A light scattering study on gelatin gels chemically crosslinked in solution

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(Received 9 October 1992; revised 2 December 1992)

Light scattering measurements were made of glutaraldehyde (GA)-cured gelatin gels. Gel modulus, which was found by intensity measurements of scattered light, decreased with increasing GA content, and the autocorrelation function of dynamic light scattering could be reproduced by a double-exponential fitting. The diffusion constant of the fast mode was proportional to gelatin concentration, C_e, to the power of 0.79, while the correlation length of the slow mode hardly depended on C_e. These facts suggested that GA curing may not influence the size of crystalline triple-helical structure but depresses the number of nucleation sites, which leads to lowering of the total crosslink density. Further, chemical and/or physical crosslink densities of GA-cured gelatin gels were estimated roughly by using swelling ratio data.

(Keywords: dynamic light scattering; gelatin gel; diffusion)

INTRODUCTION

Some proteins are chemically crosslinked in order to analyse a high-order structure such as a distance (~ 1 nm), relative position and configuration between amino acid residues[1] or between subunits[2]. This technique is very convenient, and is closely related to X-ray diffraction ($\lesssim 0.5$ nm) and electron microscopy ($\gtrsim 2$–3 mm) in the study of proteins[3]. In addition, chemical crosslinking is carried out to improve physicochemical properties[3] or to fix an enzyme in a protein[4].

Gelatin is a water-soluble protein resulting from the partial hydrolysis of collagen, which is found in hides, skins, sinews, bones and connective tissue[5]. Gelatin gels have been widely studied as thermoreversible gels, and a crystalline triple-helical structure like collagen is said to act as a physical crosslink site or junction zone[6]. Chemically crosslinked gelatin gels are utilized as surgical absorbent powders, hectograph films, in some types of capsules, in tanning, etc.[7]. Reagents for crosslinking are generally metal salts, formaldehyde, glutaraldehyde, aldehyde sugars, disubstituted carbodiimides, epichlorohydrin, etc[5]. However, the gel structure of chemically crosslinked gelatin gels has been studied little compared to thermoreversible gelatin gels.

In this paper, the gel structure of glutaraldehyde-cured gelatin gel will be discussed in detail by the use of dynamic light scattering (d.l.s.), which is a powerful method to investigate the dynamic properties of polymer solutions and gels[8].

EXPERIMENTAL[9]

Sample preparation

The materials used in the present study were gelatin (Sigma Chemical Company, type B, bovine skin, ~ 60 bloom), and an aqueous glutaraldehyde (GA) solution (12.5%) (Wako Pure Chemical Ind. Ltd).

Gelatin powders were swollen in water containing phosphate buffer (pH ≈ 6.9) and a small amount of sodium azide (to prevent bacterial contamination) at 5°C overnight, and the mixture was stirred to completely dissolve gelatin at 50°C for 1.5 h. The aqueous gelatin solution was further stirred at the same temperature for 0.5 h after adding a given amount of aqueous GA solution, [GA], and the resulting solution was passed immediately through a 0.45 μm Millipore Millex-HA filter and poured into a d.l.s. cell. The concentration of aqueous gelatin solution was fixed at 5 wt%. Gelatin gel samples were allowed to remain in the d.l.s. cell at 20°C for a week to maturate. Reheating for all samples was carried out at 60°C for 2 h.

The amount of aqueous GA solution added, [GA], and other experimental results are summarized in *Table 1*, in which PCGG and CCGG represent physically crosslinked gelatin gel at [GA]=0 and chemically crosslinked gelatin gel, respectively.

D.l.s. measurements and analysis

The d.l.s. apparatus and conditions of measurement are described in detail elsewhere[10]. D.l.s. was measured using homodyne spectroscopy[8] at 293.15 K. The optical system using the polarization microscope was designed and constructed so that the scattering angle θ was fixed at 90°. The photon correlator used was LPA-3000 (Ootsuka Electronics Co. Ltd).

An average decay constant, $\bar{\Gamma}$, of the normalized first-order intensity autocorrelation function of the photoelectric field, $|g^{(1)}(\tau)|$, where τ is the correlation time, and the normalized variance, $\mu_2 \bar{\Gamma}^{-2}$, were determined by the second-order cumulant method[11]. The cooperative diffusion constant D_{coop} can be calculated from the value of $\bar{\Gamma}$ as $\bar{\Gamma} = q^2 D_{coop}$, where q is the scattering vector[8]. On the other hand, so-called double-exponential (DE) fitting

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0032-3861/93/163358-04

was applied to $|g^{(1)}(\tau)|$ as follows[12]:

$$|g^{(1)}(\tau)| = A\exp(-\Gamma_f\tau) + (1-A)\exp(-\Gamma_s\tau) \quad (1)$$

where Γ_f, Γ_s and A are, respectively, the decay constants of the fast and the slow modes, and the relative amplitude (or scattering intensity) of the fast mode. One can obtain two kinds of diffusion constants, D_{fast} and D_{slow}, of each mode as well as D_{coop}. The correlation length, ξ, was given by the following relation, which is of the same type as the Einstein–Stokes equation[8,13]:

$$\xi = k_B T/6\pi\eta_0 D \quad (2)$$

where k_B, T and D are the Boltzmann constant, the absolute temperature and the diffusion constant, respectively. The dependence of the viscosity of water η_0 in gelatin gel on gelatin weight concentration at the equilibrium state of swelling, C_e, was assumed to be negligible[14], and the viscosity of pure water was taken as η_0.

All the experimental results in the present study have satisfied the requirement[8] that $q\xi < 1$. The systematic errors on calculation of the cumulant analysis and of the DE fitting were about 5–10%.

RESULTS AND DISCUSSION

Figure 1 shows the [GA] dependence of volume fraction of gelatin at the equilibrium state of swelling, ϕ_v, and of relative change of gel modulus, $E_{gel}([GA])/E_{gel}([GA]=0)$. The gel modulus was estimated from the following relation[15]:

$$i_s \propto I_0(T/E_{gel})C_e(\delta\varepsilon/\delta C_e) \quad (3)$$

where i_s and I_0 are, respectively, the photocurrent due to scattered light and the intensity of the incident light. One can calculate C_e from ϕ_v, i.e. $C_e = \rho\phi_v$, where ρ is the bulk density of gelatin ($\rho = 1.368\ g\ cm^{-3}$). Here, the increment of dielectric constant, $\delta\varepsilon/\delta C_e$, with C_e and I_0 was assumed to be constant[15]. The values of ϕ_v and $E_{gel}([GA])/E_{gel}([GA]=0)$ decreased continuously with increasing [GA], as shown in *Figure 1*. In other words, the decrease in E_{gel}, despite the introduction of a chemical crosslink site, is an entirely opposite tendency compared with ordinary crosslinked synthetic polymers and rubber vulcanizates[16]. The change of gel modulus was also suggested qualitatively from the decrease in the amount of non-frozen water in a similar gel, which was determined by d.s.c. measurement[9].

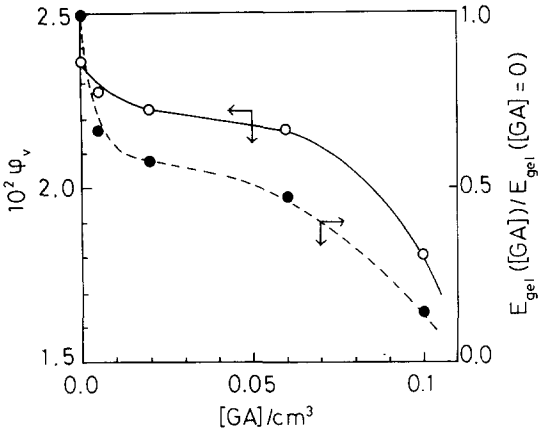

Figure 1 Relationship between volume fraction of gelatin at the equilibrium state of swelling, ϕ_v (○) and added amount of aqueous glutaraldehyde (GA) solution [GA] for GA-cured gelatin gel, and [GA] dependence of relative change of gel modulus $E_{gel}([GA])/E_{gel}([GA]=0)$ (●), which was estimated from equation (3)

Figure 2 indicates the relationship between D_{coop} and/or D_{fast} with C_e, and depicts the variations of the value of A in equation (1) with C_e. In the present study, as mentioned in the Experimental section, the initial concentration of aqueous gelatin solutions was constant and [GA] was changed in order to investigate an effect of chemical crosslinking. However, if [GA] is more than 0.10 cm^3 (see *Table 1*), the resulting sample seems to be macroscopically inhomogeneous like phase separation. As a result, the range of C_e was narrow. D_{coop} increased with C_e, since the restoring force (due to the osmotic pressure in swollen networks) is stronger at high C_e values[13]. However, D_{coop} was proportional to C_e to the power of 0.60 within the present range of C_e, which is smaller than the exponent predicted theoretically from both the dynamic scaling law and the C^* theorem[13], i.e. 0.75.

The values of $\mu_2\bar{\Gamma}^{-2}$ varied from 0.26 to 0.07 with decreasing C_e or with increasing [GA]. This implies that $|g^{(1)}(\tau)|$ deviated from a single exponential decay with decreasing [GA][8], and suggests that the gel structure of sample PCGG may be more inhomogeneous[8,17].

It should be borne in mind that the linearity of the plots of $\bar{\Gamma}$ *versus* q^2 has not been checked, because θ cannot be varied with the apparatus used[10]. However, the experimental condition of $\theta = 90°$ is frequently preferred in the case of d.l.s. measurements of gels, because

Table 1 Preparation and characterization of chemically crosslinked gelatin gels

Gelatin sample	[GA][a] (cm^3)	ϕ_v[b]	ν_e[c] (mol dm^{-3})	Reheating test[d]	$\nu_{e,chem}$[e] (mol dm^{-3})	$\nu_{e,phys}$[f] (mol dm^{-3})
PCGG[g]	0.0	2.36×10^{-2}	5.33×10^{-3}	Soluble	–	5.33×10^{-3}
CCGG-1[h]	0.005	2.27×10^{-2}	4.96×10^{-3}	Soluble	1.76×10^{-4}	4.78×10^{-3}
CCGG-2	0.02	2.23×10^{-2}	4.76×10^{-3}	Soluble	7.20×10^{-4}	4.04×10^{-3}
CCGG-3	0.06	2.17×10^{-2}	4.54×10^{-3}	Swollen	1.93×10^{-3}	2.61×10^{-3}
CCGG-4	0.10	1.81×10^{-2}	3.28×10^{-3}	Swollen	3.10×10^{-3}	1.76×10^{-4}

[a] Added amount of aqueous glutaraldehyde solution; concentration 12.5%
[b] Volume fraction of gelatin at the equilibrium state of swelling at 293.15 K
[c] Total value of crosslink density calculated by the modified Flory–Rehner equation
[d] Reheating was carried out at 60°C for 2 h
[e] Chemical crosslink density (referred to in text)
[f] Physical crosslink density (referred to in text)
[g] Physically crosslinked gelatin gel
[h] Chemically crosslinked gelatin gel

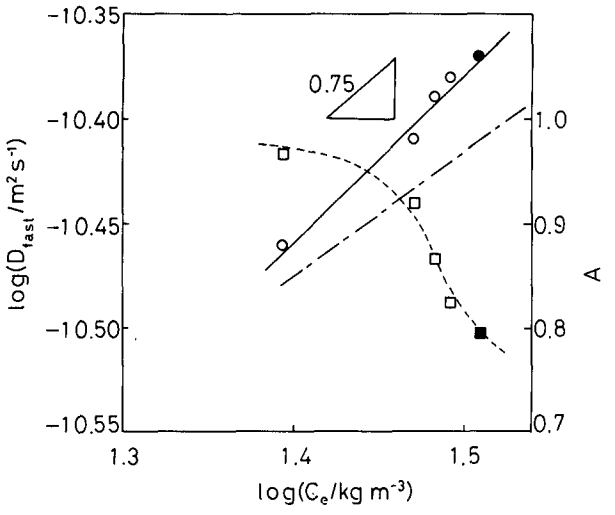

Figure 2 Variations of diffusion constant of the fast mode, D_{fast} (○, ●) and of relative amplitude (or scattering intensity) of the fast mode, A (□, ■) in equation (1) with gelatin weight concentration at the equilibrium state of swelling C_e. ———, the change of cooperative diffusion constant D_{coop} determined by the cumulant method with C_e. Solid symbols represent each physical quantity at [GA]=0. The numerical value of 0.75 implies the exponent predicted theoretically from both the dynamic scaling law and the C^* theorem

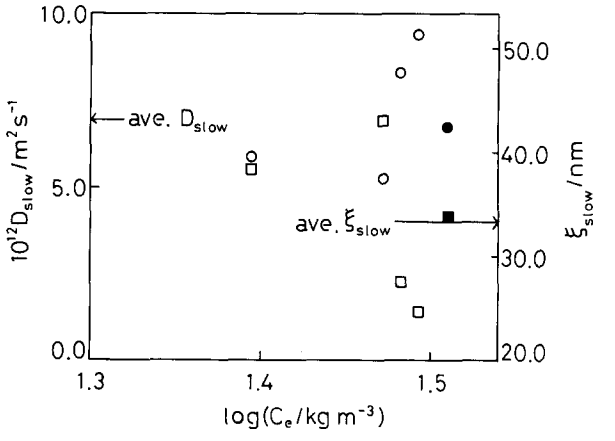

Figure 3 Plots of diffusion constant of the slow mode, D_{slow} (○, ●) and of correlation length of the same mode, ξ_{slow} (□, ■) calculated by equation (2) *versus* C_e. Arrows denote average values of D_{slow} and ξ_{slow}. Solid symbols have the same meaning as in *Figure 2*

the strength of scattering light is generally weak, dust in gels cannot be removed completely, and a stray light on the wall of the cell and/or a mode of intramolecular motion along long network chains at (much) lower C_e can be effectively negligible[10].

On the other hand, D_{fast} increased with C_e to the power of 0.79 within the present range of C_e, which is close to the theoretical prediction[13]. In addition, the value of A in equation (1) increased and became approximately unity with decreasing C_e or with increasing [GA], which corresponds well to the above-mentioned variations of $\mu_2\bar{\Gamma}^{-2}$ (refs 8 and 12). Here, the so-called heterodyne effect was assumed to be negligible in the DE fitting[18]. This seems to be experimentally reasonable, because the non-ergodic problem[19], which is the dependence of the decay curves of $|g^{(1)}(\tau)|$ on the scattering position of the incident light in one sample, could not be recognized within the experimental error, and all the samples were optically clear. It follows that hardly any voids and/or dust exist in the samples used, and that GA curing may occur randomly in gelatin gels.

The fast mode was assigned to the cooperative diffusion mode of network chains, which is also substantiated by the dependence of D_{fast} on C_e. On the other hand, the slow mode may be considered to be the diffusive motion of a certain size of 'cluster' as a whole in gels. These modes were assigned by reference to the theory proposed by Pusey *et al.*[20], by *in situ* investigation of gelation of polystyrene (PS) with d.l.s.[21], and by d.l.s. studies on transient networks of PS semidilute solutions[18,22]. In the latter two cases, the slow mode originates from clustering of branched PS chains on the process of gelation, and from centre-of-mass translational motions of entangled PS chains in a transient network, respectively.

The plots of D_{slow} and ξ_{slow}, which was determined from equation (2), *versus* C_e are shown in *Figure 3*. Although the values of ξ_{slow} are scattered to some extent, the ξ_{slow} appeared scarcely to depend on C_e: the average value was about 33 nm.

There have been a number of investigations on the size of junction zones (or physically thermoreversible crosslink sites) of gelatin chains[5,7] with a crystalline triple-helical structure like collagen[6]. The critical size is reported to be 10–20 nm, corresponding to 20–40 amino acid residues[23]. Further, the size of helical segment zones, which were selectively isolated with enzymatic treatment, was estimated to be 50–100 nm (100–200 amino acid residues) from g.p.c. analysis[24]. As another example, the correlation length in an Ornstein–Zernike approximation measured with static light scattering was about 60 nm in the case of formaldehyde-cured gelatin gels[25].

From these facts and the assignment of the slow mode[20], the 'cluster' in the present study may correspond to junction zones in gelatin gels[26], and ξ_{slow} can be roughly regarded as the size of a junction zone. The experimental results in both *Figures 1* and *3* suggest that GA curing may not affect the size of junction zone but affects the number of nucleation sites of the crystalline triple-helical structure.

The results in the reheating experiment are shown in *Table 1*. Samples CCGG-3 and CCGG-4 were merely swollen even at 60°C, while the other samples dissolved; these gel structures are described as mainly formed from chemical and physical crosslink sites, respectively, though the gel modulus decreased continuously with increasing [GA], as shown in *Figure 1*.

The authors attempted to estimate approximately the chemical and/or physical crosslink densities, $\nu_{e,chem}$ and $\nu_{e,phys}$, in chemically crosslinked gelatin gels, which are summarized in *Table 1*.

The total crosslink density ν_e, indicated in *Table 1*, was calculated by the Flory–Rehner (FR) equation[27]. The interaction parameter between polymer and solvent (Flory–Huggins constant)[28] was assumed to be 0.46, and the FR equation was modified by low-concentration approximation, because the values of ϕ_v were less than 0.1 as shown in *Table 1*[29]. As a result, one need not consider the functionality of crosslink sites in the FR equation[29].

Chemical crosslink sites are considered to be Schiff-base structures and to be formed not by a single GA molecule but by an α,β-unsaturated GA oligomer[3], which was estimated to be constituted from six GA units[30]. Aqueous GA solutions, however, are usually free from

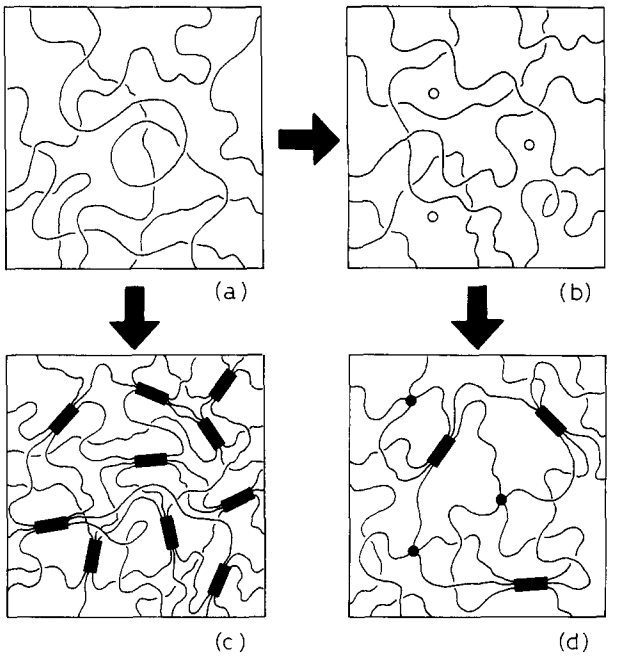

Figure 4 Proposed scheme of gel structure of an ordinary and a chemically crosslinked gelatin gel: (a) aqueous gelation solution at temperature; (b) aqueous gelatin solution, containing GA (represented by open circle symbols); (c) physically crosslinked gelatin gel; (d) chemically crosslinked gelatin gel. In (c) and (d), solid rectangles and circles represent physically thermoreversible crosslink sites formed from crystalline triple-helical structure like collagen, and chemical crosslink sites, respectively

this GA oligomer, which is speculated to be produced first in the presence of a protein like gelatin[31]. The value of $\nu_{e,chem}$ was calculated with the assumptions that chemical crosslink sites were tetrafunctional[3], 1.5 wt% of GA oligomer was formed in aqueous GA solutions used, and that the crosslinking reaction proceeded stoichiometrically. On the other hand, $\nu_{e,phys}$ was regarded as the difference in value between ν_e and $\nu_{e,chem}$. The concentration of GA oligomer was the maximum of a self-consistent value. That is to say, $\nu_{e,phys}$ for CCGG-4 becomes negative if the concentration is more than 1.5 wt%.

The trial calculations described above are fairly crude owing to the assumptions made. Unfortunately, the values of ν_e cannot be compared with those of E_{gel}, because the absolute value of scattered light could not be measured with the instrument used. In addition, the relative changes of E_{gel} in *Figure 1* disagreed completely with those of ν_e. However, ν_e, $\nu_{e,chem}$ and $\nu_{e,phys}$ have been estimated semiquantitatively for the first time, and one can understand the approximate order of crosslink density, average molecular weight between two crosslink sites, $\bar{M}_c$ ($\bar{M}_c = \rho/\nu_e$), the ratio of chemical to physical crosslink sites, and so on. The value of $\bar{M}_c$ estimated from ν_e was about 10^5 and seems to be reasonable in this case.

Consequently, it was apparent from the measurements of scattered light that E_{gel} decreased continuously with increasing [GA], and ξ_{slow} could be regarded as the size of physical crosslink sites, which was hardly influenced by GA curing, as illustrated in *Figures 4c* and *d*. However, the number of nucleation sites of crystalline triple-helical structure was suggested to decrease with the introduction of chemical crosslink sites (*Figures 4c* and *d*), which may be attributed to the lowering of E_{gel} and/or ν_e. In addition, the values of $\nu_{e,chem}$ and $\nu_{e,phys}$ could be approximately estimated.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the help received from Research Associate Dr K. Tsukamoto (Department of Mineralogy, Petrology, Economic Geology, Faculty of Science, Tohoku University) with the d.l.s. measurements. The d.l.s. equipment was made in his laboratory.

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