NMR Study of Poly(γ-Glutamic Acid) Hydrogels Prepared by γ-Irradiation : Characterization of Bond Formation and Scission

Oc Hee Han* and Hyuk Joon Choi†

Magnetic Resonance Group, Korea Basic Science Institute, 52 Yeoeundong, Yoosung-goo, Taejeon 305-333, Korea
†Doosan Technical Center, 39-3 Songbok-ri, Suji-myon, Kyonggi-do 449-840, Korea

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Hydrogels were prepared from poly(γ-glutamic acid) (PGA) solution by γ-irradiation of 90 kGy and 170 kGy. The hydrogels were more cross-linked with a higher dosage γ-irradiation and completely hydrolyzed at 85 °C within 4 hours resulting in homogeneous solution. NMR techniques were employed to clarify chemical bond formation and scission involved during γ-irradiation and hydrolysis. Characterization of these samples was carried out by taking both liquid state and solid state NMR spectra of PGA and hydrolyzed hydrogels and comparison of these spectra with the solid state NMR spectra of hydrogels. Our results indicate that complicated chemical bond formation and scission have occurred during hydrolysis and γ-irradiation. The samples prepared with higher dosage of γ irradiation showed more diverse chemical bond formation and scission.

Introduction

Hydrogels have a variety of applications such as controlled release devices,[1-3] superabsorbent materials,[4] and biomaterials like enzyme immobilization.[5] Irradiation,[2,3,6,7] repetitive freezing[8] and chemical cross-linking[9] have been representative techniques to develop new hydrogels. Among these methods, radiation processing has the advantage of no need to add chemical initiators or to do additional sterilization for the products.[6,7] In recent years, hydrogels prepared from natural polymers have received great attention in the environmental preservation aspect: hydrogels prepared from microbial poly(γ-glutamic acid) (PGA) are good examples. PGA can be cross-linked by γ-irradiation.[10] PGA hydrogels produced by γ-irradiation have shown high water sorption ability and various equilibrium swelling behaviors responding to the conditions of surrounding medium.

γ-irradiation on aqueous solution is known to produce hydroxyl radicals which mediate radical reactions.[11-14] Irradiation studies on cross-linking of polydeoxythymidylic acid[11] and modifications[12,13] of nucleobases, and DNA-protein cross-linking[14] confirm the invlovement of hydroxyl radicals in the reactions. But all the chemical reactions involved are not identified yet. The characterization of the cross-linking bonds in the hydrogels, especially PGA hydrogels is not clarified either, as far as we know. Thus, in this work, we report the results of ^{13}C NMR (nuclear magnetic resonance) study for the characterization of cross-linking and bond scission in PGA hydrogels produced by γ-irradiation. Bond scission during hydrolysis of the hydrogels is also discussed.

Experimental Section

Materials

Preparation of PGA and PGA hydrogel samples was described in detail in previous report.[15] PGA fermented by Bacillus subtilis F-02-1 was obtained from Meiji Seika kaisha, Ltd. (Japan) and was reacted with sodium hydroxide in deionized water to produce water soluble PGA. The number-average molecular weight, M_n of PGA was about 5.5×10^5. Cross-linking of PGA by γ-irradiation was carried out on 2 ml aqueous PGA solution (5 wt %) in a 10 ml glass bottle with 1.5 cm diameter under nitrogen atmosphere. A ^{60}Co γ radiation system at a dose rate of 1.6 kGy/h was employed at room temperature. PGA hydrogels were completely hydrolyzed within 4 hours at 85 °C. Sample notation in this report is summarized in Table 1.

Carbon-13 NMR Spectroscopy

Liquid State ^{13}C NMR. All liquid state ^{13}C NMR spectra were acquired at an UNITYplus 300 NMR instrument (Varian Associates Inc., U. S. A.) with 75.46 MHz and standard 5 mm NMR tubes at room temperature. The NMR samples were prepared by dissolving in D_2O solvent. Pulse length of 8.7 μs corresponding to 90° flip angle and pulse repetition delay of 10 s were employed. For peak assign-

Table 1. Summary of sample notation

Notation	Sample Description
GA	glutamic acid
PGA	poly(γ-glutamic acid) (water soluble)
PGA-90kGy	PGA hydrogel prepared with 90kGy γ-irradiation (water insoluble)
PGA-170kGy	PGA hydrogel prepared with 170kGy γ-irradiation (water insoluble)
hydrolyzed PGA-90kGy	hydrolyzed PGA-90kGy at 85 °C (water soluble, but freeze-dried before NMR study)
hydrolyzed PGA-170kGy	hydrolyzed PGA-170kGy at 85 °C (water soluble, but freeze-dried before NMR study)

ments, DEPT (Distortionless Enhancement by Polarization Transfer)[16] spectra of hydrolyzed PGA hydrogels were acquired.

Solid State ^{13}C NMR. All solid state ^{13}C NMR spectra were acquired at a DSX 400 NMR instrument (Bruker Analytik GmbH, Germany) with 100.6 MHz and a CP-MAS (cross polarization-magic angle spinning) probe for 4 mm rotors. All experiments were carried out with freeze-dried samples. Proton RF (radio frequency) field strength for CP and decoupling was 59.5 kHz and 89 kHz, respectively. Contact time for CP was 1 ms. Spinning rate was varied between 3 kHz and 12.5 kHz to distinguish center peaks from spinning side bands. All spectra were taken at room temperature.

Results and Discussion

Liquid state ^{13}C NMR spectrum of GA (Figure 1A) does not much differ from that of PGA (Figure 1B). All peak assignments[17] from chemical shift values are summarized in Table 2. Amide bond formation didn't bring difference in the spectrum because the chemical shift of carbonyl carbon (carbon #5) at the end of alkyl carbon chain in GA happen to be the same as that of amide carbon in PGA.[17] In contrast, linewidths of the peaks in the solid state ^{13}C CP-MAS NMR spectrum of PGA (Figure 1D) are much wider than those of GA (Figure 1C). Both peaks from carbonyl and methylene carbons appear as broad peaks in the CP-MAS spectrum of PGA while well resolved five peaks are shown in the GA spectrum as in its liquid state spectrum. This resolution difference comes from the difference of crystallinity. PGA is in amorphous phase in comparison with well crystallized GA.

Liquid state ^{13}C NMR spectrum of hydrolyzed PGA-90 kGy (Figure 2A) have three new peaks at 63, 72, and 174.4 ppm in addition to the five peaks from PGA. DEPT experimental results indicate that the peaks at 63, 72, and 174.4 ppm are methylene, methine, and tertiary carbons, respectively. More new peaks appeared in the spectrum of hyrolyzed PGA-170 kGy (Figure 2B). DEPT results reveal that small peaks between 60 and 64 ppm are from methylene carbons, those between 68 and 100 are from methine carbons, and 174.4 ppm peak is from tertiary carbons.[17] Amount of these new peaks in the spectrum of hydrolyzed PGA hydrogel-170 kGy is greater than that of the hydrolyzed PGA-90 kGy. Due to poor solubility, liquid state ^{13}C NMR spectra could not be obtained from PGA hydrogels before hydrolysis.

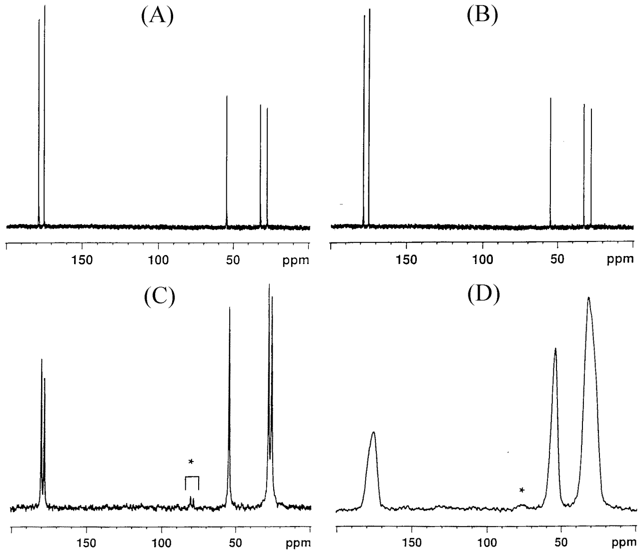
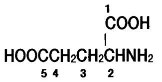


Figure 1. Liquid state ^{13}C NMR spectra of (A) GA and (B) PGA. Solid state ^{13}C CP-MAS NMR spectra of (C) GA at 10 kHz and (D) PGA at 10 kHz. Spinning side bands are marked by *.

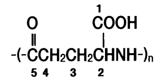
Table 2. ^{13}C peak assignment in the liquid state spectra of GA and PGA

compound	carbon site*				
	C-1	C-2	C-3	C-4	C-5
GA	175.4	55.5	27.2	31.7	178.7
PGA	175.3	55.1	28.0	32.6	178.7

*notation of carbon sites in GA and PGA:



$\underset{5\,4}{\mathrm{HOOC}}\underset{3}{\mathrm{CH_2}}\mathrm{CH_2}\underset{2}{\overset{\overset{1}{\mathrm{COOH}}}{\overset{|}{\mathrm{C}}}}\mathrm{HNH_2}$



$\text{-(-}\underset{5\,4}{\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}}\underset{3}{\mathrm{CH_2}}\mathrm{CH_2}\underset{2}{\overset{\overset{1}{\mathrm{COOH}}}{\overset{|}{\mathrm{C}}}}\mathrm{HNH\text{-})_n\text{-}}$

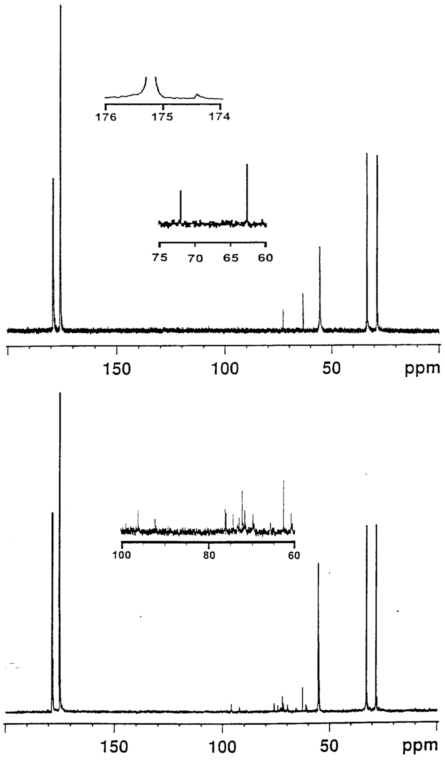


Figure 2. Liquid state ^{13}C NMR spectra of (A) hydrolyzed PGA-90 kGy and (B) hyrolyzed PGA-170 kGy. Regions with small peaks are expanded as in insets.

Solid state ^{13}C NMR spectra of hydrolyzed PGA hydrogels (Figure 3A and 3B) indicate that peak positions are qualitatively consistent with those in the liquid state ^{13}C spectra. But the small carbonyl peak at 174.4 ppm was not detected in the solid state MAS spectra: it was probably too small and close to a big peak to be detected as a well-

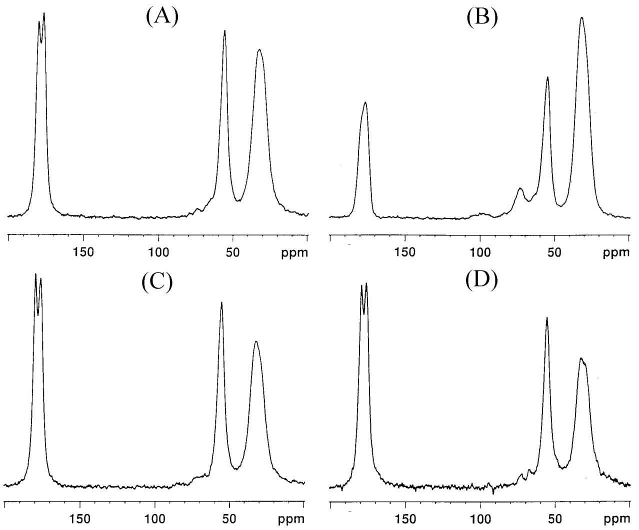


Figure 3. Solid state ^{13}C CP-MAS NMR spectra of (A) hydrolyzed PGA-90 kGy, (B) hydrolyzed PGA-170 kGy, (C) PGA-90 kGy, and (D) PGA-170 kGy. All spectra were obtained at the spinning rate of 12.5 kHz.

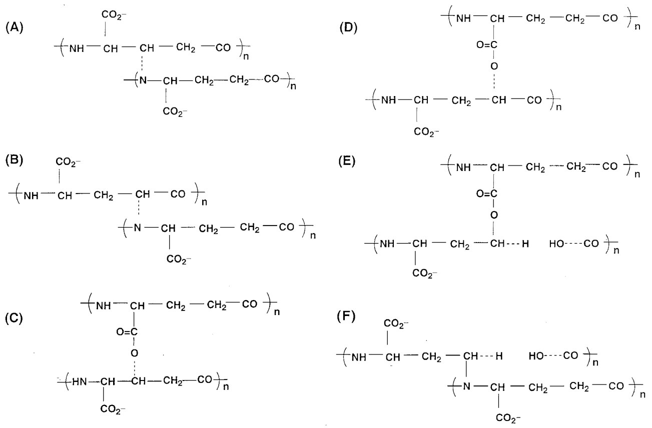


Figure 4. Some possible chemical bonds created due to cross-linking and hydrolysis created by radical reactions in PGA: only bonds between methylene or methine carbons and oxygen or nitrogen are shown. The chemical bonds are represented as dotted line.

resolved peak in the solid state spectra. In addition to the three main peaks at 32, 57, and 178 ppm from PGA, small peaks are present at 65 and 74 ppm in the MAS spectrum of hydrolyzed PGA-90 kGy (Figure 3A). In addition to these small peaks, the spectrum of hydrolyzed PGA-170 kGy (Figure 3B) has a broad peak covering 90-110 ppm. These agree with the presence of many small peaks in the region of 60-100 ppm in its liquid state spectrum. Many peaks with small differences in chemical shift can appear as a broad peak in the solid state NMR spectra due to low resolution. As in the counter liquid state NMR spectra, portion of small peaks in the spectrum of PGA-170 kGy is greater than that of PGA-90 kGy.

Carbon-13 MAS spectra of PGA-170 kGy (Figure 3D) have small peaks at 68 and 73 ppm. These peaks are smaller than the peaks at 63, 73, and 90-110 ppm in the spectrum of hydrolyzed PGA-170 kGy. The spectra of PGA-90 kGy (Figure 3C) and PGA-170 kGy (Figure 3D) have similar intensities of small peaks in the region of 63-80 ppm. However, the spectrum of PGA-90 kGy does not differ from the hydrolyzed PGA-90 kGy spectrum (Figure 3A) in small peak intensities in contrast with the case of PGA-170 kGy. The peak at 73 ppm in the spectra of PGA-170 kGy before and after hydrolysis can be from either the same or different carbon sites. In either case, greater intensity of the peak in the spectrum of hydrolyzed PGA-170 kGy suggests that this peak is mainly from the carbon sites created during hydrolysis. Likewise, peaks at 63 and 90-110 ppm can be considered to be also from the carbon sites created during hydrolysis. Chemical shift values[17] and DEPT results indicate these carbons are not amide carbons but methine (73 and 90-110 ppm) and methylene (63 ppm) carbons. Then, hydrolysis process should involve chemical bonds other than amide bonds. Thermodynamically it is difficult to explain this spontaneous covalent bond scission in water just by raising temperature above 80 °C. It is more likely that residual radicals progress in reaction during hydrolysis at temperature above 80 °C producing molecules small enough to dissolve in water. Similarity of the spectra for hydrolyzed PGA-90 kGy and PGA-90 kGy supports this explanation: smaller dose of irradiation would produce lesser amount of residual radicals.

The peaks at 68 and 73 ppm in the spectrum of PGA hydrogels are definitely created during γ-irradiation. These peaks are from methine (73 ppm) and methylene (68 ppm) carbons bonded to oxygen or nitrogen.[18] If only amide bond formation is involved in cross-linking, PGA hydrogels would not have more peaks in their solid state NMR spectra than PGA since amide carbon has similar chemical shift to that of carboxylic acid carbon. Especially in the solid state NMR spectra, carbonyl resonance does not have good resolution to distinguish carboxylic acid and amide carbons. Thus, if only amide bonds are produced by cross-linking, the amide carbon peak would be included in the carbonyl peaks of PGA in the spectra. Our NMR results indicate otherwise. It is difficult to assign all peaks exactly to specific carbon sites, however, at least we can say that chemical bonds other than amide-bond are created during γ-irradiation. During irradiation, not only cross-linking but also bond scission can happen. According to our NMR results, most possible cross-links and hydrolysis by radical reactions would be chemical bond formation of -CH$_2$---N-, -CH$_2$---O-, -CH---N-, and -CH---O-, which are summarized in Figure 4. The carbonyl carbon invloved in the cross-links and hydrolysis in Figure 4C-4F can be taken to explain the small peak at 174.4 ppm in the liquid state hydrolyzed hydrogel spectra. Chain reaction by radical created by irradiation is also possible. More diverse chemical bond formations and scissions were detected with the sample prepared with higher dosage of γ irradiation. Our results indicate that a simple model with one knid of chemical bonding is not appropriate to explain cross-linking and bond scission in PGA hydrogels prepared by irradiation. Amide bond formation and scission could not be directly detected in NMR spectra, however, the possibility of the bond formation and scission can not be completely excluded. Our results do not distinguish the initiation of the cross-linking by hydroxyl radicals capturing hydrogens of PGA from by hydrogen elimination of PGA due to direct irradiation.

Conclusions

Liquid and solid state NMR techniques were applied for characterization of cross-links and possibly also bond-scission produced by γ-irradiation in γ-PGA hydrogel. The NMR results indicate that chemical bonds other than amide-bond, especially methylene and methine carbons next to oxygen or nitrogen, are formed during γ-irradiation and consecutive hyrolysis at temperatures higher than 85 ^{o}C. For higher dosage of γ-irradiation, more diverse bonds were observed to be involved. Our results show that cross-linking in a real system can not be explained by simple models with one kind of chemical bonds.

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