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NMR Studies on Structure and Action Mechanism of Sulfated Dodecyl Laminaripentaoside with High Anti-Human Immunodeficiency Virus Activity

Kaname KATSURAYA, Kwan-Jun JEON, Hideki NAKASHIMA,* and Toshiyuki Uryu†

Institute of Industrial Science, University of Tokyo, 7–22–1 Roppongi, Minato-ku, Tokyo, 106–8558, Japan
**Kagoshima University, Dental School, 35–1 Sakuragaoka, Kagoshima 890–8544, Japan*

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ABSTRACT: Anti-HIV (human immunodeficiency virus) active sulfated dodecyl laminaripentaosides having various degrees of sulfation were synthesized and their structures analyzed by 2D NMR spectroscopic measurements such as C–H direct, C–H long-range, H–H direct, NOESY, and HOHAHA. The 6-hydroxyl was first sulfated, followed by the sulfation of the 2-hydroxyl and the 4-hydroxyl, in the sulfation of dodecyl laminaripentaoside. Interactions between sulfated dodecyl laminaripentaoside and poly- or oligo-lysine as a virus protein model compound were analyzed. The reason why high degree of sulfaton was necessary for obtaining high anti-HIV activity in vitro was strong ionic interactions existed between oligo-lysine and sulfated laminaripentaosides with high degree of sulfation. In the gel formed by the interaction the local motion of the sulfated oligosaccharide was restricted to a large extent, whereas that of the alkyl portion was not restricted so much.

KEY WORDS Nuclear Magnetic Resonance / Sulfated Alkyl Oligosaccharide / Anti-Human Immunodeficiency Virus Activity / Degree of Sulfation / Ionic Interaction / Polylysine /

Since the discovery of human immunodeficiency virus (HIV), great efforts have been made to elucidate the mechanisms of its infection and growth, as well as treatment against the aquired immunodeficiency syndrome (AIDS). Following the development of nucleic acid reverse transcriptase inhibitors,[1-8] virus protease inhibitors[9-12] were developed since 1995.

Synthesis of highly anti-HIV active compounds has been studied by use of sulfated poly- and oligo-saccharide derivatives. It has been found that, a (1→3)-β-glucan (curdlan) sulfate and a sulfated alkyl laminari-oligosaccharide have high anti-HIV activity but low cytotoxicity.[13,14] It has also been revealed that the high anti-HIV activity of curdlan sulfate, depends on the molecular weight and the degree of sulfation.[15,16] The activity of sulfated alkyl oligo-saccharides was affected by structual factors such as sugar length, type of sugar structure, type of alkyl group, and hydrophobicity.[17-21]

The degree of sulfation was the most important in producing the compound with high anti-HIV activity. Consequently, a fully sulfated dodecyl laminaripentaoside is an optimum compound which possesses the highest anti-HIV activity and lowest cytotoxicity in addition to very low anticoagulant activity.

So far, for curdlan sulfate, effects of the position and degree of sulfation on the biological activities have been examined in detail.[22-24] This study reports structural analysis of sulfated dodecyl laminaripentaosides with different degrees of sulfation using two-dimensional NMR measurements.

To examine an action mechanism of the sulfated dodecyl laminaripentaoside (L5C12S) as an anti-HIV active agent, interactions of L5C12S's having different degrees of sulfation with polylysine, a model compound for virus proteins, were measured by NMR spectroscopy. Based on the results, we discuss necessity of the high degree of sulfation to cause high anti-HIV activity.

RESULTS AND DISCUSSION

Structural Analysis of Sulfated Dodecyl Laminaripentaoside by NMR Spectroscopy

Structures of sulfated dodecyl laminaripentaoside having different degrees of sulfation were analyzed using 2D-NMR technique. NMR absorptions of unsulfated dodecyl laminaripentaoside (L5C12) were assigned on the basis of the assignment of laminaripentaoside. For persulfated dodecyl laminaripentaoside (L5C12S) with degree of sulfation (DS) of 3, NMR signals were assigned by various 2-D NMR measurements such as CHCOSY, C–H long-range (HMBC), HHCOSY, NOE, and HOHAHA. Structures of sulfated dodecyl laminaripentaosides are exhibited as well as polylysine in Figure 1. The PCHCOSY spectrum for L5C12S as a C–H direct relation is shown in Figure 2. Since secondary carbon absorptions appeared as negative signals in the PCHCOSY spectrum, the methylene carbons in both alkyl group and C6 position were explicitly assigned.

PDQF-COSY (H–H direct), PROESY, and PTOCSY spectra for L5C12S are shown in Figure 3. The PROESY spectrum showing interactions between nuclei positioned in short spatial distance was used to assign proton absorptions due to H1, H3, and H5 in the glucose residue. H1 and C1 absorptions were decided first, because these signals appeared at the lowest magnetic field. H2 and H4 protons, H3 and H5 protons, and H6 proton were assigned by PDQF-COSY, PROESY, and a combination of CHCOSY and DEPT, respectively. Subsequently, individual carbon absorptions were assigned using proton assignment and CHCOSY. ^{1}H and ^{13}C assign-

† To whom correspondence should be addressed.

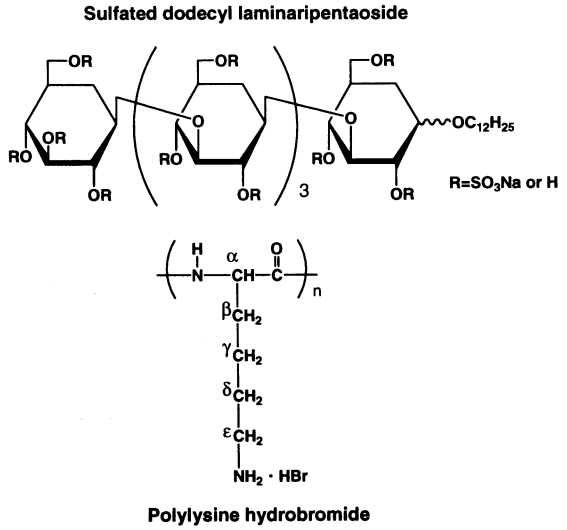

Figure 1. Structures of compounds.

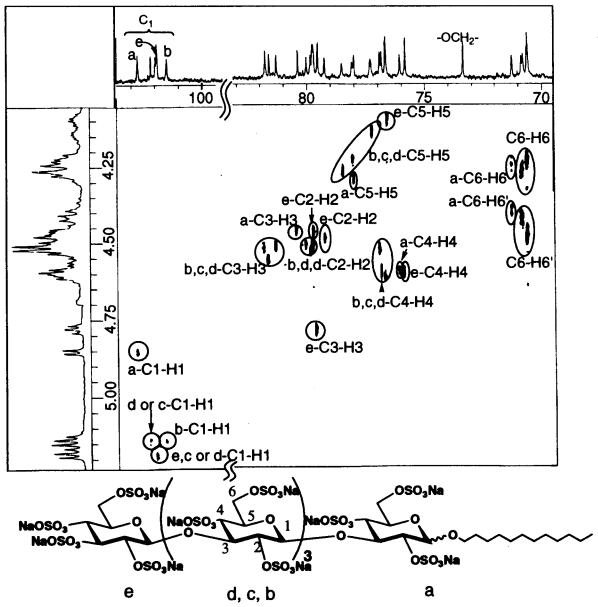

Figure 2. PCHCOSY NMR spectrum of sulfated dodecyl laminaripentaoside (DS = 3.0).

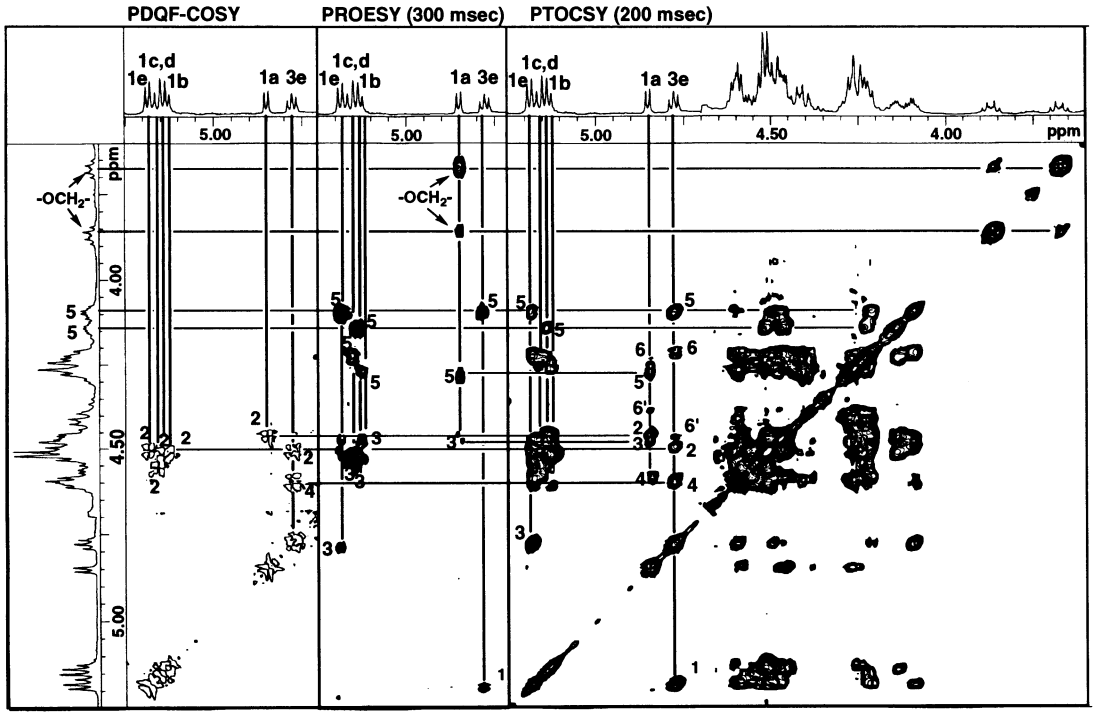

Figure 3. 500 MHz PDQF-COSY, PROESY, and PTOCSY spectra of sulfated dodecyl laminaripentaoside (DS = 3.0) in D_2O at 27°C.

ments are summarized in Table I.

The structure of sulfated alkyl oligosaccharides with DS of 0.7, 1.4, and 2.4 was analyzed. Since ^{13}C NMR failed to afford significant signals, the ^{1}H observation mode (inverse mode) was applied in C–H direct relation. To improve the S/N ratio and resolution, the phase-sensitive mode with field-gradient spin-lock purge pulse was used. Analysis of sulfated alkyl laminaripentaosides revealed the order of sulfation in the three hydroxyl groups. Results of the ^{13}C assignment in the sulfations are shown in Figure 4.

1) OH6s of primary alcohols were sulfated selectively. C6s of reducing and non-reducing ends were sulfated more easily than other OH6s. At DS of 0.7, no secondary alcohol was sulfated.

2) In the case of DS = 1.4, 95% of OH6s and partially OH2s and OH4s were sulfated, but no tri-substituted glucose unit was detected at this degree of sulfation.

3) All OH6s, about 70% of OH2s and 60% of OH4s were sulfated at DS = 2.4. Since the order of substitution was the same as the order of sulfation of curdlan, it might depend on steric hindrance mainly.

Table I. ^{1}H and ^{13}C NMR chemical shift assignments of persulfated dodesyl laminaripentaoside (L5C12S)

Glucose unit	Chemical shift/ppm						
	C_1 H_1	C_2 H_2	C_3 H_3	C_4 H_4	C_5 H_5	C_6 H_6	$H_{6'}$
a	102.70	79.69	80.31	76.05	77.97	71.25	
	4.85	4.48	4.47	4.60	4.30	4.25	4.41
b	101.49	79.69	81.25	76.93	77.41	70.58	
	5.14	4.52	4.52	4.61	4.14	4.23	4.50
c	101.92	79.77	81.56	76.85	78.15	70.76	
	5.18	4.54	4.57	4.53	4.24	4.26	4.43
d	102.13	80.00	81.73	76.80	78.56	70.81	
	5.15	4.52	4.53	4.62	4.27	4.27	4.42
e	102.92	79.26	79.43	75.75	76.68	70.58	
	5.19	4.49	4.78	4.61	4.10	4.23	4.50

Chemical shift assignments of C_1H_1 (c and d), C_2H_2–C_5H_5 (b, c, and d) still have not been completed.

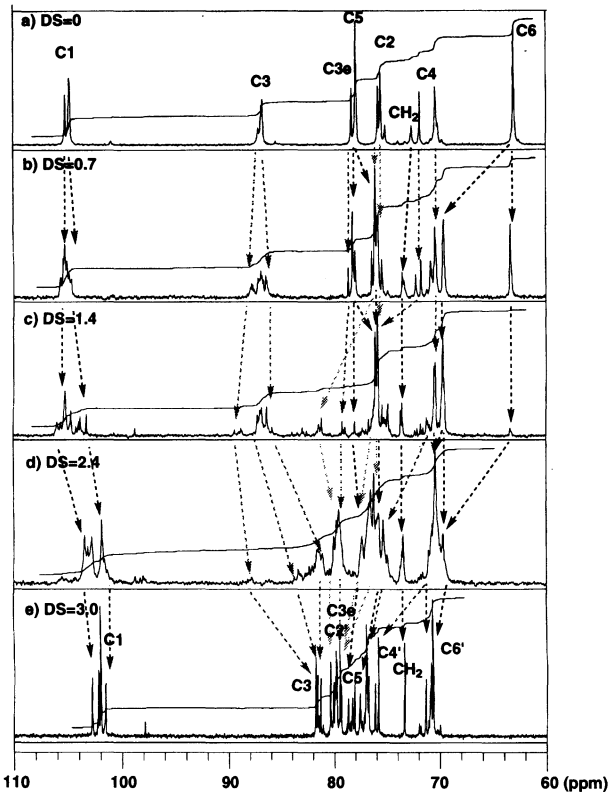

Figure 4. 125 MHz ^{13}C spectra of a) dodecyl laminaripentaoside, and sulfated dodecyl laminaripentaoside, b) DS = 0.7, c) DS = 1.4, d) DS = 2.4, and e) DS = 3.0 in D_2O at 25°C.

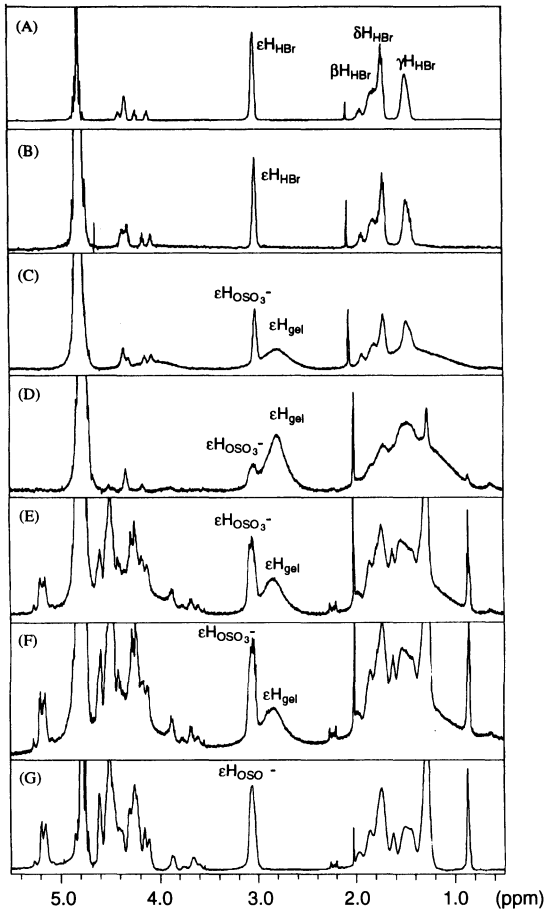

Figure 5. 500 MHz ^{1}H NMR spectra of (A) poly-L-lysine hydrobromide (PL) and polyioncomplexs between sulfated dodecyl laminaripentaoside (L5C12S, DS = 3.0) and PL on different ion ratios (B) [L5C12S]/[PL] = 0.5, (C) 0.8, (D) 1.0, (E) 1.5, (F) 2.0, and (G) 4.0 in D_2O at 22°C.

Action Mechanism Analysis of the Interaction between Sulfated Dodecyl Laminaripentaoside and Polylysine as a Virus Protein Model

The anti-HIV activity of the sulfated alkyl oligosaccharide is assumed to be caused by interactions with virus protein sequence or virus lipid bilayer. For a sulfated polysaccharide, ionic interactions between a negatively charged sulfated polysaccharide and positively charged polypeptide were assumed to be the force causing the anti-HIV activity by measuring NMR spectra of the gel formed by the interaction.[25–27] Choosing polylysines as the model compound for a positively charged virus protein sequence, interactions between sulfated dodecyl laminaripentaoside L5C12S and polylysines were examined by NMR spectroscopy.

When polylysine with $\bar{M}_n$ of 8000 was mixed with L5C12S, complex formation occurred. However, probably because the complex was almost immobile, faint NMR signals were obtained, giving no information on the structure and interaction of the complex. Polylysine

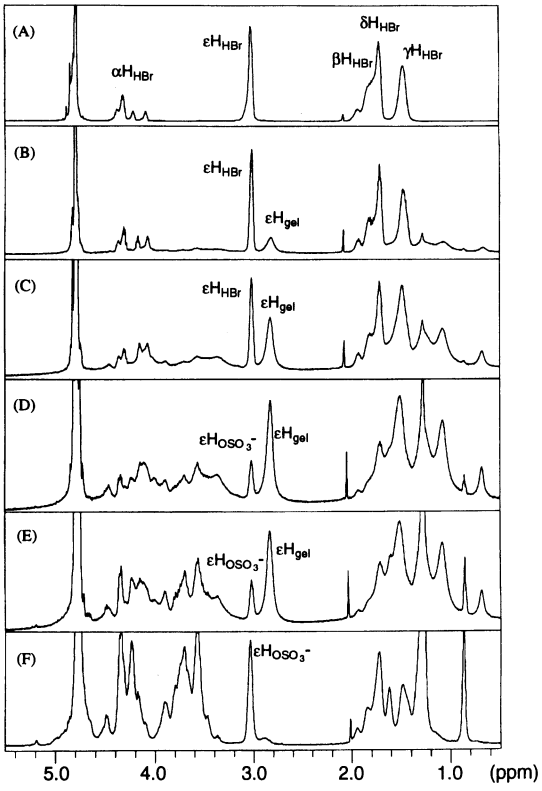

Figure 6. 500 MHz ^{1}H NMR spectra of poly-L-lysine hydrobromide (PL) and polyioncomplexes between sulfated dodecyl laminaripentaoside (L5C12S, DS = 1.4) and PL on different ion ratios (B) [L5C12S]/[PL] = 0.5, (C) 1.0, (D) 1.5, (E) 2.0, and (F) 4.0 in D_2O at 22°C.

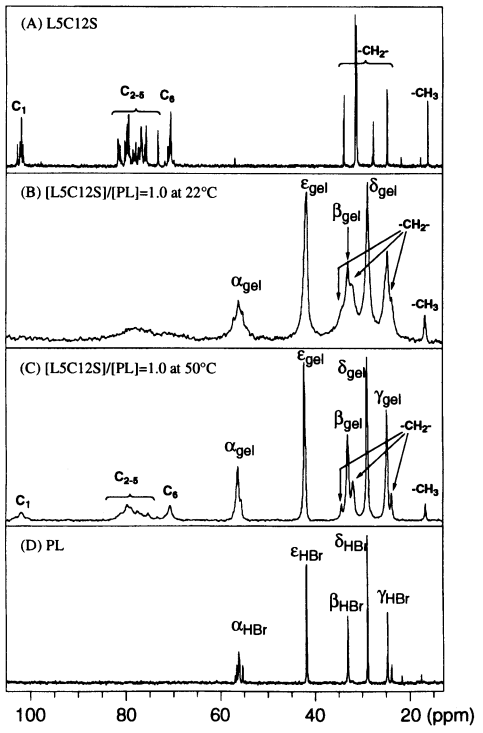

Figure 7. 125 MHz ^{13}CNMR spectra of (A) sulfated dodecyl laminaripentaoside (L5C12S, DS = 3.0) at 22°C, (B) polyioncomplexes between L5C12S and poly-L-lysine hydrobromide (PL) at 22°C, (C) polyioncomplexes between L5C12S and PL at 50°C, and (D) PL at 22°C in D_2O.

with $\bar{M}_n$ of 4000 interacted with L5C12S to produce gellike complex the NMR spectrum of which exhibited absorptions attributable to the new compound, as shown in Figure 5.

When the molar ratio [L5C12S]/[PL] was 0.8 (Figure 5(C)), a new ^{1}H NMR absorption due to ε CH_2 protons of the lysine side chain included in the gel appeared at 2.82 ppm in addition to the original peak at 3.01 ppm (Figure 5(A)). This peak corresponded to the formation of gellike material visibly observed. This assignment was confirmed by disappearance of the absorptions assingned to the gellike material by removing off the materials. As the ratio increased to 1.0, the peak intensity reached maximum, and the proportion of the gel was 92%.

In the case of the molar ratio more than 1.0, the ε methylene absorption shifted downfield by about 0.04 ppm. The downfield shift might be ascribed to change in the polarity of solvent caused by excess sulfate anions. Since the amino group of the lysine side chain existed in the form of HBr salt, the counter anion Br^- must have been changed into the sulfate anion.

To clarify the reason for low anti-HIV activity of sulfated dodecyl laminaripentaoside (L5C12S') with low degree of sulfation of 1.4, interactions with polylysine having $\bar{M}_n$ of 4000 were investigated by NMR. Similarly to the case of L5C12S with the high degree of sulfation, formation of gellike material was observed by mixing the two compounds. As shown in Figure 6, a gellike material was detected by ^{1}H NMR.

Gel absorptions such as εH_{gel} formed from L5C12S' with DS of 1.4 appeared at higher mangetic field than that of the original peak. There was a clear difference between the compound with a low DS and that with a high DS. The peak due to the former was a sharper peak possessing smaller half-height width than that of the gel formed from the compound L5C12S with a high DS. This sharp peak might indicate that weak ionic interactions occur between the sulfated compound and polylysine to produce weakly crosslinked networks. It is assumed that the weak intermolecular interaction resulted in decrease in the anti-HIV activity of L5C12S'.

As shown in Figure 7, the gel formation and information on molecular motions in the gel were obtained from ^{13}C NMR spectroscopy. For the molar ratio of 1.0 producing the maximum proportion of the gel, absorptions due to the sugar moiety of L5C12S appeared as a very broad peak around 70 to 85 ppm (Figure 7(B)). This reveals that local motions of the sugar moiety were suppressed by intermolecular interactions. Similarly, absorptions due to polylysine were fairly broad. There were thus interactions between polylysine and sulfated sugar moiety. When the temperature was raised to 50°C, all carbon absorptions became sharper than those at 22°C, showing that the molecular motion in the gel increased, with keeping the gel state (Figure 7(C)).

EXPERIMENTAL

General

All NMR spectra were recorded on a JEOL LA-500 spectrometer attached field gradient unit operating at 500 MHz. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as the internal standard. Laminaripentaose was kindly supplied from Dainippon Ink

and Chemicals, Inc. Lysine dimer hydrochloride, oligolysine acetates (from trimer to pentamer) and poly-L-lysine hydrobromide ($\bar{M}_n$=4000 and 8000, measured by viscosity) were purchased from Sigma. Deuterium oxide (D_2O, 99.8%) was purchased from E. Merck.

Structural Analysis of Sulfated Dodecyl Laminaripentaoside by NMR Spectroscopy

Sulfated dodecyl laminaripentaosides were prepared according to our previous papers.[14,17,21] A sample for NMR measurement contained 50—200 mg of sulfated dodecyl laminaripentaoside (L5C12S) dissolved in 0.55 mL of D_2O. DQF-COSY (double quantum filtered correlated spectroscopy), ROESY (rotating frame nuclear Overhauser effect spectroscopy), and TOCSY (total correlation spectroscopy) spectra were used for proton signal assignments of L5C12S. TOCSY spectra were measured at various mixing times, the most useful being 200 ms for classification of each glucose unit. ROESY spectra were also observed at mixing time of 300 ms. C–H correlations were examined by using of C–H COSY, HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear mutiple bond connectivity) spectra. DQF-COSY, TOCSY, ROESY, C–H COSY, and HSQC spectra were measured in the phase sensitive mode. DQF-COSY, HSQC, and HMBC spectra were measured with field gradient pulse. In the case of proton–proton 2D NMR, the HOD signal was suppressed by presaturation. For all 2D NMR, 256 spectra of 1024 data point or 512 spectra of 2048 data point were recorded with 1—192 scans. Apodization was carried out for each domain using exponential function with trapezoidal function of T1=0, T2=0, T3=70, T4=100% for phase sensitive measurement, sine-bell function for homonuclear 2D spectrum, and Blackman–Harris function for heteronuclear 2D spectrum. The temperature of all spectra of this section was 22°C.

Action Mechanism Analysis of Interaction of Sulfated Alkyl Oligosaccharide and Polylysine as a Virus Protein Model

For proton NMR, 250 μL of L5C12S solutions with different concentrations were added to 250 μL of 2.0% (w/v) solution of lysine compounds in D_2O in a NMR tube and mixed for a few minutes using of a test tube mixer (Model YM-121, Yamato Scientific Co., Ltd.). The solution was kept at 40°C for 1 h horizontally. For ^{13}C NMR, a 10% (w/v) solution of lysine compounds was measured in an 8 mm raised-bottom NMR tube to prevent the gellike complex from precipitating during long time accumulation.

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