65II**, 258 (in Japanese).
28. A. Amamiya (1962) *Houshasenn Kagaku Nyuumon, Vol. 2*, Maruzen, Tokyo, pp. 474, 544.
29. T. Nagai, Y. Yamamoto, and H. Takeuchi (1973) Japanese patent, 48-85687.
30. A. Goto and M. Kunioka (1992) *Biosci. Biotech. Biochem.* **56**, 1031-1035.
31. H. J. Choi, R. Yang, and M. Kunioka (1995) *J. Appl. Polym. Sci.* **58**, 807-814.