# Holographic relaxation spectroscopic study on the structure of gelatin gel doped with fluorescein as a tracer*)

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*Abstract:* The structures of gelatin gels have been studied by holographic relaxation spectroscopy (HRS) with fluorescein as a doped tracer. An HRS spectrum with double peaks has been observed. It has been experimentally proven that this "anomalous" HRS spectrum is related to the structures of the gelatin gels. It is shown that two kinds of gel networks are formed when a gelatin solution is cooled at a rate of 2 °C/min to a temperature below the gel temperature. Microviscosity inside one kind of network is about four times lower than inside the other one. One with lower microviscosity is called a "coarse" network, and it is formed through aggregation of a collagen-like, triple-stranded helix. The other is named a "fine" network and it is constructed simply by entanglements between polymer chains. The structure of gelatin gel can be considered as a blend of these two networks. By controlling the cooling speed, the structure of gelatin gels can be dominated by either "coarse" or "fine" gel networks.

*Key words:* Gelatin gels, structure, fluorescein, translational diffusion, holographic relaxation spectroscopy (HRS)

## Introduction

Gelatin converted from the collagenous component of connective tissue, such as skin, bone, and tendon, has been recognized for its gel forming, film-forming, and surface-active properties [1–3]. When a sufficiently concentrated aqueous solution of gelatin is cooled to a certain low temperature, the viscosity of the gelatin solution will progessively increase in time, and the liquid eventually turns into a gel nearly without exception. Structures of gelatin gels have been studied for many years by many researchers using various physical techniques, including holographic relaxation spectroscopy (HRS) [4]. Not only for basic research, but also for quality control of gelatin, there is still a need for an efficient method to characterize gelatin. Therefore, elucidating the structures of gelatin gels still remains as a challenge to researchers.

HRS, which is also known as forced Rayleigh scattering (FRS), has been demonstrated by many researchers to be a very useful method for measuring translational diffusion coefficients of small photochromic dye molecules [5–9] or macromolecules labeled by photochromic dye molecules [10–13].

In this investigation, fluorescein molecules were doped into the gelatin gels as a tracer. Structures of gelatin gels were studied by observing the translational diffusion of the tracer. An HRS spectrum with double peaks is reported in this article. It has been experimentally demonstrated that this "anomalous" HRS spectrum is related to the structures of the gelatin gels. According to the x-ray diffraction pattern [14–19], very small locally ordered crystallites exist in a well-matured gelatin gel. These crystallites have a collagen-like structure, which is re-naturalized from gelatin molecules during the gelation process through triple-stranded helix formation [20]. During the gelation process, a gel network with solvent entrapped in the meshes is built up inside the solution through the aggregation of the helix where the small crystallites act as the "links" [2, 15–17]. At the

*) Dedicated to Prof. Dr. W. Pechhold on the occasion of his 60th birthday.

same time another network is also formed inside the solution, simply through the entanglements between gelatin molecules when gelatin concentration is sufficiently high. The first network is called the "coarse" network because of its larger mesh size in comparison with the second one which is called the "fine" network. It is confirmed in this study that the gel structure is a blend of "coarse" networks and "fine" networks. Microviscosity inside the "coarse" network is found to be four times lower than inside the "fine" one. By controlling the cooling speed, the ratio between the amount of "coarse" network and the "fine" network can be varied almost from 0 % to 100 % inside the gelatin gels, i.e., the structure of gelatin gels can be dominated by either "coarse" or "fine" gel networks.

## Experimental

### *Materials*

Gelatin used in this experiment was purchased from Deutsche Gelatine-Fabriken Stoess AG, Eberbach, FRG. It is a pharma grade B-type gelatin (Bloom value is 200), i.e., it is obtained from alkaline-treated bone stock. The GPC measurements (courtesy of Dr. Klaus Bräumer and Dr. Wilfried Babel, Deutsche Gelatine-Fabriken Stoess AG, Eberbach, FRG) show that the weight-averaged molecular weight ($M_w$) and the number-averaged molecular weight ($M_n$) are $1.4 \times 10^5$ g/mol and $6.3 \times 10^4$ g/mol, respectively. Fluorescein is from Riedel de Haen Co., FRG. Both gelatin and fluorescein were used without further purification.

### *Sample preparation*

The gelatin gels were prepared by first dissolving a proper amount of gelatin in a buffer solution (pH = 10, $Na_2B_4O_7$/NaOH) at 50 °C for at least 1 h in order to form a uniform gelatin solution, then adding a solution of fluorescein in water until a final fluorescein concentration ($\sim 10^{-4}$ g/mL) was reached. Because the fluorescein concentration in the gelatin solution is so dilute, we can neglect the interaction between fluorescein molecules and have a self-diffusion measurement. After the dissolution process, the gelatin solution is cooled to the desired temperature and matured at this temperature for about 8 h until the measured waveforms no longer change.

### *HRS Method*

The detailed instrumentation and principle of HRS can be found elsewhere [4, 6–13]. For convenience of discussion, the basic principle of HRS measurement is outlined in the following: A spatial modulation of refractive index and/or absorption coefficient is artificially created by a photochromic reaction of the labels by crossing two coherent laser beams (writing beams) on the same spot in the sample for a very short time. After this writing pulse, the periodic concentration distributions of the reacted and unreacted molecules will gradually be smeared out by translational diffusion. The relaxation process can be directly followed by measuring the transient diffraction intensity at Bragg's angle with another laser beam (reading beam) passing through the same spot.

In this study, a 400 mW Argon ion laser from Coherent (INNOVA 90) operated at 488 nm was used to produce writing beams and a 1 mw He-Ne laser from Spectra-Physics (Model 155 A) operated at 632.8 nm was used to produce a reading beam. The sample was placed between two flat optical glass plates with a 0.5 mm Teflon spacer. The sample temperature from 5 °C to 80 °C, with a precision of ± 0.1 °C, can be controlled by a home-made cell holder with a thermostat. The fringe spacing can be varied from 0.8 μm to 37 μm by changing the crossing angle between the two writing laser beams. Since the reading beam is far from the absorption band of fluorescein, the diffraction intensity is mainly from the phase change, i.e., "phase HRS".

### *Analysis of HRS spectrum*

In principle, if the rate of thermal decay is much faster and the intrinsic intramolecular photochromic back-reaction is much slower than the translational diffusion, the relaxation process, measured by the output signal $I(t)$ of a photomultiplier, should be represented by a single exponential decay [6],

$$I(t) = [A \exp(-DK^2t) + B]^2 + C^2 \quad (1)$$

where $A$ is the amplitude of the diffraction intensity, which is mainly related to the photochromic properties and the concentrations of the labels, and also to the intensity and the pulse length of the writing laser beams; $B$ and $C$ are the coherent and the incoherent background scattering, respectively; $D$ is the averaged translational diffusion coefficient; and $K (= 2\pi/L)$ is a constant at a fixed fringe spacing ($L$) of the optical grating.

However, it has been found by some researchers that the diffraction intensity cannot be described by the simple relation of Eq. (1). The intensity has rather a non-exponential single peak shape [21–26]. The origin of this deviation from single-exponential of the diffraction intensity decay has not been fully understood. There still remain some contradictions in explaining this non-exponential spectrum form the experimental data [24–26]. But, it is generally accepted that this non-exponential spectrum is due to one pair of optical gratings formed in the sample during the writing process. The photochromic labels falling into the "bright area" of the interference pattern of the writing beam will undergo a photochromic reaction and become a different molecule, the so-called photochromic reaction product. The molecules falling into the "dark-area" of the interference pattern will remain unchanged. These two sets of optical gratings are spatially out of phase by 180° for a "phase HRS". Therefore, the electric fields at the detector diffracted from each optical grating have opposite signs. The detector will sum and square these two contributions because of the quadratic nature of the photocathode of the photomultiplier. In this way, the diffraction intensity can be represented by

$$I(t) = [A_1 \exp(-D_1K^2t) - A_2 \exp(-D_2K^2t) + B]^2 + C^2, \quad (2)$$

where subscripts 1 and 2 are denoted to two optical gratings. For multiple sets of gratings, Eq. (2) can be generalized as [27]

$$I(t) = [\Sigma_i A_i \exp(-D_i K^2 t) + B]^2 + C^2 \qquad i = 1, \cdots, N, \tag{3}$$

where $A_i$ can be either positive or negative.

## Results and discussion

In this study, a HRS curve with double peaks was found for a gelatin gel formed by cooling the solution from 50 °C to 25 °C at a speed of ~ 2 °C/min. Hollow circles in Fig. 1 show a typical experimental HRS spectrum for a 15 weight-% solution of gelatin in the buffer. The sample was matured and measured at 25 °C, which is lower than the gelation temperature ($T_{gel} \simeq 32$ °C). The reasons for this "anomalous" HRS curve could be the following: As mentioned in the introduction, gelatin gel is not uniform microscopically, but rather it is a blend of "coarse" and "fine" networks. Each network will provide fluorescein molecules with one local "environment", because the averaged mesh size in the "coarse" network is larger than the one in the "fine" network, i. e., the microviscosities in these two networks are different. Therefore, the translational diffusions of fluorescein molecules will be different. For each "environment", the writing laser beam will create one pair of optical gratings. Then, there should be four sets of optical gratings existing inside the sample. The line

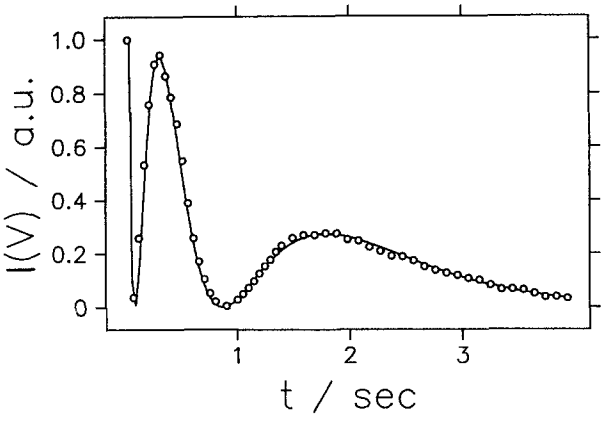


Fig. 1. Typical HRS curve of a 15 weight-% gelatin gel which was prepared by cooling a solution of gelatin in the buffer from 50° to 25 °C at a speed of 2 °C/min. The sample was matured and measured at 25 °C. The Hollow circles repesent the experimental data and the continuous line represents a best fitt by using Eq. (3) with $N = 4$

in Fig. 1 represents a best least squares fitting by using Eq. (3) with $N = 4$. The following parameters are derived from the fitting:

$$A_{1,c} = 9.27, \; A_{2,c} = -9.02;$$
$$A_{1,f} = 10.5, \; A_{2,f} = -10.6$$

and

$$D_{1,c} = 1.39 \times 10^{-10} \text{ m}^2/\text{s},$$
$$D_{2,c} = 1.36 \times 10^{-10} \text{ m}^2/\text{s};$$
$$D_{1,f} = 3.38 \times 10^{-11} \text{ m}^2/\text{s},$$
$$D_{2,f} = 3.97 \times 10^{-11} \text{ m}^2/\text{s},$$

where subscripts $c$ and $f$ denote the environments of "coarse" and "fine" networks, respectively. It has to be pointed out that the fitting is not unique, because the resolution of sums of exponential functions is an ill-posed mathematical problem. Before taking these parameters seriously, the fitting has to be well justified physically and experimentally. In this study, the relative values of these parameters are of more interest. Relatively, $D_{1,f}$ ($D_{2,f}$) is called a "slow" diffusion, which is assumed to be the diffusion of fluorescein molecules in the environment of a "fine" network, and $D_{1,c}$ ($D_{2,c}$) is named "fast" diffusion, which is related to the diffusion of fluorescein molecules in the environment fo a "coarse" network. The fitting parameters $D$ and $A$ for each "environment" are similar. Physically, it is reasonable because fluorescein molecules and their photochromic products are similar in terms of size and density. By varying the fringe spacing from 6 μm to 36 μm, $D$ was found to be independent of $K$. The uncertainty is about 10 %, which mainly comes from the fitting.

From the observation of two distinct $D$, we conclude that there are two "local environments" in the gelatin gel for the diffusion of the probe molecules. It is also observed that all of our gelatin gels are transparent. This means that either the sizes of the inhomogeneous domains are smaller than the wavelength or the different domains have the same refractive index. The latter is unlikely to be true because the gelatin aggregates should have a higher density and a higher refractive index. If the sizes of the "environments" are smaller than the wavelength, we should observe only one averaged diffusion coefficient because our grating spacing is much larger than the wavelength. Based on these contradicting

experimental observations, it could be concluded that the sizes of the "environments" are not necessarily the same as the sizes of the inhomogeneous domains and the gelatin gels do not have a "cluster-like or island-like" structure. A possible structure model could be described in the following way:

During the gelation process, part of gelatin molecules are renaturalized into the collagen-like triple helix, which further aggregates to form a three-dimensional infinite network, i.e., the "gel phase". Within this framwork, a lot of "holes" with dimensions smaller than the wavelength are evenly distributed. Part of the "holes" are occupied by the entangled gelatin molecules which are still in the "sol phase". The rest of the "holes" are simply occupied by water. The probe molecules trapped within the "holes" occupied by the entangled gelatin molecules will move much slower than those trapped within the "holes" occupied by water. During the experimental time (or experimental length scale), there is certainly an exchange of probe molecules between the two types of "holes". An averaged diffusion coefficient will be observed because of the exchange, corresponding to the observed $D_f$. However, some of the probe molecules will move only between the "holes" occupied by water. The restriction is expected to be alleviated for such a movement, which may correspond to the observed $D_c$. Therefore, we have to realize that the path through those "holes" occupied by water could be much longer than the wavelength even though the dimension of each "hole" is smaller than the wavelength. At the same time, it has to be kept in mind that $D_f$ is not only connected to the diffusion process within the "environment" of the entangled gelatin molecules but also involves the diffusion process within the "holes" occupied by water. This would explain why $D_f$ is only about four times smaller than $D_c$. The reality of the above model consideration should be subjected to further experiment.

If the two-"environments" assumption is correct, the HRS spectrum measured at a higher temperature ($\gg T_m$) should lack these "anomalous" double peaks, because the "coarse" networks will have been molten. Hollow circles in Fig. 2 show a typical HRS curve at 60 °C for the same sample as in Fig. 1. This decay-growth-decay spectrum is typical for one pair of optical gratings with 180 ° phase difference. It is expected, because there is only one "environment" in the sample when $T \gg T_m$. The line in Fig. 2 shows a best fitting by using Eq. (2) with $A_1 = 61.6$, $D_1 = 1.10 \times 10^{10}$ m$^2$/s, $A_2 = 61.2$ and $D_2 = 1.09 \times 10^{10}$ m$^2$/s. It was noticed that the value of $D_1$ (or $D_2$) is larger than the value of "slow" diffusion coefficient, $D_{1,f}$ (or $D_{2,f}$), in Fig. 1. The reason for this could be that the microviscosity of the solution is lower at higher temperature, which enables the flourescein molecules to diffuse quickly.

Figure 3 shows an HRS curve (continuous line) for a 6.67 weight-% gelatin gel, which was prepared in the same way as described in Fig. 1. The sample was matured and measured at 25 °C. For comparison, the HRS curve in Fig. 1 is also plotted in Fig. 3 (broken line). By fitting Eq. (2) to the spectra, it is found that both the "slow" and the "fast" diffusions are faster in the 6.67 weight-% sample than in the

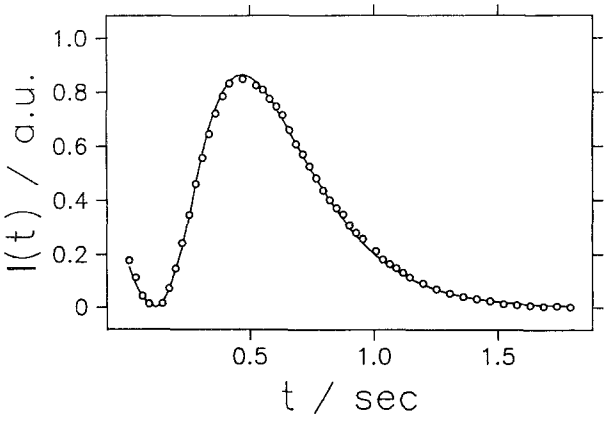


Fig. 2. HRS curve for the same sample shown in Fig. 1. All experimental conditions were identical, except the measuring temperature was 60 °C instead of 25 °C. The gel melting temperature was ~ 32 °C

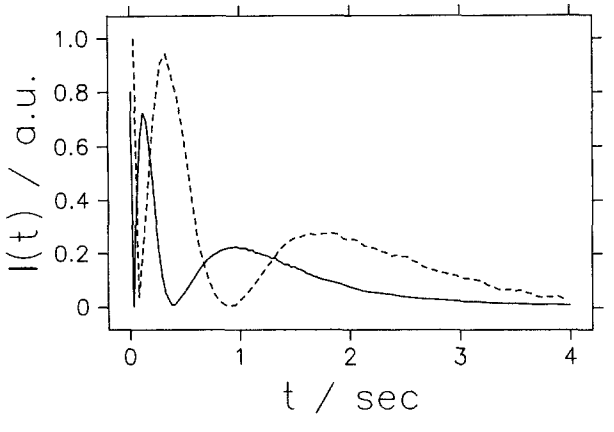


Fig. 3. HRS curves for two different concentrations. The continous line represents a 6.67 weigth-% solution of the gelatin in the buffer and the broken line is a 15 weight-% solution. Both samples were matured and measured at 25 °C

15 weight-% sample. This is expected, too, because the mesh size of both "coarse" and "fine" networks should be larger in a lower gelatin concentration than in a higher one.

In order to further confirm the assumption of "two network environments" as a base for explaining the curve in Fig. 1, two samples were prepared and cooled down from 50 °C to 25 °C with different speed. According to Stainsby [14], if the cooling speed is extremely slow, the gelatin will have more opportunities to form the triple-stranded helix, and the gel will mostly consist of the "coarse" network. In contrast, in a quick cooling process, there is not enough time for the gelatin molecules to form the triple-stranded helix and the solution will be "frozen" to a "fine" network structure through entanglements between the polymer chains. Figure 4 shows the cooling speed dependence of HRS curve. The continuous line represents a very slow cooling speed of 1 °C per day and the broken line represents a quickly quenched sample. Both samples were 15 weight-% solutions of gelatin in the buffer; both were measured at 25 °C. In comparison with Fig. 1, both spectra mainly contain one peak, which is expected, because the samples mainly contain one kind of structure (either the "coarse" or the "fine" networks) during an extremely slow or fast cooling process. In Fig. 4, the curve of the slowly cooled sample represents a diffusion process similar to the "fast" one in Fig. 1, and the curve of the quenched sample represents a diffusion process similar to the "slow" one in Fig. 1.

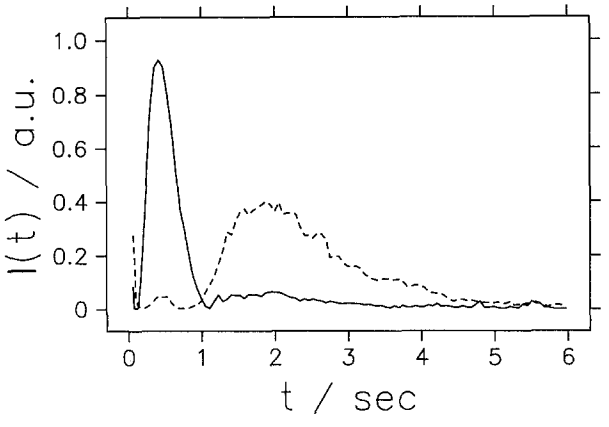


Fig. 4. HRS curves for two gelatin gels prepared at different cooling rates, but with the same gelatin concentration (15 weigth-% in the buffer). The continuous line is for a sample prepared at a cooling rate of 1 °C per day from 50° to 25 °C, and the broken line is for the sample quenched from 50° to 25 °C within 5 min

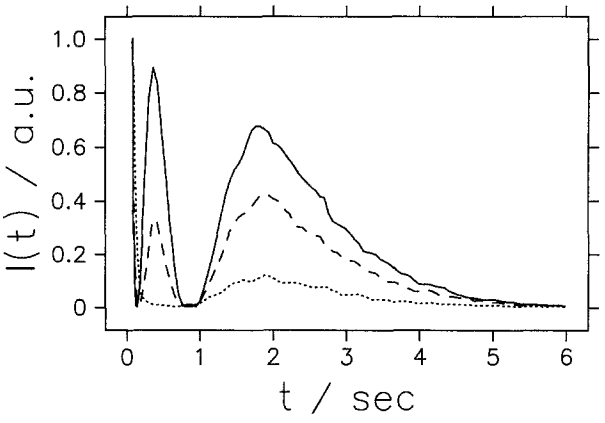


Fig. 5. HRS curves from three sequential measurements on the same spot in a 15 weight-% gelatin gel which was prepared in the same way as described in Fig. 1. After each measurement, the spot was intentionally heated by one of the writing beams for 500 ms. The continuous broken, and dotted lines represent the first, second, and thrid measurements, respectively. The sample was matured and measured at 25 °C

Both of them are in agreement with the above discussion.

Figure 5 shows HRS curves from three sequential measurements on the same spot in a 15 weigth-% gelatin gel, which was prepared in the same way as described in Fig. 1. The sample was matured and measured at 25 °C. After each measurement, the spot was intentionally heated by only one writing beam for 500 ms. The continuous, broken, and dotted lines in Fig. 5 represent the first, second, and third measurements, respectively. It is found that the first peak, which corresponds to the "fast" diffusion in the "coarse" network, becomes relatively smaller and smaller after each heating. This change further support our assumption of "two network environments". One possible explanation for this change is: During heating, the "coarse" network in the spot will be partially molten. After switching off the writing beam, the melted "coarse" network could not be restored because the spot (~ 0.7 mm in diameter) was quickly cooled to the measuring temperature by the surrounding. Therefore, the amount of "coarse" network in the gel becomes increasingly less after each heating cycle, which gives a relative, increasingly lower peak height. After each heating and writing pulse, the absolute probe concentration in the spot was becoming lower because part of fluorescein molecules change to other molecules through the photochromic reactions. This is why the heights of both peaks become lower after each measurement.

For supporting this explanation about the changes of HRS curves with the local heating, an experiment similar to that in Fig. 5 was carried out. This time, the sample was a 15 weight-% solution of gelatin in the buffer, and the prepared solution was cooled from 40 °C to 20 °C at a speed of 1 °C/day. The continuous line in Fig. 6 represents the HRS curve of the sample before the heating. As expected, the curve is mainly a single peak, as in Fig. 3. Then the sample was heated by only one writing beam for 1 s. After heating, the curve turned into the double peaks shown in Fig. 6 by a broken line. After repeating the heating on the same sample spot, HRS curve finally again turned into a single peak again, but with different peak position, as is shown in Fig. 6 by a dotted line. This change of the curve experimentally confirms our assumption: The "coarse" network can change into the "fine" network if the sample is quickly cooled after the heating.

Combining all experimental results in this study, we believe that inside gelatin gels there really exist two kinds of local "environments" for the diffusion of fluorescein molecules. The observed "anomalous" feature in the HRS spectrum of the gelatin gel is attributed to this two "environments" nature. Recently, Yu et al. [28] also used HRS to study the dynamic structure of the gel network of gelatin with a much larger tracer molecule, a photochromic-labeled polyelectrolyte. They also observed two components of $D$ below $T_{gel}$, and at the same time, they found a significant retardation of the translational diffusion of the tracer below the gel temperature. Our observed diffusion constants are much larger than their values because there is basically no retardation for the diffusion of fluorescein molecules below $T_{gel}$. The reason for the differences could be explained by the different probe sizes. The size of their polyelectrolyte is much larger than the size of small fluorescein molecule. So our sensor detects a much smaller length scale of the network. The concept of two "environments" and its detailed temperature dependence should be tested in further experiments.

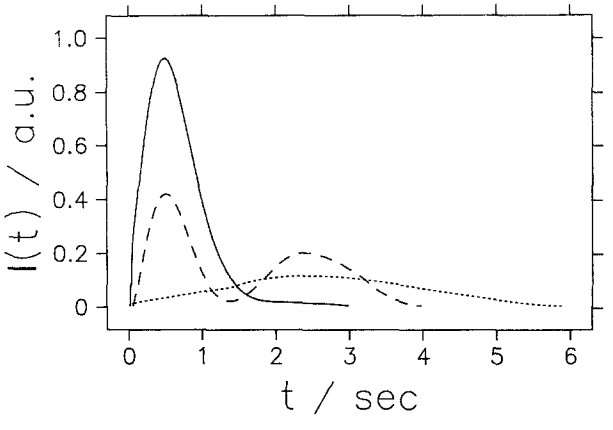


Fig. 6. HRS curves from three sequential measurements on the same spot in a 15 weight-% gelatin gel was prepared by cooling the solution of gelatin in the buffer from 50 ° to 20 °C at a rate of 1 °C per day. The measurements were performed in the same way as in Fig. 5, except the heating period was 1 s

## Conclusions

An HRS curve with double peaks has been observed for the first time. It has been experimentally demonstrated that these double peaks are related to the structure of gelatin gel. Inside gelatin gels, there are "two environments" existing for the diffusion of fluorescein molecules. Relatively, microviscosity in one environment is about four times larger than in the other one. The one with the lower microviscosity could be formed through the aggregation of a triple-stranded helix, and the other with higher microviscosity could be formed simply through the entanglements between polymer chains. These are called "coarse" network and "fine" network, respectively, because of the difference in their averaged mesh size. By controlling the cooling speed, the structure of gelatin gel can be dominated by either "coarse" or "fine" networks. A gelatin gel could be qualitatively described as a blend of those two networks. In order to quantitatively study the structure of gelatin gel by utilizing this "anomalous" feature of double peaks in HRS curve, the signal-to-noise ratio of the measurements and the data reduction have to be improved. It is also suggested that a set of probe molecules with different sizes should be used to monitor the gel formation process and structure.

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