Collapse and Association of Denatured Lentinan in Water/Dimethlysulfoxide Solutions

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Triple helical lentinan, β-(1→3)-D-glucan from *Lentinus edodes*, was denatured in dimethlysulfoxide (DMSO) into single random coils. The DMSO solutions of randomly coiled lentinan were diluted with pure water to different w_H (the weight fraction of water in the mixed solvent), and their specific optical rotation $[\alpha]_D$, reduced viscosity $(\ln\eta_r)/c$, and hydrodynamic radius R_h were investigated as a function of w_H and storage time t. With an increase of w_H from 0.1 to 0.2, $[\alpha]_D$ increased sharply, suggesting that transition of conformation of the macromolecules has occurred. When w_H was lower than 0.1, $(\ln\eta_r)/c$ of lentinan in water-diluted DMSO exhibited the almost same value as that in pure DMSO and changed hardly with increasing t. Interestingly, $(\ln\eta_r)/c$ decreased to reach a minimum with a further increase of w_H from 0.1 to 0.25 and then increased with a continuous increase of w_H from 0.25 to 0.5. Both $(\ln\eta_r)/c$ and R_h of the denatured lentinan in water-diluted DMSO with w_H of ~0.25 both exhibited a minimum, indicating that collapsed coil chains have occurred. All of the experimental findings revealed that the behaviors of lentinan in water-diluted DMSO solution with $w_H < 0.1$ were consistent with that in good solvent, DMSO. When $w_H = 0.25$, the quality of the mixed solvents became worse, and the dominant intramacromolecular hydrogen-bond interaction enhanced, leading to minimum of viscosity and size of the chains as a result of the collapsed coils. When $w_H > 0.25$, the quality of the mixture weakens further, and the intermolecular hydrogen-bond interaction enhanced and was dominant, leading to aggregation of the collapsed chains.

Introduction

Lentinus edodes is now cultivated and is the second most commonly produced edible mushroom in the world.[1] Recently, *Lentinus edodes* have attracted much attention because of the application of its extracts in an antitumor effect,[2−4] antimicrobial properties,[5−6] improved liver function and a reduction of viremia in patients with chronic hepatitis B,[7] and an inhibition of human immunodeficiency virus infection in vitro.[8] Among the extracts, lentinan (LNT) is a fully purified β-(1→3)-D-glucan with two β-(1→6) branches for every five glucose residues, sharing the same chemical structure with schizophyllan.[9−10] This family of polysaccharides is often referred to as β-(1→3)-D-glucans, which display antitumor, antibacterial, antiviral effects, and anticachectic actions, and has been used clinically in Japan as a host immunopotentiator for cancer therapy.[11−14] Furthermore, molecular weight, degree of branching, conformation, and intra- and intermolecular association of the polysaccharide chains have been exhibited to influence the immune stimulating effect to various extents.[15−20] It has been reported that immunopharmacological activities of schizophyllan in mice

have conformation dependence.[21] Therefore, an understanding of the conformational states for the polysaccharides in solutions is essential for the successful application.

Recently, we have confirmed that lentinan dissolves in water as a triple helix, whereas it disperses in dimethlysulfoxide (DMSO) as a single random coil by using light scattering, viscometry combined with wormlike chain model and ^{13}C NMR.[22,23] Moreover, lentinan directly dissolved in water/DMSO mixtures undergoes all or none dissociation from the triple helix to single expanded chains when the weight fraction w_H (w/w) of water in the binary mixtures is lowered to about 0.15 at 25 °C.[23] Furthermore, the viscosity results showed that the single lentinan chains dispersed in DMSO would not be re-natured to the triple helix when water was added to the solvent to $w_H = 0.5$. The values of intrinsic viscosity $[\eta]$ in water-diluted DMSO solution were significantly lower than those from directly dissolved in water/DMSO mixtures with corresponding w_H, but they slightly increased with an increase of w_H, suggesting an irreversible transition.[23] What happened to the lentinan single chains in "water-diluted" DMSO solutions? Four decades ago, it was suggested that a flexible linear chain can change from an expanded coil to a collapsed globule.[24] This prediction has aroused considerable interests experimentally and theoretically since then, and useful experimental results were obtained using viscometer, static, and dynamic light

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scattering.[25−30] It has been proven that DMSO is a powerful hydrogen bond acceptor and works by breaking associative hydrogen bonds in the polysaccharide and in water.[22,23,25,31] In the present work, we attempted to study the intra- and intermacromolecular interactions and solution behavior of lentinan in DMSO solutions diluted with water to various w_H ranging from 0 to 1.0 by optical rotation, viscometer, static light scattering combined with size exclusion chromatography, and dynamic light scattering.

Experimental Section

Sample Preparation. Lentinan was isolated from fruiting bodies of *Lentinus edode*s, a commercial product cultivated in Hubei of China. The detailed procedure of extraction was reported previously.[22] To get pure lentinan with relatively narrow molecular weight distribution, the original sample was dissolved in water and centrifuged at 10000 rpm for 1 h. The resulting samples were further fractionated by repeated reprecipitation from water to acetone at room temperature to obtain several fractions. The precipitates were dissolved in water again to obtain clear solution, filtered, and lyophilized for one week. The middle part was chosen in the present work. DMSO was distilled and treated with a molecular sieve to further dehydrate. Water-diluted DMSO solutions of lentinan with desired w_H (weight fraction of water in the mixed solvent, w/w) and given polysaccharide concentration c (0.95×10^{-3} g/mL) were prepared by diluting a polysaccharide solution in DMSO with a desired amount of water. Lentinan was dissolved in DMSO to be destroyed into single random coil chains, and then the behaviors of lentinan in water-diluted DMSO solutions were investigated by viscometer and dynamic light scattering.

Optical Rotation. The order−disorder transitions or association-dissociation for helix or associating polymers are often accompanied by changes, which may be monitored by optical rotation.[32] The specific optical rotations at 589 nm for lentinan in water-diluted DMSO solution with desired w_H were measured on a Perkin-Elmer 341 polarimeter in a jacketed standard cell (10 cm/6.2 mL, Perkin-Elmer). All of the solutions were kept at 25 °C during the measurements.

Viscometry. Viscosities of water-diluted solutions were measured at 25 °C by using Ubbelohde capillary viscometer. The kinetic energy correlation was always negligible. Huggins and Kraemer equations were used to estimate the intrinsic viscosity [η]. The changes of reduced viscosity $(\ln\eta_r)/c$ with time for lentinan in water-diluted DMSO solutions with desired w_H were measured at 25 °C.

Static Light Scattering Combined with Size Exclusion Chromatography (SLS−SEC). The weight-average molecular weight M_w was determined by using size exclusion chromatography with multi-angel laser light scattering, which is convenient for determination of molecular weight without standard samples. SEC-LLS measurements were carried out on a DAWNDSP multi-angel laser photometer with a P100 pump (Thermo Separation Products, San Jose, USA) equipped with TSK-GEL G6000 PWXL with a G4000 PWXL column (7.8 mm × 300 mm, TOSOH Corporation) for aqueous solution and G4000-H8 with G3000-H8 column for DMSO

Table 1. Molecular Weights and Intrinsic Viscosities of Lentinan in Water and DMSO at 25 °C

	$M_w \times 10^{-4}$		$M_{w,in\ water}/$	[η] (mL/g)	
sample	in water	in DMSO	$M_{w,in\ DMSO}$	in water	in DMSO
lentinan	172.2	56.4	3.05	1975	243

(TOSOH Corporation) and a differential refractive index detector (RI-150, Thermo Separation Products, Thermo Finnigan, USA) at 25 °C. The eluent was water and DMSO at a flow rate of 1.00 mL/min. DMSO was distilled and treated with a molecular sieve to further dehydrate. The samples were dissolved in water and DMSO overnight with stirring, respectively. All solutions were filtered with a 0.45 μm filter (CA, Puradisc 13 mm Syringe Filters, Whattman, England) for water and a 0.45 μm filter (PTFE, Puradisc 13 mm Syringe Filters, Whattman, England) for DMSO and degassed before use. The injection volume was 200 μL. Astra software was utilized for data acquisition and analysis.

Dynamic Light Scattering (DLS). Dynamic light scattering (DLS) has been used to ascertain the size, shape, and dynamics of biological macromolecules and supermacromolecular assemblies and to characterize flow and other properties in physiological and biomedical situations.[33] In this work, a modified commercial light scattering spectrometer (ALV/SP-125, ALV, Germany) equipped with an ALV-5000/E multi-τ digital time correlator (ALV, Germany), and a He−Ne laser (at $\lambda = 632.8$ nm) was used to characterize the size of lentinan macromolecules in water-diluted DMSO solutions at 25 °C. Test solutions were made optically clean by filtration through 0.45 μm Millipore filters.

The precisely measured intensity−intensity time correlation function $G^{(2)}(q,\tau)$ in the self-beating mode can be related to the normalized field-field autocorrelation function $g^{(1)}(q,\tau)$ via the Siegert relation as[34]

$$G^{(2)}(q,\tau) = A[1+\beta|g^{(1)}(q,\tau)|^2] \tag{1}$$

where A is the measured baseline and β is a constant related to the coherence of the detected optics. For a polydisperse system, $g^{(1)}(q,\tau)$ is related to the distribution of the characteristic line width $G(\Gamma)$ by[34,35]

$$|g^{(1)}(q,\tau)| = \int_0^\infty G(\Gamma)e^{-\Gamma\tau}d\Gamma\theta\gamma \tag{2}$$

Thus, $g^{(1)}(q,\tau)$ can be resulted in a line width distribution $G(\Gamma)$ by the CONTIN Laplace inversion algorithm in the correlator according to eq 2. For a pure diffusive relaxation, Γ is related to the translational diffusion coefficient D and $G(\Gamma)$ can be converted to a translation diffusion coefficient distribution $G(D)$ by

$$\Gamma = Dq^2 \tag{3}$$

or a hydrodynamic radius distribution $f(R_h)$ using the Stokes−Einstein equation

$$R_h = \frac{k_B T}{6\pi\eta_0 D} \tag{4}$$

where k_B is the Boltzmann's constant, T is the temperature in unit of K, and η_0 is the solvent viscosity. For the present
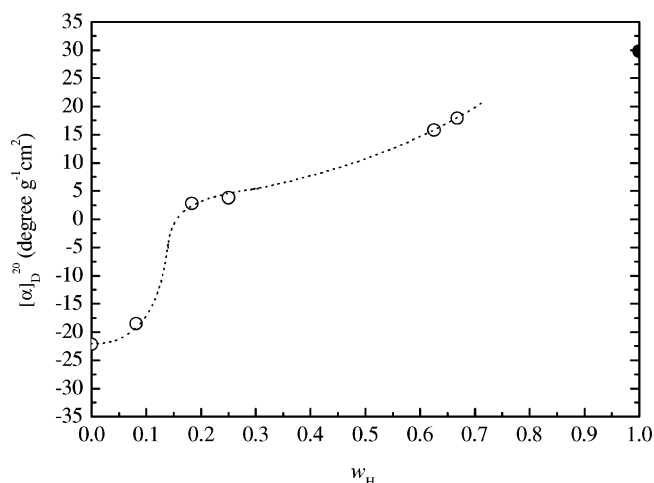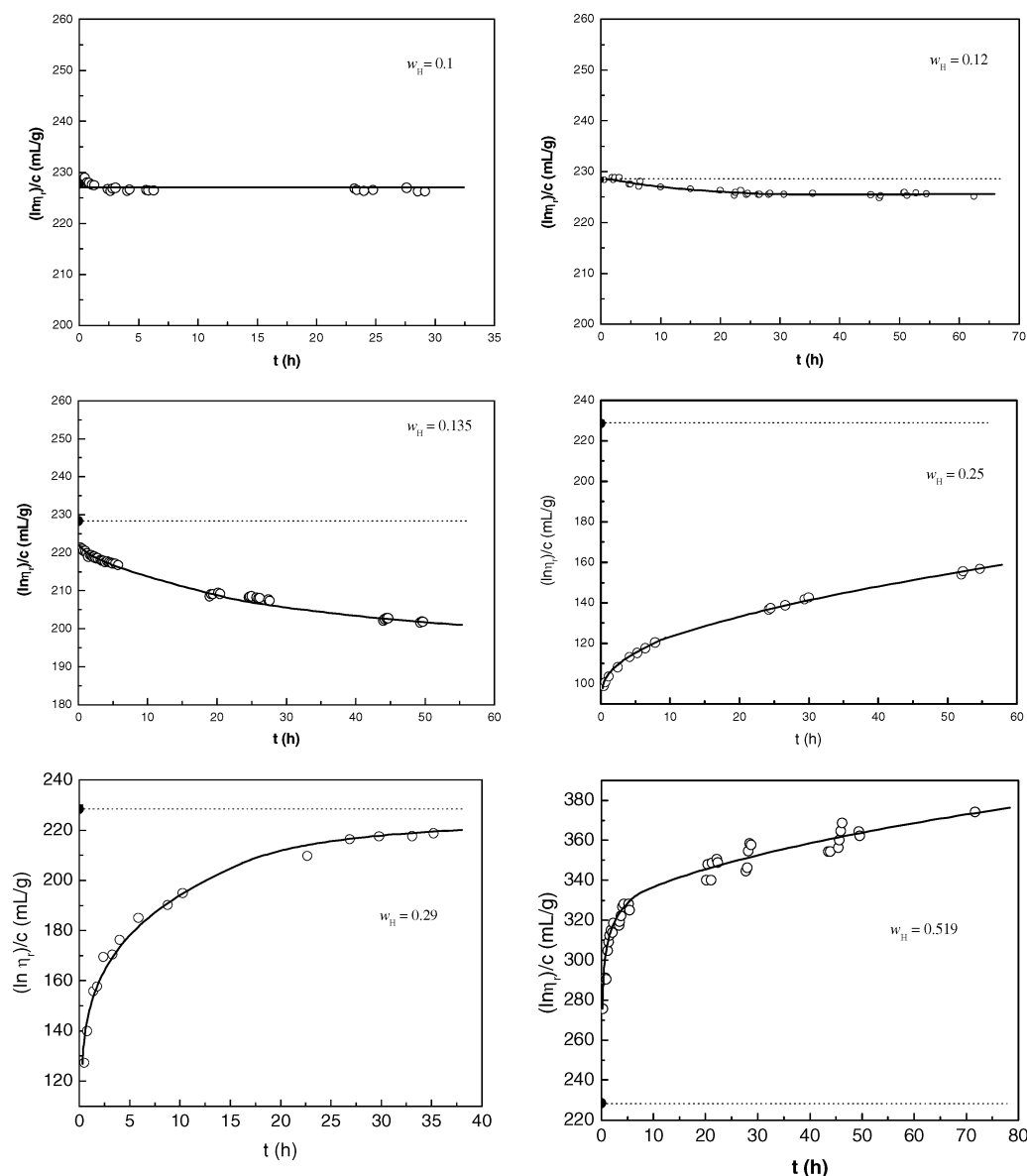
Figure 1. w_H dependence of $[\alpha]_D$ for lentinan in water-diluted DMSO solutions at 25 °C. The filled circle stands for the data of lentinan in pure water.

system, the measured R_h is apparent at given polymer concentration.

Results and Discussion

Table 1 summarizes the values of M_w and $[\eta]$ for the purified lentinan in water and dry DMSO at 25 °C determined by light scattering and viscometer. The ratio of M_w in water and in DMSO was determined to ~3, whereas that of $[\eta]$ in water and in DMSO to ~8, confirming that lentinan exists as triple helix in water and single random coil in pure DMSO. Figure 1 shows the w_H dependence of $[\alpha]_D$ for lentinan in water-diluted DMSO solutions at 25 °C, similar to that for lentinan directly dissolved in water/DMSO mixtures. $[\alpha]_D$ increases with an increase of w_H, especially in the range of w_H from 0.1 to 0.2, and gives a sharp rise. This indicates that an obvious transition takes place between 0.1 and 0.2 of w_H, implying a change in the conformation of macromolecules.[32] Namely, the shape of single chains for lentinan in DMSO changed once water was added. The filled circle represents the data of lentinan in pure water. Figure 2 shows the storage time t dependence of $(\ln\eta_r)/c$ for lentinan in water-diluted DMSO solutions of different w_H at a fixed

Figure 2. Time dependence of $(\ln\eta_r)/c$ for lentinan in water-diluted solutions of different w_H at a fixed c of 0.95×10^{-3} g/mL. Each of the filled circles represents $(\ln\eta_r)/c$ of the DMSO solutions with the same c.
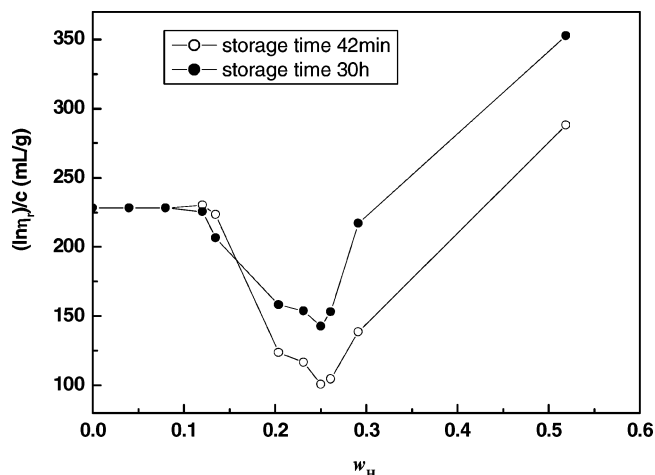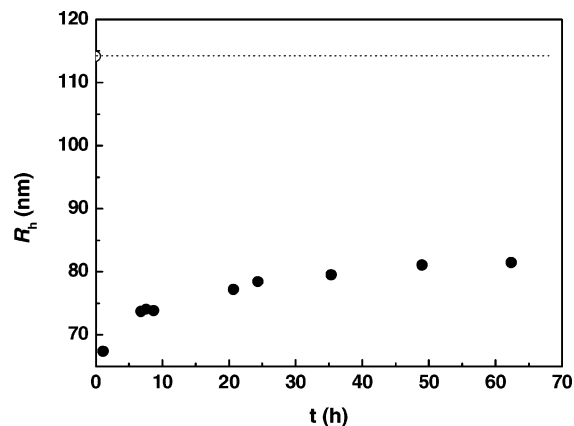
Figure 3. w_H dependence of $(\ln\eta_r)/c$ for lentinan in water-diluted solutions with a fixed c of 0.95×10^{-3} g/mL at an elapsed time of 42 min and 30 h.
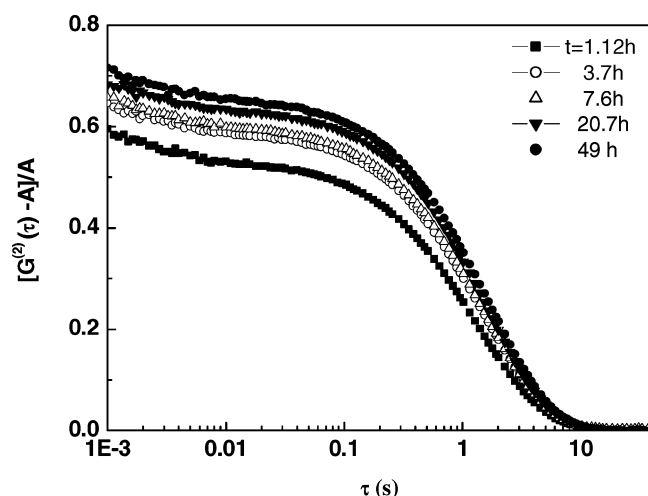
Figure 4. Intensity-intensity time correlation function of lentinan in water-diluted DMSO solution at $w_H = 0.25$ with a fixed c of 0.95×10^{-3} g/mL at different storage time, where $\theta = 90°$.

c. For $w_H < 0.1$, the values of $(\ln\eta_r)/c$ almost keep consistent with that in pure DMSO, and change hardly with t, suggesting that the quality of the mixed solvent with $w_H < 0.1$ is almost same as that of good solvent, DMSO. When w_H is between 0.1 and 0.29, all of the $(\ln\eta_r)/c$ values undergo a discontinuous decrease at the beginning in comparison with that in DMSO. Furthermore, the change mode of reduced viscosity with t changed with w_H. For $w_H = 0.12$ and 0.135, $(\ln\eta_r)/c$ decreases with t, whereas for $w_H \geq 0.25$, $(\ln\eta_r)/c$ increases with t, similar to the viscosity behavior of schizophyllan in water-diluted DMSO solutions reported by Sato et al.[25] It is well-known that viscosity reflects the hydrodynamic properties of polymers in solution and is proportional to the hydrodynamic volume of particles. Therefore, the magnitude and change mode of viscosity can reflect the size and interaction of the particles, respectively. The decrease of viscosity implies a reduction of the size of the particles because of the collapse of coil chains, owing to the formation of intramolecular hydrogen bonding.[25,36,37] On the other contrast, the increase in viscosity suggests the enhancement of particle size as a result of the formation of association between chains or chain expansion in good solvent. There-
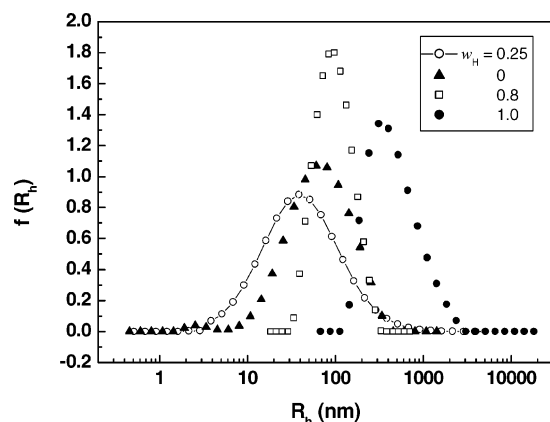

Figure 5. Time dependence of R_h for lentinan in water-diluted DMSO solution with $w_H = 0.25$ at a fixed c of 0.95×10^{-3} g/mL and 25 °C, where $\theta = 90°$.

fore, that $(\ln\eta_r)/c$ decreases with an increase of t for w_H between 0.1 and 0.135 suggests the occurrence of chain shrinkage as a result of the formation of intramolecular hydrogen bonding. Interestingly, for $w_H = 0.25$ and 0.29, there occurs a monotonic increase in $(\ln\eta_r)/c$ following a discontinuous initial decrease, showing coinstantaneous association as a result of the formation of intermolecular hydrogen bonding. Despite increase of $(\ln\eta_r)/c$ with t, the values are lower than that in pure DMSO, implying that the intramolecular interaction dominates over the intermolecular interaction. For $w_H = 0.52$, $(\ln\eta_r)/c$ is much higher than that in pure DMSO and increases monotonically with t, but much lower than that in water, giving further evidence of association between unlike chains, but not restoration of triple helix.

The triple helical structures of schizophyllan[38] and lentinan[22] are sustained by the intra- and interchain hydrogen bonds to be stabilized in aqueous solution, and can be disrupted by strong solvents such as DMSO.[23,39] Usually, polysaccharides possess a strong tendency of association caused by the abundant hydroxyl groups, which easily undergo hydrogen bonding.[40] With addition of water into DMSO, the quality of the mixed solvent decreases, which weakens the interactions between solvent and macromolecules, resulting in intra- or interchain interaction of macromolecules. From Figure 2, it can be seen clearly that $(\ln\eta_r)/c$ first decreases with t, and then increases with t for $w_H > 0.1$, implying the existence of minimum. The values of $(\ln\eta_r)/c$ at given t for lentinan as a function of w_H are illustrated in Figure 3. With an increase of w_H, $(\ln\eta_r)/c$ of water-diluted DMSO solutions remains constant up to $w_H \sim 0.1$, and then abruptly decreases to reach a minimum, and finally continues to increase markedly, similar to that for schizophyllan.[25] The above viscosity behavior indicates that two possible processes occur in the water-diluted DMSO solutions of lentinan. In the first process, the hydroxyl groups of random coil chains more easily form intramolecular hydrogen bonding, resulting in the shrinkage to a higher density coil. Namely, the so-called chain collapse takes place in the water-diluted DMSO solution, leading to the decrease of viscosity. This may be explained as that the intramolecular hydrogen bonding is more easily formed than the intermolecular hydrogen bonding, and the intramolecular interactions occurs first, leading to the collapse of the macromolecules

Figure 6. Distributions of hydrodynamic radius R_h for lentinan in desired water-diluted DMSO solutions, pure DMSO and water at a fixed c of 0.95×10^{-3} g/mL at 25 °C, where $\theta = 90°$.
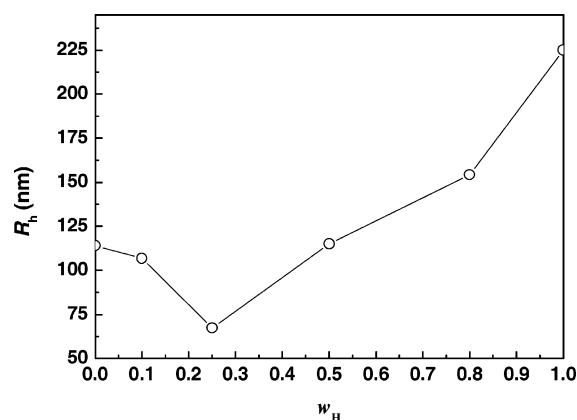
Figure 7. w_H dependence of R_h for lentinan in water-diluted DMSO solutions with a fixed c of 0.95×10^{-3} g/mL at storage time of about 2 h, where $\theta = 90°$.

when a little amount of water was added into the mixed solvent making the quality of the solvent bad. Takigawa et al.[36,37] examined the viscosity behavior of poly(vinyl alcohol) (PVA) in DMSO/water mixtures and found that the shrinkage of the PVA chains in dilute solutions resulted from the formation of intrachain hydrogen bonding. In the second, hydroxyl groups of two or more chains, either collapsed or not, are hydrogen bonded intermolecularly to form aggregates, namely, interchain association occurs, resulting in an increase of viscosity. When w_H increases further, the association should be enhanced as a result of the decrease in quality of mixed solvent. As shown in Figure 3, when w_H is higher than 0.5, interchain association surpasses the chain collapse, resulting in the higher viscosity than that in pure DMSO.

Moreover, the two curves at different t in Figure 3 show the same trend of changes and undergo the same minimum at $w_H \sim 0.25$ for lentinan in water-diluted DMSO solutions. Sato et al. has also studied the viscosity behavior of schizophyllan in water-diluted DMSO solutions, and found the same two processes occurred.[25] It is noted that the tendency of changes for viscosity and specific optical rotation with w_H is different from each other. It may be explained that specific optical rotation is less sensitive than viscosity to chain size. However, the detailed reason is not clear at present.

Motions (translational, rotational or internal motion) of macromolecules or colloidal particles in solution can be conveniently studied by using DLS, giving information on the dimension of macromolecules or colloidal particle. Figure 4 shows the intensity–intensity time correlation function of lentinan in water-diluted DMSO solution with $w_H = 0.25$ at scattering angle $\theta = 90°$ and different storage time. With an increase of t, the relaxation shifts to a longer delay time, and the value of the ordinate increases, indicating the formation of large aggregates. The time dependence of R_h for lentinan in water-diluted DMSO solution with $w_H = 0.25$ at $\theta = 90°$ and 25 °C is shown in Figure 5. R_h is much lower than that in pure DMSO (see dotted line), further confirming the occurrence of the chain shrinkage in the dimension as a result of intrachain association. The R_h values increase monotonically with an increase of t, implying the formation of interchain aggregates, similar to the viscosity behavior in Figure 2. Figure 6 shows the distributions of R_h for lentinan in desired water-diluted DMSO solutions with $t \sim 2$ h, pure DMSO and water at $\theta = 90°$ and 25 °C. Obviously, the R_h in water is much higher than those in other systems, indicating the extended and stiff conformation of lentinan. With an increase of w_H from 0 to 0.25, the R_h peak shifts to smaller size, suggesting the decrease of hydrodynamic volume as a result of chain collapse. With further increase of w_H from 0.25 to 1.0, interchain association dominates in the system, and makes the peak shift to larger size. Figure 7 shows w_H dependence of R_h for lentinan in water-diluted DMSO solutions at $t \sim 2$ h. Interstingly, R_h change tendency is similar to that of viscosity. The R_h values pass through a minimum at $w_H \sim 0.25$, and then increases sharply with further an increase of w_H. However, the values of viscosity and hydrodynamic radius of lentinan in water-diluted DMSO solution are much lower than the corresponding values in pure water, indicating that the random

$w_H = 1.0$ $w_H = 0$ $w_H = 0.25$ $w_H = 0.52$
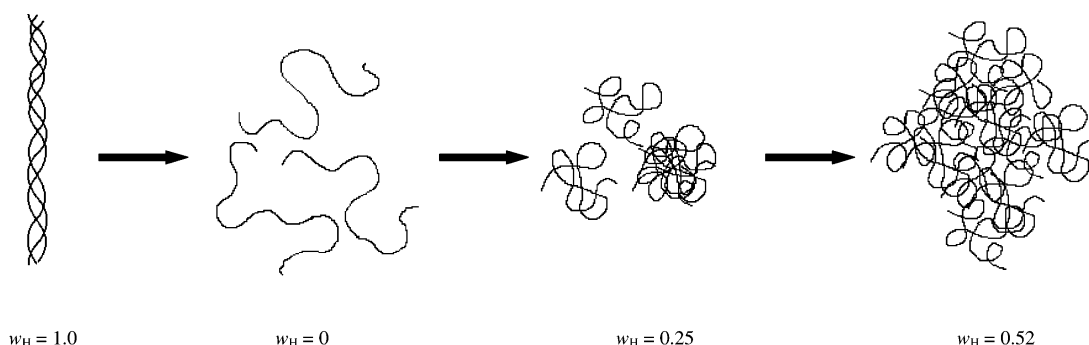
Figure 8. Scheme of solution behavior for lentinan in DMSO/water mixtures.

aggregation occurs in the water-diluted DMSO solution rather than renaturation of the triple helix conformation.

On the basis of the above results, a molecular model describing the process of the chain collapse and the aggregation of the collapsed chains is shown in Figure 8. When w_H < 0.1, the quality of DMSO/water mixture is same as that of pure DMSO, and lentinan exists as random coil chain. When $w_H = 0.25$, the quality of the mixture weakens, leading to the enhancement of intra- and intermolecular interactions, which results in shrinkage and association of chains. When w_H increases further, the interaction between macromolecules and solvent is weaken more, and the interaction among macromolecules is built up, leading to association of the collapsed chains.

Conclusions

Lentinan, triple helical polysaccharide in water, has been broken into single random coil in DMSO. The collapse and association behaviors of the denatured random coil chains in water-diluted DMSO solutions take place successively with an increase of w_H. When w_H is lower than 0.1, the quality of DMSO/water mixture is same as that of pure DMSO, and the viscosity and R_h change hardly. Namely, the interaction between the macromolecules and solvent keeps constant. When $w_H = 0.25$, the values of viscosity and R_h reach to the minimum, suggesting the occurrence of shrinkage of the chains as a result of the dominant intramolecular hydrogen bonding. When w_H > 0.25, the quality of the mixture weakens further, and the intermolecular interaction enhances more, leading to association of the collapsed chains to large aggregates.

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