

Radiation-Induced Cross-Linking of Gelatin by Using γ-Rays: Insoluble Gelatin Hydrogel Formation

Masahiko Bessho,*[1] Takao Kojima,[2] Shuichi Okuda,[2] and Masayuki Hara[1]

[1]Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570

[2]Radiation Research Center, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570

Received June 15, 2006; E-mail: bessho@b.s.osakafu-u.ac.jp

Gelatin hydrogels with various concentrations were cross-linked by the irradiation with ^{60}Co γ-rays, and we investigated the radiation-induced cross-linking of gelatin hydrogels by estimating the physical properties of irradiated hydrogels. In case of the 1, 5, and 10% (w/v) gelatin solutions, the specific water content of the irradiated hydrogels, the index showing the extent of cross-linking depended on the absorbed dose, and that of the irradiated gelatin hydrogels with the lower concentration decreased drastically. The breaking strength correlated to the absorbed dose, irrespective of the initial gelatin concentration. More than 8 kGy irradiation induced insolubility due to the cross-linking of the gelatin hydrogels. Besides, γ-ray irradiation to gelatin with free amino acids revealed that amino acids, which have side chains of hydrocarbon groups that are more than two carbon atoms, obstructed the cross-linking of gelatin hydrogels. It is thought that the hydrocarbon groups, such as an alkyl or a phenyl group of the side chains, are the cross-linking sites of gelatin hydrogels.

Gelatin, which is denatured fractions of collagen (mostly Type-I collagen), is an important fibrillary protein and can be obtained from acid or alkaline extraction processing of animal skin or bone containing collagen matrices. Unlike collagen, gelatin is characterized by high water-solubility and a thermo-reversible sol–gel transition. Thus, hydrogels with various concentrations of gelatin can be prepared easily. Gelatin has been widely used as the foods, water-soluble capsules and coating materials for oral drugs, and as a stabilizer of photo-sensitive reagents in photographic films and adsorbent for diluted chemicals.[1]

Recently, the use of gelatin as a carrier for drug-delivery system (DDS) and controlled release of growth factors for tissue engineering has attracted much attention.[2–4] In spite of their biodegradability and biocompatibility, like collagen, it is difficult to use gelatin hydrogels as biomaterials at body temperatures (approx. 40 °C), because of their thermo-reversibility. In order to restrain dissolution or decomposition of hydrogels at body temperatures, the cross-linking of gelatin hydrogels is required. It is well known that bifunctional cross-linker reagents, such as glutaraldehyde,[5,6] and carbodiimides,[7] can be used to cross-link the amino-groups in gelatin. However, the use of bifunctional chemical reagents can cause toxic side effects due to residual reagents.[8]

We investigated gelatin or collagen hydrogels cross-linked by the irradiation with ^{60}Co γ-rays. Radiation-induced cross-linking of the gelatin by using γ-rays is more attractive, because there are no residual chemical reagents in the irradiated gelatin hydrogels. As well as being cross-linked, hydrogels can be sterilized during the γ-ray irradiation process. The γ-ray-induced cross-linking process has been known for many years. It is considered that polymer radicals generated by the reaction to hydroxyl radicals account for the cross-linking of gelatin hydrogels.[9,10] However, the reaction mechanism has not been clarified in detail.

In our previous paper,[11,12] it has been suggested that the gelatin hydrogel cross-linked by γ-ray irradiation has the potential to be used as a carrier for controlled release. Naturally, the properties of cross-linked gelatin hydrogels are influenced by γ-ray irradiation. We have proposed a schematic model of the collagen gel cross-linked by γ-ray irradiation.[13] The gel formation of gelatin is different from that of collagen. Gelatin consists of polypeptides in random coils, while a collagen molecule has a triple-helical structure that consists of three polypeptides (α-subunits). Thus, in case of gelatin hydrogels, it is imperative to clarify the cross-linking process by the γ-ray irradiation process. However, it is very difficult to monitor the cross-linking process of the irradiated gelatin hydrogels, because the lifetime of polymer radicals or hydroxyl radicals is very short. The molecular assembly state of gelatin molecules in the irradiated hydrogels is difficult to observe directly.

In this paper, Type-A gelatin hydrogels with various concentrations were irradiated with ^{60}Co γ-rays. Some of the physical properties of the irradiated gelatin hydrogels were investigated. From these results, we estimated the cross-linking process of the irradiated gelatin hydrogels indirectly. Type-A gelatin, purified by an acid extraction process, has nearly the same side chains as collagen without hydrolysis during the extraction process and an isoelectric point (IEP) of 8–9 close to that of collagen.[6] Finally, we propose a model for formation of insoluble gelatin hydrogel induced by the cross-linking during the γ-ray irradiation process.

Published on the web May 14, 2007; doi:10.1246/bcsj.80.979

Experimental

Materials. Type-A gelatin isolated from porcine skin by an acid extraction process, was purchased from Sigma-Aldrich Japan Co., Tokyo. It had an IEP of 8–9 and approx. 300 Bloom strength.

Preparation of Gelatin Hydrogels. A typical example of preparation of an aqueous gelatin solution is as follows: gelatins (1–10 g) were dissolved in distilled water (100 mL) at 40 °C for 30 min. The gelatin solution (25 mL, 1–10% (w/v)) was poured into a round glass vial (diameter 40 mm, height 80 mm) with a plastic screw cap. The aqueous gelatin solution was transformed into a hydrogel by storage at 4 °C before irradiation.

Cross-Linking of Gelatin Hydrogels by γ-Ray Irradiation. The gelatin hydrogels in covered glass vials were irradiated using the ^{60}Co γ-ray irradiation facility at Radiation Research Center, Osaka Prefecture University, at an absorbed dose rate of 11.8–13.6 kGy h^{-1}.

Specific Water Content of Cross-Linked Gelatin Hydrogels. The extent of cross-linking was estimated via the specific water content. Cross-linked gelatin hydrogels were immersed in 30 mL of distilled water, and swelled for 48 h at room temperature. The wet weight of the hydrogel (W_w) after swelling and the dry weight (W_d) after freeze-drying were measured. The specific water content was calculated according to the following equation:

$$\text{Specific water content} = (W_w - W_d)/W_d. \quad (1)$$

Scanning Electron Microscopy (SEM). Scanning electron microscopic images were recorded using a SM-300 scanning electron microscope (TOPCON Corporation, Tokyo) operated at 15 kV. Prior to observation of gelatin hydrogel samples, the solvent in them was changed from water to ethanol (Wako Pure Chemical Industries, Ltd., Osaka), and then to *t*-butyl alcohol (Wako) by successive incubation in order to remove moisture. After the hydrogels were freeze-dried, platinum was coated on the surface of the freeze-dried samples under vacuum using a IB-3 ion coater (EIKO Engineering Co., Ltd., Ibaraki).

Mechanical Strength of Cross-Linked Gelatin Hydrogels. Cubic blocks (1 cm on a side) of the cross-linked gelatin hydrogels after swelling were cut out, and the breaking strength of the hydrogel blocks was measured at 25 °C by using a FUDOH-Rheometer (RT-2002DD, Rheotec Co., Ltd., Tokyo, Japan), equipped with a round flat plate adaptor (ϕ5 mm). The moving speed of the stage was 6 cm min^{-1}. Penetration length of the adaptor was set up as 4 mm.

Heat Treatment for Estimation of the Insolubility. In order to estimate the insolubility of gelatin hydrogels, the heat treatment of hydrogels was performed. After γ-ray irradiation, the cross-linked gelatin hydrogels in covered glass vials were incubated at 50 °C in a shaking bath. When gelatin sols were confirmed in the vials after the heat treatment, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 10% polyacrylamide gel with a 10% stacking gel, according to Laemmli's method.[14] Sol samples for SDS-PAGE were boiled for 3 min in a sample buffer solution. Proteins were stained in 0.25% Coomassie Brilliant Blue R-250 in 10 methanol/1 acetic acid/9 distilled water, and then destained in 2 methanol/1 acetic acid/7 distilled water.

Absorption of Hydrophilic Compounds into Cross-Linked Gelatin Hydrogels. Methyl orange (MW 326.74) was purchased from Wako Pure Chemical Industries, Ltd., Osaka. It has anionic properties because it is deprotonated in aqueous solution. Absorption of Methyl orange into the gelatin hydrogels was performed at room temperature, using the following procedure. A cubic block (7 mm on a side) of the cross-linked gelatin hydrogel, after swelling, was cut out and incubated with 1 mL of Methyl orange solution at room temperature, and then, the time course in Methyl orange concentrations was monitored by measuring the absorbance at 450 nm with a reference at 750 nm, using a microplate-reader (Model 680, Bio Rad, Melville, NY, U.S.A.). In this experiment, 200 mM of Methyl orange solution was used. From these data, the amount of absorption into the cross-linked gelatin hydrogels was calculated.

γ-Ray Irradiation of Gelatin Hydrogels with Free Amino Acids. In order to estimate the effect of amino acids on radiation-induced gel formation, gelatin hydrogels including each free amino acid were irradiated with γ-rays. To the gelatin solution (10 mL, 5% (w/v)) was added an amino acid (10 mL, 100 mM). Then, 3 mL of the mixture was introduced into each well of a 24-well microtiter plate. Stored for 24 h at 4 °C to form hydrogels, the microtiter plate was irradiated with γ-rays at absorbed dose of 25 kGy using the ^{60}Co γ-ray irradiation facility. In this experiment, 17 amino acids (Gly, Pro, Ala, Hyp (hydroxyproline), Glu, Arg, Asp, Ser, Leu, Lys, Val, Thr, Phe, Ile, Met, His, and Tyr) were used. The pH of each amino acid solution was adjusted to 6–8 with HCl or NaOH.

Results

Change of Gelatin Hydrogels by γ-Ray Irradiation. After γ-ray irradiation, each Type-A gelatin remained in the hydrogel state in the vials. However, depending on the initial gelatin concentration of aqueous solution, it was observed that irradiated gelatin hydrogels gradually shrank with exclusions of a liquid phase at higher absorbed doses. Figure 1 shows their volume change upon irradiation with absorbed dose rates of 11.8–13.6 kGy h^{-1}. The absorbed dose for the beginning of shrinking decreased with a decrease in the initial gelatin concentration. In case of 1% (w/v) of gelatin hydrogels, the irradiation over 80 kGy gave volume reduction of about 90% against the un-irradiated one. But, even at the absorbed dose of 300 kGy, 5 and 10% (w/v) gelatin hydrogels shrank about 40 and 60% of the un-irradiated, respectively. These results indicate that the shrinkage of irradiated hydrogels is affected by the initial concentration of gelatin hydrogels.

Specific Water Content of the Irradiated Gelatin Hydrogels. Figure 2 shows un-irradiated and irradiated

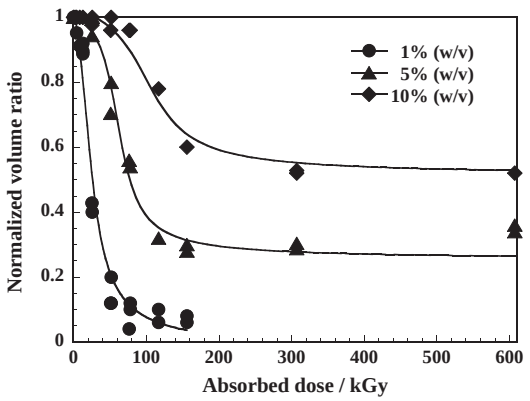

Fig. 1. Effect of absorbed dose on the shrinking of irradiated Type-A gelatin hydrogels. The volume of un-irradiated gelatin hydrogel is defined as 1.

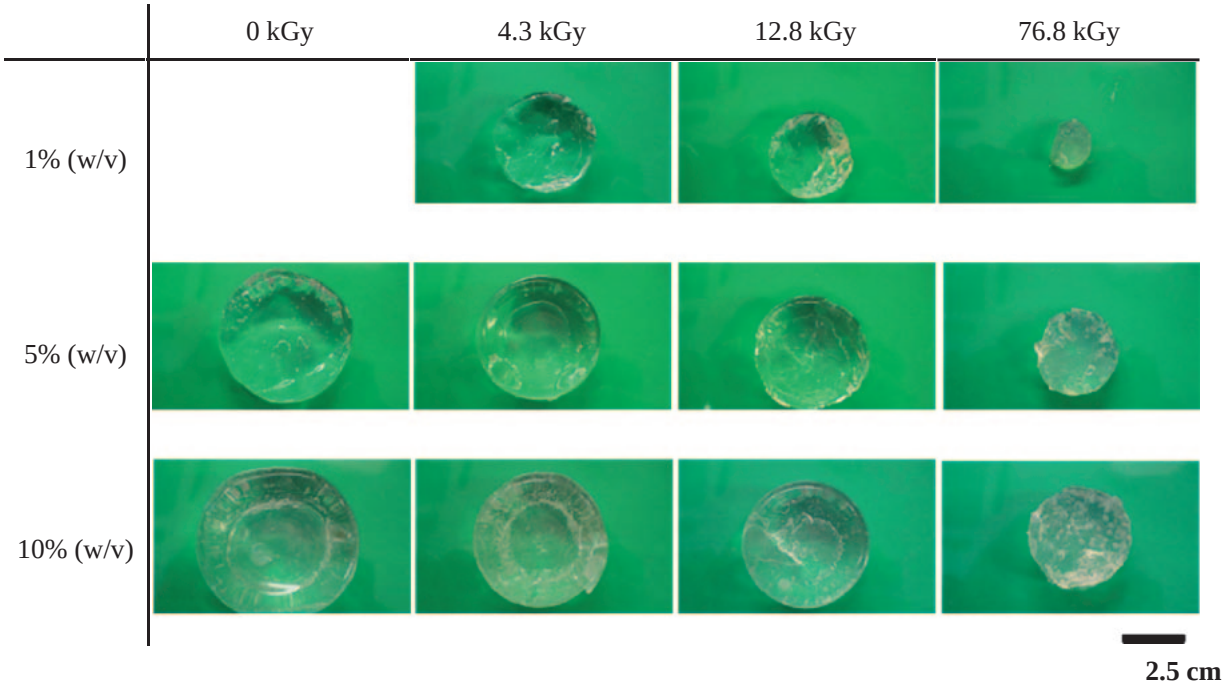

Fig. 2. Photograph of Type-A gelatin hydrogels cross-linked by γ-ray irradiation with 12.8 kGy h^{-1} of absorbed dose rate. The gelatin hydrogels were irradiated at 0–76.8 kGy of absorbed dose (from left to right).

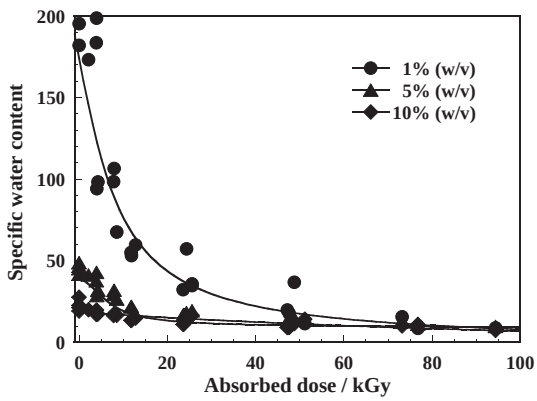

Fig. 3. Effect of absorbed dose on specific water content of Type-A gelatin hydrogels cross-linked by γ-ray irradiation at an absorbed dose rate of 12.8 kGy h^{-1}.

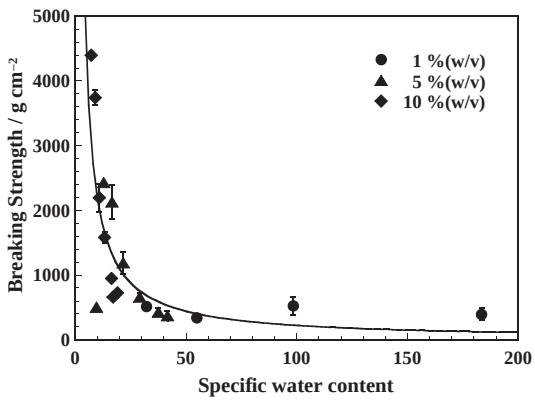

Fig. 4. Relationship between of specific water content and the breaking strength of gelatin hydrogels.

Type-A gelatin hydrogels after immersion in distilled water. Un-irradiated hydrogel prepared from 1% (w/v) gelatin solution dissolved during immersion. Gelatin hydrogels un-irradiated or irradiated at lower doses swelled due to absorption of surrounding water. On the contrary, the irradiated ones at the higher dose tended to exclude water. In general, hydrogels can include water through weak hydrogen-bonds.[15] However, it is thought that the irradiated gelatin hydrogels cannot expand themselves with an increase of cross-linking points.

This result was reflected by the specific water content of cross-linked Type-A gelatin hydrogels by γ-ray irradiation at different absorbed doses. Specific water content was plotted against absorbed doses as shown in Fig. 3. The water content in 1, 5, and 10% (w/v) gelatin hydrogels before immersion corresponded to approx. 99, 19, and 9, respectively. However, the specific water content of hydrogels irradiated at lower doses is higher than that. The specific water content of cross-linked gelatin hydrogels decreased with an increase in an absorbed dose. Specific water content is often used as an index showing the extent of cross-linking. Therefore, γ-ray irradiation promoted the cross-linking reaction. Especially, that of 1% (w/v) gelatin hydrogels decreased drastically. However, more than 80 kGy irradiation gave almost the same value of the specific water content, irrespective of the initial gelatin concentration.

Breaking Strength of the Cross-Linked Gelatin Hydrogels. The breaking strength of the cross-linked gelatin hydrogels was plotted against specific water content as shown in Fig. 4. The cross-linked hydrogels were irradiated at up to 94.4 kGy. The breaking strength correlated to the specific water content of the irradiated hydrogels regardless of the initial gelatin concentration. In the range of less than 50, it drastically increased with a decrease in the specific water content. This

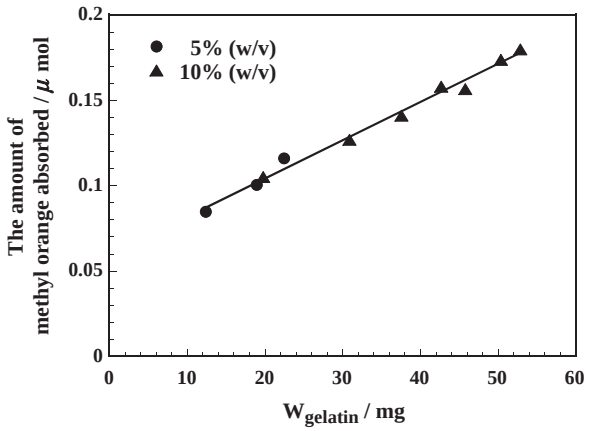

Fig. 5. Relationship between the amount of gelatin and that of absorption of Methyl orange into Type-A gelatin hydrogels cross-linked by γ-ray irradiation at an absorbed dose rate of 13.6 kGy h^{-1}.

indicates that hydrogels were reinforced due to the cross-linking by γ-ray irradiation.

Absorption of Dyes into Cross-Linked Type-A Gelatin Hydrogels. The absorption test of Methyl orange into cross-linked gelatin hydrogel blocks was performed. Cross-linked gelatin hydrogels prepared from 5 or 10% (w/v) aqueous solutions, were irradiated with γ-rays at an absorbed dose rate of 13.6 kGy h^{-1}, and offered for specimens of hydrogel blocks. Roughly speaking, absorption of Methyl orange increased with an increase in the absorbed dose or the initial gelatin concentration. We have reported in our previous paper[11] that of Methylene blue shows the same trend as Methyl orange. The amount of gelatin in hydrogels has an influence on the absorption into irradiated hydrogels. So, relationship between the amount of absorption and the weight of gelatin included in cross-linked hydrogels at various absorbed doses was examined. The weight of gelatin included in hydrogels ($W_{gelatin}$) was calculated according to the following equation:

$$W_{gelatin} = \text{(Weight of hydrogel)}/((\text{Specific water content}) + 1). \quad (2)$$

The amount of absorbed Methyl orange was plotted against $W_{gelatin}$, as shown in Fig. 5. It was obvious that the amount of absorbed Methyl orange was almost proportional to $W_{gelatin}$, irrespective of gelatin concentrations. Because of cross-linking, higher irradiation with γ-rays might cause the increase in the amount of gelatin in hydrogels, which would lead to the increase of adsorption sites into them for Methyl orange. Therefore, it might be imply that the γ-ray irradiation process affected absorption into the gelatin hydrogels indirectly. In addition, it suggests that the adsorption sites rarely took part in the cross-linking points of gelatin during the γ-ray irradiation process.

Heat Treatment of the Irradiated Gelatin Hydrogels. In order to confirm the irreversibility of sol/gel transition, gelatin hydrogels irradiated at 2.1–12.8 kGy were heated for 1 h at 50 °C, during which gelatin fully dissolved in water. As shown in Table 1, all hydrogels irradiated at 2.0 kGy dissolved completely to be transformed into gelatin sols. These sols reverted back to hydrogels on storage at 4 °C. These gel formations are

Table 1. Sol/Gel Transition of the Irradiated Gelatin Hydrogels by Heat Treatment at 50 °C

	1% (w/v)	5% (w/v)	10% (w/v)
2.0 kGy	sol	sol	sol
4.1 kGy	gel	gel	sol
8.2 kGy	gel	gel	gel
12.2 kGy	gel	gel	gel

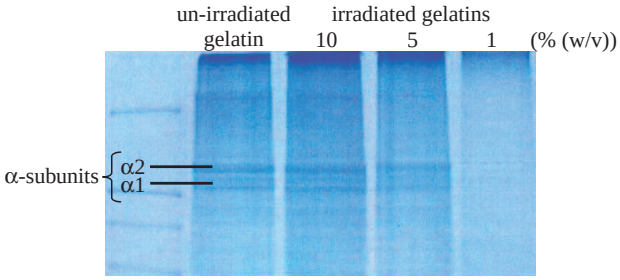

Fig. 6. SDS-PAGE of 1, 5, and 10% (w/v) gelatin sols after the heat treatment at 50 °C with 2.0 kGy irradiation.

derived from the gelation property peculiar to gelatin. The absorbed dose at 8.2 kGy was required to cause the irradiated hydrogels with a 10% (w/v) gelatin concentration to become insoluble, while 4.1 kGy was in case of 1 and 5% (w/v). This indicates that the amount of gelatin in hydrogels is affected by insoluble hydrogel formation during irradiation.

Gelatin molecules (polypeptide chains) in the sols after the heat treatment were investigated. The SDS-PAGE of the sols is displayed in Fig. 6. Un-irradiated Type-A gelatin has the band patterns corresponding to α- and β-bands, those are monomer and dimer of α-subunit of collagen, respectively. However, the 1% (w/v) gelatin hydrogel irradiated at 2.0 kGy did not show the bands corresponding to the α-subunit, that is, the gelatin molecules had to connect each other by radiation-induced cross-linking. In case of 5 or 10% (w/v) gelatin hydrogels irradiated at 2.0 kGy, the band patterns of the sol samples were almost same as those of un-irradiated Type-A gelatin (Fig. 6). This result indicates that only a part of gelatin molecules cross-linked. It was confirmed that the gelatin molecules were cross-linked to each other by γ-ray irradiation. However, for the irradiation at 2.0 kGy, the cross-linking of gelatin molecules was so insufficient that the irradiated hydrogel did not become insoluble.

SEM Observation of Cross-Linked Gelatin Hydrogels. Figure 7 shows SEM images of 5% (w/v) Type-A gelatin hydrogels un-irradiated and irradiated at 122.4 kGy. Scale bars in images represent 1 μm. That of un-irradiated collagen hydrogel prepared by pH adjustment is also presented in Fig. 7c by way of comparison. A three-dimensional tangled network of collagen microfibril was observed in the collagen hydrogel, but not in the gelatin hydrogels in the same magnification, as shown in Fig. 7a and Fig. 7b. Nor was it in case of 1 or 10% (w/v). Gelatin basically consists of the finer denatured fractions of collagen (polypeptide chains). It is considered that gelatin hydrogel has a three-dimensional network structure of polypeptide chains with the partial triple-helical structures.[16,17] The polypeptide chains were cross-linked through the γ-ray irradiation process, but cross-linking did not form collagen-like

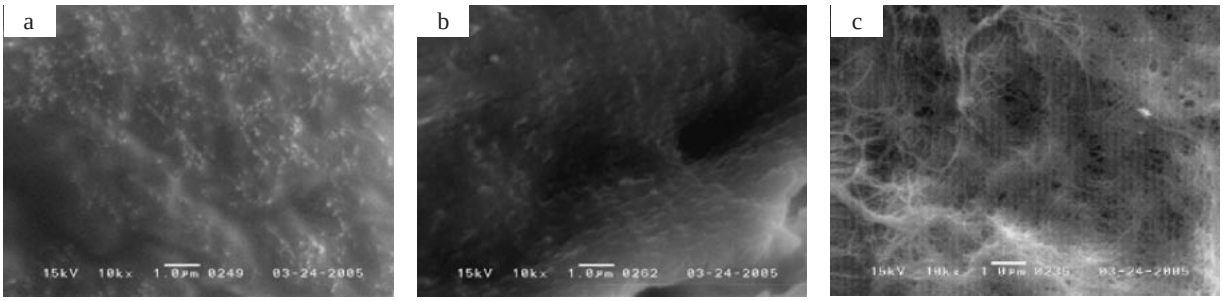

Fig. 7. SEM images of 5% (w/v) Type-A gelatin hydrogels, un-irradiated (a) or irradiated at 122 kGy of absorbed dose (b). SEM image of collagen hydrogel prepared by neutralization of pH, is also presented in (c).

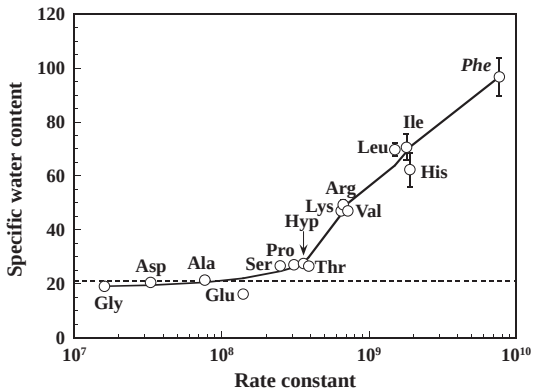

Fig. 8. The correlation between specific water content of the irradiated gelatin hydrogels including amino acids and the rate constant of hydroxyl radical and amino acids. The specific water content of the irradiated hydrogel without amino acid is also presented by dashed line, and that of the irradiated hydrogels including Met and Tyr could not be presented because of dissolution during immersion in water.

fibrils from them.

Effect of Amino Acids on the Cross-Linking of Irradiated Gelatin Hydrogels. We found that addition of each amino acid affected the efficiency in cross-linking of gelatin hydrogels. Specific water content of the irradiated gelatin hydrogels including each amino acid was plotted against the rate constant of hydroxyl radical and amino acids[18,19] in Fig. 8. That of the irradiated hydrogel without amino acid is also presented by dashed line, and that of the irradiated hydrogels including Met and Tyr is not presented because of dissolution during immersion in water. Un-irradiated gelatin hydrogel also dissolved. Thus, higher specific water content implies that the cross-linking of gelatin hydrogels is suppressed. It could be correlated to the rate constant. The amino acids were classified into five categories with regard to the tendency to suppress the cross-linking. Tyr, Phe, and Met amino acids with aromatic side chains or sulfur, were the strongest. Three amino acids His, Ile, and Leu belonged to the second category group of those suppressing the cross-linking. Three other amino acids, Val, Arg, and Lys, were in the third category. Four amino acids, Thr, Hyp, Pro, and Ser that weakly suppressed cross-linking were placed in the fourth category. The remaining amino acids (Glu, Ala, Asp, and Gly) showed no or little suppression of the cross-linking. It is thought the free amino acids with the higher rate constant could trap the unpaired electron from the hydroxyl radical and worked as a radical scavenger.

Discussion

Insoluble Hydrogel Formation of Gelatin by Cross-Linking. An irradiation process with γ-ray chiefly causes the cross-linking and decomposition in proteins and polysaccharides, respectively. However, it has been reported that γ-ray irradiation promotes the decomposition of gelatin or collagen in a dry state.[20,21] On the contrary, the irradiation of aqueous solutions including gelatin or collagen promoted the cross-linking of them. This accounts for hydroxyl radicals generated by the reaction of γ-ray irradiation to water molecules.[9,10,21,22] In the aqueous solution, γ-ray irradiation indirectly caused cross-linking of gelatin molecules. Briefly, generated by γ-ray irradiation process, hydroxyl radicals react with polypeptide chains. Then, they are transformed into polymer radicals, which cross-link with covalent bonds each other.[9,10]

Gelatin dissolves in the aqueous solution with the formation of random coils (polypeptide chains), and then on cooling, gelatin hydrogel forms. It can be regarded as a three-dimensional network structure of polypeptide chains with the partial triple-helical structures.[16,17] Irradiation with γ-rays has the potential to induce cross-linking of inter- or intra-triple-helical structures, or that of polypeptides, which do not form a triple-helical structure. The 4.0 kGy irradiation caused the 1% (w/v) gelatin hydrogel to become insoluble (Table 1). However, we confirmed that of gelatin aqueous solution could not be transformed into the insoluble hydrogel after γ-ray irradiation of the same dose at 50 °C at which gelatin can dissolve (data not shown). This indicates that the radiation-induced cross-linking of the inter- or intra-partial triple-helical structures promotes the insoluble hydrogel formation effectively. Naturally, it should be noted that a part of polypeptide chains, which are not included in a triple-helical structure, is cross-linked by γ-ray irradiation. But, the partial triple-helical structures play a more important role in insoluble hydrogel formation.

A schematic model of the insoluble gelatin hydrogel formation by radiation-induced cross-linking is proposed in Fig. 9. It is assumed that the formation of insoluble cross-linked gelatin hydrogels has the following steps: 1) the polymerization of gelatin molecules (polypeptides) due to the major cross-linking of partial triple-helical structures, and 2) the successive connections of the polymerized gelatin molecules including the cross-linked triple-helical structures. Consequently, the connected gelatin molecules form an insoluble "framework" of

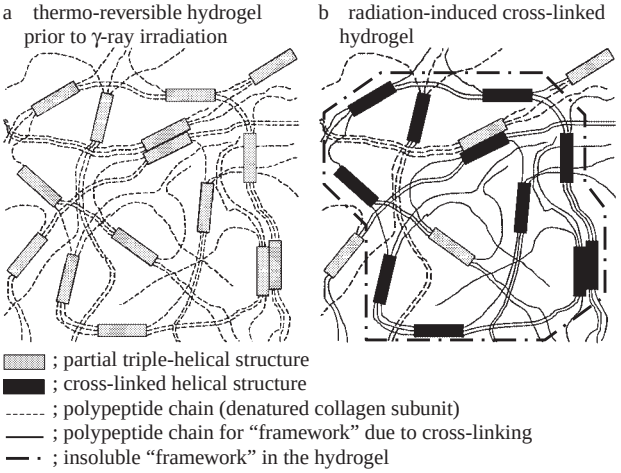

Fig. 9. Schematic illustrations of insoluble gelatin hydrogel formation by the radiation-induced cross-linking.

irradiated hydrogel structure (Fig. 9b). Irradiated hydrogels might include non-crosslinked gelatin molecules with the non-covalently bonds. As shown in Table 1, the gelatin hydrogels irradiated with a dose of 2.0 kGy transformed into gelatin sols. It is implied that the framework cannot be organized. When the concentration of gelatin hydrogels increases, the more absorbed dose is required to form the insoluble "framework" of gelatin hydrogels.

Predicted Cross-Linking Sites in Irradiated Gelatin Hydrogels. It is important to estimate the sites where hydroxyl radicals react to generate polymer radicals. Bowes and Moss have reported that γ-ray irradiation causes the loss of Phe, Tyr, and His, which have a ring structure, to collagen polypeptides.[20] In our laboratory, it has been confirmed that the content of three amino acids, Tyr, Phe, and His in collagen gels, significantly decreases with an increase in the absorbed dose, and that Phe and Tyr, amino acids with aromatic side chains, suppress the cross-linking of collagen by irradiation the strongest.[13] Thus, amino acids suppressing the radiation-induced cross-linking of collagen gels correspond to those in cross-linked collagen gels, which decrease after γ-ray irradiation. Gelatin has almost the same composition of amino acids as collagen. As shown in Fig. 8, it is predicted that 13 amino acids, Tyr, Phe, Met, His, Ile, Leu, Val, Arg, Lys, Thr, Hyp, Pro, and Ser are concerned with the cross-linking sites of gelatin hydrogels. These amino acids have side chains of hydrocarbon groups including more than two carbon atoms. It is suggested that polymer radicals are generated with the reaction of these hydrocarbon groups and hydroxyl radicals, supporting that the amount of absorbed Methyl orange was almost proportional to that of gelatin in irradiated hydrogels (Fig. 5). Methyl orange is anionic in aqueous solution. Electrolytic compounds can be adsorbed mainly by pertinent polar groups, i.e., $-COO^-$ or $-NH_3^+$. These results suggest that the cross-linking by γ-ray irradiation leads to a rare reaction of hydroxyl radicals with the polar groups, while bifunctional cross-linkers, such as glutaraldehyde and carbodiimide, react with the free amino groups and carboxyl groups on the side chain of various amino acids.[23,24] Therefore, we inferred that hydroxyl radicals mainly attacked hydrocarbon groups, alkyl or phenyl groups of side chains, and that the connection (cross-linking) of hydrocarbon radicals served as a foundation for the insoluble "framework" formation of gelatin hydrogels.

Conclusion

In order to estimate the cross-linking process of the irradiated gelatin hydrogels, some of the physical properties of the irradiated gelatin hydrogels were investigated. The breaking strength was correlated with specific water content, and the absorption of Methyl orange into the irradiated hydrogels was almost proportional to the amount of gelatin in the hydrogels. The proposed model of the insoluble gelatin hydrogel formation by radiation-induced cross-linking has the following steps: the polymerization of gelatin molecules due to the major cross-linking of partial triple-helical structures, and the successive connections of the polymerized gelatin molecules, including the cross-linked triple-helical structures. It is suggested that the insoluble "framework" of irradiated hydrogel structure thus forms. From the result of irradiated gelatin hydrogels including amino acids, we think that the amino acids, which have side chains of hydrocarbon groups including more than two carbon atoms, are involved in the cross-linking sites of gelatin hydrogels.

This research is partly supported by 21st Century, COE program 24403, E-1, Ministry of Education, Culture, Sports, Science and Technology. We express our gratitude to Prof. Naofumi Morita (Graduate School of Life and Environmental Sciences, Osaka Prefecture Univ.) for instructing rheological measurements.

References

1 A. I. Van Den Bulcke, B. Bogdanov, N. De Rooze, E. H. Schacht, M. Cornelissen, H. Berghmans, *Biomacromolecules* **2000**, *1*, 31.
2 C. H. Lee, A. Singla, Y. Lee, *Int. J. Pharm.* **2001**, *221*, 1.
3 E. M. Noah, J. Chen, X. Jiao, I. Heschel, N. Pallua, *Biomaterials* **2002**, *23*, 2855.
4 T. Shimizu, M. Yamato, A. Kikuchi, T. Okano, *Biomaterials* **2003**, *24*, 2309.
5 Y. Tabata, A. Nagano, M. Muniruzzaman, Y. Ikada, *Biomaterials* **1998**, *19*, 1781.
6 Y. Tabata, Y. Ikada, *Adv. Drug Delivery Rev.* **1998**, *31*, 287.
7 C. M. Ofner, W. A. Bubnis, *Pharm. Res.* **1996**, *13*, 1821.
8 C.-H. Yao, B.-S. Liu, C.-J. Chang, S.-H. Hsu, Y.-S. Chen, *Mater. Chem. Phys.* **2004**, *83*, 204.
9 Y. Tomoda, M. Tsuda, *J. Polym. Sci.* **1961**, *54*, 321.
10 Y. Tomoda, M. Tsuda, *Nature* **1961**, *190*, 905.
11 M. Bessho, M. Furuta, T. Kojima, S. Okuda, M. Hara, *J. Biomater. Sci., Polym. Ed.* **2005**, *16*, 715.
12 T. Kojima, M. Bessho, M. Furuta, S. Okuda, M. Hara, *Radiat. Phys. Chem.* **2004**, *71*, 235.
13 N. Inoue, M. Bessho, M. Furuta, T. Kojima, S. Okuda, M. Hara, *J. Biomater. Sci., Polym. Ed.* **2006**, *17*, 837.
14 U. K. Laemmli, *Nature* **1970**, *227*, 680.
15 H. Muta, M. Miwa, M. Satoh, *Polymer* **2001**, *42*, 6313.
16 C. Joly-Duhamel, D. Hellio, A. Ajdari, M. Djabourov, *Langmuir* **2002**, *18*, 7158.
17 C. Joly-Duhamel, D. Hellio, M. Djabourov, *Langmuir*

2002, *18*, 7208.
18 G. E. Adams, J. W. Boag, J. Currant, B. D. Michael, *Pulse Radiolysis*, ed. by M. Ebert, J. P. Keene, A. J. Swallow, J. H. Baxendale, Academic Press, San Diego, CA, **1965**, p. 131.
19 G. Scholes, P. Show, R. L. Willson, M. Ebert, *Pulse Radiolysis*, ed. by M. Ebert, J. P. Keene, A. J. Swallow, J. H. Baxendale, Academic Press, San Diego, CA, **1965**, p. 151.
20 J. H. Bowes, J. A. Moss, *Radiat. Res.* **1962**, *16*, 211.
21 F. F. Vieira, N. L. Del Mastro, *Radiat. Phys. Chem.* **2002**, *63*, 331.
22 B. Liu, R. Harell, R. H. Davis, M. H. Dresden, M. Spira, *J. Biomed. Mater. Res.* **1989**, *23*, 833.
23 S. F. Badylak, *Methods in Tissue Engineering*, ed. by A. Atala, R. P. Lanza, Academic Press, San Diego, CA, **2002**, p. 505.
24 K. Watanabe, *Recent Res. Dev. Macromol. Res.* **1999**, *4*, 157.