博 士 論 文
Doctoral Thesis

Elucidation of anti-HIV mechanism of
sulfated glycodendrimers and alkyl oligosaccharides

硫酸化アルキルオリゴ糖鎖および糖鎖デンドリマーの
抗 HIV **メカニズムの解明**

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Preface

This thesis deals with the elucidation of anti-HIV mechanism in regard to the cluster effect of sulfated oligosaccharides and the role of long-chain alkyl group in sulfated alkyl oligosaccharides with potent anti-HIV activity. Oligosaccharide is a key word through this thesis. Since Schuerch reported for the first time in 1966 ring-opening polymerization of an anhydro glucose monomer, benzylated 1, 6-anhydro α-D-glucopyranan into a stereoregular polysaccharide after debenzylation to recover hydroxyl groups, (1→6)-α-D-glucopyranan (synthetic dextran) bearing stereoregularity, many synthetic polysaccharides are reported. Because synthetic polysaccharides have well-defined structures by distinct from naturally occurring polysaccharides bearing complex structures, it is convenient to know the relationship between the structure of polysaccharides and biological activities. The biological activities of sulfated polysaccharides such as blood anticoagulant and anti-HIV activities are dependent on their molecular weights, in general, the higher molecular weights give the higher biological activities. Sulfated polysaccharides exhibited the potent antiviral activities such as HIV, influenza, and dengue viruses and the biological mechanism was elucidated attributed to the electrostatic interaction between negatively-charged sulfated groups in sulfated polysaccharides and positively-charged amino acids in the viral surface glycoproteins.

There are still two undissolved problems on biological activities of sulfated poly- and oligosaccharides such as antiviral mechanism of sulfated polysaccharides and role of long-chain alkyl group on the antiviral activity. The answer of the first problem should be an electrostatic interaction and elucidated by SPR measurements between oligopeptides and sulfated polysaccharides. The second problem is assumed by the interaction with lipid bilayer of viruses. However, antiviral mechanism of oligosaccharides doesn't completely dissolved, especially, cluster

effect and cytotoxicity. Therefore, the author wants to dissolve the two unclear problems of sulfated oligosaccharides.

The thesis consists in four chapters. The first chapter is instruction. The second chapter is on the elucidation of anti-HIV mechanism of sulfated cellobiose–polylysine dendrimers. The third chapter is on the relationship between anti-HIV activity and cytotoxicity of sulfated alkyl oligosaccharides. The last chapter is conclusions. In the second chapter, the author investigate the elucidation of anti-HIV mechanism of sulfated glycodendrimers by using newly synthesized sulfated cellobiose–polylysine dendrimers 1st, 2nd, and 3rd generations. Sulfated oligosaccharide-bearing dendrimers also had potent anti-HIV activity although sulfated oligosaccharide alone had low anti-HIV activity. It was found that the EC_{50} (50% effective concentration of sulfated polysaccharides on prevention of virus infection) values were 3.7, 0.6, and 1.5 μg/mL, respectively. The second-generation dendrimer was the most active, suggesting that the moderate distance between the terminal sulfated cellobiose units in the second generation dendrimer favored the high anti-HIV activity owing to the most effective electrostatic interactions developed due to the cluster effect of the sulfated cellobiose unit revealed by the SPR measurements. These biological results suggest that the distance between sulfated sugar units plays an important role in the anti-HIV. In the third chapter, the author exactly investigated the anti-HIV activity and cytotoxic mechanism of sulfated alkyl oligosaccharides. Anti-HIV mechanism of sulfated alkyl oligosaccharides was recently reported by our lab that the long-chain alkyl group was penetrated into the lipid bilayer of HIV and then sulfated maltoheptaoside portion was electrostatically interacted with HIV gp120 to inhibit the infection of HIV in vitro. However, it was reported previously that the cytotoxicity increased with increasing the length of the long-chain alkyl groups. The cytotoxic mechanism of sulfated alkyl oligosaccharides is still unclear. therefore, several sulfated alkyl maltoheptaosides were newly synthesized by using click reaction and the interaction

with liposome as a model of HIV gp120 was investigated, indicating that the longer chain alkyl group gave higher association- (k_a) and lower dissociation-rate (k_d) constants by SPR measurements. These results suggest that the longer chain alkyl group in sulfated alkyl maltoheptaosides was strongly penetrated into the lipid bilayer of cells and the cytotoxicity was expressed.

In this thesis, high anti-HIV and cytotoxic mechanisms of sulfated cellobiose polylysine dendrimers and sulfated alkyl oligosaccharides were evaluated. The results lead to a more extensive investigation into the adaptability to the development of safety biomaterials.

Song Weiyue

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Literature of this thesis

1. Song, W., Li, Y., Kanamoto, T., Asai, D., Takemura, H., Nakashima, H., Miyazaki, K., Yoshida, T. (**2020**). Elucidation of anti-HIV mechanism of sulfated cellobiose−polylysine dendrimers by SPR and DLS. *Carbohydr*. *Res*., **495**, 108084.

2. Song, W., Asai, D., Takemura, H., Nakashima, H., Miyazaki, K., Yoshida, T. (**2021**). Relationship between anti-HIV activity and cytotoxicity of sulfated alkyl oligosaccharides. *Carbohydr*. *Polym*., to be submitted.

Table of Contents

Chapter 1
Background of this research

1.1. Sulfated polysaccharides with biological activities.

Professor Yoshida of my supervisor recently appeared a review on anti-HIV mechanism of sulfated poly and oligosaccharides, which review focuses on the previous and recent results as well as related literatures regarding the anti-HIV mechanism of sulfated alkyl poly- and oligosaccharides [Yoshida, 2020]. Polysaccharides as well as proteins and nucleotides are one of three biomacromolecules and are also the most abundant natural resource on the earth. All of the biomacromolecules are produced in living organisms and play significant and functional roles in a vital support. Polysaccharides have been used from ancient time as foods, fibers, and construction materials. In particular, a naturally occurring sulfated polysaccharide with potent blood anticoagulant activity, heparin, which found in liver, lung, intestine, and other tissues, is the most popular sulfated polysaccharide for medical purposes [Lane and Lindahl, 1989]. In Captor 1, the author describes on functionality of sulfated poly- and oligosaccharides related to his doctoral researches based on Professor Yoshida's review [Yoshida, 2020; 2019].

Figure 1-1 shows the pentasaccharide residue in the polysaccharide chain of heparin. The

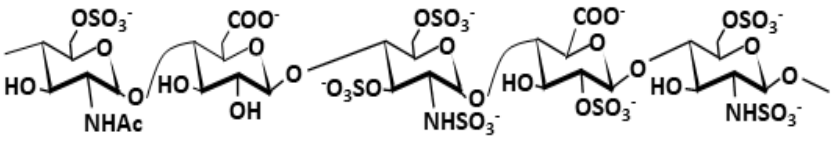


Figure 1-1.
Structure of heparin pentasaccharides residue.
Heparin is electrostatically interacted with antithrombin III, a protease inhibitor, to form heparin-antithrombin III complex that inhibits the activity of thrombin.

blood anticoagulant mechanism of heparin was reported by Lindahl [Lindahl, 1983], indicating that the negatively charged pentasaccharide residue was electrostatically interacted with the positively charged lysine site of a protease inhibitor Antithrombin III to form the heparin- Antithrombin III

complex that inhibits the activity of a blood coagulant factor Thrombin to prevent blood coagulation [Ginsberg and Robbins, 1991]. The blood anticoagulant activity of heparin was firstly reported by Rosenberg and Damus in 1973 [Rosenberg and Damus, 1973]. The Antithrombin III and thrombin form a 1: 1 stoichiometric complex which cannot be dissociated with denaturing and reducing agents. Addition of heparin, a widely used anticoagulant which specifically accelerates the action of inhibitor, increases the rate of formation of this complex without altering its stoichiometry or its dissociability. Heparin binds to lysyl residues of the inhibitor and accelerates this reaction as illustrated in Figure 1-2. In the first step of

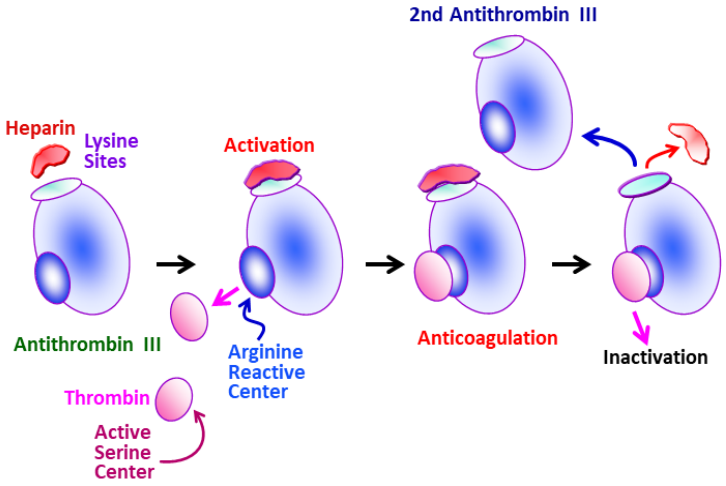


Figure 1-2
Anticoagulant mechanism of heparin.
Antithrombin III is activated by heparin to express potent blood anticoagulant activity.
(Marcam and Rosenberg, Adapted from Biology of Carbohydrates (1991) Vol. 3.)

the blood anticoagulation, heparin electrostatically binds to the lysyl site on the antithrombin III, a protease inhibitor, to activate the antithrombin III, which activated heparin-antithrombin III complex interacts with thrombin through the arginine reactive center of the complex and active serine center of thrombin to reduce the activity of thrombin, a blood coagulation factor. After stopping the activity of thrombin, the antithrombin III detached the thrombin and heparin to be back

original antithrombin III. The dramatic increase in the rate of interaction is probably due to a heparin-dependent conformational alteration of the inhibitor which renders the reactive site arginine more accessible to the active center serine of thrombin.

In 1958, sulfated polysaccharides isolated from sea alga were found to inhibit the increase of influenza B virus [Gerber, 1958]. Polysaccharides extracted from the seaweed, *Gelidium cartilagenium*, were found to exert an inhibitory effect on growth of influenza B and mumps viruses in embryonated chicken eggs, however, no effect on the multiplication of influenza A and Newcastle disease viruses. The results suggest that the polysaccharides may act intracellularly and were rather specific.

Sulfated polysaccharides are known to have specific biological activities and revealed recently on the inhibitory effect of HIV multiplication. Nakashima and Yamamoto found for the first time in 1987 that naturally occurring sulfated polysaccharides extracted from sea red alga showed potent anti-HIV activity [Nakashima and Yamamoto, 1987a; 1987b]. An aqueous extract was tested for the inhibitory effect of reverse transcriptase (RT) from avian and mammalian retroviruses, respectively, indicating that the extract was found to inhibit the activity of the RTs. The extracts were sulfated polysaccharides bearing high molecular weights approximately 2×10^6 Da. Chondroitin, dermatan, heparin, keratin sulfates, and heparin also inhibited the RT of avian mycloblastosis viruses. Sulfated polysaccharides may suppress HIV infection by interfering with virus adsorption as well as by inhibiting RT.

It was found for the first time in 1987 that sulfated synthetic polysaccharides completely inhibited the infection (EC_{100}) of HIV in the concentration as high as $EC_{100}<3.3$ μg/mL, respectively, (Figure 1-3) [Nakashima, 1987c]. The completely inhibited concentration (EC_{100}) is equal to below 1.0 μg/mL for 50% effective concentration (EC_{50}). Sulfated polysaccharides, ribofuranan, xylofuranan, and dextran sulfates were synthesized by the ring-opening polymerization of the

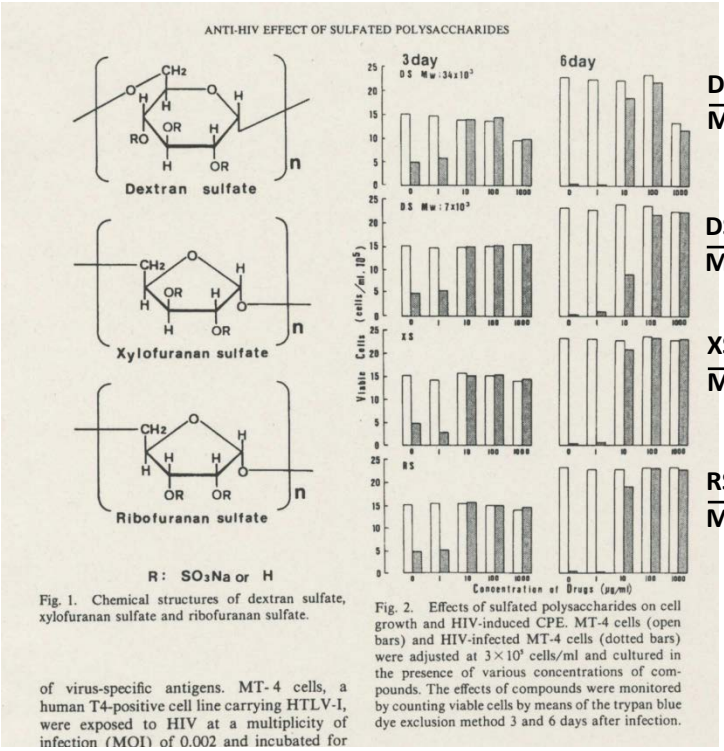


Fig. 1. Chemical structures of dextran sulfate, xylofuranan sulfate and ribofuranan sulfate.

of virus-specific antigens. MT-4 cells, a human T4-positive cell line carrying HTLV-I, were exposed to HIV at a multiplicity of infection (MOI) of 0.002 and incubated for

Fig. 2. Effects of sulfated polysaccharides on cell growth and HIV-induced CPE. MT-4 cells (open bars) and HIV-infected MT-4 cells (dotted bars) were adjusted at 3×10^5 cells/ml and cultured in the presence of various concentrations of compounds. The effects of compounds were monitored by counting viable cells by means of the trypan blue dye exclusion method 3 and 6 days after infection.

Figure 1-3.
Anti-HIV activity of synthetic sulfated polysaccharides.

corresponding 1, 4-anhydro ribose, xylose, and 1, 6-anhydro glucose monomers, and subsequent deprotection to recover hydroxyl groups and then sulfation, respectively. These sulfated synthetic polysaccharides were found to have high anti-HIV activity and low cytotoxicity. However, the blood anticoagulant activity was relatively higher compared with that of standard dextran sulfate (AA=22.7 unit/mg). For cytotoxicity, although dextran sulfate showed 20−30% growth inhibition of HIV-uninfected MT-4 cells at 1000 μg/mL, xylofuranan and ribofuranan sulfates exhibited no effect on the MT-4 cell growth at this concentration. All of the synthetic sulfated polysaccharides efficiently inhibited the reverse transcriptase activity of avian myelobiastosis virus and HIV.

1.2. Curdlan sulfate as an potent inhibitor of HIV.

It was found previously curdlan sulfate has both potent anti-HIV activity and low cytotoxicity

in vitro in a MT-4 cell that is a HIV sensitive cell line from dog's kidney cell [Pauwels, 1988], and low blood anticoagulant activity that is a side-effect on the anti-HIV drugs, Therefor, curdlan sulfate as well as dextran sulfate has been used as a positive standard for sulfated polysaccharides with antiviral activities. The high anti-HIV activity gives small EC_{50} values and low cytotoxicity large CC_{50} values. The completely inhibited concentration (EC_{100}) is equal to below 1.0 μg/mL for 50% effective concentration (EC_{50}). In addition, the large blood anticoagulant activity (AA) represents large AA values.

Naturally occurring curdlan has a linear (1→3)-β-D-glucopyranosidic structure produced by *Alcaligenes faecalis var*. *myxogenes* 10C3 strain [Harada, 1992]. As described in the section 1-2, based on the findings of inhibitory effect of naturally occurring and synthetic sulfated polysaccharides against HIV infectivity, curdlan was sulfated to give curdlan sulfates bearing several molecular weights and different sulfur contents (Figure 1-4) [Yoshida, 1990; Kaneko, 1990].

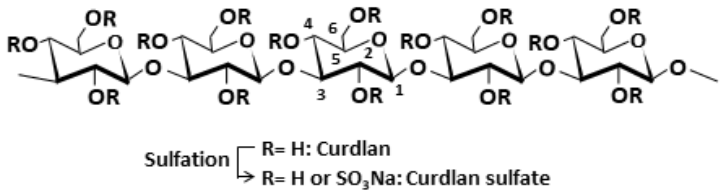


Figure 1-4.
Synthesis of curdlan sulfate by sulfation of naturally occurring curdlan. Curdlan is a naturally occurring polysaccharide and curdlan sulfate was synthesized by sulfation of curdlan. Curdlan sulfate had high anti-HIV activity (EC_{100}=3.3 μg/mL), low cytotoxicity

It was found that curdlan sulfate bearing a sulfur content of 14.1% and molecular weight of 2.1×10^3 completely inhibited the infection of HIV into MT-4 cell in the concentration as low as 3.3 μg/mL (EC_{100}). The cytotoxicity was quite low against MT-4 cell up to 5000 μg/mL. The sulfate groups were introduced to C6, C4, and C2 hydroxyl groups in the glucose unit in the proportion of ~100%,

~5%, and ~40%, respectively, by the high resolution two dimensional NMR measurements. The hydroxyl group at the C6 position is a primary alcohol and has a relatively lower steric hindrance than those of the C2 and C4 hydroxyl groups.

In addition, to investigate the relationship between the position of sulfate group and anti-HIV activity, three regioselective sulfate-substituted curdlan sulfates were synthesized [Gao, 1997]. That are, curdlan sulfate bearing sulfate groups at all C6 and some C2 positions (62S), at all C4 and some C2 positions (42S), and at some C6, C4, and C2 positions (642S) were prepared, which curdlan sulfates had the number average molecular weights in the range of $\overline{M}_n$=6.2×10^3 to 10.8×10^3. It was revealed that the curdlan sulfates exhibited high anti-HIV activity in the range of the EC_{50}=0.04-0.4 μg/mL when the degree of sulfation was more than 1.3 and low cytotoxicity. It was found that the anti-HIV activity was depended on the degree of sulfation rather than the position of sulfate groups.

Curdlan sulfate was found to be a superior candidate for a HIV drug because of high activity and low cytotoxicity and low blood anticoagulant activity. In addition, the price is also low. The phase I/II study of curdlan sulfate exhibited that CD4 positive helper T lymphocytes were observed to increase by the intravenous injection to HIV-infected patients [Godon, 1994; 1997]. Namely, curdlan sulfate worked effectively for the HIV-infected patients because CD4 positive helper T lymphocytes decrease after infection of HIV.

Therefore, curdlan sulfate is expected to bear improved stability as a biomaterial after modification. Four branched curdlan sulfates were synthesized by D- and L-glucosyl and mannosyl orthoacetates followed by sulfation. These branched curdlan sulfates had high anti-HIV activity of the EC_{50}=0.3–1.2 μg/mL in vitro and low cytotoxicity as well as low anticoagulant activity. For L-glucosyl-branched curdlan sulfates, the retention time in rate in vivo calculating from their anticoagulant activity (ATPP) were few hours [Yoshida, 1994]. The disappearance of curdlan sulfates from blood was due to absorption mainly in such tissues as liver, bone marrow, kidney, and

lymph node without degradation for 10d [Uryu, 1993]. This fact might be preferred to protect from infection of HIV because HIV is found often in the first step of infection in the lymph node.

Graft copolymerization of methyl methacrylate (MMA) onto curdlan was first investigated to prepare a chromatographic carrier after cross-linking, sphering, and subsequent sulfation [Yoshida, 1996]. The Tg of the grafted copolymers increased to reach 270°C with increasing grafting percentage more than 1012%. From IR spectra, the OH stretching and H-O-H bending vibration bands at 3500 and 1635 cm^{-1} decreased with increasing the grafting percentage, suggesting that the hydrophobicity increased. It was revealed that the graft copolymers swelled in ordinary organic solvents to form a white or colorless gel even though curdlan does not dissolve of swell in organic solvents.

The grafting of curdlan was carried out with styrene initiated with ammonium persulfate (APS) in H_2O at 60°C to give curdlan-graft-polystyrenes [Sawada, 1997]. The maximum grafting percentage was 209%. The grafting polystyrene had high molecular weights and very narrow molecular weight distributions. The degree of swelling of the polystyrene-grafted copolymers in DMF were 2 times higher than that of curdlan-graft-PMMA having grafting percentage of 1620%.In addition,, the polystyrene-grafted copolymers after sulfation were insoluble in water and formed gels depending on the degree of sulfation. Thus, sulfated graft copolymers are expected to be a biomaterial having selective removal of HIV and other envelope viruses from blood or air working outside of the human body.

For an AIDS medicine, azidothymidine (AZT)-branched curdlan sulfate at the C6 position were synthesized to carrier in lymphoid tissues [Gao, 1999]. AZT was bound through a long-chain dicarboxylate bond onto curdlan sulfate to produce biodegradable AZT-dicarboxylate-curdlan sulfates. When the carbon number of the alkylene group was 2–12, the AZT-spacer-curdlan sulfates exhibited high anti-HIV activity of the EC_{50}=0.04–0.21 μg/mL and low cytotoxicity. The

AZT-dodecane dicarboxylate-curdlan sulfate was easily hydrolyzed by esterase to release free AZT. An acidically released AZT from the curdlan sulfate also exhibited high anti-HIV activity. The AZT-spacer-curdlan sulfates may not only be expected to act as a desirable AIDS drug delivery system, but also it may become a highly active anti-HIV agent.

As described in this section, because curdlan sulfate has both high anti-HIV activity and low cytotoxicity as well as low anticoagulant activity, curdlan sulfate was a candidate of HIV medicine. It was indicated that the modified curdlan sulfates are expected to be a biomaterial to remove viruses working outside of human body.

1.3. Anti-HIV mechanism of sulfated polysaccharides

Anti-HIV mechanism of sulfated polysaccharides was investigated. Curdlan sulfate was mainly used as a sulfated polysaccharide. The electrostatic interaction of sulfated polysaccharides is an important role in the specific biological activities as described in the 1-1 section. Here, poly-L-lysine was used as a models HIV gp120, and the interaction with curdlan sulfate was investigated. Until now, there are several papers appeared on the anti-HIV mechanism of sulfated polysaccharides.

Taking into account the anticoagulant mechanism between heparin and Antithrombin III as described in the section 2-2, the anti-HIV mechanism of sulfated polysaccharides has been assumed the electrostatic interaction to inhibit the infection of HIV [Uryu, 1992]. Figure 1-5 shows the proposed electrostatic interaction of curdlan sulfate with HIV gp120 at the C-terminus that is one of positively charged lysine and arginine accumulated portions. In the C-terminus, there are seven positively charged amino acids, lysine and arginine, in the sequence of twenty amino acids. The negatively charged sulfate groups of curdlan sulfate might be electrostatically interacted into the

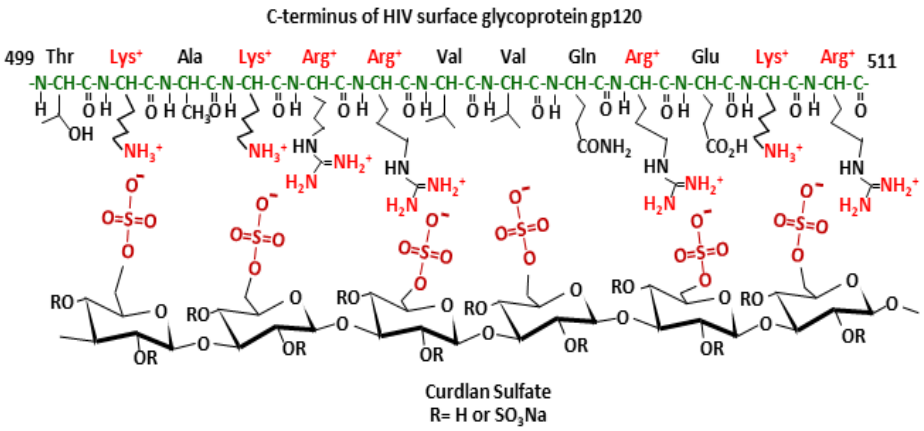


Figure 1-5.
Proposed electrostatic interaction of curdlan sulfate with HIV surface glycoprotein gp120. Negatively charged sulfate groups of sulfated polysaccharides were interacted with positively charged amino acid residues of HIV gp120 to prevent the infection of HIV to human cells. (Uryu et al., *Biochem. Pharmacol,*. 43 (1992) 2385–2389)

positively charged amino acids to prevent the binding of HIV to CD4 positive helper T lymphocytes.

To reveal the anti-HIV mechanism, the inhibitory effect of curdlan sulfate was analyzed against HIV-1 infection of CD4 positive sup T-1 lymphoma cells and peripheral blood lymphocytes, resulting that HIV was bound to the V3 loop of HIV gp120 and T-cell tropic HIV had over 10-fold more sensitive to neutralization by curdlan sulfate than macrophage-tropic HIV, because surface glycoproteins of macrophage had a relatively less positively-charged amino acid composition [Jagodzinski, 1994; 1996]. The effect of curdlan sulfate on binding functionality of HIV neutralizing antibodies to HIV gp120 demonstrated that both continuous epitopes on the V3 loop and discontinuous CD4 binding site of HIV gp120 represent targets for curdlan sulfate. These results suggest that curdlan sulfate interferes with the membrane fusion process during HIV-1 infection. In addition, the effect of curdlan sulfate on the binding of neutralizing antibodies to monomeric and oligomeric HIV gp120 mutants of T and MT tropic HIV-1 clones was examined, revealing that the amino acid composition of the V3 loop appears to determine the extent of

interaction of curdlan sulfate with the V2 and CD4 binding regions. The automated fitting procedure by software Chem X reveals that the V3 domain represents the most probable site for the interaction with curdlan sulfate as shown in Figure 1-6.

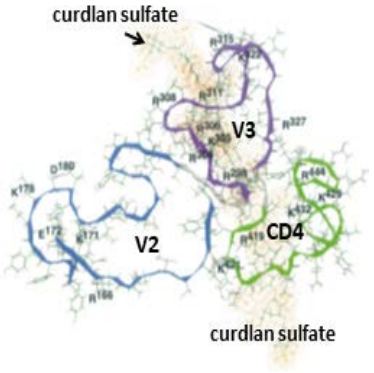


Figure 1-6.
Proposed electrostatic interaction of curdlan sulfate with V2, V3, and CD4 domains of HIV gp120. The V3 domain represents the most probable site for the interaction with curdlan sulfate because of having six positively charged amino acid residues located near. The V2 and CD4 domains have less positively amino acid residues compered with the V3 loop. (Jagodzinski et al., *Virology* 225 (1996) 217–223)

It is assumed that sulfate anions in curdlan sulfate interacted with ammonium cations in poly-L-lysine to form an insoluble gel-like complex. In ^{13}C NMR spectra as shown in Figure 1-7B, signals due to curdlan sulfate disappeared as background when the molar ratio was smaller than 0.8. Regarding the absorptions of poly-L-lysine, α and β carbon signals of the poly-L-lysine side chain were broadened by the formation of the complex. Disappearance of the absorption due to curdlan sulfate and broadening of α and β carbon signals clearly demonstrate a lack of local motion of the polymer backbone due to the formation of high molecular weight polyion complexes. All absorptions due to γ and ε carbons of poly-L-lysine were split individually into two peaks due to the coexistence of unreacted free poly-L-lysine and the gel-like complex. It is assumed that the gel formation would be affected by changing several reaction conditions such as the ratio, concentration, molecular weight of curdlan sulfate, pH, and temperature. The NMR measurements

indicated that curdlan sulfate has a strong interaction with poly-L-lysine consisted in the positively charged amino group amino group known to act as a cation in biological systems. It is presumed that the anti-HIV activity of curdlan sulfate originates from its ionic interactions with a viral envelope glycoprotein.

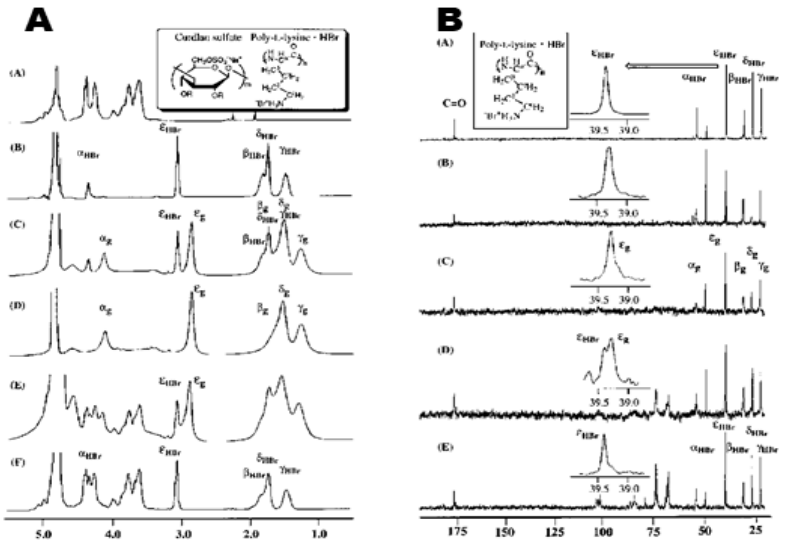


Figure 1-7.
400 MHz ^{1}H and 100 MHz ^{13}C NMR spectra of curdlan sulfate (CS), poly-L-lysine·HBr (PL), and polyion complexes (PIC) between CS and PL at different molar ratios (CS/PL).
Fig.7A: (A) curdlan sulfate; (B) poly-L-lysineâHBr; (C) PIC at a molar ratio of 0.5; (D) PIC at a molar ratio of 0.8; (E) PIC at a molar ratio of 1.0; (F) PIC at a molar ratio of 2.0. The concentration of PL is 2.8%(w/v).
Fig. 7B: (A) poly-L-lysine·HBr; (B) PIC at a molar ratio of 0.5; (C) PIC at a molar ratio of 0.8; (D) PIC at a molar ratio of 1.0; (E) PIC at a molar ratio of 2.0. The concentration of PL is 2.8% (w/v). (Jeon et al., *Macromolecules*, 30, 1997 (1997))

To further elucidate the mechanism of anti-HIV activity of curdlan sulfate, an oligopeptide sequence of a dimer (D518) from nos. 506 to 518 at the C-terminus of HIV gp120 was synthesized and the interaction was measured by NMR [Jeon, 2000]. As shown in Figure 1-5, the C-terminus sequence has several positively charged lysine and arginine residues located nearby [Uryu, 1992]. In the ^{1}H and ^{13}C NMR spectra (Figure 1-8), signals due to positively charged lysine (K) and arginine (R) were moved to higher and lower magnetic fields and broadly compared with those

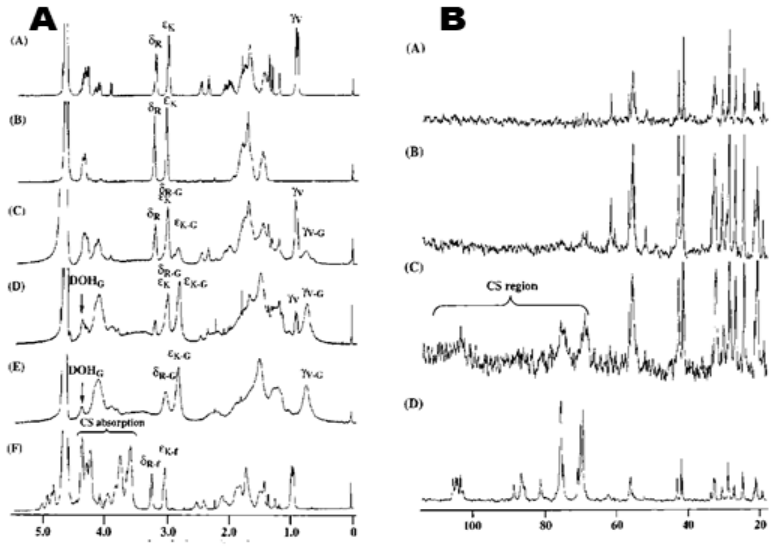


Figure 1-8.
500 MHz ^{1}H and 125 MHz ^{13}CNMR spectra of D518 and mixtures of curdlan sulfate (CS) with D518 in different molar ratios.
Fig. 1-8A: D518 (A) and a mixture of polylysine (PL) with polyarginine (PA) in the molar ratio of PL to PA of 4:3 (B) and in the molar ratio ([CS]/[D518]) of 0.12 (C), 0.23 (D), 0.27 (E), and 1.16 (F).
Fig. 1-8B: D518 (A) and in the molar ratio of0.12 (B), 0.27 (C), and 1.16 (D).
(Jeon et al, *J. Am. Chem. Soc.*, 122, 12536 (2000))

before the addition of curdlan sulfate, based on assumptions that ionic interactions occurred between negatively charged curdlan sulfate and positively charged lysine and arginine residues in the oligopeptide. When the oligopeptide of the V3 loop region of HIV, nos. 309 to 331, was mixed with curdlan sulfate, precipitates were produced, indicating that which no structural information by NMR spectroscopy afforded. These results suggest that the interactions between curdlan sulfate and the V3 loop region in HIV gp120 also occurred, however, the NMR detection method could not be applied to such the peptide sequence.

Since the quantitative elucidation of the anti-HIV mechanism of sulfated polysaccharides by NMR analysis was limited, it was used SPR and DLS to further analyze the mechanism [Tungalag, 2019]. The three oligosaccharides were synthesized according to the reference of HIV gp 120 as

shown in Figure 1-9 [Hansen, 1996; Kwong, 1998; Crublet, 2008],

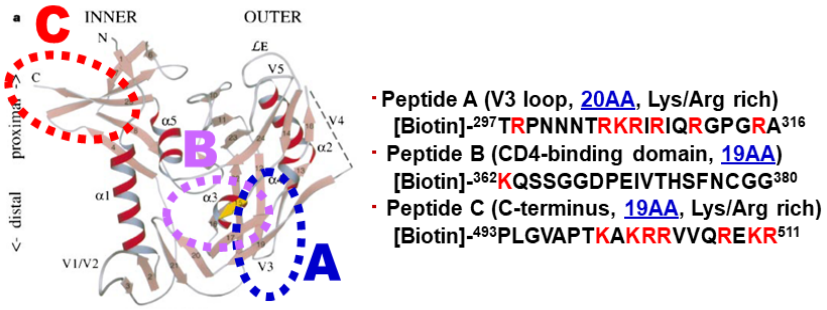


Figure 1-9.
HIV gp120 and oligopeptides.
Three oligopeptides in HIV gp120 were synthesized and the interaction with sulfated polysaccharides were measured by SPR, indicating that the oligosaccharides bearing positively charged amino acids ()peptides A and C) were electrostatically interacted with sulfated polysaccharides. (Hansen et al., *Proteins*, (1996) 25, 1; Kwong *et al.*, *Nature* (1998) 393, 648-659; Crublet *et al.*, *J. Biol. Chem.* (2008) 283, 15193-15200; Tungalag, Yoshida, *Int. J. Biol. Macromol*. 125 (2019) 909–914.)

^{297}TRPNNNTRKRIRIQRGPGRA^{316} with several lysine (K) and arginine (R) residues in the V3 loop region, ^{493}PLGVAPTKAKRRVVQREKR^{511} with several K and R in the C-terminus region, and ^{362}KQSSGGDPEIVTHSFNCGG^{380} with few basic amino acids in the CD4 binding domain. Curdlan and dextran sulfates were found to exhibit strong interactions against the two oligopeptides from the V3 and C-terminus regions, (k_a=5.48×10^4–2.96×10^6 1/Ms and k_d=1.74×10^{-4}–6.24×10^{-3} 1/s, respectively). No interaction was observed against the oligosaccharide from the CD4 binding domain, probably due to the small number of positively charged amino acids. The interaction of curdlan and dextran sulfates with poly-L-lysine was also measured by SPR to provide apparent association-rate (k_a) and dissociation-rate (k_d) constants of k_a=6.92×10^4–2.17×10^6 1/Ms and k_d=4.29×10^{-5}– 2.22×10^{-4} 1/s, respectively. The particle size and zeta potential by DLS also indicated an interaction between curdlan and dextran sulfates with poly-L-lysine. Therefore, the anti-HIV activity of sulfated polysaccharides was revealed to be induced by electrostatic interactions at the

V3 loop domain from the C-terminus of HIV gp120.

1.4. Sulfated alkyl poly and oligosaccharides with potent anti-HIV activity.

Since lower molecular weight oligosaccharides are expected to have lower cytotoxicity than polysaccharides with high molecular weights, the anti-HIV activity of sulfated oligosaccharides was examined. However, the anti-HIV activity of sulfated oligosaccharides was low due to their low molecular weights. Therefore, a long-chain-alkyl group was attached at the reducing end of oligosaccharides [Katsuraya, 1994b; 1994b; 1995], because the structure of the obtained sulfated alkyl oligosaccharides is similar to that of surface active agents, namely, sulfate alkyl oligosaccharides have the hydrophilic sulfate and hydrophobic long-chain alkyl groups.

Laminari- and malto-oligosaccharides composed of glucopyranosidic units from tetraose to nonaose were obtained by acetolysis of curdlan and starch with (1→3)-β-D- and (1→4)-α-D-

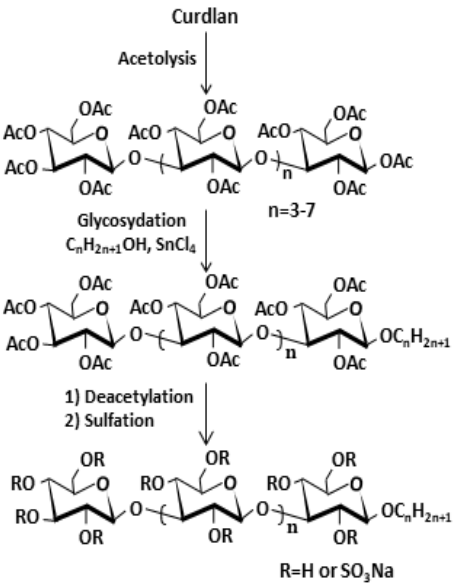


Figure 1-10.
Synthesis of sulfated laminari-oligosaccharides.
Acetolysis of curdlan followed by glycosylation with a long-chain aliphatic alcohol and then sulfation after deacetylation gave sulfated alkyl oligosaccharides.

glucopyranosidic structures, respectively, followed by column chromatographic purification. Figure 1-10 exhibits the synthetic route for sulfated alkyl laminari-oligosaccharides. Acetolysis of curdlan, glycosylation by n-dodecyl alcohol, deacetylation, and then sulfation gave sulfated n-dodecyl laminaripentaoside. Sulfated alkyl malto-oligosaccharides were prepared *via* the same procedures. Sulfated oligosaccharides without the long-chain alkyl group exhibited low anti-HIV activity as described above. When the long-chain alkyl group was introduced at the reducing end of oligosaccharides, the sulfated alkyl oligosaccharides exhibited potent anti-HIV activity in concentrations below 1 μg/mL, which was as high as that of high molecular weight curdlan sulfate. The potent anti-HIV activity of sulfated alkyl oligosaccharides might be ascribed to the formation of hydrophilic and hydrophobic structures like surfactants, since sulfated alkyl oligosaccharides are easily oriented and aggregated [Uryu, 1996]. The high anti-HIV activity of sulfated alkyl oligosaccharides is due to the amphiphilic structure bearing hydrophilic sulfate groups and a hydrophobic long-chain alkyl group at the reducing end of oligosaccharides. It was presumed that the long-chain alkyl group was destructed the virus lipid bilayer to inhibit the infection of HIV because sulfated alkyl oligosaccharides have similar structure to surfactants. The high anti-HIV activity of sulfated alkyl oligosaccharides is due to the amphiphilic structure bearing hydrophilic sulfate groups and a hydrophobic long-chain alkyl group at the reducing end of oligosaccharides.

In addition, sulfated octadecyl ribofuranans bearing low molecular weights were prepared by ring-opening polymerization of a benzylated ribose monomer, 1, 4-anhydro-2, 3-di-*O*-benzyl-α-D-ribopyranose, and then debenzylation and glycosylation followed by sulfation [Choi, 1996]. The anti-HIV activity is depended on the molecular weights, the low molecular weight sulfated ribofuranan had low anti-HIV activity. After the long-chain alkyl group was introduced at the reducing end of the low molecular weight ribofuranans, the anti-HIV activity improved. Figure 1-11 exhibits the structure of sulfated octadecyl ribofuranan. Sulfated octadecyl ribofuranan bearing low

molecular weight of $\overline{M}_n=3\times10^3$ was found to exhibit relatively high anti-HIV activity at a concentration of EC_{50}=2.5 μg/mL. The enhancement of the anti-HIV activity of the low molecular weight sulfated alkyl ribofuranans was due to the introduction of octadecyl group. The cytotoxicity of sulfated octadecyl ribofuranans was low, CC_{50}>1000 μg/mL. As described above, it is necessary to balance the length of hydrophobic long-chain alkyl groups and hydrophilic oligosaccharide moieties for high anti-HIV activity and low cytotoxicity [Katsuraya, 1994b; 1994b; 1995]. In the case of the sulfated octadecyl ribofuranan compared with that of sulfated octadecyl oligosaccharides

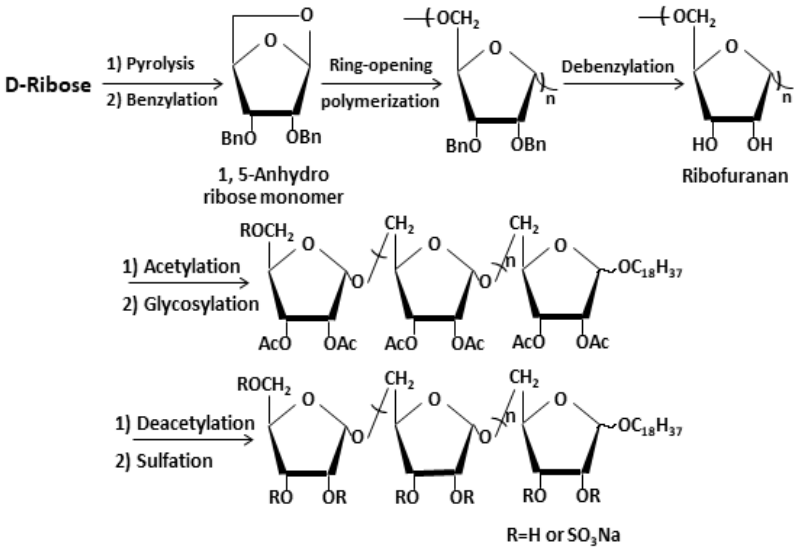


Figure 1-11.
Synthesis of sulfated octadecyl ribofuranan with medium-range molecular weights. Sulfated octadecyl ribofuranan was prepared by ring-opening polymerization of anhydro ribose monomer and glycosylation followed by sulfation. (Choi, Yoshida, Uryu, 282 (1996) 113–123.)

as described above, the molecular weight of ribofuranan moiety was relatively higher than that of laminari- and malto-oligosaccharides, so sulfated octadecyl ribofuranans might exhibit low cytotoxicity.

Recently, water-soluble sulfated 3-*O*-octadecyl dextrans bearing the DSUB of 2.8−4.7 mol%

and lower molecular weights of $\overline{M}_n$=2.5×10^3–5.1×10^3 were synthesized and and the anti-HIV activity were measured by comparison with that of standard dextran and curdlan sulfate (EC_{50}=0.05 μg/mL and 0.18 μg/mL). These sulfated 3-*O*-octadecyl dextrans were found to have potent anti-HIV activity at EC_{50}=0.05 μg/mL−1.25 μg/mL [Bai, 2015]. The interaction with poly-L-lysine as a model of HIV gp120 was analyzed by SPR, indicating that sulfated 3-*O*-octadecyl dextrans with $\overline{M}_n$=5.1×10^3 and $\overline{M}_n$=2.5×10^3 had high association- and low dissociation-rate constants calculated from SPR measurements, k_d=3.1×10^{-4} and k_d=1.0×10^{-3}, respectively. The particle size measured by DLS increased after adding poly-L-lysine, suggesting that sulfated 3-*O*-octadecyl dextrans were electrostatically interacted with poly-L-lysine to expand the particle size. For the interaction with poly-L-lysine, the octadecyl group could be independent of the binding of sulfated 3-*O*-octadecyl dextrans to poly-L-lysine because of having no charges. However, since the anti-HIV activity of sulfated 3-*O*-octadecyl dextrans bearing low molecular weight increased, the hydrophobic octadecyl group and hydrophilic dextran sulfate moiety were assumed to be induced synergistic effect against HIV.

1.5. Anti-HIV mechanism of sulafted alkyl polysaccharides.

As mentioned above, the anti-HIV mechanism of sulfated polysaccharides is originated from the electrostatic interaction between negatively charged sulfate groups in sulfated polysaccharides and positively charged amino acids in HIV gp120. For sulfated alkyl poly and oligosaccharides, a pioneering research on the interaction of synthetic dextran bearing long-chain alkyl group and liposome was reported by Kobayashi in 1986 [Kobayashi 1986]. Figure 1-12 exhibits the synthesis of 3-*O*-octadecyl dextran (A) and proposed interaction between long-chain alkyl group in dextran and liposome. The interaction between 3-*O*-octadecyl dextran bearing a degree of substitution (DSUB) of 0.03 and liposome was analyzed by a gel chromatography using Sephadex G-50The

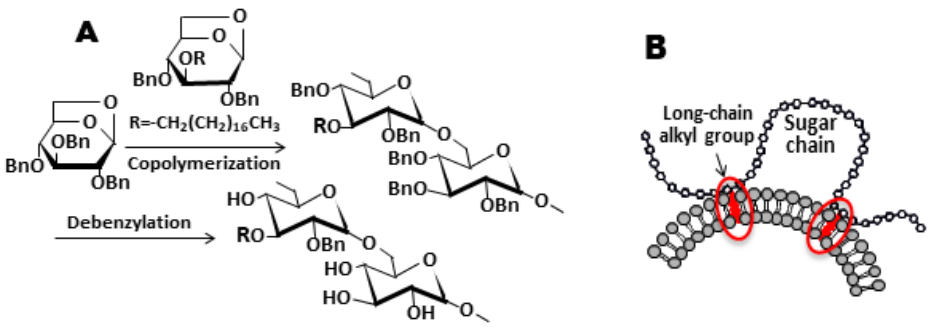


Figure 1-12.
Proposed interaction of long alkyl group in polysaccharide with liposome. (A) Synthesis of octadecyl dextran by ring-opening copolymerization and (B) Interaction of long-chain alkyl group with liposome. Long-chain alkyl group was penetrated into liposome. (Kobayashi et al., *Macromolecules* 19 (1986) 529-534)

long-chain alkyl group was found to interact with liposome to produce complex. The dissociation was suppressed when the long-chain alkyl groups attached to dextran anchored deeply into the lipid bilayer of liposome. The octadecyl group exerted a stronger anchoring effect against liposome than the shorter dodecyl group. In addition, no dextran-coated liposomes were isolated when 3-*O*-octadecyl dextran at more than DSUB=0.07 was used. The reasons for decreasing interaction were considered as follows: (1) Although the octadecyl groups exhibited potent anchoring effect, too many octadecyl groups (DS>0.07) disordered the structure of the liposomes, which became unstable. (2) Fluorescence polarization measurement of 3-*O*-octadecyl dextran revealed that the mobility of the dextran molecules was restricted due to the anchoring of octadecyl groups into the liposome.

As described above, the potent anti-HIV activity of sulfated alkyl poly- and oligosaccharides is assumed to involve the penetration of the long-chain alkyl group into the lipid bilayer of HIV and then the sulfated oligosaccharide moiety should electrostatically interact with HIV surface

glycoprotein gp120 to inhibit the infection of HIV into cells. Thus, the interaction of sulfated 3-*O*-octadecyl dextrans with liposomes was directly investigated by SPR and DLS analysis [Budragchaa, 2020].

Two liposomes bearing diameters of 58±20 nm and 107±28 nm as models of HIV were used. SPR measurements of sulfated 3-*O*-octadecyl dextran with 2.8 mol% of the octadecyl group and the liposome (diameter=58±20.0 nm and ζ=0 mV) resulted in an apparent association- and dissociation-rate constants of k_a=6×10^5 1/M and k_d=4×10^{-4} 1/s, respectively. The particle size of sulfated 3-*O*-octadecyl dextran (67±14 nm) measured by DLS increased to 104±25 nm, whereas the ζ potential (-29 mV) was unchanged (-33 mV). These results suggest that the long octadecyl group penetrated into the liposome lipid bilayer and sulfated glucopyranan covered the liposome. The 107 nm liposome exhibited similar results.

Figure 1-13 shows the proposed illustration of the interaction of (A) liposome and dextran sulfate and (B) liposome and sulfated 3-*O*-octadecyl dextran. In Figure 1-13A, for dextran sulfate, the particle size of the liposomes did not change and the ζ potential decreased, suggesting that

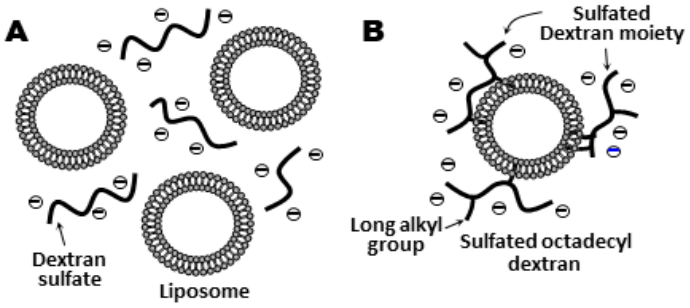


Figure 1-13.
Illustration of the interaction between (A) liposome and dextran sulfate and (B) liposome and 3-*O*-octadecyl dextran sulfate. The octadecyl groups penetrated into the lipid bilayer of liposomes to increase particle size and the ζ potential changed. (Budragchaa, Yoshida, *Carbohydr. Polym*. 245 (2020), 116022)

dextran sulfate was present in the system without any interaction. For sulfated 3-*O*-octadecyl dextran in Figure 1-13B, the long octadecyl group penetrates into the liposome, and the fixed sulfated dextran moiety covers the surface of the liposome to expand the particle sizes, and the ζ potential became similar to that of sulfated 3-*O*-octadecyl dextran alone. Taking into account the results, the long-chain alkyl group works effectively by penetrating or anchoring into the lipid bilayer of HIV to improve anti-HIV activity, and subsequent electrostatic interaction between negatively charged sulfated polysaccharide moieties and positively charged HIV gp120 portions occurred and also contributes to the anti-HIV activity. This consideration aligns with the previous research as described in this section [Uryu, 1992]. The role of the long-chain alkyl group in sulfated alkyl oligosaccharides with potent anti-HIV activity might be similar to the results of this section. The precise considerations are presented in the next section.

1.6. Anti-HIV mechanism of sulafted alkyl oligosaccharides.

The role of the long-chain alkyl group in sulfated alkyl oligosaccharides was also investigated by the combination of SPR and DLS measurements [Bai, 2020]. Sulfated maltoheptaoside with a

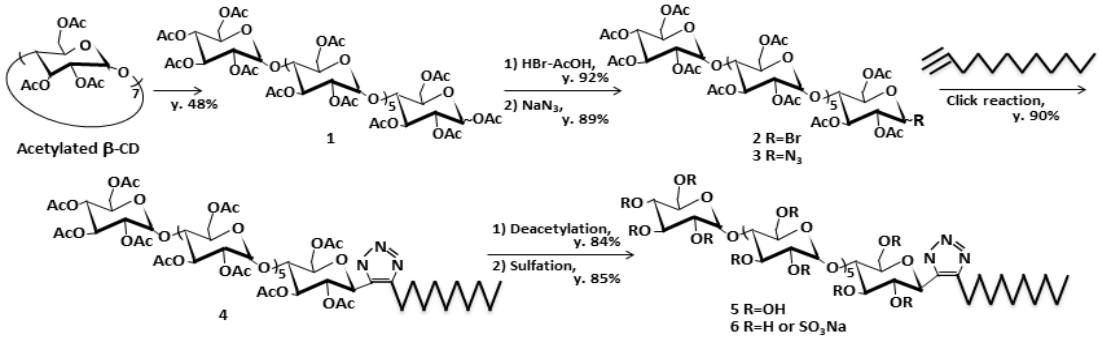


Figure 1-14.
Synthesis of sulfated oligosaccharides with a long-chain alkyl group at the reduced end. Click reaction is the key reaction of the synthesis. (Bai, Yoshida, *Carbohydr. Polym*. 245 (2020) 116518.)

long-chain alkyl group was newly synthesized by using click reaction in good yield as exhibited in Figure 1-14. The long-chain alkyl group was attached at the reducing end of maltoheptaose through the triazole ring. Both alkyl oligosaccharides, 1-(decadecyl-1, 2, 3-triazoyl)-1-deoxy-maltoheptaoside (dodecyl maltoheptaoside) and sulfated 1-(decadecyl-1, 2, 3-triazoyl)-1-deoxy-maltoheptaoside (sulfated dodecyl maltoheptaoside) gave the SPR signals, respectively. However, maltoheptaose and sulfated maltoheptaose without long-chain alkyl group afforded no SPR signals. The particle size of the maltoheptaosides bearing long-chain alkyl group increased in the presence of liposome, however, for the maltoheptaosides without long-chain alkyl group, the particle size was not changed. The ζ potential of sulfated maltoheptaosides exhibited negative values and maltoheptaosides without sulfation gave approximately zero mV. These results are similar to 3-*O*-octadecyl dextran as described in section 1-5. Figure 1-15 shows the proposed interaction between sulfated dodecyl maltoheptaoside and liposome. Considering of the SPR and DLS results on sulfated octadecyl maltoheptaosides, the long-chain alkyl group penetrated into the lipid bilayer of HIV and then the sulfated maltoheptaoside portion electrostatically interacted with HIV gp120 to inhibit HIV infection in vitro.

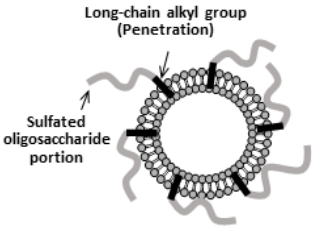


Figure 1-15.
Proposed model of the interaction of sulfated alkyl oligosaccharides with liposome. Long-chain alkyl group was penetrated into liposome and then sulfated oligosaccharide portion having negative charges was electrostatically interacted with positively charged HIV gp120 to cover the surface of HIV. (Bai, Yoshida, *Carbohydr. Polym*. 245 (2020) 116518)

As descreibed above, although the long-chain alkyl group in sulfated oligosaccharides enhanced anti-HIV activty due to the interaction with lipid bilayer of HIV, Katsuraya reported on the cytotoxicity of sulfated alkyl oligosaccharides. Sulfated alto- and laminari-oligosaccharides bearing pentoside and nonaoside had high anti-HIV and low cytotoxicity represented by large CC_{50}

values. However, when sulfated alkyl oligosaccharides had too long alkyl groups compared to those of the carbohydrate portion, the cytotoxicity considerable increased to ranging from CC_{50} of 380 to 810 μg/mL (Table 1-1) [Katsuraya et al., 1994; 1995; 1996].

Table 1-1. Anti-HIV activity of sulfated laminara- and malto-oligosaccharides[a)]

Sulfated alkyl oligosaccharides				Anti-HIV activity[b)]		
Sample[c)]	No. of glucose	C no. of alkyl chain	DS[d)]	EC_{50} μg/mL	CC_{50} μg/mL	AA[e)] unit/mg
L5C12S	5	12	3.0	0.10	>1000	0
L5C18S	5	18	3.4	0.63	220	0
L7C12S	7	12	n.d.	0.14	>1000	0
L7C18S	7	18	2.8	0.20	180	2
L9C12S	9	12		0.18	>1000	3
L9C18S	9	18		0.59	240	6
CS[f)]		0	1.6	0.18	>1000	10
M4C12S	4	12		13	>1000	0
M4C14S	4	14		10	810	n.d.
M4C18S	4	18		9.3	510	0
M5C12S	5	12	3.4	0.53	>1000	0
M5C18S	7	18	4.0	0.43	410	0
M7C12S	7	12	3.1	0.19	>1000	3
M7C18S	7	18	3.3	0.37	380	2
CS[f)]		0	1.6	0.4	>1000	10

a) Original data are in references [Katsuraya et al., 1994; 1995; 1996].
b) Anti-HIV activity was evaluated by EC_{50} and CC_{50} that defied as 50% effective and cytotoxic concentrations, respectively.
c) L5C12S means sulfated dodecyl laminara-pentoglycoside, for example.
d) DS means degree of sulfation that designated the number of sulfate groups in a glucose unit.
e) Anticoagulant activity measured according to a modification of the United States Phamacopoeia using bovine plasma and dextran sulfate (21.0 unit/mg) was used as a reference [U.S. Pharmacopoeia].
f) Curdlan sulfate with $M_n=7.9\times10^4$ was used as a reference for anti-HIV activity.

It is important for potent anti-HIV and low cytotoxic sulfated alkyl oligosaccharides to balance between the length of hydrophobic long-chain alkyl group and hydrophilic sulfated oligosaccharide moiety, which sugar units bearing more than five (pentose) produce high anti-HIV activity and low cytotoxicity.

1.7. Potent anti-HIV activity by cluster effect of dendric sulfated oligosaccharides

Since biopolymers bearing sugar moieties play important roles in biological activities as cellular recognition and adhesion, dendrimers bearing mono or oligosaccharides units at the terminals attract much interest for biomedical applications to expect potent biological activities owing to the cluster effect of the terminal mono and oligosaccharide units.

The enhancement of biological activities by the cluster effect is an effective method to improve biological activities of mono or oligosaccharides with low or no biological activities due to the low molecular weights. For sulfated polysaccharides with antiviral activity, the cytotoxicity in general depends on their molecular weights. Sulfated polysaccharides with low molecular weights, for example, sulfated oligosaccharides, exhibit low cytotoxicity, however, also low antiviral activity. Therefore, for HIV agents, it is preferred low molecular weights polysaccharides with high inhibitory effects against HIV and related virus infections. To dissolve the conflicting problems, sulfated alkyl oligosaccharides are one of the answers as described above. Another one is the cluster effects of glycodendrimers bearing mono or oligosaccharides at their terminals.

The cluster effects were first reported by Lee in 1978 and recognition of mono or oligosaccharides by cells or proteins may require local concentration or clustering of sugar residues [Lee, 1978]. Recognition of carbohydrates by cells or proteins may require local concentration of clustering of sugar residues. Glycosides consisting of more than one sugar residue at the terminal were synthesized. The structure is a triglycoside possessing a terminal amino or hydrazide group for attachment to carboxyl or amino group of proteins.

Glycodendrimers with biological activity was reported for the first time in 1993 by Roy, which dendrimers had the inhibitory effect against influenza A virus as shown in Figure 1-16 [Roy, 1993]. Influenza A Viruses are infected by the recognition and binding of a viral envelope glycoprotein, haemagglutinin, to α-sialosides on the host cell. The glycodendrimers bearing poly-L-lysine

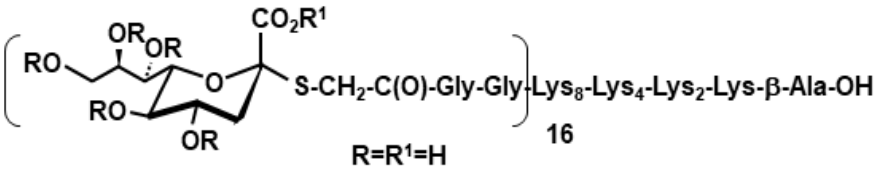


Figure 1-16.
Solid-phase synthesis of a new water-soluble dendric a-thiosialosides with hyper branched fractal structures. Preliminary experiments with influenza A virus showed powerful inhibition of haemagglutination of erythrocytes at concentrations of 625-19 μoml dm^{-3}.
(Roy et al., *J. Chem. Soc., Chem. Commun*. (1993) 1869–1872)

scaffold with α-sialoside at the terminal were synthesized. The divalent dendrimer even at a low level of clustering exhibited five times more potent (625 μmol dm^{-3} equal to 50% inhibition at 40 μmol dm^{-3}) than a monosialoside (3 mmmol dm^{-3}). The dendrimer bearing 16 terminals gave the highest activity as potent as previous sialylated glycopolymers.

A sphere-type monodispersed oligosaccharide-polylysine dendrimer as an antigen carring candidate for glycopeptide-type HIV vaccine was synthesized from 1, 4-aminobutane as a core initiator. The terminal amino groups were substituted by β-alanine with highly reactive amino groups and oligosaccharides were attached at the reactive amino groups by reductive amination [Baigude, 2002]. On the other hand, a cellobiose-polylysine dendrimer with reducing sugar terminals was synthesized. Hexa-*O*-benzyl-4'-(1-carboxyethyl)-cellobiose was synthesized and reacted with polylysine dendrimer generation 3 to produce a dendrimer bearing cellobiose at the terminals. For the preparation of a HIV vaccine, tripeptide and cyclic oligopeptide from HIV gp120 were attached, respectively, by reactive amination. The dendric peptides are expected to new HIV vaccine to inhibit the infection of HIV [Baigude, 2003].

Although sulfated polysaccharides bearing high molecular weights had potent anti-HIV activity, sulfated mono and oligosaccharides had no or low anti-HIV activity because of their low molecular weights [Choi, 1996], however, the cytotoxicity was low. It was found that dendric

poly-L-lysine bearing sulfated mono or oligosaccharides at the terminal inhibited effectively infection of HIV in MT-4 cells in vitro [Han, 2010].

Polylysine dendrimer generation 3 bearing sulfated cellobiose was prepared and the anti-HIV activity was EC_{50}=3.2 μg/mL, which was as same as that of a clinical used ADIS drug, 2', 3'-dideoxycytidine (ddC), EC_{50}=3.5 μg/mL. The cytotoxicity was low, CC_{50}=1000 μg/mL [Han, 2010]. The anti-HIV activity was improved by a cluster effect of sulfated cellobiose at the terminal of dendric scaffold. In addition, a semi-spherical and third generation dendrimer was synthesized from strearylamine by repeated condensations of lysine, and then cellobiose at the terminal (Figure 1-17) [Han, 2012]. After sulfation, the sulfated cellobiose semi-spherical dendrimer had anti-HIV

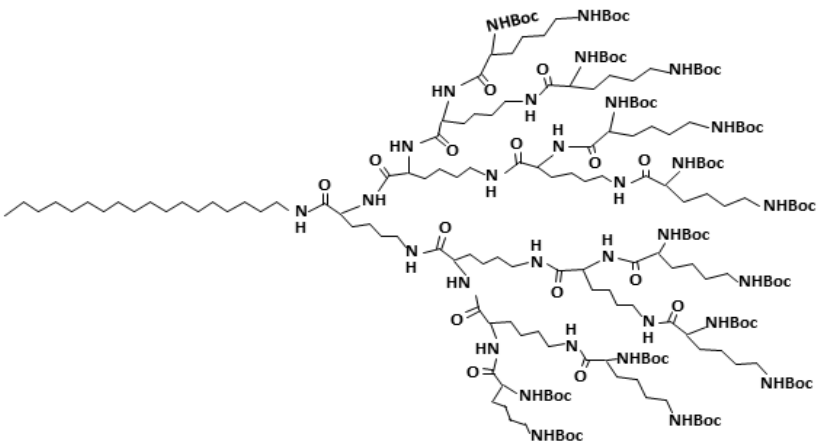


Figure 1-17.
Stearylamide lysine dendrimer 3rd generation. After deprotection and sulfation, the dendrimer exhibited potent anti-HIV activity as high as 6.4 mg/mL and low cytotoxicity. (Han, Yoshida, Carbohydr. Polym. 90 (2012) 1061–1068.

activity at EC_{50}=6.4 μg/mL and low cytotoxicity. The semi-spherical dendrimer bearing hydrophobic long-chain alkyl group is expected to provide a new biomaterial with the surface functionality of hydrophilic sulfated oligosaccharides.

Above spherical and semi-spherical dendrimers bearing third generation structures gave the

enhancement of the anti-HIV functionality of oligosaccharides. However, the anti-HIV activity was medium values or relatively lower than that (EC_{50}=0.1 μg/mL) of standard curdlan sulfate bearing high molecular weight. One of the reasons for the medium anti-HIV activity is expected to crowded sulfated cellobiose units at the terminal. Therefore, to reveal the relationship between generation and anti-HIV activity, the new first, second, and third generation spherical dendrimers with cellobiose units at their terminals were synthesized from 1, 4-diaminobutane as a starting compound by repeated condensation and deprotection of di-boc- lysine in 75%, 82%, and 83% yields, respectively [Li, 2015]. A methylene spacer with six methylene carbons was introduced between the polylysine dendrimer and cellobiose to increase the flexibility of attached cellobiose unit at the terminal. After sulfation of the terminal cellobiose units, it was found that the 2nd generation polylysine-cellobiose dendrimer had the highest anti-HIV activity among the three sulfated dendrimers, suggesting that the 2nd generation dendrimer had a prefer distance between sulfated cellobiose units for the interaction with HIV gp120 [Song, 2020]. The results for the anti-HIV activity after sulfation are presented in Chapter 2.

In this thesis, the interaction between sulfated oligosaccharides and HIV is described. Chapter 1 is the introduction of this thesis. In Chapter 2, potent anti-HIV activity of sulfated cellobiose by the cluster effect originated from the dendric core structures is presented. The inhibitory effects of the long-chain alkyl group attached with sulfated maltoheptaoside against HIV will be described in Chapter 3 and the relationship between the length of long-chain alkyl groups and cytotoxicity is also exhibited. The author evaluated the anti-HIV mechanism of sulfated glycodendrimers and sulfated alkyl oligosaccharides in this thesis. The cluster effect of sulfated glycodendrimers and the long-chain alkyl group in sulfated alkyl oligosaccharides are found to exhibit a key role in their potent anti-HIV activity.

Chapter 2

Elucidation of anti-HIV mechanism of sulfated cellobiose–polylysine dendrimers

2.1. Abstract of Chapter 2

Three new spherical sulfated cellobiose–polylysine dendrimers of increasing generations bearing negatively charged sulfate groups were prepared by sulfating the corresponding cellobiose–polylysine dendrimers. The first, second, and third-generation derivatives exhibited potent anti-HIV activity with EC_{50} values of 3.7, 0.6, and 1.5 μg/mL, respectively, in constant to sulfated oligosaccharides with low anti-HIV activity, while the second-generation sulfated dendrimer was the most active. Surface plasmon resonance measurements with poly-L-lysine bearing positively charged amino acids as a model of the HIV surface glycoprotein gp120, indicated that the second-generation dendrimer had the lowest dissociation constant ($K_D=1.86\times10^{-12}$ M). Both the particle size and ζ potential increased in the presence of poly-L-lysine. It was proven that the moderate distance between the terminal sulfated cellobiose units in the second-generation dendrimer favored the high anti-HIV activity, owing to the electrostatic interactions developed due to the cluster effect.

2.2. Introduction

Since the first report of dendrimers by Tomallia and Fréchet in 1985 [Tomalia, 1985], several dendrimers and glycodendrimers have been disclosed as functional and biological macromolecules. Glycodendrimers, which bear a core hyper-branched polymer scaffold with mono or oligosaccharides attached to its terminal end as functional groups, are expected to have potent biological activities due to the cluster or multivalent effect [Lee, 1978].

Several studies and reviews have already explored the functionality of glycodendrimers [Newkome 2001; Roy 1993; Roy 2003; Roy 2013]. The synthesis of polylysine core dendrimers with carbohydrate antigens at their terminal ends has been reported by Roy et al., indicating glycodendrimers as vaccine candidates [Tze 2012]. The applications of dendrimers and glycodendrimers as drug delivery system (DDS) have demonstrated that HIV chemical drugs could be transported into the cells, thus improving the anti-HIV activity [Peng 2013]. Dendrimers β-cyclodextrin (β-CD) moiety including methotrexate drug were found to be suitable DDS for the prevention of cancer [Yousef 2015]. Glycodendrimers bearing carbohydrates or glycomimetic dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) at the terminal ends exhibited potent anti-HIV and anti-dengue virus activities [Varga 2014]. The mechanism of the antiviral activity was evaluated by surface plasmon resonance (SPR), suggesting that it depended on the charge of the scaffold. Wrobel reported the synthesis of maltose-modified poly(propylene-imine) core dendrimers and their interaction with lipid model membranes was examined by fluorescence spectroscopy and nuclear magnetic resonance (NMR), indicating that the glycodendrimers strongly interacted with the model liposomes, while the hydrogen bonds significantly contributed to these interactions [Wrobel 2015].

Given that glycodendrimers are being used as biopolymers in a wide range of application, our research group has disclosed a series of glycodendrimers with anti-HIV activity. More specifically,

a spherical polylysine third-generation dendrimer with sulfated cellobiose at its terminal end was synthesized from tris(2-ethylamino)amine, followed by condensation and deprotection reactions of di-*tert*-butoxycarbonyl lysine (di-boc-lysine), which were both repeated in triplicate to obtain the third-generation core dendrimer [Han 2010]. Cellobiose was then attached to the terminal end by a long alkyl spacer (C12). After sulfation, the sulfated cellobiose dendrimer exhibited anti-HIV activity at a 50% effective concentration (EC_{50}) of 3.2 μg/mL, which was comparable to that of an AIDS drug, 2', 3'-dideoxycytidine (ddC), EC_{50}=3.51 μg/mL, while its 50% cytotoxic concentration (CC_{50}) was low, i. e., CC_{50}>1000 μg/mL. Moreover, its blood anticoagulant activity (AA) (19.4 units/mg) was not high in the same range as that of curdlan and dextran sulfates (19 and 22.9 units/mg, respectively). Another new semi-spherical and amphiphilic glycodendrimer third-generation bearing a long-chain alkyl group and cellobiose at their terminal ends of the dendrimer was also synthesized using strearylamine as the starting long-chain alkyl group. Repeated condensation and deprotection reactions of di-boc-lysine followed, and cellobiose, a functional oligosaccharide, was attached to the terminal ends by a peptide bond [Han 2012]. The sulfated semi-spherical glycodendrimer exhibited anti-HIV activity (EC_{50}=6.7 μg/ml) and low cytotoxicity (CC_{50}>1000 μg/ml). The long stearyl group was expected to immobilize on the hydrophobic surfaces, while the hydrophilic cellobiose terminals would cover the surface. Therefore, these semi-spherical type glycodendrimers are expected to become a new biomedical material with hydrophilic sulfated oligosaccharides on the hydrophobic surface [Tegshi 2011].

Recently, we prepared new spherical polylysine glycodendrimers of generations. The cellobiose unit was attached to the terminal ends of polylysine dendrimers by the peptide bond through a C6 methylene spacer [Li 2015]. Using sulfated cellobiose as a model of functional oligosaccharides, the new glycodendrimers bearing clustered sulfated cellobiose units with the C6 methylene spacer at each terminal were expected to have high anti-HIV activity due to the increased mobility of the

cellobiose units provided by the spacer. However, the moderate anti-HIV activity (EC_{50}=3.2−6.7 μg/mL) of the reported third-generation dendrimers compared to dextran and curdlan sulfates with EC_{50} values of <1 μg/mL suggested that the generation must contribute to the high antiviral activity of glycodendrimers. Therefore, in this study, we disclose the sulfation of first-, second-, and third-generation spherical type cellobiose–polylysine glycodendrimers to reveal the relationship between their generation and the anti-HIV activity. The biological mechanism was also investigated by SPR and dynamic light scattering (DLS) measurements, and the structure of the sulfated glycodendrimers was analyzed by Fourier-transform infrared (FT-IR) and high-resolution NMR spectroscopies.

2.3. Materials and methods

2.3.1. Materials

Sulfur trioxide-pyridine (SO_3-pyridine) complex, anhydrous dimethyl sulfoxide (DMSO), and poly-L-lysine with a molecular weight of 1000–5000 were purchased from Sigma-Aldrich Japan Co. The SPR reagents, a CM5 sensor chip, an amine coupling kit, HBS-EP+ buffer, and solution of ethylenediaminetetraacetic acid (EDTA) (30 mM), surfactant P20, and NaOH (50 mM) were obtained from GE Healthcare Japan, Co. Ltd. The first-, second-, and third-generation cellobiose–dendrimers were synthesized from 1, 4-diaminobutane by the repeated stepwise condensation and deprotection of di-boc-lysine following the procedure of our previous paper [Li 2015]. The cellulose dialysis tube (3500 molecular weight cut-off) was obtained from Spectrum Laboratories, Inc. Japan.

2.3.2. Measurement

The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM ECA-600 spectrometer at 600 MHz and 150 MHz, respectively, in D_2O at 40°C, and the chemical shift was expressed as ppm using 4, 4'-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard at 0 ppm. The FT-IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer by a KBr pellet. The SPR measurements were performed with a Biacore X100 instrument at 25°C using a CM5 sensor chip. The particle size and the zeta (ζ) potential were calculated by an ELSZ-1000ZS DLS analyzer (Otsuka Electric Co. Ltd., Japan).

2.3.3. Sulfation of cellobiose–polylysine dendrimers

The first generation cellobiose–polylysine dendrimer (0.1 g) was dissolved in anhydrous DMSO (8 mL), followed by the addition of the SO_3-pyridine complex (0.31 g, 1.89 mmol). The resulting mixture was stirred at 40 °C for 30 h. After the reaction completion, the reaction mixture was neutralized with a 10% NaOH aqueous solution and then dialyzed for 2 days. After freeze-drying, the first-generation sulfated cellobiose–polylysine dendrimer (**SCLDG1**) was obtained in 67% yield. The second- and third-generation sulfated cellobiose–polylysine dendrimers (**SCLDG2** and **SCLDG3**) were obtained in 89% and 93% yield, respectively, following the same process.

2.3.4. Anti-HIV activity

The anti-HIV activity of the sulfated cellobiose–polylysine dendrimers was estimated by the MTT method according to literature [Pauwels 1988] using MT-4 cells. The anti-HIV activity was evaluated by the 50% effective concentration (EC_{50}) of HIV infection to the MT-4 cells. Accordingly, the cytotoxicity (CC_{50}) was defined by the 50% cytotoxic concentration.

2.3.5. SPR and DLS measurements

Poly-L-lysine (M_w=1000–5000) was immobilized on the Biacore CM5 sensor chip according to the Biacore protocol using an amine coupling kit. Each sulfated cellobiose–polylysine dendrimer was added in buffer solution and the resulting mixture was then injected to the SPR instrument and flowed on the sensor chip in a concentration order of 500, 250, 125, 62, and 31 μg/mL, respectively. The apparent association- (k_a) and dissociation- (k_d) rate constants were calculated from the response signals by 1:1 binding mode, and the association constant (K_D) was calculated by $K_D=k_d/k_a$.

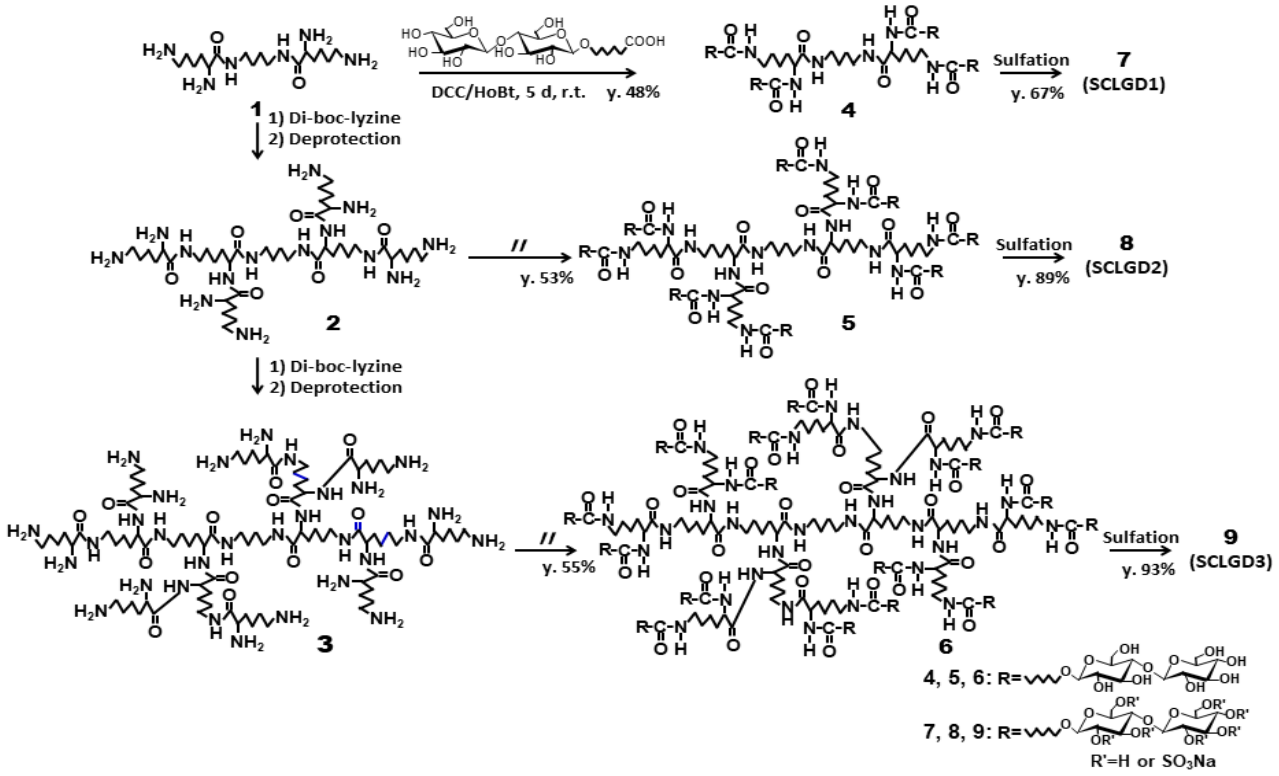
The particle size and the ζ potential were measured by DLS using sulfated cellobiose–polylysine dendrimers and poly-L-lysine concentrations of 0.5 mg/mL each.

2.4. Results of discussion

2.4.1. Sulfation of the first-, second-, and third-generation cellobiose–polylysine dendrimers

The synthesis of the currently investigated spherical type cellobiose cellobiose–polylysine dendrimers (Scheme 2.1) has been recently reported by our research group [15 Li 2015]. The cellobiose unit with a C6 alkyl spacer, 1-*O*-(6-carboxylpentoxy)-β-D-cellobioside, was attached to the terminal end of each dendrimer through a methylene spacer to improve the molecular flexibility of cellobiose. Although the first-, second-, and third-generation dendrimers have four, eight, and sixteen terminal ends, respectively, 4, 6.8, and 11 cellobiose units were attached, respectively, according to the NMR data. The number of the introduced cellobiose units decreased with increasing generation of dendrimers, probably due to the steric hindrance of the terminal ends. Based on the sulfation (Table 2.1), the dendrimers were sulfated with the SO_3-pyridine complex in DMSO at 85°C to afford the three desired sulfated cellobiose–polylysine dendrimers of the first (**SCLGD1**), second (**SCLGD2**), and third (**SCLGD3**) generation in 67%, 89%, and 93% yield,

respectively. Their degrees of sulfation (DS) were 1.8, 2.1, and 2.1 (maximum 3.0), respectively, as calculated by elementary analysis, due to the facile introduction of sulfate groups into the small cellobiose units.



Scheme 2. 1. Synthesis of the first-, second-, and third-generation sulfated cellobiose–polylysine dendrimers.

Table 2.1. Sulfation and anti-HIV activity of cellobiose–poly-L-lysine dendrimers

Sulfated dendrimer[a]	Sugar moiety[b] Found	(Theoretical)	Yield %	M_W[c] ×10^3	$[\alpha]_D^{25}$ deg	Elemental analysis (%) C	H	N	S	DS[d]	EC_{50}[e] μg/ml	CC_{50}[f] μg/ml
SCLDG1	4	(4)	67	3.3	-6.1	20.79	2.98	0.60	16.56	1.8	3.7	>200
SCLDG2	6.8	(8)	89	7.0	-6.9	26.65	3.48	2.65	15.91	2.1	0.6	>200
SCLDG3	11	(16)	93	12.8	-7.2	32.38	4.66	6.32	13.26	2.1	1.5	>200
Dextran sulfate[f]				8.5					18.4	2.1	0.45	691
Curdlan sulfate[f]				79.0					14.1	1.4	0.22	>1000

a) SCLDG1: Sulfated cellobiose-lysine dendrimer generation 1, SCLDG2: Sulfated cellobiose-lysine dendrimer generation 2, SCLDG3: Sulfated cellobiose-lysine dendrimer generation 3.
b) Number of cellobiose units in one dendrimer, which was calculated from NMR measurement.
c) Calculated molecular weight. d) Degree of sulfation in cellobiose moiety.
e) 50% Effective concentration against HIV. f) 50% Cytotoxic concentration against MT-4 cell.
f) Standard dextran and curdlan sulfates were used.

*2.4.2. Structure of **SCLDG1**, **SCLDG2**, and **SCLDG3** dendrimers*

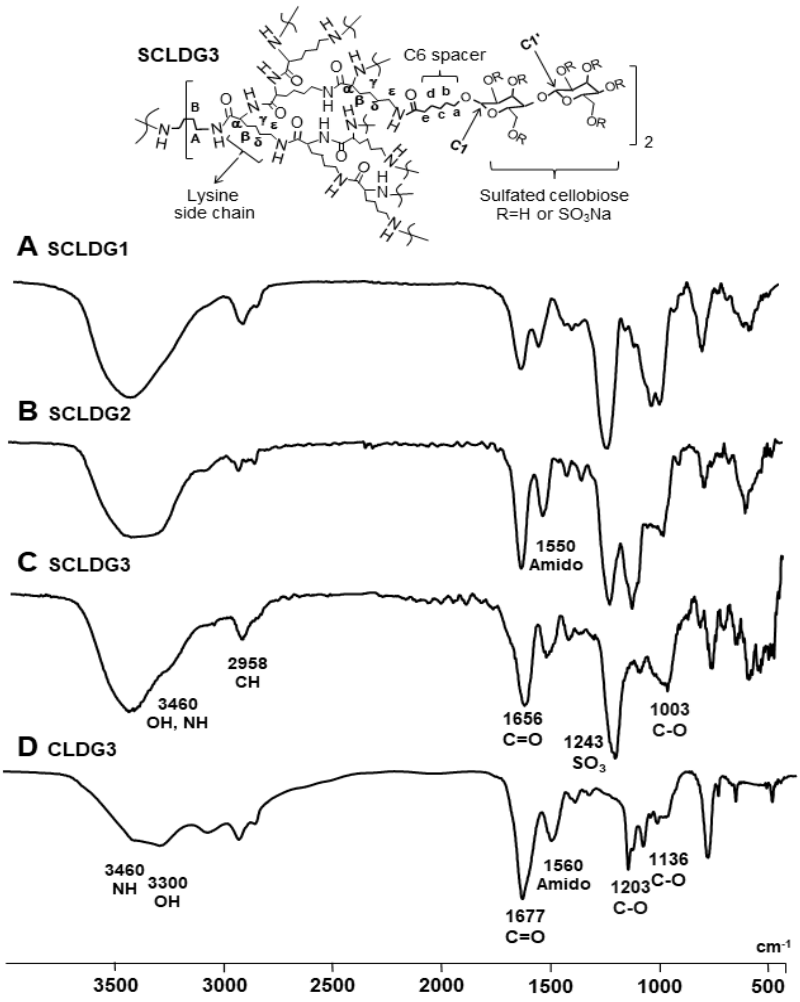


Fig. 2.1. FT-IR spectra of the (A) first-, (B) second-, and (C) third-generation sulfated cellobiose–polylysine dendrimers. Spectrum (D) is the third-generation cellobiose–polylysine dendrimer before sulfation.

hydroxyl and amide NH absorptions of the sulfated dendrimers due to stretching vibrations around 3300–3400 cm^{-1} increased, and a new signal was detected at 1243 cm^{-1} due to the stretching The structure of the sulfated cellobiose–polylysine dendrimers was analyzed by FT-IR and high-resolution NMR spectra. The corresponding FT-IR spectra of **SCLDG1**, **SCLDG2**, and

SCLDG3 are presented in Fig. 2.1. Compared to spectrum D before sulfation, the shape of the vibration of the sulfate group was changed. The characteristic two large signals of the amide group could be clearly identified at 1656 and 1550 cm^{-1} due to the amide carbonyl vending and the NH stretching vibrations, respectively. The signals at 2950 cm^{-1} were attributed to the stretching vibration of the C6 methylene spacer and the lysine side chain. In addition, the signals in the region 1000–1200 cm^{-1} could be attributed to the cellobiose unit and the covalent oxygen bonding between the cellobiose unit and the methylene spacer. In the fingerprint region, the absorptions of **SCLDG1**, **SCLDG2**, and **SCLDG3** exhibited different and intricate patterns each other probably due to the difference of the generations and number of branched sulfated cellobiose units.

The ^{13}C NMR spectra of the sulfated cellobiose−dendrimers **SCLDG1**, **SCLDG2**, and **SCLDG3** were also recorded (Fig. 2.2) and corresponding to those in Fig. 2.1. In the spectrum of the third-generation cellobiose–polylysine dendrimer before sulfation (spectrum D), the ^{13}C signals were assigned by the combination of H–H COSY and HMQC measurements. After sulfation, the resolution of the spectra was lower, probably due to the increased number of sulfate groups at the terminal cellobiose units, and did not allow the assignment of all the complex and overlapped signals. The two singlet signals of C1 and C1' shifted to a higher magnetic field from 104 ppm to around 102 ppm due to the sulfated cellobiose units. The rest of the carbon signals of cellobiose unit appeared as complex and overlapped signals between 60–85 ppm. The methylene spacer and lysine side chain signals were detected between 20–60 ppm, in where the methylene spacer signals were observed as several sharp singlet signals, whereas those of the lysine side chain (α–δ) were small and broad. Furthermore, the signal at 56 ppm was attributed to the α carbon atom of the lysine side chain. The carbonyl carbon signals due to several peptide bonds were observed at 180 ppm.

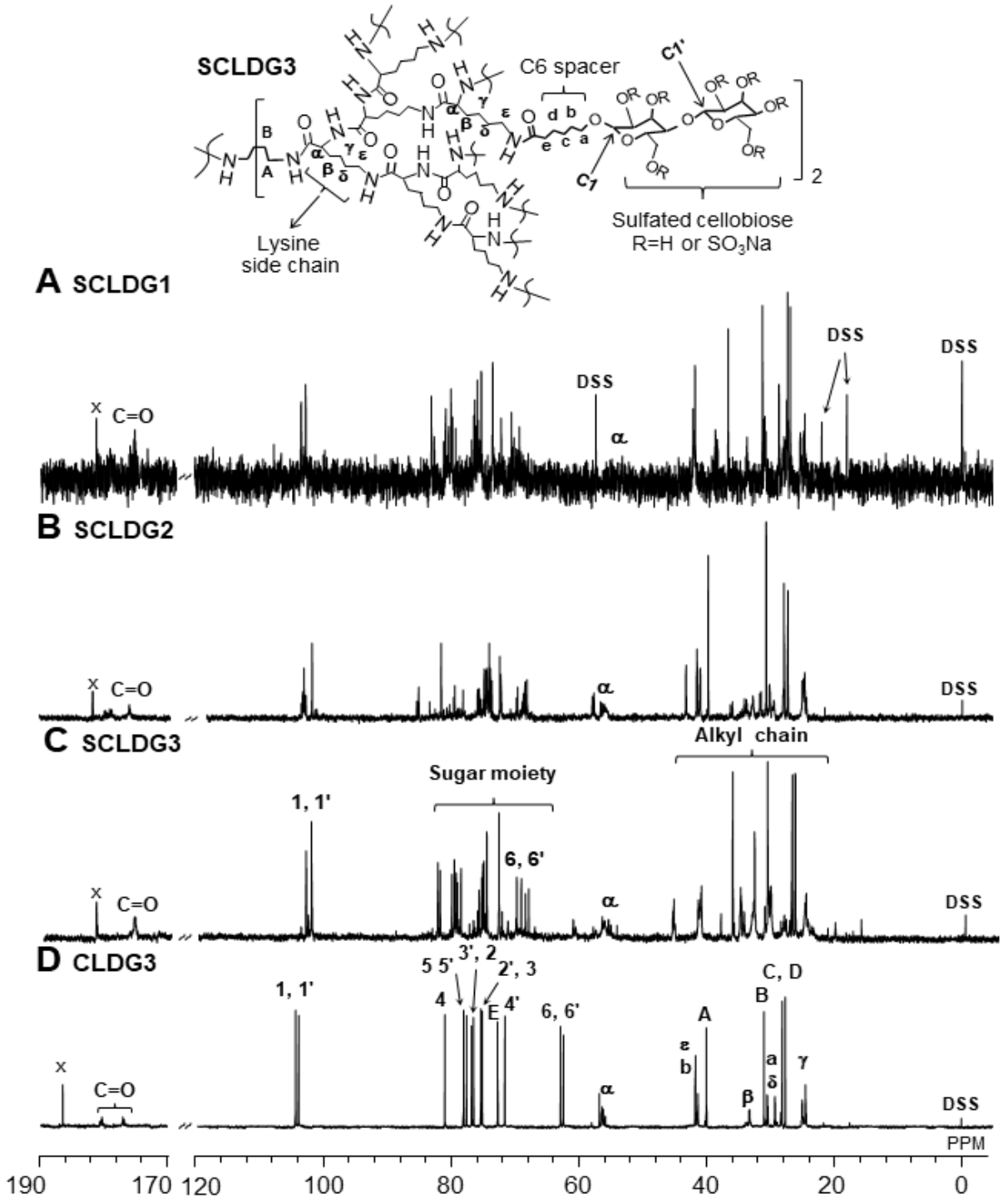


Fig. 2.2. 150 MHz ^{13}C NMR spectra of the (A) first-, (B) second-, and (C) third-generation sulfated cellobiose–polylysine dendrimers. Spectrum (D) is the third-generation cellobiose–polylysine dendrimer before sulfation. The spectra were measured in D_2O at 40°C and DSS (δ=0 ppm) was used as an internal standard.

Table 2.2. Interaction of sulfated cellobiose–poly-L-lysine dendrimers with poly-L-lysine as a model peptide[a, b]

	Before sulfation		After sulfation						
			With poly-L-lysine			Without poly-L-lysine		With poly-L-lysine	
Sulfated	Particle size	ζ	Apparent kinetic constant			Particle size	ζ	Particle size	ζ
dendrimer	nm	mV	k_a (1/Ms)	k_d (1/s)	K_D (M)	nm	mV	nm	mV
SCLDG1	1.5±0	2.77	6.62×10^{3}	4.37×10^{-3}	6.60×10^{-8}	21.5±3.4	-27.5	164.3±22.3	0
SCLDG2	22.8±7.0	5.24	2.45×10^{4}	4.55×10^{-8}	1.86×10^{-12}	65.8±0	-25.6	248.5±13.2	0.2
SCLDG3	46.4±4.9	20.02	5.77×10^{4}	2.37×10^{-4}	4.10×10^{-9}	86.8±10.2	-0.7	324.2±54.0	0.6
Dextran sulfate[c]			1.03×10^{5}	5.29×10^{-4}	5.14×10^{-9}	50.3±9.5	-10.0	161.2±42.9	-9.4

a) Commercially available poly-L-lysine with $M_W = 1\times10^3$–5×10^3, particle size = 26.5±4.5 nm, and ζ potential = 0.22 mV was used.
b) The particle size (nm) and zeta potential (mV) of the sulfated dendrimers in the presence or absence of poly-L-lysine were determined at 25°C by a dynamic light scattering measurement in phosphate buffer solution (pH=7.4) at a concentration of 1 mg/ml or 0.5 mg/ml.
c) Standard dextran sulfate with $\overline{M}_n=8.5\times10^3$ and DS=2.1 was used.

*2.4.3. Anti-HIV activity of **SCLDG1**, **SCLDG2**, and **SCLDG3***

It is known that the high molecular weight and DS of sulfated poly and oligosaccharides significantly contribute to potent anti-HIV activity, while sulfated oligosaccharides with low molecular weight exhibited low anti-HIV activity [Yoshida 1990]. Sulfated oligosaccharides attached to the terminal ends of dendrimers were also found to have high anti-HIV activity due to the cluster effect (Table 1) [Lee 1978]. In this study, the relationship between the generations of a dendrimer and the antiviral activity was investigated. The anti-HIV activity was represented by the 50% effective concentration against HIV (EC_{50}) and the cytotoxicity by the 50% cytotoxic concentration (CC_{50}) against MT-4 cell. Lower EC_{50} values indicated a higher anti-HIV activity, whereas increased CC_{50} values implied low cytotoxicity. The anti-HIV activity was also compared to that of standard sulfated polysaccharides with large molecular weights, namely, curdlan and dextran sulfates, which exhibited in vitro potent anti-HIV activity (EC_{50}=0.22 and 0.45 μg/mL, respectively) and low cytotoxicity (CC_{50}>1000 μg/mL). In particular, the anti-HIV activity of **SCLDG1** was low with an EC_{50} value of 3.7 μg/mL, and increased to EC_{50}=0.6 μg/mL for the second generation **SCLDG2**. Nevertheless, the anti-HIV activity of the next generation dendrimer, **SCLDG3**, was lower (EC_{50}=1.5 μg/mL). These high to moderate anti-HIV activities were attributed to the cluster effect of the sulfated cellobiose units at the terminal ends of the dendrimers, considering that previously reported sulfated oligosaccharides had low or no anti-HIV activity [Katsuraya 1994a; 1994b; 1995]. Thus, **SCLDG2** exhibited the highest anti-HIV activity among the three dendrimers, suggesting that the number of the sulfated cellobiose units at the terminal end in **SCLDG2** favored the interactions between the negatively charged sulfate groups in the cellobiose units and the positively charged amino groups in the HIV surface glycoprotein gp120. Furthermore, the relatively low anti-HIV activity of **SCLDG1** could be attributed to the weak interaction with HIV gp120, due to the lower number of sulfated cellobiose units. The third-generation dendrimer

SCLDG3 did not exhibit any electrostatic interaction, probably due to the crowded sulfated cellobiose units at the terminal end. None of the reported third-generation sulfated glycodendrimers has exhibited high anti-HIV activity (EC_{50}=3.2–6.7 μg/mL) as mentioned in the introduction part. These phenomena were similar to previously reported anti-HIV activity of sulfated branched polysaccharides [Han 2009]. Sulfated polysaccharides bearing branches in every monomeric saccharide unit in the main chain were less active (EC_{50}=1.3–5.9 μg/mL) than sulfated polysaccharides (EC_{50}=0.3 μg/mL) bearing less branches (ca. 15 mol%). In addition, it was found that the distance between branched saccharides in the sulfated branched polysaccharides significantly contributes to the high anti-HIV and blood anticoagulant activities. Therefore, the number of the sulfated cellobiose units at the terminal ends as defined by the generation, determined the anti-HIV activity of the **SCLDG1**, **SCLDG2**, and **SCLDG3** dendrimers. It should also be noted that the cytotoxicity of the dendrimers was low at concentrations higher than 200 μg/mL.

*2.4.4. Interaction of **SCLDG1**, **SCLDG2**, and **SCLDG3** with poly-L-lysine*

On the anti-HIV mechanism, the reverse transcriptase (RT) and protease inhibitors against HIV work in the infected cells to suppress the replication of HIV virions. For sulfated alkyl oligosaccharides, the long-chain alkyl group plays a key role in the enhancement of anti-HIV activity [Katsuraya 1994a; 1994b; 1995], namely, the long-chain alkyl group was fixed into the lipid bilayer of HIV and then the sulfated oligosaccharide moiety was electrostatically interacted with HIV gp120 to prevent the infection of HIV [Uryu 1992; Bai 2020]. On the other hand, sulfated polysaccharides blocks HIV adsorption to the target cell surfaces by the electrostatic interaction as described in the 3.3 section [Battulga 2019]. The inhibitory mechanism of the sulfated cellobiose–polylysine dendrimers is most likely to that of sulfated polysaccharides. The interaction between the sulfated cellobiose–polylysine dendrimers and poly-L-lysine

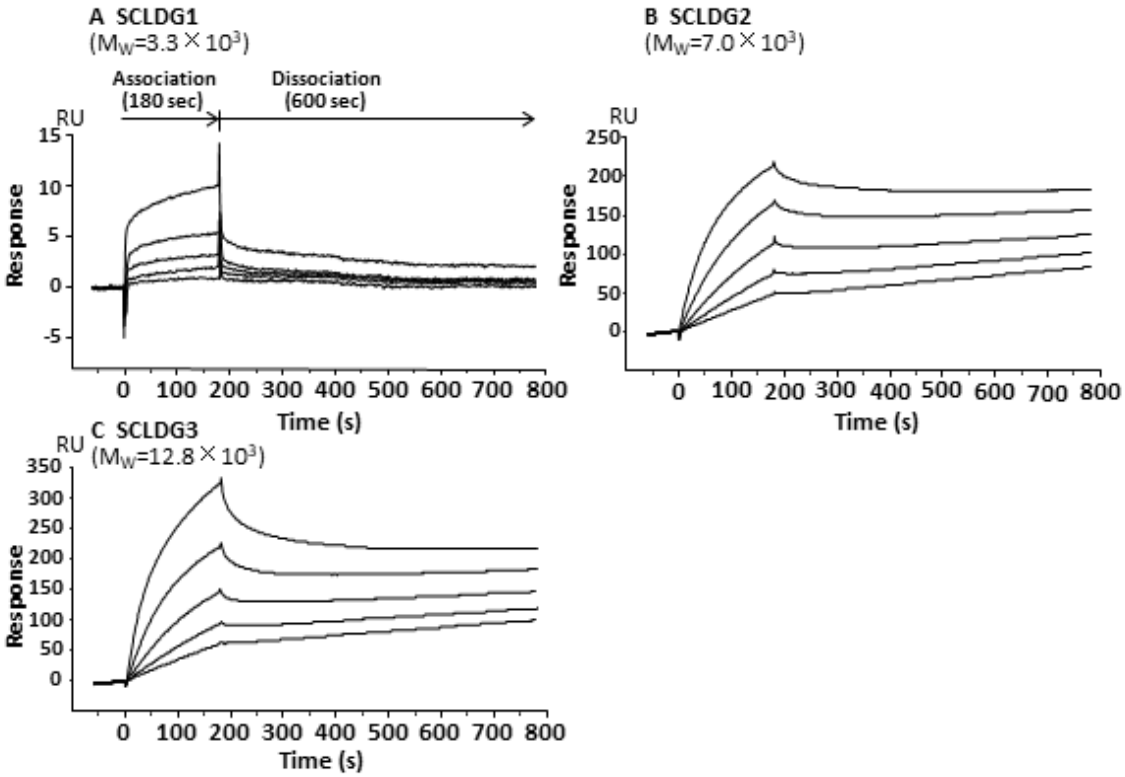


Figure 2-3. SPR profiles of the (A) first-, (B) second-, and (C) third-generation sulfated cellobiose–polylysine dendrimers. The degree of sulfation was (A) 1.8, (B) 2.1, and (C) 2.1, respectively. The apparent k_a and k_d were (A) k_a=6.62×10^3 1/Ms and k_d=4.37×10^{-3} 1/s, (B) k_a=2.45×10^4 1/Ms and k_d=4.55×10^{-5} 1/s, and (C) k_a=5.77×10^4 1/Ms and k_d=2.37×10^{-4} 1/s, respectively.

(M_w=1000−5000) as a model of HIV gp120 was investigated by SPR to reveal the anti-HIV mechanism. Poly-L-lysine bearing positively charged amino acids within the side chain was immobilized on the Biacore CM5 sensor chip. Afterwards, each sulfated dendrimer (**SCLDG1**, **SCLDG2**, and **SCLDG3**) bearing negatively charged sulfate groups within the terminal sulfated cellobioses, was passed through the sensor chip at five concentration steps diluted from 500 to 0.31 μg/mL to give the corresponding response signals (Fig. 2.3). The ordinate is the SPR response

intensity represented by the response unit. The response SPR signals were also used to calculate the apparent association- (k_a) and dissociation- (k_d) rate constants were calculated (Table 2.2).

The k_a value of the second-generation dendrimer **SCLDG2** (k_a=2.45×10^4) was higher than that of **SCLDG1** (k_a=6.62×10^3) and slightly lower than that of **SCLDG3** (k_a=5.77×10^4), suggesting that the rate of association between the sulfated cellobiose–polylysine dendrimers and poly-L-lysine was enhanced with increasing the generations of dendrimers. In comparison, **SCLDG2** had the lowest k_d value (4.55×10^{-8}) compared to **SCLDG1** (k_d=4.37×10^{-3}) and **SCLDG3** (k_d=2.37×10^{-4}). Since the dissociation-rate indicates the strength of the interaction, the calculated values implied that the association between **SCLDG2** and poly-L-lysine was not affected over time. The dissociation constant (K_D) of **SCLDG2** was also the lowest with a value of 1.86×10^{-12}, further confirming that the second-generation sulfated dendrimer **SCLDG2** exhibited the strongest interaction with poly-L-lysine compared to the first- and third-generation dendrimers **SCLDG1** and **SCLDG3**. This strong interaction was attributed to the electrostatic interaction between the negatively charged sulfate groups in the sulfated cellobiose units and the positively charged amino acids in the poly-L-lysine side chains. Thus, the cluster effect of **SCLDG2** was found to be the most effective for potent anti-HIV activity. The SPR results were also consisted with the anti-HIV activity results, which indicated **SCLDG2** as the most active (Table 2.1).

The particle size and ζ potential of the synthesized dendrimers measured by DLS (Table 2.2). In particular, the particle size increased from 21.5 to 86.8 nm as the generations increased, while it was further increased to 164.3, 248.5, and 324.2 nm for **SCLDG1**, **SCLDG2**, and **SCLDG3** respectively, by the addition of poly-L-lysine, confirming the electrostatic interaction between the negatively charged sulfated groups in the terminal sulfated cellobiose units and the positively charged amino groups in poly-L-lysine. The ζ potential values of **SCLDG1**, **SCLDG2**, and **SCLDG3** without poly-L-lysine were -27.45, -25.55, and -0.7 mV, respectively, with **SCLDG3**

exhibiting a relatively higher value among the three dendrimers. Given that the ζ potential indicates the state of the electric charges around the molecules, the higher value of **SCLDG3** might be due to the neutralization of the third-generation core by an increase number of amide bonds and to the five remaining unsubstituted positively charged amino groups in the dendrimer. Furthermore, the degree of substitution of the cellobiose units was 11 in the 16 terminals **SCLDG3**. After the addition of poly-L-lysine with ζ=0.2 mV, the ζ potential of all dendrimers increased to 0, 0.2, and 0.6 mV, respectively, due to the neutralization of the negatively charged sulfate groups by the positively charged amino groups in poly-L-lysine. Hence, the DLC results also highlighted the strong electrostatic interaction between the sulfated cellobiose–polylysine dendrimers bearing negatively charged sulfate groups at their terminal ends and the positively charged amino groups of the poly-L-lysine side chains.

2.5. Conclusion

New sulfated cellobiose−polylysine dendrimers of the three generations were synthesized and investigated for their anti-HIV activity. Compared to **SCLDG1** and **SCLDG3**, that the second-generation sulfated dendrimer (**SCLDG2**) exhibited the highest anti-HIV activity (EC_{50}=0.6 μg/mL) and the strongest interaction with poly-L-lysine, which was used as a model of HIV gp120. The anti-HIV mechanism was also explored by SPR and DLS and the obtained results were consisted with the anti-HIV activity assay. The high anti-HIV activity of **SCLDG2** was attributed to the cluster effect that originated from its dendric structure, which had a moderate distance between the sulfated cellobiose units at its terminal ends. Furthermore, it was revealed that the electrostatic interaction between the negatively charged sulfated groups in the cellobiose units at the terminal of dendrimers and the positively charged amino acids in HIV gp120 was the driving force of the high anti-HIV activity. Further studies are currently underway to elucidate the cluster effect of low

molecular sulfated oligosaccharides against HIV and other viruses, and to develop new oligosaccharide materials with high biological activity and lower cytotoxicity.

Chapter 3

Relationship between anti-HIV activity and cytotoxicity of sulfated alkyl oligosaccharides

3.1 Abstract of Chapter 3

Sulfated alkyl oligosaccharides with long-chain alkyl group bearing carbon number of C6 and C18 were synthesized by click reaction between the corresponding 1-alkynes and acetylated 1-azide-1-*O*-deoxy-maltoheptaoside, respectively, to elucidate the relationship between the length of long-chain alkyl groups and anti-HIV as well as cytotoxicity. SPR measurements of the alkyl oligosaccharides before sulfation with liposome suggested that the apparent association-rate constant (k_a) of sulfated alkyl oligosaccharides bearingC6 and C18 alkyl groups gave smaller and larger k_a values and the dissociation-rate constant (k_d) afforded lager and smaller k_d values. These results indicate the longer-chain alkyl groups are interacted strongly and penetrate to liposome. The long-chain alkyl group should be interacted with lipid bilayer of both HIV and MT-4 cells to appear high anti-HIV activity and also cytotoxicity as a side effect. The balance between hydrophobic long-chain alkyl group and hydrophilic sulfated oligosaccharide moiety was found to be important for high anti-HIV activity and low cytotoxicity.

3.2. Introduction

Since sulfated polysaccharides, which were synthesized by sulfation of synthetic and naturally occurring polysaccharides, were found to have potent anti-HIV activity and low cytotoxicity [Nakashima et al., 1987], many sulfated polysaccharides were prepared and the relationship between structure and anti-HIV activity was investigated [Yoshida, 2001; 2020]. The anti-HIV activity of sulfated polysaccharides was expected to the electrostatic interaction between negatively charged sulfate groups in sulfated polysaccharides and positively charged amino group in HIV surface glycoprotein gp120[Uryu et al., 1992; Tungalag et al., 2019]. Several papers reported on the anti-HIV mechanism, for example, interaction between a mutant of HIV gp120 and curdlan sulfate [Jagodzinski et al., 1996], analysis by a software Chem X [Jagodzinski et al., 1994], and NMR analysis referenced by the interaction between heparin and antithrombin III [Lindahl et al.; 1983],. Recently, we elucidated the anti-HIV mechanism by the interaction of oligopeptides from HIV surface glycoprotein gp120 and sulfated polysaccharides using SPR and DLS [Tungalag et al, 2019]. The three oligopeptides were synthesized by the reference of the three regions in HIV gp 120 at C-terminus, V3 loop region, and CD4 binding domain. It was found that sulfated polysaccharides were strongly interacted to oligosaccharides from C-terminus and V3 loop regions having positively charged amino acids, and no interaction occurred at the CD4 binding domain without positively charged amino acids, indicating that sulfated polysaccharides were electrostatically interacted to the C-terminus and V3 loop of HIV gp120 to inhibit the infection of HIV.

On the other hand, sulfated oligosaccharides bearing ling-chain alkyl group (sulfated alkyl malto-oligosaccharides) have been reported to have potent anti-HIV activity, even though sulfated oligosaccharides without long-chain alkyl group had low anti-HIV activity. Therefore, long-chain alkyl group plays a significant role in the potent anti-HIV activity of sulfated oligosaccharides

[Uryu et al.; 1997]. Sulfated laminari-oligosaccharides composed of 4−9 glucose residues were prepared by acetolysis of a naturally occurring polysaccharide, curdlan, bearing linear 1, 3-β-D-pyranosidic structure and then HPLC purification. Glycosylation with long-chain alkyl group, deacetylation, and subsequent sulfation, sulfated alky laminari-oligosaccharides were obtained, in which sulfated alkyl laminari-oligosaccharides composed of pentaoside to nonaoside bearing dodecyl group at the reduced end had potent anti-HIV activity of EC_{50}=0.10−0.18 μg/mL and low cytotoxicity of CC_{50}>1000 μg/mL, respectively. However, when the further long alkyl group was introduced, for example, octadecylated sulfated laminari-oligosaccharides composed of 5−9 glucopyranoses increased cytotoxicity, CC_{50}=180−240 μg/mL. For sulfated alkyl malto-oligosaccharides with 4−7 glucose residues, the similar results were obtained [Katsuraya et al., 1994; 1995, 1996]. Previously, professor Kobayashi reported that 3-*O*-dodecyl dextran was interacted with liposome lipid bilayer by anchoring the long-chain dodecyl group [Kobayashi et al.; 1986]. For the elucidation of the role of the long-chain alkyl group in sulfated polysaccharides on the anti-HIV activity, sulfated 3-*O*-dodecyl dextran was synthesized and anti-HIV mechanism was investigated by using SPR and DLS [Bai et al.; 2015, Budragchaa et al.; 2020]. Liposome was used as a model of HIV. The dodecyl group in sulfated 3-*O*-dodecyl dextran was penetrated and fixed into lipid bilayer of liposome and then the sulfated dextran moiety was covered over the liposome, because the zeta potential and particle size of liposome were changed measured by DLS and the interaction with liposome gave large k_a and small k_d obtained by SPR. The cytotoxicity of sulfated 3-*O*-dodecyl dextran was low. In addition, sulfated dodecyl maltoheptaoside was newly synthesized by click reaction of 1-dodecyne and acetylated 1-azide-1-*O*-deoxy-maltoheptaoside followed by deacetylation and sulfation. Newly synthesized sulfated 1-(decadecyl-1, 2, 3-triazole)-1-deoxy-maltoheptaoside bearing long-chain alkyl group was found to have potent anti-HIV

activity, EC_{50}=0.03 μg/mL, and to interact with liposomes. The apparent associate- (k_a) and dissociate-rate (k_d) constants were k_a=1.11×10^4–8.62×10^5 1/Ms and k_d=7.66×10^{-7}–8.87×10^{-4} 1/s measured by SPR. The particle size increased and the zeta potential was negative, indicating that the sulfated alkyl maltoheptaoside was interacted with liposomes through the long-chain alkyl group. However, the sulfated alkyl maltoheptaoside exhibited cytotoxicity against MT-4 cell, a HIV sensitive cell, at CC_{50}=91 μg/mL, probably due to strong interaction of HIV lipid bilayer, by comparison with that of a standard dextran sulfate (H-39), CC_{50}=712 μg/mL (EC_{50}=0.03 μg/mL). Smaller values show lower cytotoxicity.

In this study, we report the synthesis of sulfated alkyl maltoheptaosides by click reaction of acetylated 1-azide-1-*O*-deoxy-maltoheptaoside and 1-alkynes and 1-hexyne (C6) and 1-octadecyne (C18) and elucidate the relationship between the length of long-chain alkyl groups and cytotoxicity, by SPR and DLS. The anti-HIV activity of them also investigated.

3.3. Materials and methods

3.3.1. Materials

1-Alkynes were purchased from Kishida Chemical, Co., Ltd., Japan. SO_3-pyridine complex was obtained from FUJIFILM Wako Pure Chemical Industries, Ltd. Dialysis tube with molecular weight cut-off ranging 100−500 Da (Spectrum Laboratories, Inc., USA) was used. 1-Azide-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside was synthesized by azidation of 1-bromo-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside with sodium azide according to our recent paper [Bai et al., 2020]. The liposome having the diameter of 100 nm was prepared according to the manufacture's protocol [GE healthcare, 2019].

3.3.2 Measurement

600MHz ^{1}H and 150MHz ^{13}C NMR spectra were taken on a JEOL JMN AEC-600 spectrometer in DMSO-d_6 or D_2O as solvent at 40°C using DSS (δ=0.00 ppm) as an internal standard. IR spectra were recorded with a Perkin Elmer Spectrum One FT-IR spectrometer. Elemental analysis was obtained by a CE440 elemental analyzer (Exeler Analytical, Ltd., UK). SPR spectra were measured by a Biacore X100 instrument (GE Healthcare UK) at 25°C using a L1 sensor chip and the signal was corrected by the 1:1 binding model. DLS was used for particle size and zeta (ζ) potential measurements by using an Otsuka Electronics ELSZ-1000ZS in phosphate buffer solution (pH=7.4) or Milli-Q ultrapure water at the sample concentration of 0.5 mg/mL or 1 mg/mL at 25°C.

3.3.3 Click reaction between 1-azide-1-O-deoxy-docosa-O-acetyl-maltoheptaoside and 1-hexcyne

A solution of $CuSO_4 \cdot 5H_2O$ (10 mg, 0.03 mmol) and sodium ascorbate (18 mg, 0.06 mmol) in H_2O (5 mL) was added to a mixture of 1-azide-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside **1** (0.5 g) and 1-hexcyne (0.6 g, 5 mmol) in CH_2Cl_2 (15 mL). The mixture was stirred for 24 h at 35°C and then the product was extracted with $CHCl_3$. The organic layer was washed with water several times, dried over anhydrous $MgSO_4$, and concentrated. The obtained precipitate was purified by recrystallization from an ethyl acetate/hexane solution to give pure 1-(tetracyl-1, 2, 3-triazole)-1-deoxy-docosa- *O*-acetyl-maltoheptaoside **2a** (1.45 g) in 90% yield.

3.3.4 Sulfation of alkyl maltoheptaoside

Typical procedure of sulfation of alkyl maltoheptaoside is as follows. 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside **3a** (0.38 g, 0.3 mmol), which was obtained by deacetylation

of **2a** in 90% yield, was dissolved in dry DMSO (10 mL) and then SO_3-pyridine complex (0.86 g, 5.4 mmol) was added. The mixture was stirred for 2 h at 85°C. After cooling at room temperature, the mixture was neutralized by 10% NaOH solution and dialyzed against deionized water for 2 d. The water solution was concentrated to 20 mL by using rotary evaporator at 40°C and the concentrated solution was freeze-dried to give sulfated 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside **4a**. Sulfated 1-(hexadecacyl-1, 2, 3-triazole)-1-*O*-deoxy- maltoheptaoside **4b** was obtained by the same procedures.

3.3.5 Anti-HIV activity and cytotoxicity of sulfated alkyl maltoheptaoside

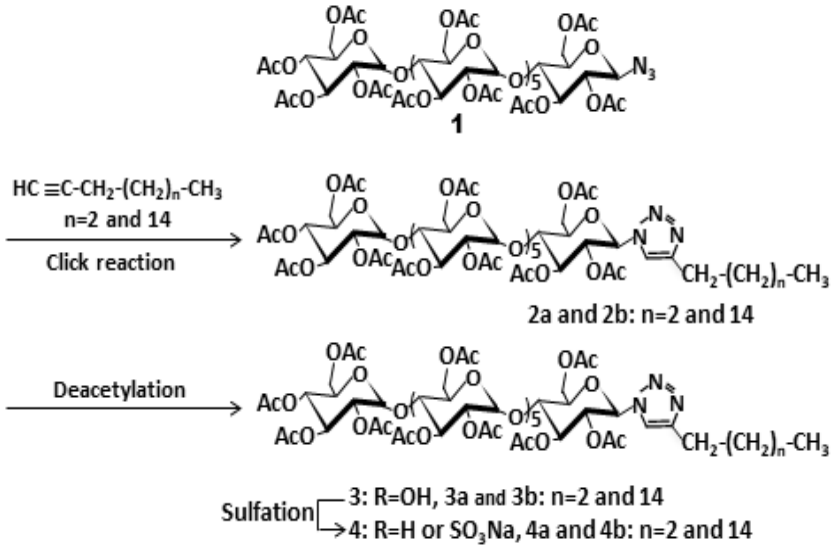
Anti-HIV activity of sulfated alkyl maltoheptaosides was assayed by MTT method using HIV_{IIIB} virus MT-4 cells according to the reported method [Pauwels et al., 1988].

3.4. Results and Discussion

3.4.1. Synthesis of sulfated alkyl maltoheptaosides

Scheme 1 shows the synthetic route of sulfated alkyl maltoheptaosides by click reaction. 1-Aside-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside (**1**) was synthesized by acetolysis of β-cyclodextrin followed by bromination at the reduced end using HBr-acetic anhydrite and subsequent azidation with sodium aside (NaN_3) according to the previous paper [Bai et al., 2020]. For the introduction of long-chain alkyl group, two 1-acetylenes with difference from the carbon numbers (length) of alkyl group, such as 1-hexcyne (C6) and 1-octadecyne (C18) were used for the elucidation of the cytotoxicity of sulfated alkyl maltoheptaosides on the length of alkyl chain. The click reaction proceeded smoothly to give 1-alkyl triazoyl maltoheptaosides (**2a** and **2b**) in 90%

yield. After deacetylation to recover hydroxyl groups and then sulfation with SO_3-pyridine complex to produce sulfated 1-(tetracyl- and hexadecadecyl-1, 2, 3-triazole)-1-*O*-deoxy-



Scheme 3-1.
Synthesis of sulfated alkyl heptamaltosides by click reaction.
The long-chain alkyl group is bound at the reduced end of maltoheptaose through triazole ring.

maltoheptaosides (**3a** and **3b**) in 90% yields, respectively. By the same procedure, we recently prepared sulfated 1-(decadecyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside bearing a C12 long-chain alkyl group at the reduced end and the anti-HIV activity was investigated [Bai et al., 2020].

Figure 3-1 shows the structure of sulfated alkyl maltoheptaosides bearing long-alkyl groups of C6 and C18 lengths, respectively, which long-chain alkyl groups were bounded at the reduced end of maltoheptaoside through triazole ring. In this research, the relationship between the length of the

long-chain alkyl groups and anti-HIV activity and cytotoxicity was described in sections 3.4.3 and 3.3.4.

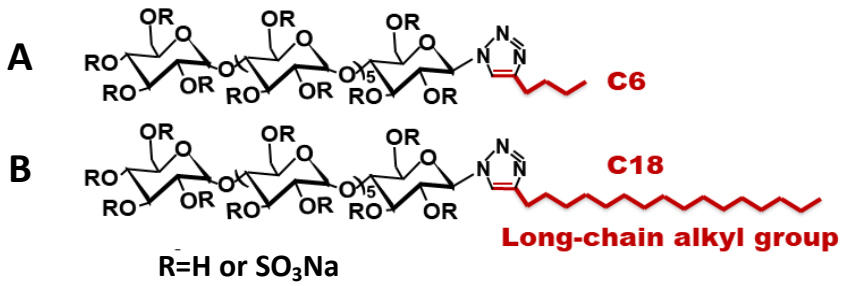


Figure 3-1.
Sulfated alkyl maltoheptaosides bearing long-chain alkyl group, which is bound at the reduced end through triazole ring.

3.4.2. Structure of acetylated alkyl maltoheptaosides

Structure of 1-(tetracyl- and hexadecyl-1, 2, 3-triazole)-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaosides **2a** and **2b** was determined by ^{13}C and ^{1}H NMR and FT-IR spectra. Figure 3-2 shows FT-IR spectra of **2a** and **2b**, respectively. After click reaction of 1-hexyne with acetylated 1-aside-1-*O*-deoxy-maltoheptaoside **1**, the addition product **2a** was obtained in good yield. In the IR spectrum of **2a**, alkyne and aside signals at 2100 and 2200 cm^{-1} disappeared, suggesting that 1, 4-disubstituted 1, 2, 3-triazoyl product **2a** was obtained. The large carbonyl signal due to stretching vibration of acetyl groups appeared at 1750 cm^{-1}. Figure 3-3 shows ^{13}C NMR spectrum of 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside (**2a**), in which the methylene in the alkyl chain and alkene carbon signals in the triazole ring, a, b, and c appeared at 62, 148.5, and 118.5 ppm, respectively. The C1 and C1' signals in maltoheptaoside moiety were assigned at 95.5 and 85 ppm, respectively. The two methylene and terminus methyl signals due to

alkyl chain appeared at 31, 26, and 13.5 ppm, respectively. Large acetyl signals were absorbed at 21 and 169 ppm due to methyl and carbonyl carbons, respectively.

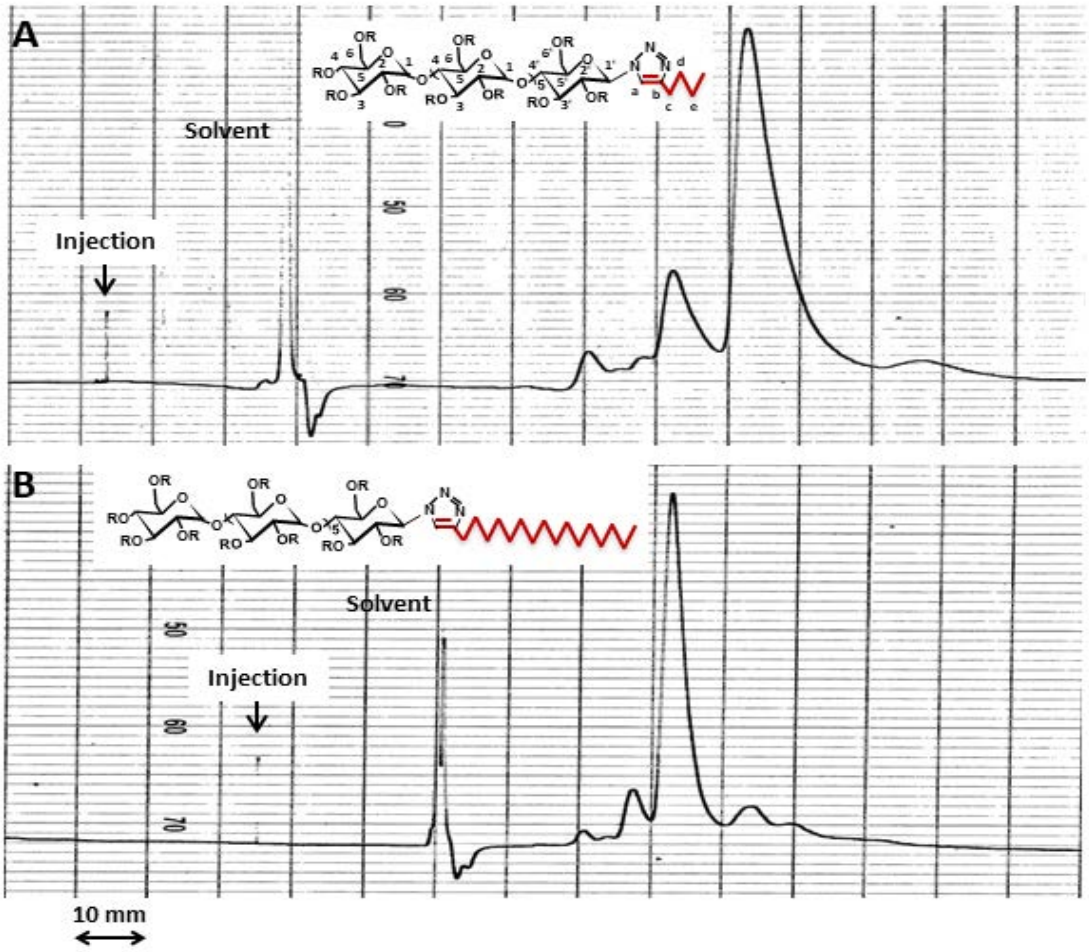


Figure 3-2.
HPLC profiles of (A) 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside and (B) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-docosa-*O*-acetyl maltoheptaoside. (Column: Silica 60, Solvent: Hexane-Ethyl acetate 6:4, 0.6 mL/min, Chart speed: 2 min/10 mm)

In addition, HPLC analysis (Figure 3-2) represented one absorption due to 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-docosa-*O*- acetyl maltoheptaoside, in which main absorptions around 12 and 10 min appeared due to the acetylated alkyl maltoheptaosides.

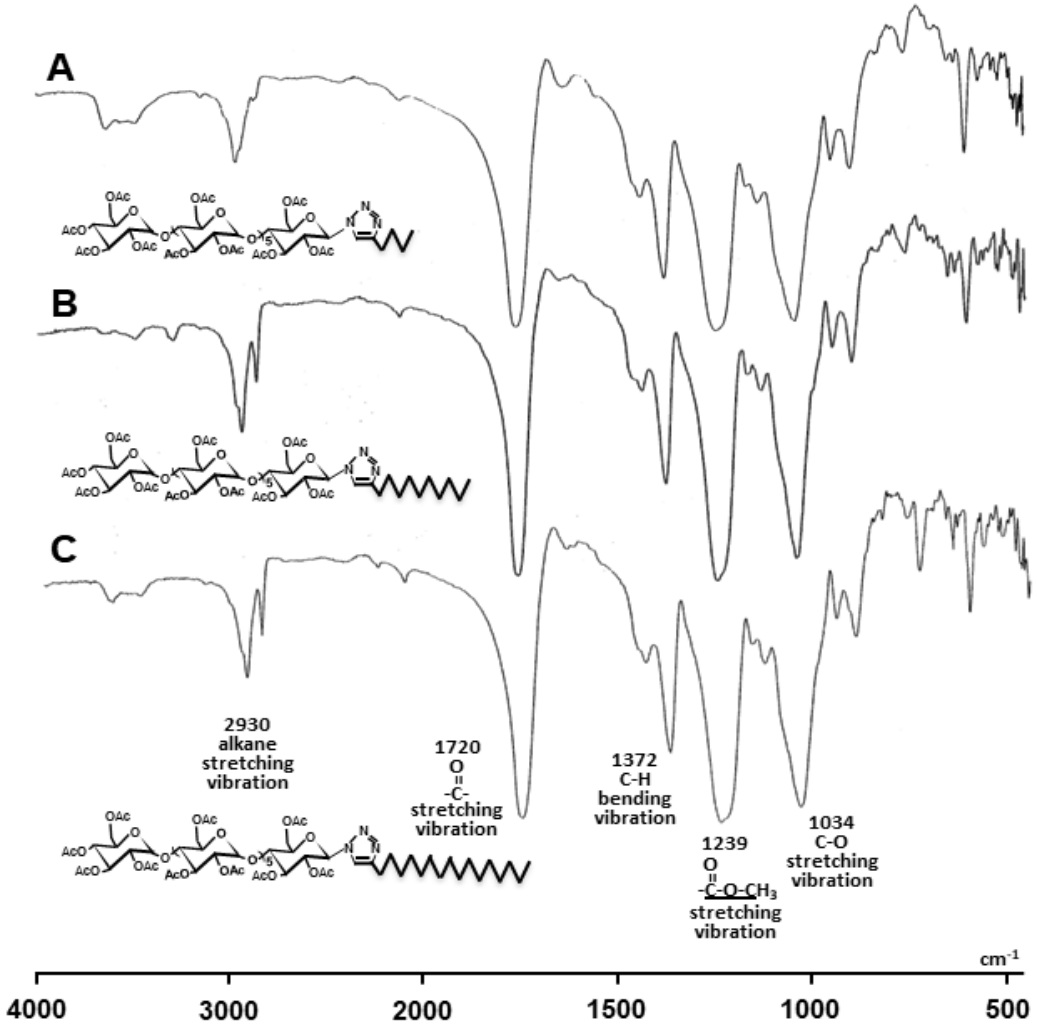


Figure 3-3.
FT-IR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-, (B) 1-(decacyl-1, 2, 3- triazole), and (C) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-docosa-*O*-acetyl maltoheptaosides.

Figure 3-3 shows FT-IR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-, (B) 1-(decacyl-1, 2, 3-triazole), and (C) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-docosa-*O*-acetyl maltoheptaosides, respectively. Strong absorption at 1720 cm^{-1} is caused by stretching vibration of carbonyl group C=O of the acetyl group and the C-H, C-O, and C-O-CH_3 vending and stretching vibrations due to maltoheptaoside, acetyl, and long-chain alkyl groups appeared as large absorptions at 2930, 1372, 1329, and 1034 cm^{-1}, respectively.

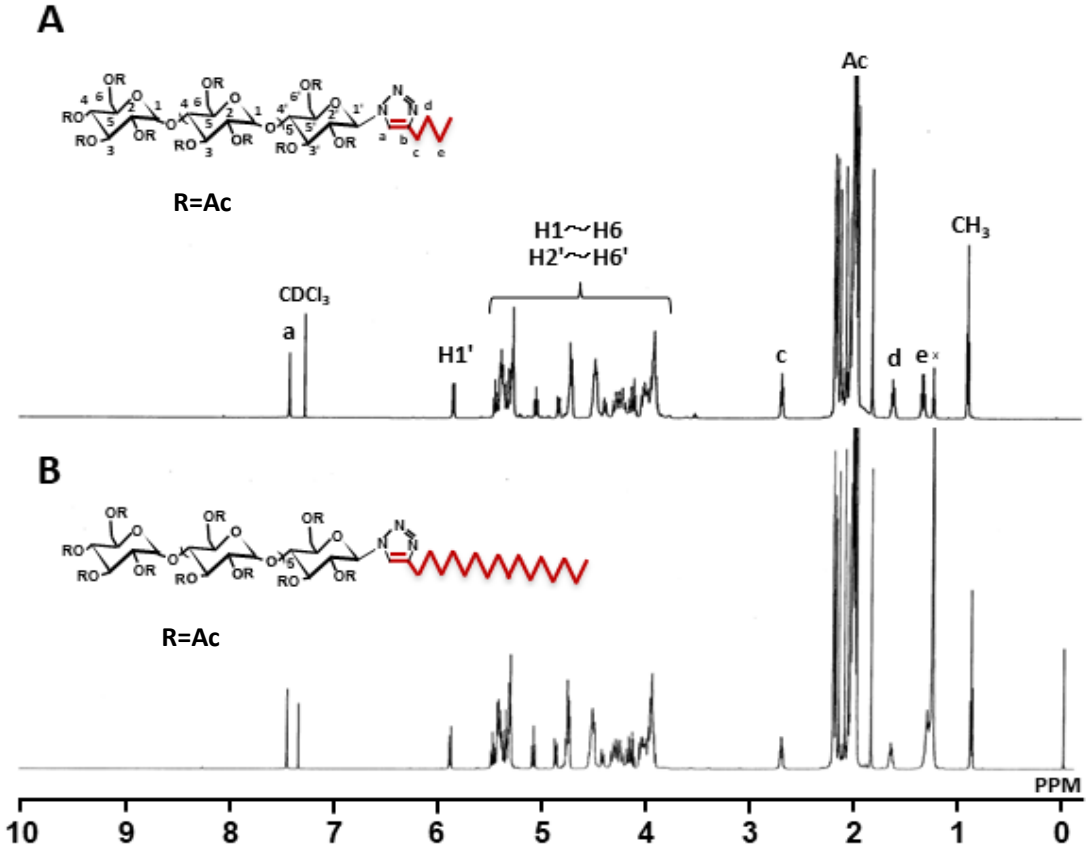


Figure 3-4.
600 MHz ^{1}H NMR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-docosa-*O*-acetyl-maltoheptaoside and (B) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-docosa-*O*-acetyl maltoheptaoside ($CDCl_3$, 30 C).

Figure 3-4 represents 600 MHz 1H NMR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)- and (B) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-docosa-*O*- acetyl maltoheptaosides, respectively. The characteristic signal at 7.45 ppm was assigned as a proton signal in the triazole ring and the H1' signal of acetylated maltoheptaoside appeared at 5.9 ppm. Other proton signals of acetylated maltoheptaoside appeared as overlapped signals between 5.5 and 3.9 ppm. In spectrum A, the proton signals due to side-chain C4 alkyl group are present at 2.5, 1.7, 1.4, and 0.85 ppm owing to

methylene protons of c, d, e, and terminal methyl proton, respectively. Acetyl protons appeared as large signals around 2 ppm.

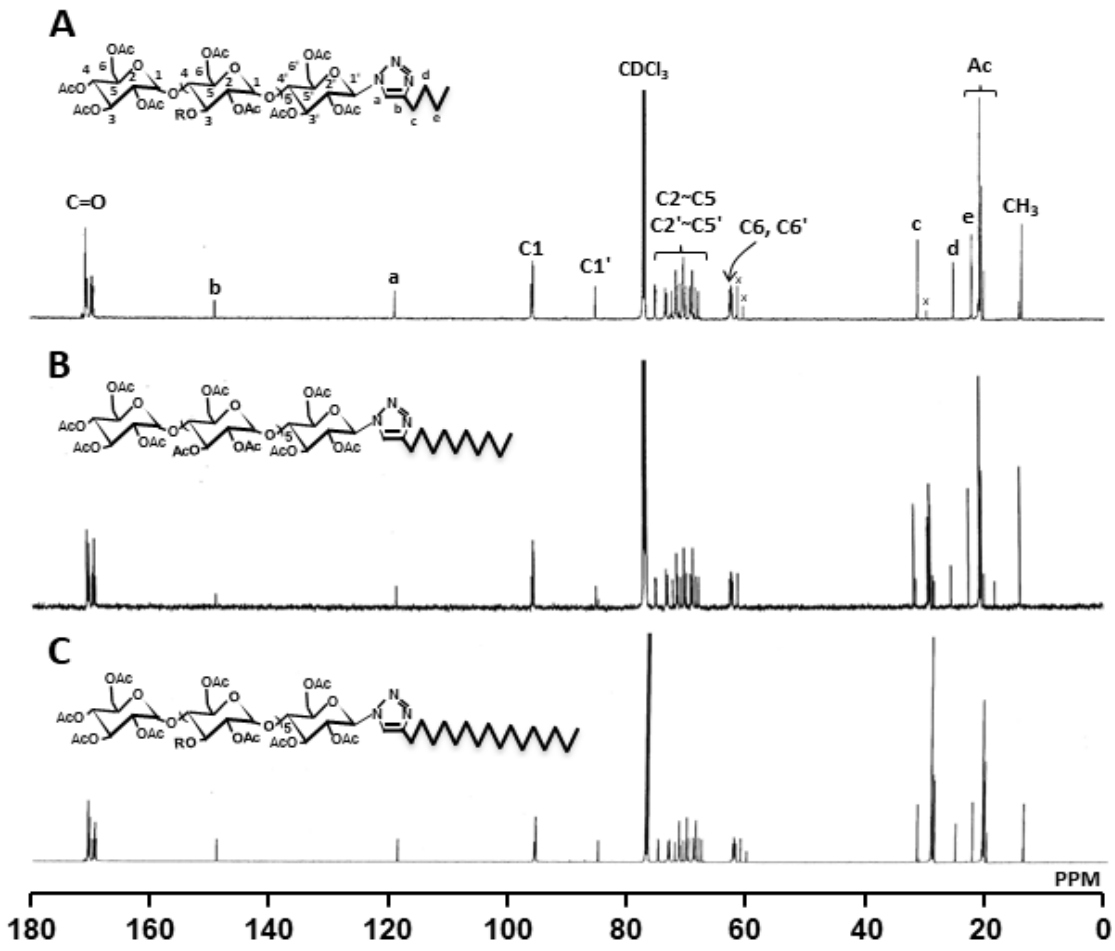


Figure 3-5.
150 MHz ^{13}C NMR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-, (B) 1-(dodecacyl-1, 2 ,3-triazole)-, and (C) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-docosa-*O*-acetyl maltoheptaosides ($CDCl_3$, 30 C).

Figure 3-5 shows ^{13}C NMR spectra of acetylated alkyl maltoheptaosides, (A) 1-(tetracyl-1, 2, 3-triazole)-, (B) 1-(dodecacyl-1, 2 ,3-triazole)-, and (C) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-

deoxy-docosa-*O*-acetyl maltoheptaosides, respectively. The characteristic carbon signals due to triazole ring a and b appeared clearly at 119 and 149 ppm. The alkyl signals in Figure 3-5A were assigned by the 2D NMR measurements owing to the overlapping. Although all of the carbon signals in sugar moieties were not determined, the C1 and C6 signals could be assigned by the 2D NMR measurements. We synthesized acetylated alkyl maltoheptaosides having different length of long-chain alkyl groups by click reaction of 1-acetylenes and 1-azide maltoheptaoside in good yields.

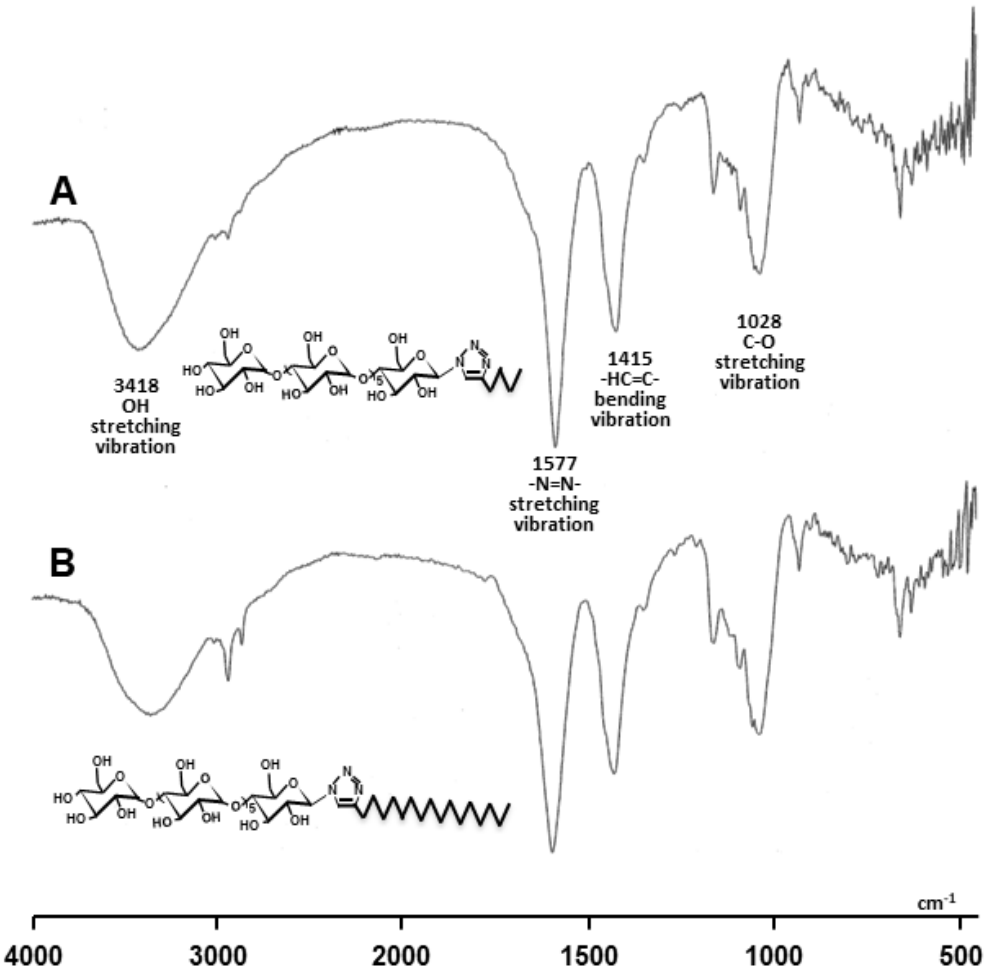


Figure 3-6.
FT-IR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside and (B) 1-(hexadecacyl- 1, 2, 3- triazole)-1-*O*-deoxy-maltoheptaoside.

Next, deacetylation of the acetylated alkyl maltoheptaosides was carried out with sodium in methanol to give alkyl maltoheptaosides having free hydroxyl groups in good yields. Figure 3-6 shows the FT-IR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside and (B) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-maltoheptaoside, respectively. After deacetylation, a large absorption due to hydroxyl group at 3418 cm-1 appeared and acetyl signals at 1720 cm^{-1} disappeared.

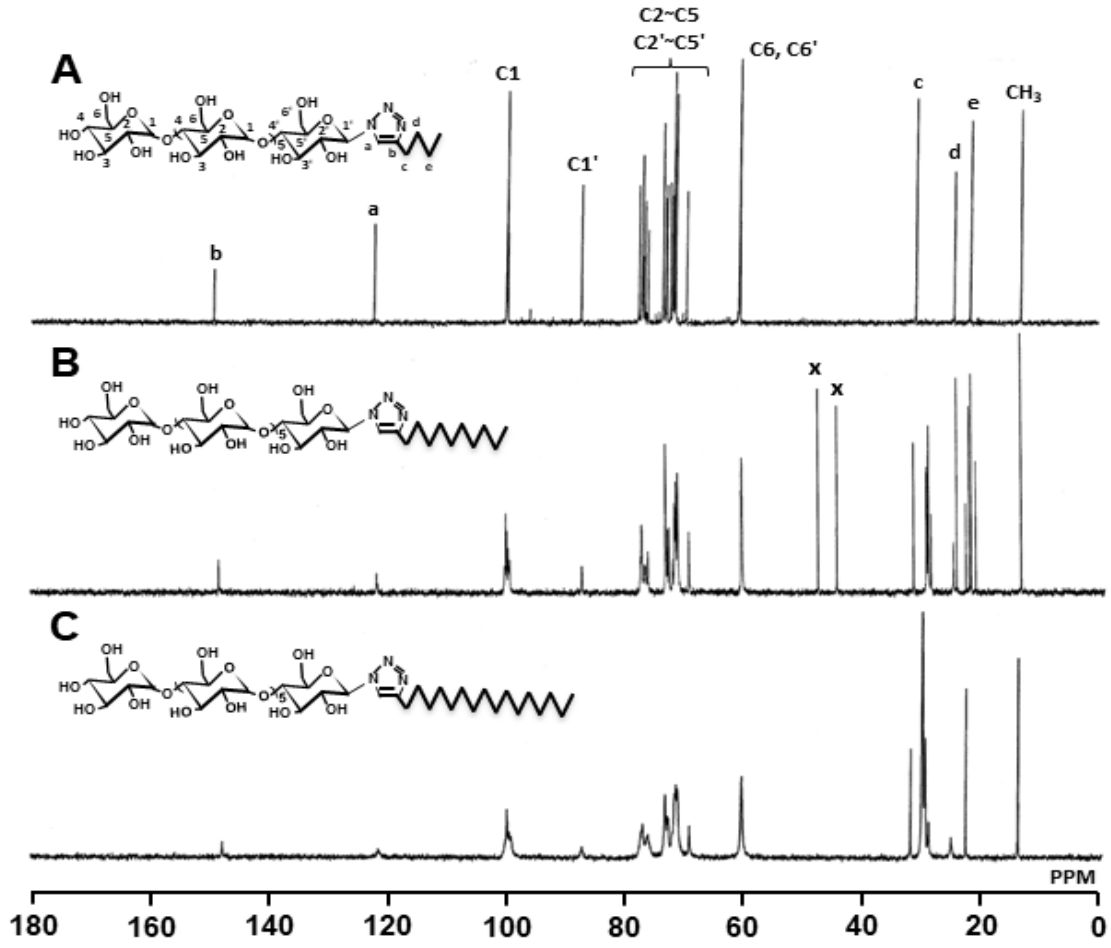


Figure 3-7
150 MHz ^{13}C NMR spectra of (A) 1-(tetracyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside, (B) 1-(dodecacyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside, and (C) 1-(hexadecacyl-1, 2, 3-triazole)-1-*O*-deoxy-maltoheptaoside. (D_2O, 30 C)

Figure 3-7 shows ^{13}C NMR spectra of deacetylated alkyl maltoheptaosides, (A) 1-(tetracyl-1, 2, 3-triazole)-, (B) 1-(dodecacyl-1, 2, 3-triazole)-, and (C) 1-(hexadecacyl-1, 2, 3- triazole)-1-*O*-deoxy-maltoheptaosides, respectively. The characteristic two carbon signals in triazole ring clearly appeared at 121 and 147 ppm, respectively, due to **a** and **b**. The C1 and C1' signals were absorbed separately at 85 and 100 ppm, and the C6 and C6' signals appeared at 60 ppm. The alkyl methylene and methyl carbons were assigned in Figure 3-7A.

3.4.3 Interaction of alkyl maltoheptaosides and liposome

Table 3-1 Association and dissociation constants of alkyl triazoyl maltoheptaosides

Alkyl triazoyl maltoheptaoside			Kinetic result[a]		
	Number of carbon	$\overline{M}_n$ [b] x10^3	k_a 1/Ms	k_d 1/s	K_D M
1	6	1259	$3.11x10^4$	$4.56x10^{-2}$	$1.47x10^{-6}$
2	12	1343	$6.37x10^4$	$1.64x10^{-6}$	$2.57x10^{-11}$
3	18	1427	$1.20x10^5$	$7.30x10^{-7}$	$6.08x10^{-12}$

a) k_a: Association- and k_d: dissociation-rate constants
b) Calculated molecular weight.

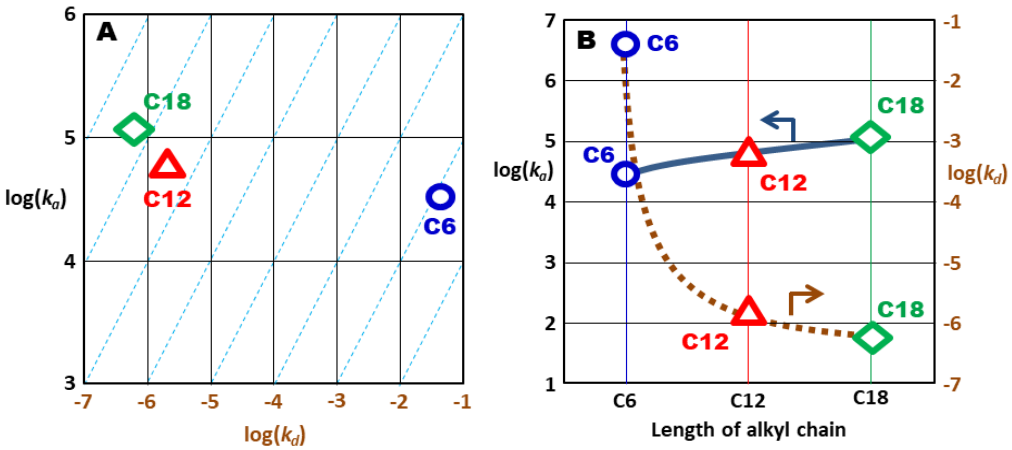


Figure 3-8. Interaction of alkyl maltoheptaoses with liposome. (A) Relationship between association- (k_a) and dissociation- (k_d) rate constants and (B) between k_a and k_d on length of alkyl chain, respectively.

Before sulfation, interaction of alkyl heptamaltoses having C6, C12, and C18 alkyl chains was performed with liposome with an particle size of 100 nm. Table 3-1 shows the results of association- (ka) and dissociation-rate (kd) constants. It was found that the ka and kd increased and decreased with increasing the alkyl chain. Figure 3-8 presents the relationship between the ka and kd (Figure 3-8A) and between the ka and kd on the length of the alkyl chain (Figure 3-8B) respectively. In Figure 3-8A, alkyl heptamaltose having C18 long-chain alkyl group was found to be interacted strongly with liposome, indicating that the long-chain alkyl group was penetrated deeply into liposome. In Figure 3-8B, alkyl maltoheptaose having C18 long-chain alkyl group had the strongest interaction with liposome.

As mentioned above, it was previously reported that laminari- and malto-oligosaccharides exhibited potent anti-HIV activity in the EC_{50} range of 0.4–0.7 μg/mL in vitro using MT-4 cell line and HIV-1_{III} virus, although sulfated oligosaccharides without alkyl groups had low anti-HIV activity. The inhibitory mechanism of HIV infection was assumed to be the interaction of the alkyl group with the lipid bilayer of HIV and then sulfated oligosaccharide moiety should electrostatically bind to the surface glycoprotein HIV gp120 [Katsuraya et al., 1994; 1994; 1995]. Recently, we elucidated the anti-HIV mechanism of sulfated alkyl oligosaccharides by using SPR and DLS measurements with liposomes as a model of HIV, suggesting that the long-chain alkyl group penetrated into the lipid bilayer of HIV to fix sulfated oligosaccharide moiety, which was covered on HIV and then electrostatically interacted with the surface glycoprotein HIV gp120. However, the role of the long-chain alkyl group on the cytotoxicity was still unclear.

3.4.4 Sulfation

Alkyl maltoheptaosides were sulfated with pyridine-SO_3 complex in anhydrous DMSO to give sulfated alkyl maltoheptaosides. Figure 3-9 shows the ^{13}C NMR spectrum of sulfated alkyl maltoheptaosides having C18 long-chain alkyl group through triazole ring.

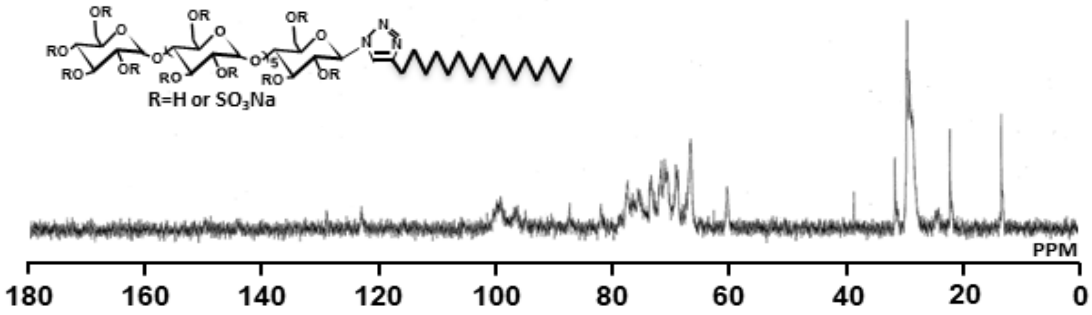


Figure 3-9. ^{13}C NMR spectrum of sulfated alkyl maltoheptaoside having C18 long-chain alkyl group in D_2O at 40 C. DSS was used as an internal standard.

After sulfation, the resolution decreased and the triazole carbon due to b was unclear. However, sulfated alkyl maltoheptaoside should be obtained.

3.4.5. Anti-HIV and cytotoxicity of sulfated alkyl maltoheptaosides

The anti-HIV activity and cytotoxicity are under measured and the interaction with liposome is also investigated.

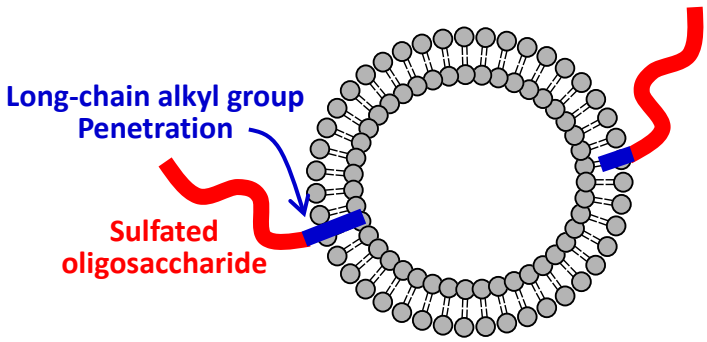


Figure 3-10.
Proposed interaction of sulfated alkyl oligosaccharides bearing C6 and C18 long-chain alkyl groups, respectively, with liposome. The C18 long-chain alkyl group was interacted stronger than that of shorter C6 alkyl group probably to enhance both anti-HIV activity and cytotoxicity.

Figure 3-10 shows the proposed and schematically interaction of the long-chain alkyl groups in sulfated alkyl oligosaccharides with liposome based on the results of SPR and DLS measurements represented in Figure 3-8. The C18 long-chain alkyl group in **4b** was anchored deeply into the lipid bilayer compared with the C6 alkyl group in **4a**. As the results, the lipid bilayer was destroyed by the long-chain alkyl group and the cytotoxicity increased. On the other hand, the shorter C6 alkyl group in **4b** was shallowly interacted with liposome to reduce cytotoxicity.

3.5. Conclusion

The long-chain alkyl group was necessary to produce potent anti-HIV activity, however, the cytotoxicity also increased. It was revealed that the alkyl groups bearing too long length caused the increase in cytotoxicity. For high anti-HIV activity and low cytotoxicity of sulfated alkyl oligosaccharides, the balance between a hydrophobic long-chain alkyl group and a hydrophilic sulfated oligosaccharide moiety is a significant factor.

The SPR results were agreed with the biological results. Thus, the length of long-chain alkyl group was found to be the significant role in the biological activities. At the same time, too long alkyl group caused an increase of cytotoxicity. Because the difference from the anti-HIV activity of **4a** and **4b** will be small and the cytotoxicity of **4a** is low, the balance of hydrophobic long-chain alkyl group and hydrophilic sulfated oligosaccharide moiety is found to be an important factor. Sulfated alkyl maltoheptaosides are synthesized soon and the interaction with liposome is investigated.

Chapter 4
Conclusions and future remarks

4.1. Conclusions and future remarks

In this thesis, the author has researched anti-HIV and cytotoxic mechanisms of sulfated polysaccharide dendrimers and sulfated oligosaccharides bearing a long-chain alkyl group at the reduced end. In the first Chapter, the author described recent reports on biological activities of sulfated polysaccharides as an introduction. Since Gaber found the anti-influenza virus activity of naturally occurring sulfated polysaccharides in marine red algae, many papers have appeared. Professors Yamamoto and Nakashima (Yamaguchi University School of Medicine at that time) found in 1987 that naturally occurring sulfated polysaccharides exhibited potent anti-HIV activity and synthetic sulfated polysaccharides had also potent anti-HIV activity found by Professors Uryu and Yoshida (University of Tokyo at that time) in 1987. Uryu and Yoshida also presumed that the anti-HIV mechanism of sulfated polysaccharides is electrostatic interaction of negatively charged sulfate groups in sulfated polysaccharides and positively charged amino acids in surface glycoprotein gp120 on HIV. From that time, Professor Yoshida has worked on the elucidation of antiviral mechanism of sulfated poly- and oligosaccharides. Recently, Tungalag and Yoshida found the electrostatic interaction using curdlan sulfate, a potent anti-HIV polysaccharide, and oligopeptides from HIV gp120 by SPR and DLS. Therefore, the author has researched the relationship between generations and anti-HIV activity of sulfated oligosaccharide dendrimers in the second Chapter, which relationship was still unclear. In the third Chapter, the author elucidates the relationship between cytotoxicity and length of long-chain alkyl groups of sulfated alkyl oligosaccharides.

In Chapter 2, three new spherical sulfated cellobiose–polylysine dendrimers bearing first, second, and third generations were synthesized by sulfating the corresponding cellobiose–polylysine dendrimers. Cellobiose was used as a model oligosaccharide. The first, second, and third generation sulfated cellobiose dendrimers exhibited potent anti-HIV activity with EC_{50} values of 3.7,

0.6, and 1.5 μg/mL, respectively, in contrast to sulfated oligosaccharides with low anti-HIV activity. The author found that the second-generation sulfated cellobiose dendrimer was the most active. SPR measurements with poly-L-lysine bearing positively charged amino acids as a model of the HIV surface glycoprotein gp120, indicated that the second generation dendrimer had the lowest dissociation constant (K_D=1.86×10^{-12} M). Both the particle size and ζ potential increased in the presence of poly-L-lysine measured by DLS. From these results, the moderate distance between the terminal sulfated cellobiose units in the second-generation dendrimer favored the high anti-HIV activity, owing to the electrostatic interactions developed due to the cluster effect.

In Chapter 3, the author researched on the relationship between cytotoxicity of sulfated alkyl oligosaccharides and length of long-chain alkyl groups. For sulfated oligosaccharides, a long-chain alkyl group at the reduced end of sulfated oligosaccharides enhanced the antiviral activity. However, the cytotoxicity increased with increasing the length of long-chain alkyl groups. Sulfated laminari-oligosaccharides with a C12 alkyl chain had anti-HIV activity of EC_{50}=0.10 μg/mL and cytotoxicity CC_{50}>1000 μg/mL. For sulfated laminari-oligosaccharides with a C18 alkyl chain, the anti-HIV activity was high, EC_{50}=0.63 μg/mL, however, cytotoxicity increased to CC_{50}=220 μg/mL. It was found the hydrophobic and hydrophilic balance for anti-HIV and cytotoxicity of sulfated alkyl oligosaccharides were important factor, however, the precise mechanism on cytotoxicity was still unclear. Bai and Yoshida recently appeared the role of the long-chain alkyl groups on anti-HIV activity analyzed by the interaction of sulfated dodecylated maltooligosaccharide and liposome using SPR, indicating that the long-chain alkyl group penetrates and is fixed into the lipid bilayer of HIV and the sulfated maltoheptaose moiety with negatively charged sulfate groups was electrostatically interacted with HIV gp120 molecule with positively charged amino acids to achieve the inhibition of HIV infection. Thus, two sulfated alkyl malto-oligosaccharides bearing C6 and C18 long-chain alkyl groups were synthesized, respectively, and anti-HIV and cytotoxicity

were measured, indicating that both oligosaccharides were found to exhibit potent anti-HIV activity as high as 0.5 μg/mL. However, the cytotoxicity gave different results, that is, sulfated malto-oligosaccharide bearing C6 alkyl chain had lower cytotoxicity than that bearing C18 alkyl chain. In addition, the interaction with liposome of sulfated malto-oligosaccharide bearing C6 alkyl chain was weaker than that bearing C18 alkyl chain measured by SPR. These results suggest that the longer alkyl chain might be tend to strongly destroy the lipid bilayer of HIV like surface active reagents to exhibit potent anti-HIV activity.

As mentioned above, in the doctoral researches, the author studied on the elucidation of anti-HIV and cytotoxic mechanism of sulfated cellobiose dendrimers and alkyl oligosaccharides, revealing that the distance between sulfated groups was important for the potent anti-HIV activity of sulfated oligosaccharide dendrimers and the length of long-chain alkyl groups played significant role in anti-HIV activity and cytotoxicity of sulfated alkyl oligosaccharides. In future remarks, sulfated oligosaccharide dendrimers and alkyl oligosaccharides with potent antiviral activities and low cytotoxicity will be developed, and then apply to biomaterials to create virus-removing functionality that works effectively outside of the human body, for example, masks, fibers, filters, resins, and so on.

Chapter 5
References of this thesis

Chapter 1

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