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Note

Low M_w sulfated curdlan with improved water solubility forms macromolecular complexes with polycytidylic acid ☆

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Abstract—The water solubility of curdlan was enhanced by partial sulfation at O-6. Circular dichroism measurements suggest that the sulfated curdlan with the degree of substitution (DS) from 0 to 8.7 mol% forms macromolecular complexes with polycytidylic acid (poly(C)). Although the thermal stability of the complexes decreased with increase in DS, this could be overlapped by addition of NaCl in the concentration above that of serum. The results clearly indicate that the drawback arising from the electrostatic repulsion between the anionic charges can be partially compensated by the presence of salt. Furthermore, the polynucleotide chain complexed with the sulfated curdlan was protected from the enzymatic hydrolysis, corroborating the assumption that the sulfated curdlan has an ability to bind poly(C).

Keywords: Curdlan; β-(1→3)-Glucan; Sulfation; Macromolecular complex; Polycytidylic acid

1. Introduction

We recently reported that schizophyllan (SPG, Fig. 1a), a β-(1→6)-branched β-(1→3)-glucan, can specifically form macromolecular complexes with certain polynucleotides.[2–5] As far as we know, this is the first example of a specific polysaccharide–polynucleotide interaction. This complex has several novel properties inherent to the macromolecular complex: they are; (i) the complex is dissociated in a cooperative manner upon heating similarly to DNA duplexes;[4] (ii) this complexation ability is induced only by the β-(1→3)-glucan skeleton;[6,7] (iii) the polynucleotide chain incorporated into the complex resists the enzymatic hydrolysis[8,9] and; (iv) the polynucleotide chain bound to the complex can be immediately replaced when it meets the complementary nucleotide chain.[10]

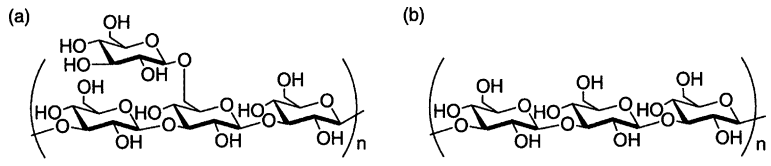

Figure 1. Comparative chemical structure of (a) schizophyllan and (b) curdlan.

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Among β-(1 → 3)-glucans, curdlan (Fig. 1b) is an inexpensive polysaccharide, which could find application in this area. We recently found that a low-molecular-mass curdlan prepared by acid hydrolysis is partially water soluble and forms complexes with certain RNAs and DNAs, although the commercially available curdlan ($M_w > 1{,}000{,}000$) is virtually insoluble in water and cannot form any complex with polynucleotides.[6,7] Taking its low cost into consideration, an appropriate modification of curdlan should improve its capability as polynucleotide binder.

From a viewpoint of application to gene technology, improvement of the water solubility is among the most crucial problems to be solved. In this report, we partially introduced sulfate groups in curdlan and evaluated its ability as polynucleotide binder.

2. Results and discussion

2.1. Synthesis of a sulfated curdlan

According to our recent results,[11] the complexation ability of curdlan appears in a limited M_w range window; the affinity for polycytidylic acid (poly(C)) was observed only in the M_w range from 1700 to 87,000 at $V_w = 0.75$, where V_w is the water volume fraction in the water–dimethylsulfoxide mixture. Taking this molecular mass effect and the purification easiness into account, it was desirable that curdlan with the molecular mass mentioned above could be prepared by acid hydrolysis.[7] In this study, the low-molecular-mass curdlan with $M_w = 24{,}800$ was used.

The synthesis of the sulfated curdlan was carried out in 0.25 M LiCl/Me_2SO solution using the pyridine–sulfur trioxide complex as the sulfation reagent (Scheme 1).[12] The degree of substitution (DS) was estimated by inductively coupled plasma (ICP) analysis using the sulfur oil standard solution (Aldrich) as a standard.[†] Thus, we could synthesize five sulfated curdlan samples with DS from 1.7 to 76 mol % based on glucose (Table 1). It should be emphasized that, as shown in Table 1, the DS values for our samples are very low compared with those obtained by Uryu and coworkers where DS values were ranging over 100 mol % in order to induce a biological response.[13,14]

It is already known that the thermal stability of the curdlan/polynucleotide complexes is affected by the M_w of curdlan and the strongest affinity appears in the M_w range from 8100 to 22,000.[11] Therefore, we compared the M_w values of the sulfated curdlans by size exclusion chromatography (SEC). The results are summarized in Table 1. The M_w value suddenly dropped from 24,800 to 13,000 by introduction of 1.7 mol % sulfate group and then linearly decreased with increase in the DS. It is known that *p*-toluenesulfonic acid hydrolyzes the main chain of the β-(1 → 3)-glucan above 50 °C.[7,11] Taking into consideration that (i) pyridinium sulfate was generated during the sulfation and; (ii) the reaction was carried out at 80 °C, the drop in M_w by slight sulfation is quite reasonable. As mentioned above, the M_w's for the DS from 1.7 to 8.7 mol % are among the M_w range useful as a polynucleotide binder, whereas the M_w for CUR-S(76) is too low to bind poly(C). Accordingly, the subsequent discussions are mainly performed for the modified curdlans bearing the DS from 1.7 to 8.7 mol %.

2.2. Structural analysis of the sulfated curdlan

Uryu and coworkers reported that the reactivity of the OH groups in β-(1 → 3)-glucans with the sulfation reagent obeys the following order: 6-OH > 2-OH ≫ 4-OH.[12,15] On the other hand, our previous studies indicated that the 2-OH group in the β-(1 → 3)-glucan main chain plays important roles in the complex formation with polynucleotides.[5,6] Therefore, if a significant amount of sulfate groups is introduced at the O-2 group, the complexation ability should be diminished by the steric bulkiness of the sulfate group.

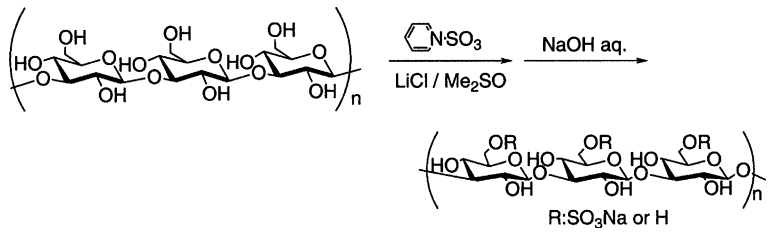
Nuclear magnetic resonance is a useful technique to determine the sulfated position in polysaccharides.[11,16] Assignment of the ^{13}C chemical shifts for the sulfated curdlan has already been reported by Uryu and coworkers.[16] When the OH groups are sulfated, the peaks (C-2′, C-4′, and C-6′) shift toward lower magnetic field by ca. 10 ppm.[16] We confirmed that in the ^{13}C NMR spectrum of CUR-S(76), only the C-6′ peak shifts to lower magnetic field. Therefore, we concluded that our modification took place at the C-6 hydroxyl group.

2.3. Water solubility of the sulfated curdlan samples

In order to estimate the water solubility after the sulfation, we measured the turbidity of the sulfated curdlan in water solution. The concentration of the sample was 0.77 mM based on the repeating glucose unit; this concentration is usually used in our complexation studies with polynucleotides. The change in the turbidity of the solution, which is induced by the precipitation and the intermolecular aggregation, was monitored by a change in the transmittance at 500 nm.

Table 1 summarizes the transmittance of the solutions. In the DS below 4.4 mol %, it was still difficult to dissolve curdlan in water completely; the sample solutions turned turbid after heating. On the other hand, when the DS increased up to 4.4 mol %, the samples dissolved well in water and the solutions were clear for at least 3 days. Furthermore, the sulfated curdlan samples with DS 8.7 and 76 mol % readily dissolved without

[†] DS is too low to give a reliable value of S for elemental analysis.



Scheme 1. Synthesis of the sulfated curdlan.

Table 1. Molecular mass data, DS, and transmittance of the sulfated curdlan

Sample code	M_n	M_w	M_w/M_n	DS (mol%) for the glucose unit[a]	Transmittance at 500 nm (%)
CUR-S(0)	18,000	24,800	1.38	0	59 (P)
CUR-S(1.7)	10,000	13,000	1.31	1.7 ± 0.2	73 (P)
CUR-S(2.2)	10,000	13,200	1.32	2.2 ± 0.3	69 (P)
CUR-S(4.4)	8500	12,200	1.44	4.4 ± 0.2	100 (S)
CUR-S(8.7)	8000	11,700	1.46	8.7 ± 0.3	100 (S)
CUR-S(76)	3100	5900	1.89	76 ± 1	100 (S[a])

P: precipitation, S: soluble, S[a]: soluble without heating.
[a]The DS was estimated by ICP analysis.

heating. It is worthy of note that introduction of only a few percent of sulfate groups changes the water solubility drastically.

2.4. Comparison of the complexation behaviors

When poly(C) is mixed with β-(1 → 3)-glucan polysaccharides, the circular dichroism (CD) spectra specifically change due to complex formation; the intensity at 275 nm is enhanced and a new band appears at around 242 nm.[4–6] The same spectral change was also observed for the 2-aminoethanol-modified SPG system,[17,18] indicating that the spectral changes are common among the complexes formed between β-(1 → 3)-glucan polysaccharides and poly(C).

Figure 2 compares the CD spectra at 5 °C for the sulfated curdlan sample, where [θ] is the molecular ellipticity. The CD spectra for the mixtures of poly(C) and the sulfated curdlan (except for CUR-S(76)) show the same spectral changes as mentioned above, although the increment in their CD intensity becomes smaller in the DS up to 4.4 mol%. This result suggests that these sulfated curdlans can also form complexes with poly(C). To get a better insight into the complexation ability, we compared the melting behaviors as shown in Figure 3. Although the CD spectra of the poly(C) complexes formed from CUR-S(0), CUR-S(1.7), and CUR-S(2.2) are the same at 5 °C (see Fig. 2), the melting temperatures (T_m) are slightly different; the T_m values decreased with an increase in the DS. When the DS further increased, CUR-S(4.4) and CUR-S(8.7) showed a decrement in T_m. On the other hand, the melting curvature of CUR-S(76) completely merged with that of poly(C) itself, indicating that no complex formation took place.

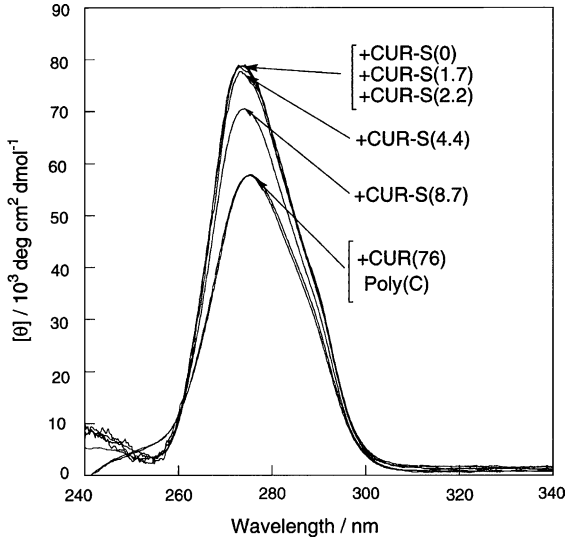


Figure 2. Comparison of the CD spectra at 5 °C for poly(C) and the sulfated curdlan samples.

Taking into consideration that (i) low-molecular-mass curdlans form complexes with poly(C) in the DS range from 1700 to 87,000 and; (ii) the modification does not take place at the C-2 hydroxyl group, it is likely that a high level of anion into curdlan inhibits the complexation. To estimate the modification effect quantitatively, we plotted the T_m values of the sulfated curdlan/poly(C) complex against their DS as shown in Figure 4. The T_m value decreases linearly with the increase in the DS indicating that the introduced sulfate groups destabilizes the complexes. Generally speaking, the electrostatic repulsion is much stronger in the nonsalt solutions; it is

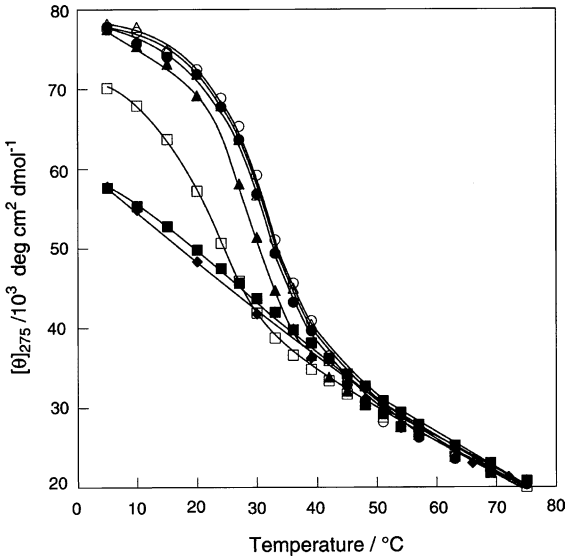


Figure 3. Comparison of the melting behaviors of the sulfated curdlan/poly(C) complexes. In the panel, poly(C) (◆), CUR-S(0)/poly(C) (○), CUR-S(1.7)/poly(C) (●), CUR-S(2.2)/poly(C) (△), CUR-S(4.4)/poly(C) (▲), CUR-S(8.7)/poly(C) (□), and CUR-S(76)/poly(C) (■), respectively.

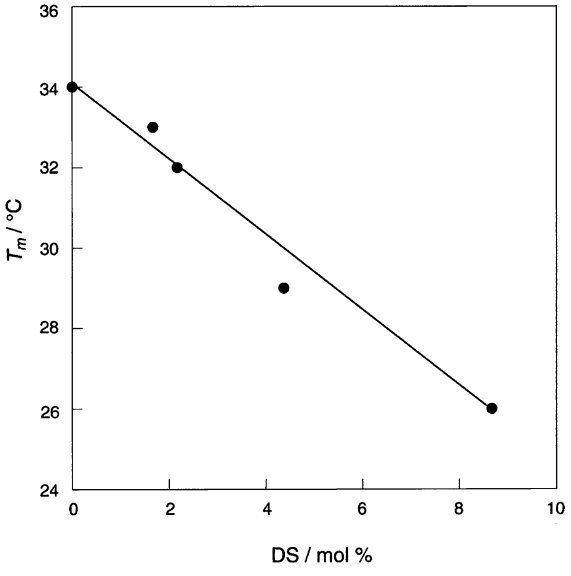


Figure 4. Relation between T_m of the complexes and their DS.

known that even DNA duplex cannot be formed in the nonsalt condition. Namely, the stability decrease shown in Figure 4 should be related to the electrostatic repulsion between the charges in the sulfate groups and poly(C) phosphate groups.

2.5. Salt concentration effect on the complex stability

To clarify whether the electrostatic repulsion destabilizes the complexes, we compared the T_m values from a relation between the thermal stability and the salt concentration. Since the electrostatic repulsion became smaller with the increase in the salt concentration, it is expected that the T_m could be enhanced with increasing NaCl concentration. Figure 5 shows the influence of the NaCl concentration on the T_m of the complexes formed between poly(C) and CUR-S(0), CUR-S(4.4), and CUR-S(8.7). In CUR-S(0), the increase in the NaCl concentration resulted in a maximum at around 50 mM similarly to the SPG/poly(C) system.[19] Although the T_m of CUR-S(4.4) was lower by 5 °C than that of CUR-S(0) at [NaCl] = 0 mM, the difference of T_m between CUR-S(0) and CUR-S(4.4) became smaller with an increase in the salt concentration and finally merged with that of CUR-S(0) at [NaCl] = 200 mM. The same phenomenon was also observed for CUR-S(8.7); the T_m increased with the concentration and merged with that of CUR-S(0) at [NaCl] = 200 mM. The results allow us to conclude that the electrostatic repulsion between the introduced sulfate group and the poly(C) phosphate group destabilizes the complex, but the effect becomes smaller with an increase in the salt concentration.

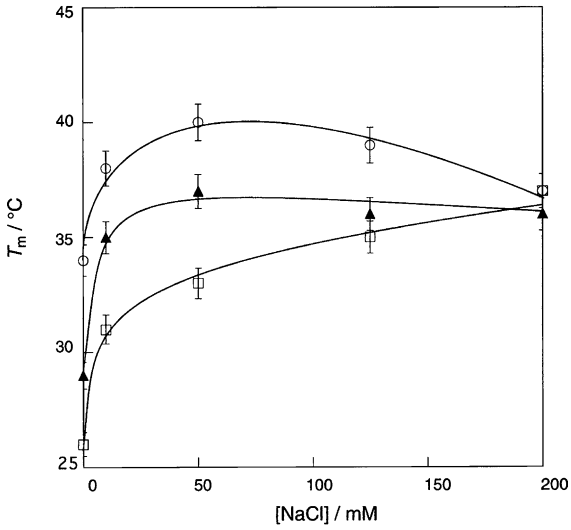


Figure 5. Salt concentration dependence of T_m. In the panel, CUR-S(0)/poly(C) (○), CUR-S(4.4)/poly(C) (▲), and CUR-S(8.7)/poly(C) (□), respectively.

By the way, when seeking to apply the sulfated curdlan to gene carrier problems, it is important to keep the salt concentration to some level and, furthermore, serum contains sodium chloride in the concentration range of 140–150 mM.[20] Such a high salt condition should suppress the electrostatic repulsion. Therefore, the stability decrease observed for the nonsalt condition should be inevitably negligible in serum; in other words, the present result supports the view that the partial sulfation is still a useful method in improving the water solubility without losing the complexation ability.

2.6. Resistance of the complexed polynucleotide chain against the enzymatic hydrolysis

To apply the sulfated curdlan/polynucleotide complex to gene carriers, we evaluated whether the complexed polynucleotide chain can resist the enzymatic hydrolysis.

The hydrolysis rate of the poly(C) chain bound to the sulfated curdlan by RNase A was evaluated as an increment of the absorbance at 260 nm according to the reported method,[21] which is ascribed to the production of cytidine-3′-monophosphate (CMP). In order to convert the absorbance at 260 nm to the CMP concentration, we used the following values for the extinction coefficient (ε): 9360 $cm^{-1}\,M^{-1}$ for CMP, 4850 $cm^{-1}\,M^{-1}$ for poly(C), 4530 $cm^{-1}\,M^{-1}$ for the CUR-S(4.4)/poly(C) complex, and 4560 $cm^{-1}\,M^{-1}$ for the CUR-S(8.7)/poly(C) complex, respectively. Figure 6 shows the CMP concentration versus incubation time plots at [NaCl] = 10 mM for poly(C), the CUR-S(4.4)/poly(C) complex, and CUR-S(8.7)/poly(C) complex. As can be seen, the complexation with the sulfated curdlan adequately suppressed the enzymatic hydrolysis although the protection effect was different between these two sulfated curdlan complexes. The hydrolysis rate constant was estimated from the initial slope in Figure 6 to be $4.7\times10^{-9}\,M\,s^{-1}$ for poly(C), $8.1\times10^{-10}\,M\,s^{-1}$ for the CUR-S(4.4)/poly(C) complex, and $1.3\times10^{-9}\,M\,s^{-1}$ for the CUR-S(8.7)/poly(C) complex. The present hydrolysis reaction was carried out in [NaCl] = 10 mM. At this salt concentration, the thermal stability brought by the added salt is not so high (vide ante). Therefore, the conformational perturbation due to the electrostatic repulsion still remains in the complex and the perturbed segment should be easier to be hydrolyzed by RNase A.

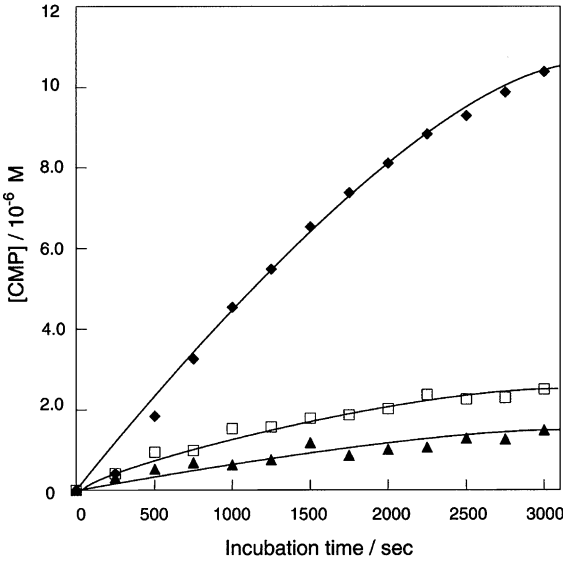


Figure 6. CMP concentration plotted against incubation time during hydrolysis by RNase A. In the panel, poly(C) (◆), CUR-S(4.4)/poly(C) (▲), and CUR-S(8.7)/poly(C) (□), respectively.

However, the hydrolysis rate of the sulfated curdlan complexes is still suppressed compared with that of the naked poly(C); the hydrolysis rate constants of the CUR-S(4.4)/poly(C) and CUR-S(8.7)/poly(C) complexes are smaller by one-sixth and one-fourth than that of poly(C), respectively. The present resistance effect against the enzymatic hydrolysis indicates that the sulfated curdlan can be well applicable to gene carriers.[‡]

3. Conclusion

Five sulfated curdlan samples with DS ranging from 0 to 76 mol% were prepared. The sulfated curdlan samples have improved water solubility. CD measurements revealed that those with DS from 1.7 to 8.7 mol% can form complexes with poly(C) as schizophyllan does. Although the thermal stability of the complex decreased by the introduction of sulfate groups due to electrostatic repulsion, it could be regained by salt addition. Furthermore, the complexed polynucleotide chain showed a significant resistance against enzymatic hydrolysis. Taking these results into consideration, we believe that curdlan could be used as functional material for gene technology.

4. Experimental

4.1. General

Curdlan was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Me_2SO with a spectroscopic grade and lithium chloride were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan), poly(C) and ribonuclease A (RNase A, E.C.3.1.27.5) from Amersham (NJ, USA), and pyridine–sulfur trioxide complex from Aldrich (WI, USA). All reagents were used without further purification. An RNase free and sterile water was used for all measurements. Sample solutions were prepared according to our previous reports.[5,16]

4.2. Syntheses of the sulfated curdlan

Curdlan used in this study ($M_w = 24{,}800$, $M_w/M_n = 1.38$) was prepared by acid hydrolysis according to the reported method.[7] The curdlan (200 mg) was dissolved in Me_2SO (50 mL) in the presence of LiCl (0.25 M) and stirred at 80 °C overnight. The pyridine–sulfur trioxide

[‡] We have confirmed that addition of CUR-S(76), which does not bind poly(C) but may interact with RNase A, scarcely changes the hydrolysis rate of poly(C). This means that in the present kinetic study the contribution of the sulfated curdlan–RNase A interaction is almost negligible.

complex was added to the solution while maintaining the temperature and stirred for 4 h under N_2. The reaction mixture was immediately cooled to room temperature and neutralized with 0.1 M aq. NaOH solution. The solution was dialyzed with distilled water for 1 week using a cellulose tube membrane ($M_w < 3500$ cut) and then lyophilized using an EYELA freeze dryer FD-5N. We thus obtained sulfated curdlan samples. To confirm the countercation, we carried out atomic absorption spectroscopy (Shimadzu AA-6700) for all samples and found that the molar concentration of sulfur determined by ICP is almost equal to that of sodium determined by atomic absorption spectroscopy. This coincidence indicates that the countercation of the sulfate group is sodium.

4.3. Determination of the content of the introduced sulfate group

The content of sulfate group was evaluated as the amount of sulfur% in the sample obtained from ICP analyses (Perkin Elmer Optima 3100RL). The calibration curve of sulfur was built using the sulfur oil standard solution (9949 ppm, ref. 44, 173-2 from Aldrich). The ICP analyses were carried out nine times for each sample and the average was used for the final value of the DS. The sample solutions for the ICP analysis were prepared as follows: the sulfated curdlan sample (2.0 mg) was dissolved in DMF (0.3 mL) and the mixture was diluted with distilled water (9.7 mL).

4.4. SEC analysis

The molecular mass was evaluated by SEC using a Tosoh HLC-8020; apparatus: two α-4000 columns are connected in series, LiBr (20 mM)/DMF was used as elution solvent, and the instrument was calibrated by a polyethyleneoxide standard (Tosoh Co., Japan).

4.5. Water-solubility test

The water solubility of the sulfated samples was estimated as a function of transmittance of the sulfated curdlan in water solution. The transmittance was recorded on a Shimadzu UV-2500 spectrometer with a 1 cm cell. The sample was prepared as follows: after dissolution of the curdlan sample (1.0 mg) in distilled water upon heating, the sample was cooled to room temperature, aged for 3 days, and then the transmittance was estimated at 500 nm.

4.6. ^{13}C NMR spectra

The NMR spectrum was recorded on a Bruker DRX600 spectrometer at 37 °C. A sample for the NMR measurements contained 70 mg of the curdlan sample dissolved in 0.75 mL of D_2O and 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as an internal standard. Assignment of each peak was confirmed using ^1H–^{13}C correlation spectroscopy (COSY).

4.7. Enzymatic hydrolysis of poly(C) chain

The increment of the CMP concentration arising from the hydrolysis of poly(C) chain by RNase A was monitored on a UV–vis spectrometer with a 1 cm cell (JASCO V-570UV/VIS/NIR spectrometer). The stoichiometric curdlan/poly(C) complex (six glucose units form complex with three cytosine residues), including Tris (pH 8.0), Me_2SO, and NaCl, was prepared and aged at 4 °C for at least 3 days. After the RNase A soln was added to the sample soln, the incubation was started at 10 °C. This temperature was chosen so as to be lower than the dissociation temperature of the corresponding complexes. The final concentrations were adjusted as follows: $V_w = 0.92$, where V_w is the water vol fraction in the Me_2SO water mixture, [poly(C)] = 0.10 mM/repeating unit, [curdlan] = 0.24 mM/repeating glucose unit, [Tris] = 0.8 mM, [NaCl] = 50 mM, and [RNase A] = 2.0×10^{-4} g L^{-1}.

Acknowledgements

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