Gamma-Crosslinked Collagen Gel without Fibrils: Analysis of Structure and Heat Stability

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plate (Falcon 353043, Becton Dickinson, Franklin Lakes, NJ). The 12-well plate was irradiated with gamma rays (absorbed dose, 0–25.6 kGy; dose rate, 10–12 kGy/h) in air at room temperature (25–30°C) using the 60Co gamma-ray irradiation facility at the Radiation Research Center of Osaka Prefecture University. A more concentrated porcine collagen solution (0.6% w/v, Collagen BM, Nitta Gelatin) was used in the structural analysis by AFM and SAXS.

In the preparation of the neutral gel, 3 ml of the acidic collagen solution (pH 3.2) was neutralized (pH 6.5) by the addition of 0.3 ml of a 10-fold concentrated solution of phosphate-buffered saline (×10 PBS[-]) to the 12-well plate. Opaque collagen hydrogel was formed during incubation for 2 h at 37°C before irradiation.

Measurement of specific water content. Collagen gels were swollen for 24 h at room temperature in 20 ml of distilled water twice and then freeze dried. Salts in phosphate-buffered saline (PBS[-]) was mostly removed and were not crystallized in the dried sample. Deterioration of the sample by bacterial contamination was not observed in the swelling procedure. The conditions were similar to those described previously, except that 0.1% w/v NaNO3 was not present in the solution for the swelling. The wet weight (Ww) and dry weight (Wd) were measured before and after freeze-drying to calculate the specific water content.

Specific water content \( = (W_w - W_d)/W_d \)

Measurement of breaking strength. The acidic and neutral gels were prepared in 12-well plates from 3 ml of collagen solution (Cellmatrix), as described above. The samples were set in a rheometer (RHEO Win. ver. 2.05, Rheotech, Tokyo, Japan) with a round plate adapter (19.6 cm²) for compression. The breaking strength was measured at room temperature at a compression speed of 6 cm/min.

Measurement by differential scanning calorimetry (DSC). Differential scanning calorimetry (DSC) measurements were performed with a Micro DCS VII (Setaram Instrumentation, Caluire, France) at a scan rate of 0.5°C/min.

The acidic and neutral gels were prepared in each well of a 96-well microtiter plate instead of a 12-well plate. Collagen solution (0.3% w/v × 200 ml) was added to each well with and without the addition of 20 μl of ×10 PBS[-] to the neutral and acidic gel respectively. The irradiated gels were swollen in 20 ml of distilled water for 24 h at room temperature (approximately 25°C) twice before DSC measurement. The sample cell for the DSC measurement contained 0.5 g of sample composed of 0.2% w/v collagen and distilled water. The reference cell contained 0.5 g distilled water only.

Scanning electron microscopy (SEM). The acidic and neutral gels were prepared from 3 ml of 0.3% w/v collagen solution (Cellmatrix) as described above in 12-well plates. These gels were swollen for 24 h at room temperature (approx. 25°C) in distilled water twice to remove the salts in PBS[-]. The solvent was changed from water to ethanol and then t-butyl alcohol by successive incubation in order to remove moisture. After the samples had been freeze-dried, platinum was coated on the surfaces of the samples in vacuum using a coater (JFC-1600, JEOL, Akishima, Tokyo). The coated samples were observed using a Fe-SEM (SM-6700F, JEOL).

Atomic force microscopy (AFM). The acidic and neutral gels were prepared from 3 ml of collagen solution (Collagen BM) as described above in 12-well plates and swollen as described above. The gel was homogenized with a glass-Teflon homogenizer into small pieces and suspended in distilled water at a concentration of 2% w/v. A drop of the suspension was placed on the flat surface of a mica crystal and dried under a flow of nitrogen gas. That condition was suitable for observation of partially fractured collagen fibrils as strings on an atomically flat stage of mica. The surface structure of the sample was observed with an AFM (MMAFM Multi-Mode, NanoScope IIIa, SPM unit, Veeco Instruments, Woodbury, NY) in tapping mode.

Small angle X-ray scattering (SAXS). Small angle X-ray scattering (SAXS) was examined for the neutral and acidic gels. They were prepared from 1.5 ml of collagen solution (Collagen BM, Nitta Gelatin) as described above in 24-well plates. The irradiated gel was disected into a piece 1 mm × 2.5 mm × 8 mm that was put in the cell for measurement. SAXS measurements were carried out at a synchrotron facility (BL-10, Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan). The wavelength of the incident X-ray beam was 1.49 Å, and the scattered X-rays were detected with a one-dimensional position-sensitive proportional counter positioned approximately 1 m from the sample holder.

Results

Appearance and mechanical properties of the gels

Figure 1A shows an image of neutral and acidic gel irradiated at various doses (3.2–25.6 kGy). The appearance of the semi-transparent neutral gel and that of the transparent acidic gel were similar to our previous data. However, the acidic gel shrank less in the present case than in our previous data (see Figs. 1 and 2 in ref. 5). The relationship between the specific water content and absorbed dose of gamma rays in Fig. 1B was very different from our previous data (see Fig. 3 in ref. 5) in which the values of the specific water content of the acidic gel were always lower than those of the neutral gel. The specific water content of the acidic gel irradiated at low doses (3.2–6.5 kGy) was higher than that of the neutral gel in our present data, as shown in Fig. 1B. We want to explain this discrepancy, because the data under for both conditions were sound and reproducible. The main difference between the experimental conditions in our present data and for the previous data is the absence and presence of 0.1% w/v NaNO3 in the solution for swelling. Collagen molecules in a random orientation were crosslinked in the acidic gel.

Fig. 1. Acidic Gel and Neutral Gel Irradiated at 3.2–25.6 kGy (A) and the Relationship between Absorbed Dose and Specific Water Content (B) of the Acidic Gel (solid circles) and the Neutral Gel (solid triangles).

Each data point is the average of duplicate determinations. Variations in each data point at the same dose are less than 10.
according to the model shown in Fig. 9 of ref. 5. One explanation for the discrepancy is as follows. When the acidic gel was swollen in pure distilled water of low ionic strength, intrinsic charges on the collagen molecules induced electrostatic repulsion and then hampered the shrinkage of the acidic gel. When the acidic gel was incubated in an aqueous salt solution, the charges on the collagen molecules were canceled by ions in the presence of salts such as NaNO₃. Therefore the electrostatic repulsion between the collagen molecules did not work so much.

We also measured the breaking strength of the acidic and neutral gels irradiated at various doses using a rheometer, as shown in Table 1. The acidic gel showed an approximately 4-fold higher value of breaking strength in a dose range of 3.1–12.4 kGy. Both types of gel became very brittle after irradiation at 24.8 kGy, with the result that proper rheological measurement was difficult because the shape of the gel was not retained in such a sample, as described in the legend to Table 1.

**Observation by SEM**

We studied the gels by SEM, and this clearly showed that collagen fibrils were present in the neutral gel but not in the acidic gel, as shown in Fig. 2. The results presented in this paper have image resolution much improved over our previous report. The tangled network of collagen fibrils in the neutral gel did not change much with the irradiation dose, as shown in Fig. 2A–C. The orientation of the fibrils was random. This suggests that the network of collagen fibrils mechanically supports the shape of the neutral gel, which shrinks very little dose-dependently. The acidic gel irradiated at 6.2 kGy had a smooth surface (Fig. 2D). On the other hand, the acidic gel irradiated at 24 kGy had many cracks (Fig. 2E). This heterogeneity of the acidic gel irradiated at high dose, must have been caused by regional shrinkage of the gel along many directions, and explain

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**Table 1. Breaking Strength of Gel Irradiated at Various Doses of Gamma Rays**

<table>
<thead>
<tr>
<th>Dose of gamma-rays (kGy)</th>
<th>Breaking strength of acidic gel (g/cm²)</th>
<th>Breaking strength of neutral gel (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>964</td>
<td>223</td>
</tr>
<tr>
<td>3.1</td>
<td>830</td>
<td>198</td>
</tr>
<tr>
<td>6.2</td>
<td>882</td>
<td>193</td>
</tr>
<tr>
<td>6.2</td>
<td>1042</td>
<td>266</td>
</tr>
<tr>
<td>12.4</td>
<td>623</td>
<td>184</td>
</tr>
<tr>
<td>12.4</td>
<td>736</td>
<td>304</td>
</tr>
<tr>
<td>24.8*¹</td>
<td>197</td>
<td>—</td>
</tr>
</tbody>
</table>

*¹ All of the samples were measured in duplicate, but one of the two samples of the acidic gel irradiated at 24.8 kGy, and both of the samples of the neutral gel irradiated at the same dose, were too brittle to obtain rheological parameters properly.
why the acidic gel irradiated at a higher dose was brittle with lower breakage strength, as described above.

**Observation by AFM**

Resolution in the SEM images was sufficient to show a tangled network of collagen fibrils, but was not good enough to show the D-period in each fibril. We observed the surface structure of the neutral gel and the acidic gel by AFM as shown in Fig. 3A and B respectively. A piece of the neutral gel fragmented in the homogenization and had a fibrillary shape with a pattern of stripes (Fig. 3A). The inter-peak distance of the stripes was approximately 70 nm. The value was roughly equal to 67 nm, the interval of the typical pattern of stripes (D-period) in the collagen fibrils caused by the regular arrangement of Type I collagen molecules.8,9) On the other hand, only an amorphous featureless image was obtained from the acidic gel (Fig. 3B). Our AFM data as described above resembled the results reported by others,8,9) and so proved the validity of the model proposed in our previous paper in the presence and absence of collagen fibrils in neutral and acidic gel, respectively.

**Measurement of DSC**

We have reported that both neutral and acidic gels acquired heat stability by crosslinking of collagen molecules after irradiation to become insoluble even at high temperatures.4) Therefore, we had a special interest in the question whether there is a difference in the heat denaturation of collagen molecules in the fibrillar and non-fibrillar states, in the neutral and the acidic gel respectively. We measured the DSC curves for the neutral and acidic gels with increasing temperature (Fig. 4A and B). The neutral gel showed a large endothermic peak at 53–55 °C in the DSC thermogram (Fig. 4A). On the other hand, the acidic gel showed a broad endothermic peak at 35–43 °C. We considered the explanation to be as follows: The broad peak at 35–43 °C in the acidic gel was derived from the enthalpy change (ΔH) accompanied by denaturation of collagen molecules: uncoiling of the triple helical structure. The larger sharp peak at 53–55 °C in the neutral gel was derived from ΔH accompanied by denaturation of the collagen fibrils. The peak lost its sharpness dose-dependently, and also showed a shift in peak temperature from 55 to 53 °C. It has been reported that Type I collagen under fibril-forming condition showed two endothermic peaks, at 40 and 55 °C, corresponding to the melting of monomeric collagen and of collagen fibrils respectively.10)

The apparent shape of the collagen fibrils did not change significantly with irradiation even at higher doses (6.2–24 kGy), as shown by the SEM data in Fig. 2A–C. However, as the data in Fig. 4A show, the sharpness of the endothermic peak in the DSC clearly

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**Fig. 3.** Atomic Force Microscopy (AFM) Image of the Neutral Gel (N) and Acidic Gel (A) Adsorbed on Mica.
Both samples were irradiated at 12.4 kGy.

**Fig. 4.** DSC Curves for the Neutral Gel (N) and Acidic Gel (A) Irradiated at 3.2–25.6 kGy.
A sharp endothermic peak at 53–55 °C in (N) and a broad endothermic peak at 35–43 °C in (A) are indicated by arrows.
Gamma-Crosslinked Collagen Gel 1919

decreased with irradiation at 3.2 kGy, and the peak was highly dependent on the dose. We next considered why the dose-dependence was so much different between the SEM image and the DSC data. Collagen molecules in neutral gel are thought to be covalently crosslinked to each other under strong irradiation by gamma rays. Both the decomposition of collagen fibrils into collagen molecules and the subsequent denaturation of collagen molecules were hampered by such intermolecular crosslinks. Thick collagen fibrils contain many subfibrils (sometimes called microfibrils). A typical example of this is the AFM image of fuzzy collagen fibrils in pieces of the homogenized neutral gel in Fig. 3A. Therefore intra-subfibrillary crosslinking and inter-subfibrillary crosslinking may also be considered.

It has been reported that the monomeric form of rat skin Type I collagen in solution at pH 3.0 showed an endothermic peak corresponding to the melting of monomeric collagen at 40 °C on the DSC measurement, and that fibrils of Type I collagen formed at neutral pH showed an endothermic peak corresponding to denaturation of collagen fibrils at 53 °C. These reports support our explanation of the mechanism of the thermal denaturation of collagen. The endothermic peak of the acidic gel at 35–43 °C derived from the denaturation of collagen was less clear in the 25.6 kGy-irradiated acidic gel than in the gel irradiated at lower doses (3.2, 6.4, and 12.8 kGy). We assume that the collagen molecules in the acidic gel were highly crosslinked covalently under strong irradiation by gamma rays, and that the deformation of triple helices was hampered by intramolecular (inter-subunit) crosslinks.

Small-angle X-ray scattering (SAXS)

We measured small-angle X-ray scattering (SAXS) to investigate difference, in the assembly states of collagen molecules in the neutral and acidic gels as shown in Fig. 5A and B respectively, by Kratky plots \(q^2 I(q) \) vs \(q\), where \(I(q)\) is the scattering intensity and \(q\) is the magnitude of the scattering vector defined by \((4\pi/\lambda)\sin(\theta)\), \(\lambda\) and \(\theta\) being the wavelength and half of the scattering angle respectively. Upturns were observed in the smaller angle region in the SAXS profiles of all of the neutral gels. This behavior means that large aggregates are formed for the observable range of SAXS. On the other hand, no upturn was observed in Kratky plots of SAXS from acidic gels. The maximum scattering appeared in the 24-kGy sample and so the difference in structure and/or aggregation state at the nano level between neutral and acidic gel was confirmed from these SAXS results. It is assumed that the structure of polymer gels consists of crosslinking regions and semi-dilute polymer solution regions. Since the Lorentzian-type scattering function is appropriate for Gaussian-type chain distribution, it is thought that scattering from the semi-dilute region in the gel is represented by\(^{11}\)

\[
I(q) \approx \frac{1}{1 + \xi^2 q^2} \quad (1)
\]

where \(\xi\) is the correlation length. The crosslinking region is assumed to be an inhomogeneous structure with two phases, which gives a Debye-Bueche type scattering function described by\(^{12}\)

\[
I(q) \approx \frac{1}{(1 + a^2 q^2)^{1/2}} \quad (2)
\]

where \(a\) is a measure of the extent of heterogeneity. The scattering from gel can be represented by the linear sum of eq. 1 and eq. 2.\(^{13}\) The experimental data were fitted with calculated curves (Fig. 6), and the values of \(\xi\) and \(a\) were evaluated as summarized in Table 2. The value of the correlation length \(\xi\) ranged from 10 to 17 Å. The acidic gel had a value of \(a\) that slightly decreased, from 45–46 Å to 37 Å, with increasing doses from 3 or 6 kGy to 24 kGy. The neutral gel had a value of \(a\) around 140 Å without irradiation and around 80–120 Å with irradiation. The difference in the value of \(a\) between the two types of gel is derived from the difference in the assembly states of the collagen molecules. The larger value of \(a\) with the neutral gel might reflect the tangled network structure of the collagen fibrils in the neutral gel. The result that the \(a\) value in both the neutral gel and the acidic one decreased slightly due to irradiation is thought to reflect the sensitivity of dissociation of the physical aggregation by the chemical crosslinking reaction.
Discussion

Molecular assembly of collagen molecules
Generally speaking, many hydrogels can be swollen or shrunk in aqueous solvent. The process was analyzed thermodynamically and is explained by the Flory-Huggins theory. Crosslinking between the polymer chains, permeation of solvent into the gel, electrostatic interaction between the polymer chains, and many other factors can affect the swelling and shrinking process. Collagen gel is very different from a simple chemical gel consisting of randomly crosslinked polymer chains. As reported in our previous paper and confirmed in this study, we found that the acidic gel had a different assembly state of collagen molecules without forming collagen fibrils. Figure 7 shows a schematic representation of the gel. The shape of the collagen in this figure was much simplified to emphasize the mechanism in shrinkage of the gel in comparison with Fig. 9 in our previous paper. Collagen molecules in a non-fibrillary state is rather flexible and can be easily bent or curled. The acidic gel irradiated with a relatively low dose, as in Fig. 2D, and it had a small number of crosslinking points of collagens (Fig. 7A). With a higher dose of gamma irradiation (Fig. 2E), the gel shrank significantly and had many crosslinking points (Fig. 7B). The neutral gel did not shrink as much because it is composed of collagen fibrils which firmly keep the shape of the gel (Fig. 7C). These models can well explain the results in Figs. 1 and 2 and are compatible with the results from SAXS: $a$ values (Table 1) as a measure of the extent of heterogeneity.

Conclusion

We analyzed the neutral gel and the acidic gel previously reported. We performed DSC, SEM, AFM, and SAXS. The experimental results are compatible with the model proposed in our previous paper, and confirmed that the acidic gel has no collagen fibrils and has a different molecular assembly state of Type I collagen than the neutral gel.

Acknowledgments

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