

Structural Aspect of Gelation in Schizophyllan/Sorbitol Aqueous Solution

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(Received December 9, 1998)

ABSTRACT: Although schizophyllan undergoes no gelation by itself in its aqueous solution, the addition of sorbitol results in the formation of transparent gel at lower temperatures. The mechanism of the gel formation was discussed from the structural analysis of schizophyllan in the aqueous solution with and without sorbitol, based on the results of small-angle X-ray scattering. Here sorbitol was found to disentangle a part of triple-stranded helices and bridge the disentangled parts which serve as a crosslinking domain.

KEY WORDS Schizophyllan/Sorbitol / Small-Angle X-Ray Scattering / Gel / Viscoelasticity / Broken Rod Model / Molecular Model /

Schizophyllan is an extracellular polysaccharide produced by fungus *Schizophyllum commune*, and is known to suppress tumor growth. It consists of (1→3)-D-β-glucan with a (1→6)-D-β-glucan branch per every 3 units as shown in Figure 1. Schizophyllan is non-ionic and water-soluble. It assumes a triple-stranded helical conformation in aqueous solution as confirmed from the measurements of the molecular weight and viscosity¹ and by calorimetric measurements,² whereas in dimethyl sulfoxide (DMSO) it has a single coiled conformation.³ Thus in water schizophyllan is rod-like and does not form gel by itself.

Addition of D-sorbitol induces the thermoreversible gelation of schizophyllan aqueous solution,^{4,5} where the gel temperature depends only on the sorbitol concentration. The present work aims to answer the question how schizophyllan forms gel in sorbitol aqueous solution. In an earlier work,⁵ sorbitol was considered to reduce a water activity and thus schizophyllan triple-stranded helices aggregate to form junction zones. We examine the structural change of schizophyllan caused by sol-gel transition in sorbitol aqueous solution by means of small-angle X-ray scattering (SAXS). If the above conjecture on the junction zone is correct, we should observe the thicker cylindrical objects corresponding to the aggregated triple-stranded helices in gel.

The work complements the rheological observation on gelation in the same system.⁵ Since SAXS contains the structural information in the order of a few Å to several hundreds Å, the structural change of schizophyllan induced by gelation is traced in principle on a molecular level. Here the strategy is to fit the observed SAXS profile with an appropriate molecular model, which can be constructed from the crystallographic data and other results. By doing so, we may hope to visualize the gelation process of schizophyllan in sorbitol aqueous solution.

EXPERIMENTAL

D-sorbitol was dissolved in distilled and deionized water by heating the solutions to 90°C, and the solutions of four different sorbitol concentrations (0, 1.5, 2.7, and 4.0 mol l⁻¹) were prepared. After cooling the sorbitol solutions to 60°C, NaN₃ (5 × 10⁻³ mol l⁻¹) was added in order to suppress bacteria growth. Schizophyllan ($M_w = 45 \times 10^4$) was dissolved in those solutions to the total concentration of 16.3 mg ml⁻¹ at 60°C by stirring for a week.

Rheological experiments were performed with a stress controlled rheometer Bohlin CS10 and a cone-plate shear geometry.

Synchrotron radiation provides a high-energy X-ray source, which is over 10⁸ times stronger than a conventional tube-type X-ray source in terms of brilliance and enables a quick measurement. SAXS measurements were performed at the BL-10C of the Photon Factory, Tsukuba, Japan. An incident X-ray from synchrotron radiation was monochromatized to $\lambda = 1.49 \text{ \AA}$, and focussed to the cell center with a bent focusing mirror. A well-focused X-ray beam has an almost point-like profile, and requires no desmearing. This is another advantage of synchrotron radiation X-ray, since desmearing often produces an artifact in the resulted

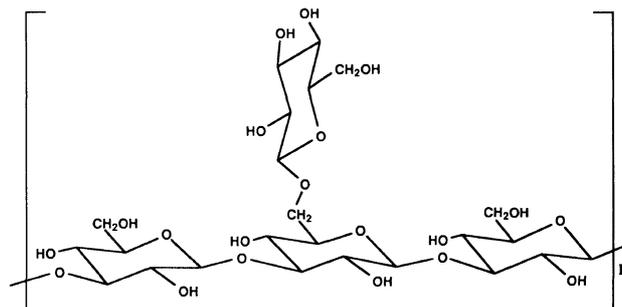


Figure 1. Chemical structure of schizophyllan.

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scattering profile.

The scattered X-ray was detected by a one-dimensional position sensitive proportional counter (PSPC) positioned at the distance of about 1 m from the sample holder. The sample solutions were injected at 60°C (in sol state) into a flat glass sample cell of 0.2 cm path-length provided with a pair of 20 μm thick quartz windows (1 cm × 0.5 cm). The samples in sol state were injected into respective cells, which were kept at 60°C. At least 10 min prior to the SAXS measurements, each cell was placed in the cell holder. The temperature of the cell was controlled by circulating water of a constant temperature through the cell holder, and the SAXS measurements were performed at 5°C (gel) and 60°C (gel). The SAXS intensities were accumulated for the total 900 s in order to assure enough statistical accuracy without degrading polysaccharide samples by X-ray irradiation. The scattering intensities were corrected with respect to the variation of the incident X-ray flux by monitoring with an ion chamber in front of the cell holder. The X-ray absorption of the solutions was compensated by the ratio of the incident to transmitted X-ray intensities through a standard polyethylene film. The excess scattering intensities were calculated by subtracting the scattering intensities of the solvent from those of the polysaccharide solutions or gels.

RESULTS AND DISCUSSION

Rheological Measurements

During gelation a material changes from a viscoelastic liquid to a viscoelastic solid, and consequently rheology is a suitable technique to determine the gel point. The results from low amplitude oscillatory shear experiments at a schizophyllan concentration of 16.3 mg ml⁻¹ in 4 M sorbitol solution are shown in Figure 2. At high temperatures, a typical liquid-like behavior and a terminal region with the loss modulus G'' being larger than the storage modulus G' was observed at low frequencies. At 40°C, however, a power law behavior was found as $G' \propto G'' \propto \omega^n$ with $n=0.31$. $\tan \delta = G''/G'$ is independent of frequency and therefore this temperature fulfills the Winter–Chambon criterion⁶ for the determination of the gel point. At lower temperatures the storage modulus approached the gel plateau in the low frequency region. The rheological data clearly show that 40°C can be regarded as the gelation temperature. Thus 5°C and 60°C at which the SAXS experiments were performed are confirmed as the gel and sol state, respectively.

Small-Angle X-Ray Scattering (SAXS)

SAXS was observed from aqueous solutions of the schizophyllan/sorbitol samples summarized in Table I. Figure 3 represents the SAXS results from schizophyllan in water at 5°C and 60°C in the double logarithmic plots. Here $I(q)$ denotes the excess scattered intensity and q is the magnitude of scattering vector given by $(4\pi/\lambda) \sin \theta$ with θ and λ being a half of the scattering angle and the wavelength of incident X-ray beam, respectively.

The cross-sectional radii of gyration R_{Gc} were determined from the slope of the linear region in the cross-sectional Guinier plots ($\ln qI(q)$ vs. q^2 , see Figure 4),

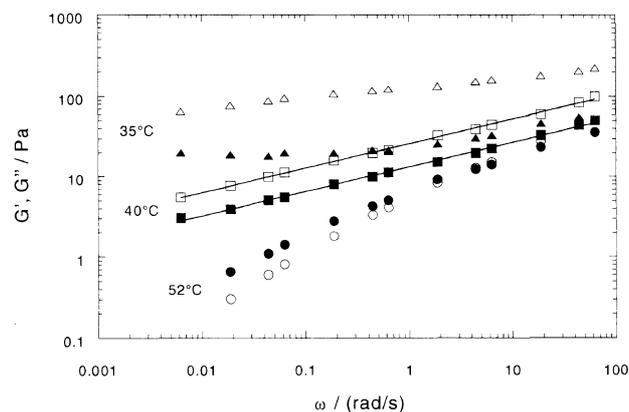


Figure 2. Frequency dependence of storage, G' , (open symbols) and loss modulus, G'' , (filled symbols) during thermoreversible gelation of 16 mg ml⁻¹ schizophyllan in 4 M aqueous sorbitol solution. The temperatures at which the three data set were recorded are given in the figure.

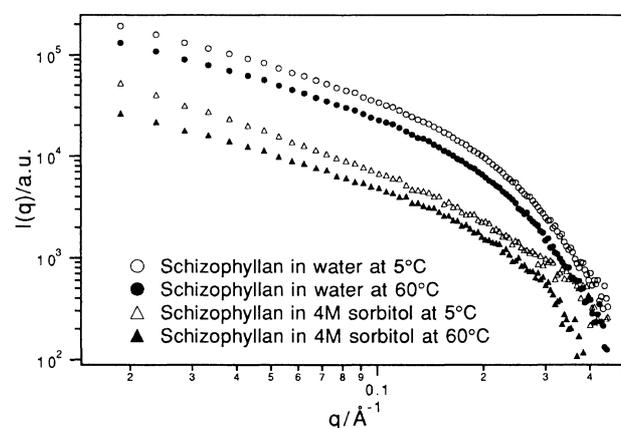


Figure 3. Small-angle X-ray scattering profiles from schizophyllan in water and in 4 M sorbitol aqueous solution at 60°C and 5°C.

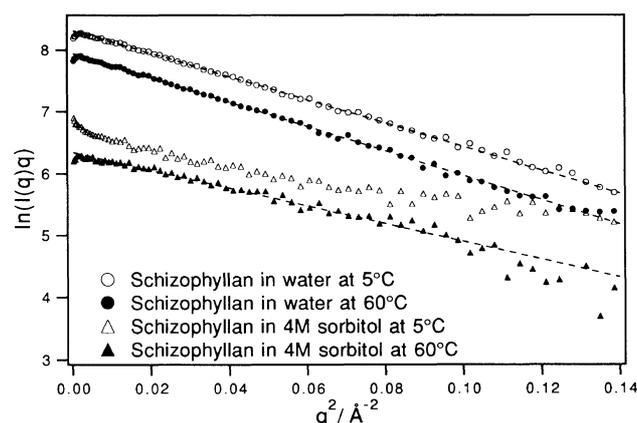


Figure 4. Cross-sectional Guinier plots from the scattering data shown in Figure 1.

since the scattering from a rod-like particle is approximately given as

$$qI(q) \approx \exp(-R_{Gc}^2 q^2 / 2) \quad (1)$$

The evaluated values were summarized in Table I. Schizophyllan in 2.7 M and 4.0 M sorbitol at 5°C (in gel state) yielded no linear region in the cross-sectional Guinier plots, so that the cross-sectional radius of

Table I. Analyzed results from SAXS in terms of a modified broken rod model

Schizophyllan in (solvent)	Measuring temperature	Sample state	R_{Gc}^a	Triple-helix : Single coil
Water	5°C	Sol	6.1 Å	1:0
	60°C	Sol	6.2 Å	1:0
Sorbitol 1.5 M	5°C	Sol	5.5 Å	1:0
	60°C	Sol	5.7 Å	1:0
Sorbitol 2.7 M	5°C	Sol	—	0.72:0.28
	60°C	Sol	5.2 Å	1:0
Sorbitol 4.0 M	5°C	Sol	—	0.42:0.58
	60°C	Sol	5.2 Å	1:0

^a R_{Gc} denotes the cross-sectional radius of gyration evaluated from the cross-sectional Guinier plots eq 1.

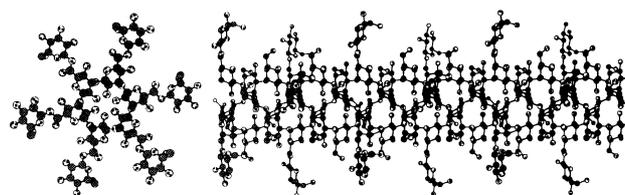
gyration was not evaluated. The result indicates that schizophyllan chain in gel may not assume a rod-like conformation of a triple-stranded helix. The value of R_{Gc} in sol was found to decrease as the sorbitol concentration increases. This effect is considered to be caused by the reduction of contrast due to sorbitol, and suggests that sorbitol is more solvated to schizophyllan at lower (gel) temperature. Thus schizophyllan chain in higher-concentration sorbitol aqueous solution looks more slender by SAXS.

Modified Broken Rod Model and Molecular Model

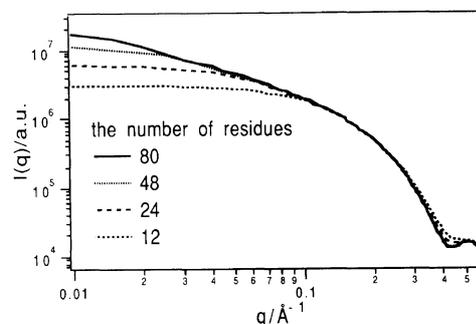
Further analysis was attempted by assuming that sorbitol disentangles partly a triple-stranded helix of schizophyllan into single coils. A modified broken rod model^{7,8} was adopted to fit the observed scattering profiles, since single coils are less rigid and act as a free joint between neighboring triple-stranded helical portions. A modified broken rod model yields a scattering function

$$q^2 I(q)/c \approx \sum_i w_i M_{Li} \Theta_i(q) + \text{const.} \quad (2)$$

Here i denotes either a triple-stranded helix component or a single chain component, and w_i is the weight fraction of the component i specified by the linear mass density M_{Li} . The scattered intensity is normalized by the concentration c (g ml^{-1}). The constant term takes into account the spatial correlation of the components.⁹ If the chain is Gaussian and has a point-like cross-section, $q^2 I(q)/c$ should become constant when q exceeds a threshold which is related with a persistent (or Kuhn) length. That is, eq 2 is valid over a certain range of q and is expressed as a hybrid of specified structure factors and a Gaussian term. Here schizophyllan in this particular example is regarded to consist of the fragments of specified structures (a triple-stranded helix and a single coil) and the spatial correlation between fragments is assumed to be random. $\Theta_i(q)$ is a corresponding particle scattering factor calculated from the atomic coordinates of the respective molecular models (Figure 5). The molecular model for a triple-stranded helix was taken from the crystallographic data,^{10,11} and that for a single coil was generated by the Monte Carlo method according to the energy map of laminari-biose.¹² A conventional Debye formula was employed for the calculation of the particle scattering factor for the

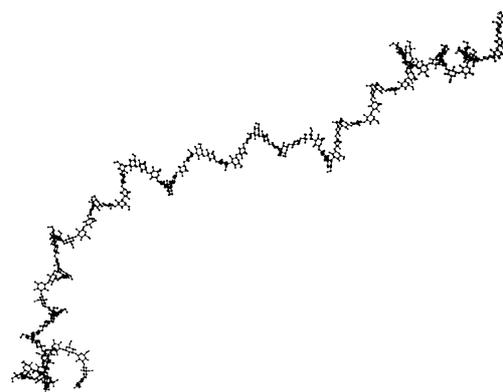


(a)

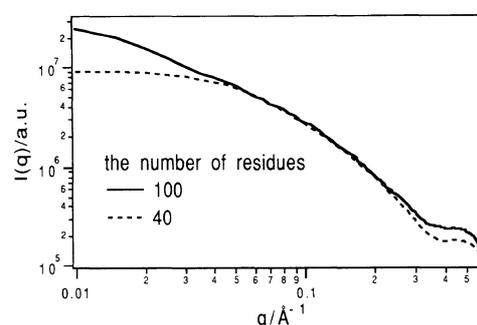


(b)

Triple-stranded helix



(a)



(b)

Single coil

Figure 5. (a) Molecular models for a triple-stranded helix and a single coil of schizophyllan, and (b) the corresponding scattering profiles calculated from those molecular models where the number of residues is as shown in the figure.

triple-stranded helix or a single coil of schizophyllan. That is,

$$\Theta_i(q) = \frac{\left[\sum_{s=1}^n f_s^2 g_s^2(q) + 2 \sum_{s=1}^{n-1} \sum_{t=s+1}^n f_s f_t g_s(q) g_t(q) \cdot \frac{\sin(d_{st}q)}{d_{st}q} \right]_i}{\left[\sum_{s=1}^n f_s^2 + 2 \sum_{s=1}^{n-1} \sum_{t=s+1}^n f_s f_t \right]_i} \quad (3)$$

Here f_s and d_{st} denote the atomic scattering factor of the s th atom and the distance between the s th and t th atoms, respectively, in either triple-stranded helix or single coil. The form factor $g_s(q)$ of a single atom is assumed to be represented by the form factor of a sphere possessing the radius equivalent to the van der Waals radius of the s th atom as

$$g_s(q) = \frac{3[\sin(R_s q) - (R_s q) \cos(R_s q)]}{(R_s q)^3} \quad (4)$$

Here R_s denotes the van der Waals radius of the s th atom, and it is set equal to 1.67 Å or 1.50 Å for a carbon or an oxygen atom, respectively. The scattering profile of a single chain was calculated as an ensemble average of the particle scattering factors over all generated chains, whereas the particle scattering factor was uniquely calculated for a triple-stranded helix from the atomic coordinates of available crystallographic data. The scattering profiles calculated from two molecular models (a triple-stranded helix and a single chain) confirm the stiff nature of (1→3)-β-D-glucan chains. A model (1→3)-β-D-glucan chain is composed of 80 residues for a triple-stranded helix or 100 residues for a single coil. No criterion is available with respect to the length of a triple-stranded helix or a single coil, but the model composed of over 80 residues was found to exhibit no difference in the scattering profile over the observable q range.

Fitting Scattering Profiles

A marked change of the scattering profile by gelation appears as an upturn at $q \rightarrow 0$ and a concave shape in the cross-sectional Guinier plot ($\ln qI(q)$ vs. q^2). This characteristic profile is only accounted for by introducing the flexible parts in the rigid triple-stranded helix, resulting in the random long-range spatial correlation between fragmented helices. The flexible part is supposed to be composed of single chains, and indeed no satisfactory fitting was achieved without a single chain component in the case of gel. Although the Kuhn length cannot be evaluated from the present analysis over a limited q range, the maximum length of a non-broken triple-stranded helix corresponds to the Kuhn length which is at least 800 Å as deduced from the molecular model composed of 80 residues.

No constant term was needed in the fitting of the SAXS profile at 60°C as verified by a sharp drop at $q \rightarrow 0$ and a slightly convex shape of the scattering profile in the cross-sectional Guinier plot (Figure 6b), which are the characteristics of a cylindrical object. The result confirms a rigid nature of a solute, and a triple-stranded helix was found to be a single component in eq 2 to fit the observed

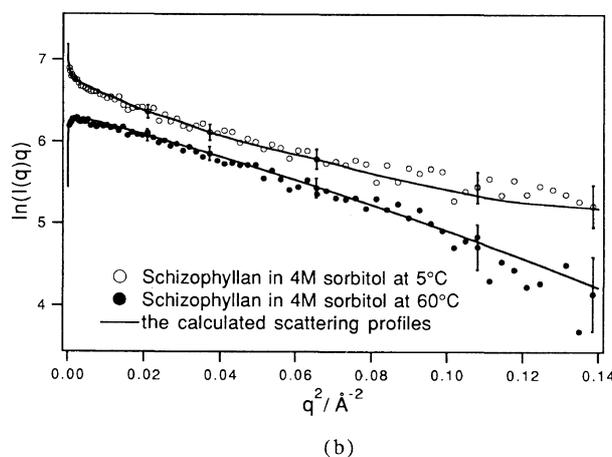
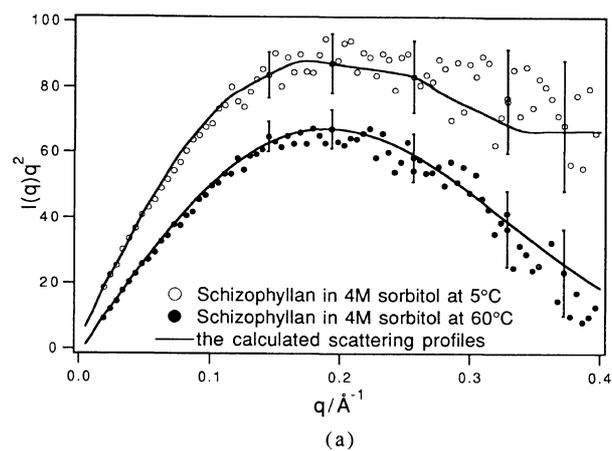


Figure 6. Fitting examples to the observed scattering profiles according to eq 2.

SAXS profile.

The observed scattering profiles were decomposed according to eq 2 in three terms including a triple-stranded helix, a single coil and a constant. The fitting by eq 2 needs two parameters w_i and a constant term. Since only relative intensities are measured, the ratio of $M_{L, \text{triple}}$ (the linear mass density of a triple-stranded helix) to $M_{L, \text{single}}$ (the linear mass density of a single chain) is a sole factor to be known *a priori* in the calculation of the scattering profile by eq 2. The ratio of $M_{L, \text{triple}}$ to $M_{L, \text{single}}$ was estimated from that of respective intercepts of the cross-sectional Guinier plots of calculated scattering intensities from the molecular models for a triple-stranded helix and a single coil (see Figure 5). In the present example, $M_{L, \text{triple}}/M_{L, \text{single}}$ was found to be 11.2, which is much larger than 3 and indicates a flexible nature of a single coil. The calculated profile was adjusted to fit the observed SAXS profile by multiply an arbitrary constant to the total calculated intensities. The fitting is satisfactory as shown in Figure 6, where the scattering profiles from schizophyllan in 4M sorbitol aqueous solution at 60°C (sol) and 5°C (gel) are fitted to those calculated from the models composed of a single coil and triple-stranded helix. It should be noted here that the model for a triple-stranded helix is slightly modified in order to take into account the apparent thinner cross-section. The model modification was

made by taking an appropriate average of the scattering profiles of respective triple-stranded helices of schizophyllan (poly(1→3)- β -D-glucan with (1→6)-D- β -glucan branches) and laminaran (poly(1→3)- β -D-glucan without branches). Here the cross-sectional radius of gyration R_{Gc} is evaluated as 6.6 Å for schizophyllan or 5.2 Å for laminaran, respectively, from respective molecular models. Observed values of R_{Gc} are found to lie between those values, so that a molecular model was adjusted to yield an observed value of R_{Gc} by taking an arithmetic average of two extremes (one with branches and another without D-glucan branches). Schizophyllan in gel (in 2.7 M or 4.0 M sorbitol aqueous solution at 5°C) revealed no visible D-glucan branch by X-ray.

As the results of the analysis by a modified broken rod model are summarized in Table I, schizophyllan was confirmed to maintain a triple-stranded helical conformation as a whole in solution. A part of triple-stranded helix seems to be disentangled into single coils by gelation, and a broken rod represents an entire chain of schizophyllan in gel.

Gelation Process of Schizophyllan

How schizophyllan forms gel in sorbitol aqueous solution? In an earlier work,⁵ sorbitol was considered to reduce a water activity and thus schizophyllan triple-stranded helices aggregate to form junction zones. However, no proof was found from the SAXS measurements to confirm the formation of the junction zone by aggregation of triple-stranded helices. The partial disentanglement of a triple-stranded helix into single coils may be caused by sorbitol breaking intermolecular hydrogen bonds that stabilize the rigid triple-stranded conformation of schizophyllan. Sorbitol melts partially a triple-stranded helix, but not necessarily from the chain ends. Since gelation induces necessarily the breakage of triple-stranded conformation, the junction zone is considered to involve the broken (disentangled) portions of triple-stranded helices. The reduction of water activity may cause the hydrogen bonding *via* sorbitol between broken portions of triple-stranded helices, and constitute junction zones to form a network. That is, the schizophyllan network is not formed by the aggregation of triple-stranded helices, but the junction zone is composed of disentangled and hydrogen-bonded schizophyllan chains. Triple-stranded network chains may be broken occasionally, as the fraction of single chains is large. In conclusion, the gelation of schizophyllan in sorbitol aqueous solution might be visualized as sketched in Figure 7.

Acknowledgments. The SAXS measurements were performed under the approval of the Photon Factory Advisory Committee (Proposal No. 94G291). KK is indebted to DAAD and JSPS for the financial support.

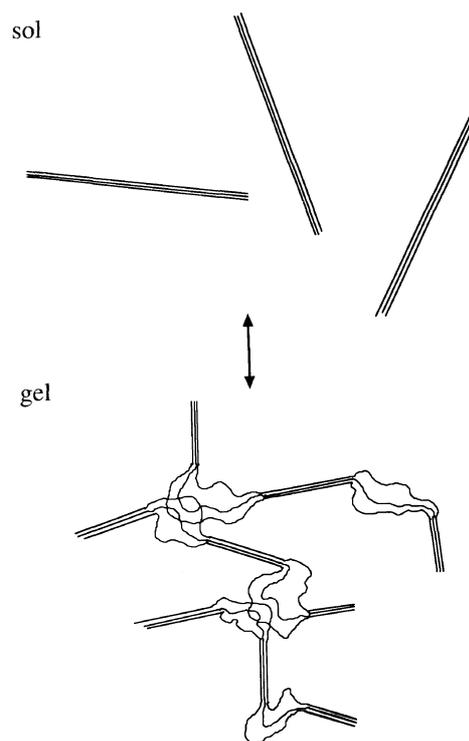


Figure 7. Gelation process of schizophyllan in sorbitol aqueous solution.

Y.Y. acknowledges a financial support of Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists. A part of the work is supported by the Grant-in-Aids from the Ministry of Education, Science, Sports and Culture of Japan.

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