# SYNTHESIS OF SULFATED ALKYL GLUCOPYRANANS BY RING-OPENING COPOLYMERIZATION OF ANHYDRO GLUCOSE DERIVATIVES AND ANTI-HIV MECHANISM

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (SCIENCE) IN KITAMI INSTITUTE OF TECHNOLOGY

2015

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Doctroral Thesis

# Synthesis of sulfated alkyl glucopyranans by ring-opening copolymerization of anhydro glucose derivatives and anti-HIV mechanism

September 2015

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Submitted to Kitami Institute of Technology for the degree of Doctor of Medical Engineering

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# *Preface*

Polysaccharides are carbohydrates that form the bulk of all food consumed by humans and that are essential for the maintenance of life and good health. It also plays an important role in the process of molecular recognition in vivo. In the last century 60's, scientists began to study the Ring-Opening polymerization method to artificial synthesis of stereoregularity polysaccharides. At the same time, after the sulfated polysaccharides have been found have anti HIV activity, a number of sulfated polysaccharides has been synthesized by past few decades. These natural or synthesized sulfated polysacchairdes can interact with viral surface proteins by electrostatic interaction to anti the virus.

The present work is an endeavor to synthesis Sulfated 3-O-Octadecy (1→6)-α-D-glucopyranans and to investigate the bioactivity mechanism of them. **Chapter 1** reports about HIV virus, polysaccharides and the type of the polysacchairde and the Ring-opening polymerization method. **Chapter 2** Describes the synthesis and structural characterization of of alkyl chain polysacchairdes. **Chapter 3** studies on the determination of anti-HIV activity and research on the mechanism of alkyl chain poslysacchairdes. **Chapter 4** summarizes the results and the significance of this experiment in this paper and future outlook.

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# *Chapter 1*

# *General introduction*

## 1.1 HIV

The human immunodeficiency virus (HIV) is a lentivirus (a subgroup of retrovirus) that causes the acquired immunodeficiency syndrome (AIDS) a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive.[1][2] First report about HIV infection in humans is from Kinshasa (Congo Republic) field serum samples stored in 1959. However, the first cases of AIDS have been officially reported in 1981.[3][4] The disease is characterized by accompanied by a reduced number of CD4 T cells, the opportunistic pathogens, Kaposi's sarcoma and B-cell lymphoma are appeared. According to the approximation of the World Health Organization in 2013, AIDS

killed 39 million people since beginning to epidemic, about 35 million people infected with HIV are alive currently. The majority live in sub-Saharan Africa, about 7.4% of the young adults in the region was infected with HIV.

Some of the countries in the region, for example, in Zimbabwe and Botswana, more than 25% of adults are infected. Epidemic of HIV infection and AIDS has shown to expand in China and India, according to a survey in several provinces, 1-2% of pregnancies was infected with HIV. Rapid rise of HIV infection prevalence is particularly pronounced in Central Asia and Eastern Europe. About one third of HIV-infected individuals in these countries is 15 years old -24 years old, most of which are not aware of their infection.[5]

The appearance of HIV-infected person looks like normal people, but their blood, semen, vaginal secretions, skin and mucous membrane damage or inflammation in ulcer exudates contain a large number of HIV virus which have a highly infectious. Milk, saliva, tears, sweat and urine also contains virus. So there are three main ways of HIV infection that sexual contact, blood transmission, mother to child transmission. HIV infection can take years or longer incubation period to develop into AIDS. Because the body resistance occur extreme decline so it would appear variety of infections. Late stage, cancer often occurs and the occurrence of long-term consumption, even systemic failure and death. In spite of many medical researchers around the world have made great efforts, they have not yet developed a specific drug to cure AIDS, and also no effective vaccine available for prevention.

Although currently it is capable of treating AIDS, but can't cure. All treatments are for control and delay disease progression and improve symptoms, improve quality of life. The US Food and Drug Administration (FDA) had approved 24 kinds of drug/ drug combination against HIV infection. These drugs can inhibit viral replication and infection of HIV and thus do not develop AIDS. However, these drugs can also affect

a patient's medication side effects. HIV reverse transcriptase is prone to error. Because he would not proofread nucleotide sequence during replication and have a high replication rate. Therefore, in this process can lead to more genetic variation. In the develop process of the drugs of treating AIDS, virus had a strong drug resistance. These drug-resistant virus spread to other people and then make these drugs more difficult to treat the offspring of HIV-infected individuals。Therefore, there is an urgent need to develop some effective anti-HIV drugs with low toxicity. Among them to develop a high activity with low toxicity carbohydrate drugs become a new area.

## 1.2 Polysaccharide

Polysaccharides are natural macromolecules derived from higher plants, animal cell membranes, Cell walls of microorganisms. It is an important component of life and a necessary structural material of living organisms. The structure of polysaccharides is the long chains of monosaccharide units are bounded together by glycosidic linkages and give the constituent monosaccharide or oligosaccharide.[6]

*Homogeneous polysaccharide*

Polysaccharide formed into the condensation of a single sugar molecule made, called homogeneous polysaccharide. The most abundant of nature homogeneous polysaccharides is starch, glycogen and cellulose. They are all composed of glucose. Starch and glycogen are glucose storing form of plants and animal, cellulose is the major structural component of the plant cell.

Starch is a storage form of plant nutrients, and also vital nutrients of plant foods. It is divided into amylose and amylopectin. Amylose: α-glucose is based $\alpha$-(1→4) glycosidic linkages connection, and the molecular weight from 150000 to 600000. It

have a structure of long and tight spiral shape, this structure is adapted to its storage capabilities. In case of iodine becomes blue. Amylopectin: On the basis of the straight-chain, every 20-25 glucose unit to form a $\alpha$-(1→6) branch. It can't form a spiral shape, in case of iodine becomes blue.

Glycogen is similar to amylopectin, but the branching degree is higher. Every 4 glucose unit will have one branch chain. The glycogen structure is more closely, and more in tune with its storage capabilities. That is an important reason for an animal as a form of energy storage. Another reason is that it contains a large number of non-immunogenic ends, which can be mobilized quickly hydrolyzed. Glycogen is also in case of iodine become blue.

Cellulose is the main structural component of plant cell walls, accounting for about 1/3 of the total weight of the plant. It is structure of $\beta$-(1→4) glycosidic linkage to linear long chains. Intact cell wall is cellulose mains, and contains hemicellulose, pectin and lignin. About 40 cellulose chains are connected to each other by hydrogen filaments into fibers, fiber filaments composed of numerous fibers intact cell wall skeletons. In addition, homogeneous polysaccharide also further comprises chitin, inulin and agar.

*Heterogeneity polysaccharide*

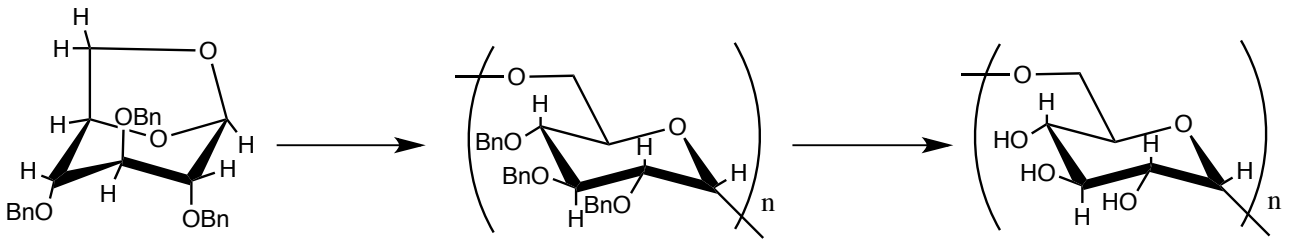
Polysaccharide condensation by different monosaccharide molecules formed, called heterogeneous polysaccharide. Heterogeneous polysaccharide has wide variety. Common are Hyaluronic acid and chondroitin sulfate.[7]

Compare to the importance of proteins and Nucleic acids, working on the life phenomenon the studies of carbohydrate started relatively late. In recent decades, researchers found the polysaccharides are not only a source of energy and a material of structure, but also it is working on many cell activities and having variety of

biological functions. Such as regulating immune function, antitumor Effect, anticoagulant effect, anti-aging effects, antiviral activity, etc. Therefore, the study of polysaccharides gradually becomes activity. Such as the extraction and purification of new natural polysaccharides, structure analysis, and structure-activity relationship, and the synthesis of polysaccharides and modifying polysaccharides is also to be valued.

## 1.3 Synthetic polysaccharide by Ring-opening polymerization method

Scherch synthesized the 2,3,4-tri-*O*-benzyl-(1→6)-$\alpha$-D-glucopyranan with the Lewis acid phosphorus pentalfuoride as catalyst from 1,6-anhydro-2,3,4-tri-*O*- benzyl -$\beta$-D-glucopyranose by ring-opening polymerization method, and then debenzylated the polymer to linear synthetic dextran, (1→6)-$\alpha$-D-glucopyranan , which is a same structure of a natural dextran confirmed by NMR and optical rotation measure-ments.[8]



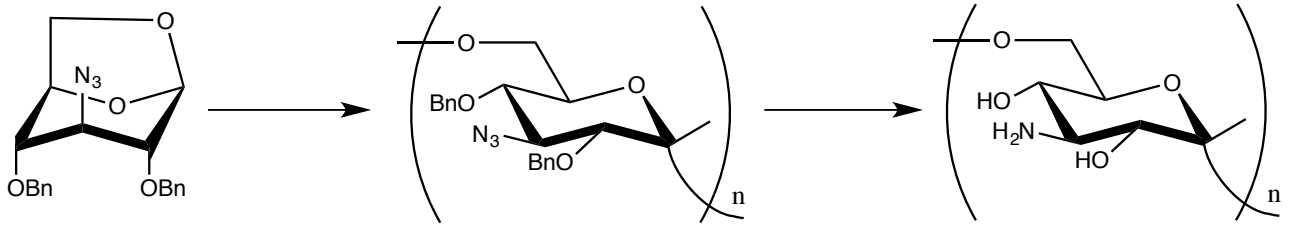

Scheme 1.1. Synthesis of dextran by ring-opening polymerization of benzylated 1,6-anhydro-glucose.

The ring-opening polymerization process via a $S_N2$ mechanism accompanied anomeric C1 inversion. The protection group of Benzyl group can be used to remove

by Birch reduction reaction against the sodium metal in liquid ammonia conditions.

Since then 1,6-anhydro-2,3,4-tri-*O*-benzyl-*β*-mannopyranose, galactopyranose and allopyranose were subsequently polymerized into 2,3,4-tri-*O*-benzylated polymers which were then debenzylated to give free polysaccharides (1→6)-*α*-mannopyranan, galactopyranan and allopyranan, respectively. They have high 1,6-*α*-stereoregularity indicated by $^{1}H$ and $^{13}C$ NMR and optical rotation measurements[9]

Polysaccharides containing azido group had been prepared by ring-opening polymerization method. 1,6-Anhydro-3-azido-3-deoxy-2,4-di-*O*-benzyl-*β*- glucopyra -nose was polymerized into high molecular weight polymers by a complex catalyst, $C_6H_5COF$-$PF_5$. After the polymer had been deprotected and converted, an amino group-containing polysaccharide was obtained.[10]

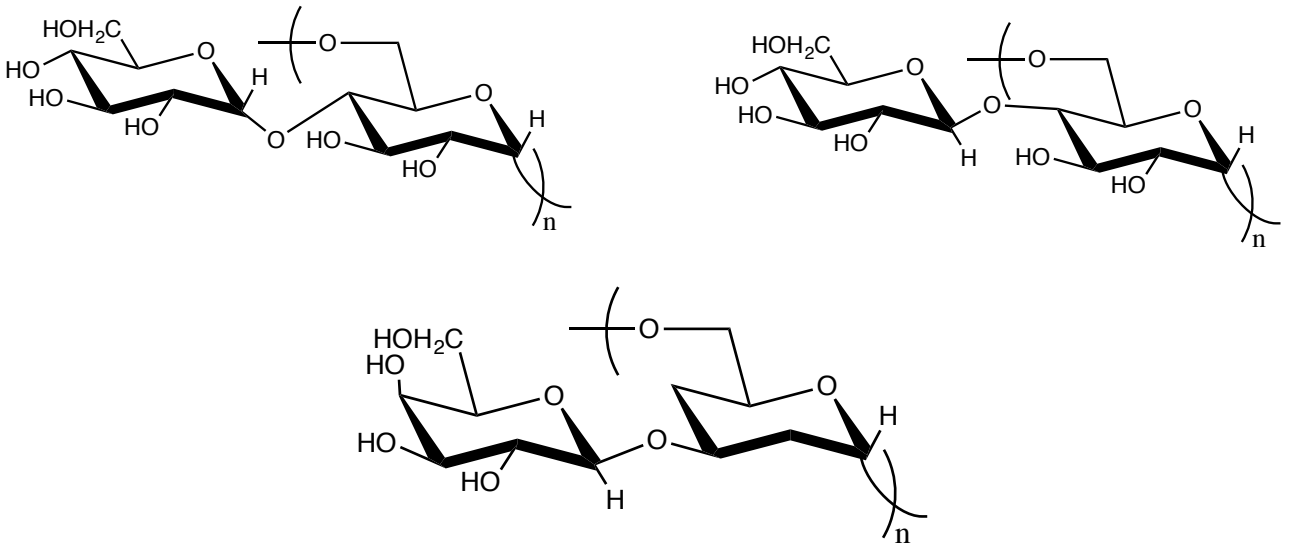



Scheme 1.2. Synthesis of 3-amino-3-deoxy-(1→6)-α-glucopyranan

(1→6)-*α* linked heteropolysaccharides of (1→6)-*α*-glucomannans were synthesized by copolymerization of tri-*O*-benzylated 1,6-anhydro-glucose and tri-*O*-benzylated 1,6-anhydro-mannose catalyzed by strong Lewis acid of phosphorus pentalfuoride with different proportions.[11]

C4 positions have a glucose branch of each backbone of glucose residues were synthesized by ring-opening polymerization of 1,6-anhydro disaccharides called Comb-type polysaccharides. Such as 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2,3,4,6- tetra -*O*-benzyl-*α*-glucopyranosyl)-*α*/*β*-glucopyranose, 1,6-anhydro-3-*O*-(2,3,4,5- tetra-*O*-benzyl-*β*-galactopyranosyl)-2,4-dideoxy-*β*-threo-hexopyranose were polymerized by

a $C_6H_5COF$-$PF_5$ complex catalyst, and then followed by debenzylation to give poly-1,6-$\alpha$-maltose,[12] poly-1,6-$\alpha$-cellobiose[13] and 2,4-dideoxy-3-*O*-($\beta$-galactosyl)-(1→6)-$\alpha$-threo-hexopyranan.[14]



Scheme 1.3. Structures of poly-1,6-a-maltose, poly-1,6-a-cellobiose and 2,4-dideoxy-3-*O*-($\beta$-galactosyl)-(1→6)-$\alpha$-threo-hexopyranan synthesized by ring-opening polymerization.

Recently, our laboratory reported Comb-type heteropolysaccharides of synthetic (1→6)-$\alpha$-D-galactomannopyranans with various proportions were copolymerized from 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2′3′4′6′-tetro-*O*-benzyl-$\alpha$-D-galacto pyrano -syl)-$\beta$-D-mannopyranose with 1,6-anhydro-2,3,4-tri-*O*-benzyl-$\beta$-D- mannopyranose by ring-opening polymerization method carried out with $PF_5$ catalyst under high vacuum at -60℃, subsequently debenzylation. Carrying out copolymerization, the yield and molecular weight were increased to 72.5% and $\bar{M}n= 22.8\times10^3$, respectively. After debenzylation, the copolysaccharides had molecular weights between $\bar{M}n= 12.6\times10^3$ and $\bar{M}n= 19.1\times10^3$ and the proportions of galactose branches from 78.2 mol% to 28.6 mol% were obtained.[15]

We have continuously investigated the ring-opening polymerization of anhydro sugar derivatives, which is a superior method to prepare stereoregular poly-saccharides with high molecular weights and defined structures, and elucidation of the relationship between the structure and biological activity of polysaccharides. We have also synthesized many anhydro pentose and hexose derivatives that were polymerized to give stereoregular polysaccharides after deprotection of the hydroxyl protective groups. It is important to obtain polysaccharides with desired molecular weights and defined structures for the investigation of the structure-biological activity relationship, because there are many naturally occurring polysaccharides that have specific biological activities; however, in general, they have very complex structures and it is difficult to know the relationship. [16]

## 1.4 Sulfated polysaccharide

Sulfated polysaccharides are a class of polysaccharides with sulfate on sugar hydroxyl groups. Including sulfated polysaccharide extracted from various plants, heparin sulfate, natural sulfated derivative of neutral polysaccharide and various synthetic sulfated polysaccharide having anti-virus, anti-tumor, anti-clotting and enhance immune biological activity. 1964, Nahmiss et al had reported that heparin inhibited herpes viruses (HSV), but did not arouse people's attention. Later, Japanese researchers study the algae sulfated polysaccharide found to have anti-tumor effects in vivo. In 1987, Nakashima and Yamamoto found the anti-human immunodeficiency virus activity of natural sulfated polysaccharides from sea alga.[17][18] Since the discovery of sulfated polysaccharide having anti-HIV activity after scientists have started studying anti-HIV sulfated polysaccharide mechanisms. There are three ways for years of study found: 1) Sulfated polysaccharides can inhibit the activity of

reverse transcriptase. 2) Can inhibit the binding of target cells to the virus. 3) Enhance immune function. The factors of influence inhibit from HIV activity including the skeletal structure of sulfated polysaccharides, the kind of substituents and substitution positions, and molecular size, branched chain and branched-chain species. According to reports, semi-synthetic sulfated polysaccharides such as dextran sulfate, poly acid pentose, sulfated lentinan and sulfated polysaccharides and other furan nuclear can inhibit the action of cell disease caused by HIV in vitro.

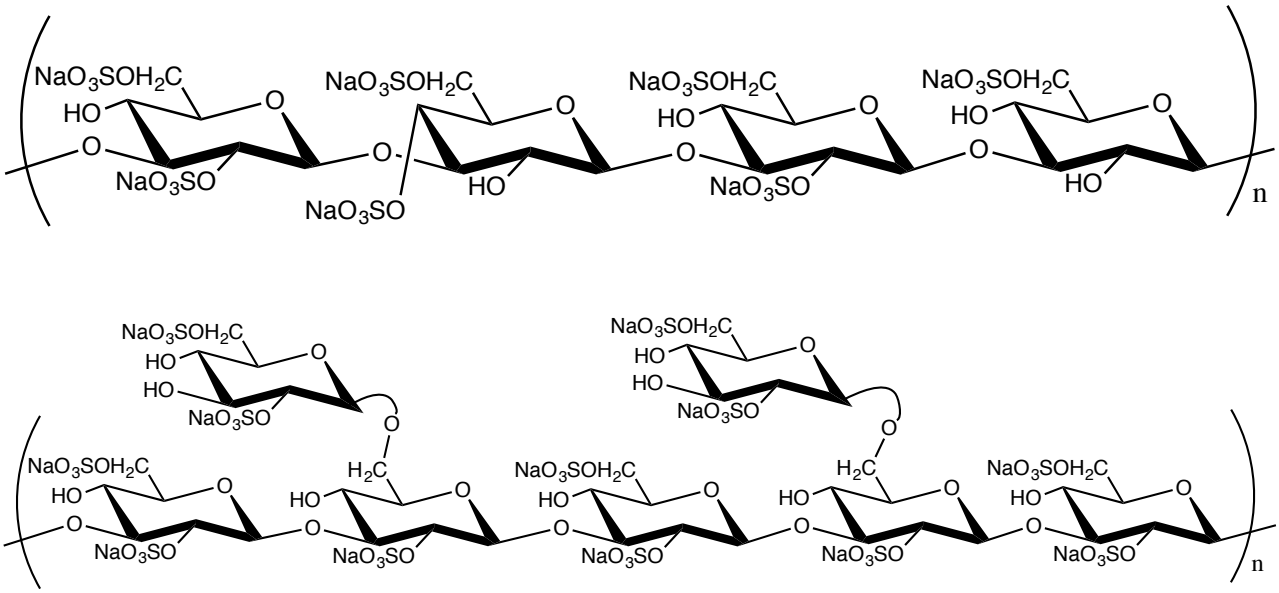
Curdlan is a natural linear 1,3-*β*-glucan. Carried out sulfonate with piperidine *N*-sulfonic acid in DMSO at 80℃, the curdlan sulfate with sulfur content of 14.4% could completely inhibit the infections of HIV in the drug concentration as low as 3.3μg/ml and had low cytotoxicity.[19][20] The anti-HIV activity was examined by MTT method. The succinate dehydrogenase in mitochondria is capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide to its insoluble formazan, however, dead cells no this function. DMSO can soluble the formazan in cell. It can be detected under 540nm and 720nm to reflect the number of viable cells.[21] The 50% effective concentration, $EC_{50}$, was calculated by the dose of sulfonated polysaccharides achieving 50% protection of HIV infections to the MT-4 cell. The cytotoxicity, $CC_{50}$ was determined by the 50% cytotoxic concentration on the MT-4 cell.

Since the new polysaccharide lentinan as an antitumor drug launched in Japan, the sulfated lentinan was obtained from the polysaccharide sulfated with piperidine N-sulfonic acid in DMSO. Lentinan sulfates ($\overline{M}n=1.8\times10^4$) with sulfur content of 16.2% could completely inhibit the infections of HIV in the drug concentration as low as 3.3μg/ml.[22] Difference of the conformation of curdlan sulfate with a flexible backbone, the lentinan sulfate might take on a more rigid rod-like conformation, thus

affording a difference in biological activites between the two sulfated poly-saccharides.

Table 1.1. Anti-HIV activity of curdlan sulfonate

| No | Scontent (%) | DS | $\overline{M}n\times10^4$ | $[a_D^{25}]$ (°) | Anti-HIV activity |
|---|---|---|---|---|---|
| 1 | 5.6 | 0.35 | | | No effect |
| 2 | 8.9 | 0.8 | 6.9 | | 1000 |
| 3 | 12.2 | 1.1 | 8.1 | -1.7 | 10 |
| 4 | 12.1 | 1.1 | 11.8 | -3.8 | 3.3 |
| 5 | 12.5 | 1.3 | 15.7 | -2.3 | 3.3 |
| 6 | 13.6 | 1.4 | 3.4 | -0.8 | 3.3 |
| 7 | 14.1 | 1.6 | 2.1 | -1.9 | 3.3 |
| 8 | 14.4 | 1.6 | 4.6 | +0.1 | 3.3 |
| 9 | 14.7 | 1.6 | 2.0 | -1.5 | 3.3 |




Scheme 1.4. The chemical structure of anti-HIV active curdlan sulfate and lentinan sulfate.

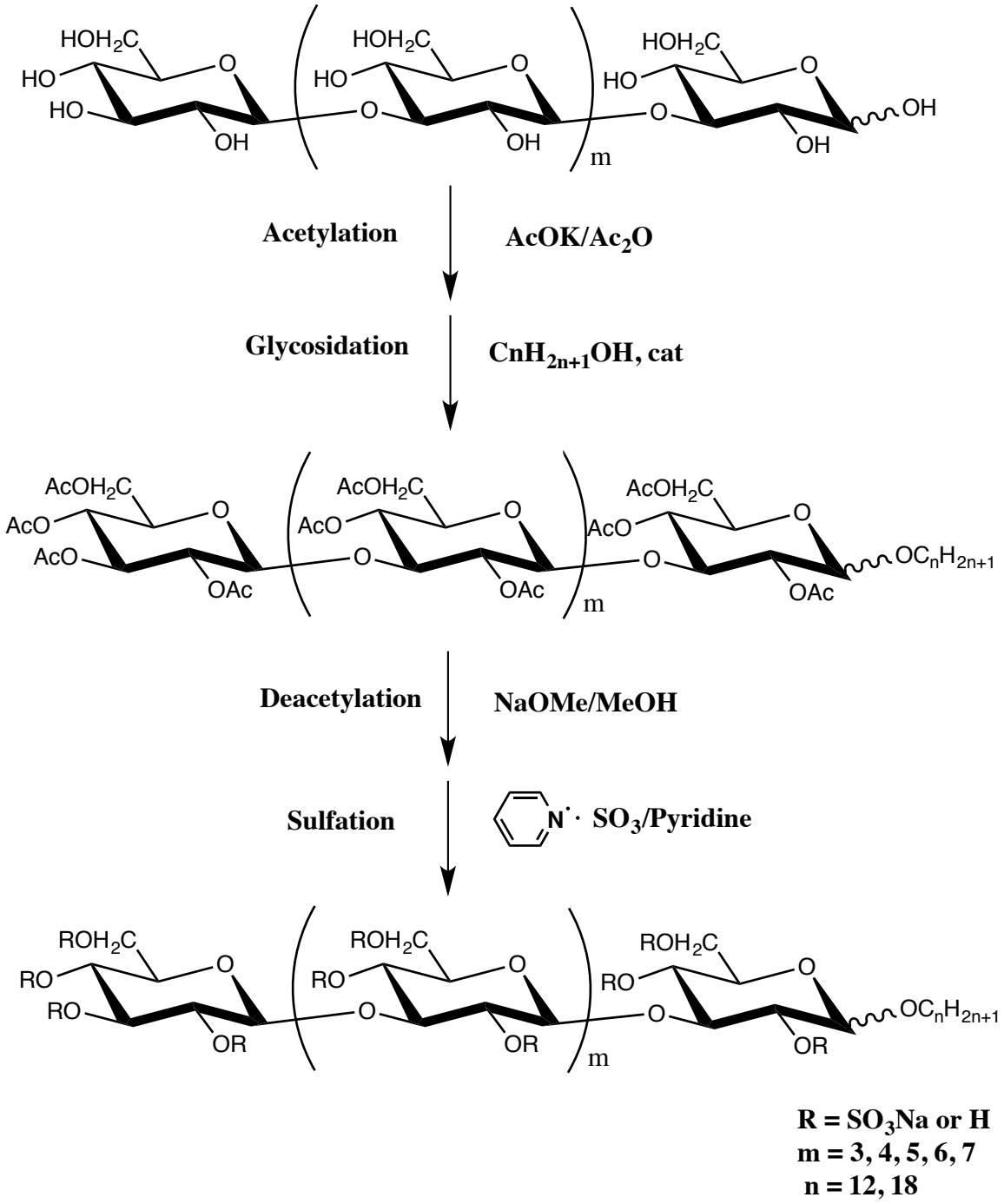
Gronwall et al. had prepared a sulfated dextran from dextran with anticoagulant activity.[23] Although it was easily synthesized, these had a considerable toxicity. Precipitation of plasma fibrinogen could cause the dextran toxicity. Ricketts through controlling the molecular weight and the degree of sulfation to prepare sodium dextran sulfate with low toxicity.[24] Linear and mannose-branched dextran sulfates

were synthesized by ring-opening polymerization method consequently sulfated by Yoshida T. et al.[25] Synthetic linear and mannose-branched dextran sulfonates with a DS more than 1.0 at concentrations of 3.3 and 10μg/ml exhibited complete inhibitory effect on HIV infection.

Anti-HIV active compounds were prepared from oligosaccharides having several glucosyl residues, however, high molecular weight sulfated polysaccharides tend to have high anti-HIV activity, the length of which corresponds to that regarded as an ordinary active site in biologically active polysaccharides. The modified *β*-cyclodextrin sulfates were synthesized by sulfation of the modified *β*-cyclodextrins with a sulfur trioxidepyridine complex in pyridine solution. The compound of 6-*O*-benzylthioleated *β*-cyclodextrin sulfate with three benzylthio substituents shown most anti-HIV-1 activity $IC_{50}$ (a concentration protecting 50% cytopathic effects) of 0.98μg/ml in MT-4 cells.[26] After oral administrated the compound to rats, the rat plasma extraction shown anti-HIV activity, suggesting that the compound was absorbed through the intestinal membrane in to blood.

Making use of sulfated oligosaccharide which have by themselves very low anti-HIV activities. recently, sulfated alkyl oligosaccharides with potent anti-HIV activities were synthesized. For example, sulfated maltopentaose and maltohexaose showed low anti-HIV activities with $EC_{50}$'s of 267 and 207μg/ml, respectively, sulfated octadecyl maltopentaoside with an octadecyl group at the reducing end of the sulfated maltopentaose exhibited a high activity, with an $EC_{50}$'s of 0.6μg/ml.[27] K. Katsuraya synthesized several sulfated alkyl laminaraoligosaccharides by reaction peracetylated laminara-oligosaccharide with n- dodecyl alcohol and n-octadecyl alcohol, subsequently sulfated with the sulfur trioxide-pyridine complex.[28] Sulfated dodecyl laminara-oligosaccharides shown anti-HIV activities shown $EC_{50}$'s of 0.10-0.18μg/ml, $CC_{50}$'s larger than 1000 μg/ml. All anti-HIV activities of sulfated

octadecyl laminara- oligosaccharides almost equivalent to those of the dodecyl analogues, $EC_{50}$'s of 0.20-0.63 μg/ml, $CC_{50}$'s of 180-240 μg/ml. Thus, it has been revealed that the binding of a terminal long alkyl group to the sulfated oligosaccharide to a great extent enhances anti-HIV activity.




Scheme 1.5. Synthetic route for sulfated alkyl laminara-oligosaccharides

We introduced a long alkyl chain into curdlan sulfates with high anti-HIV activity by the ionic interaction of sulfate groups and quaternary ammonium salt of the long alkyl chain. A membrane filter was coated with the alkyl curdlan sulfates due to the hydrophobic interaction of the long alkyl chains with the surface of the membrane filter to develop curdlan sulfate-coated membrane filters. We found that the membrane filter removed influenza A viruses at dilutions below 1: 32.[29] In addition, curdlan sulfate was shown to effectively inhibit Dengue virus replication in different cells.[30] The minimum 50% effective concentration ($EC_{50}$) was as low as 0.1 mg/ml in LLC-MK2 cells. The cytotoxicity of curdlan sulfate was quite low because no inhibitory effects on cell growth were observed in concentrations as high as 5000 mg/ml. We assumed that the biological activity of sulfated polysaccharides was due to the electrostatic interaction between negatively charged sulfate groups and positively charged envelope proteins of the viruses. The interaction between sulfated polysaccharides with poly-L-lysine as a model compound of proteins was quantitatively investigated by surface plasmon resonance (SPR) measurements, suggesting that sulfated polysaccharides had fast association and slow dissociation rates on the immobilized poly-L-lysine. These results indicate a high stability of the interaction.

Sulfated ribofuranan and ribopyranan with five- and six-membered ring structures, respectively, obtained by the ring-opening polymerization of benzylated and benzylidenated anhydro ribose monomers,[31] were found to have high anti-HIV activities.[32] Although sulfated ribofuranan showed higher blood anticoagulant activity at 56 unit/mg, sulfated ribopyranan had the lower activity of 29 unit/mg, probably because the furanosidic structure had a flexible main chain so that the interaction with proteins became stronger. Furthermore, higher molecular weights and degrees of sulfation of sulfated polysaccharides showed higher biological

activities.[33] On the other hand, sulfated polysaccharides with low molecular weights had low anti-HIV activity. However, after introduction of an octadecyl group into sulfated ribofuranans with low molecular weights, the anti-HIV activity increased to a level almost comparable to that of curdlan sulfate with a higher molecular weight and high anti-HIV activity.[34]

In this chapter, we report the synthesis of sulfated alkyl polysaccharides by the ring-opening copolymerization of LGTBE with 2, 4-di-*O*-benzyl 3-*O*-octadecyl-1, 6-anhydro-β-D-glucopyranose (LGDBE3-18) and subsequent debenzylation to recover hydroxyl groups without elimination of the 3-*O*-octadecyl group. After sulfation, we prepared sulfated 3-*O*-octadecyl glucopyranans with potent ant-HIV activity. The structure of polysaccharides obtained was determined by high resolution NMR and the interaction with poly-L-lysine as a model compound of proteins was quantitatively measured by SPR measurements. The self prepared liposome as a model of the membrane on the virus to elucidate the interaction of sulfated 3-*O*-octadecyl glucopyranans with liposome. The final purpose of the work was to develop bioactive materials such as membrane filters for removal of HIV, Dengue, and influenza viruses.

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# *Chapter 2*

# *Synthesize of Sulfated 3-O-octadecyl glucopyranans*

## 2.1 Materials and Measurement

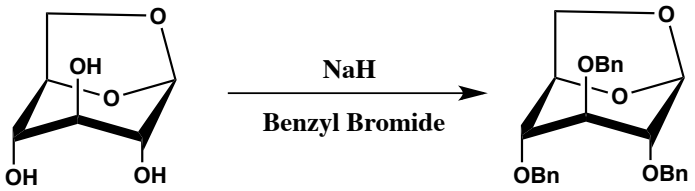
Reagents were purchased from commercially available products and used without further purification. Poly-L-lysine with a molecular weight of 6000-9000 was purchased from Sigma-Aldrich, Co. A CM5 sensor chip, HBS-EP+ buffer and 50 mM NaOH solution were supplied by GE Healthcare Japan, Co. Ltd.

The $^{1}H$ NMR (600 MHz) and $^{13}C$ NMR (150 MHz) spectra were recorded on a JEOL JNM ECX-600 spectrometer in $CDCl_3$, $D_2O$, or DMSO-$d_6$ solvents, respectively. The temperatures used were 25℃ for the $CDCl_3$ solvent and 40℃ for $D_2O$ and DMSO-$d_6$ solvents, respectively. Chemical shifts are expressed as ppm downfield from 4,4'-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal

standard. Specific rotation of benzylated copolymers was recorded in $CHCl_3$ at 25°C and other water soluble samples were recorded in $H_2O$ at 25°C in a water-jacketed 10 ml quartz cell by means of a JASCO DIP-140 digital polarimeter. Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. Elemental analysis was carried out by CE-440M CHN/O/S Elemental Analyzer, Systems Engineering, Co. Molecular weight was determined at 40°C by an organic phase GPC eluted with $CHCl_3$ equipped with TOSOH TSK-gel columns (7.6 mm × 600 mm × 3) of $G3000H_{XL}$, $G4000H_{XL}$, and $G5000H_{XL}$ for benzylated copolymers using polystyrene (Shodex standard SM-105) as a reference and by aqueous phase GPC columns (7.6 mm × 300 mm × 3) of $G2500PW_{XL}$, $G3000PW_{XL}$, and $G4000PW_{XL}$, eluted with 66.7 mmol phosphate buffer, pH=6.68) for water-soluble sulfated copolysaccharides using pullulan as a reference. The surface plasmon resonance (SPR) spectrum was taken on a Biacore X100 instrument at 25°C using a CM5 sensor chip. The dynamic light scattering (DLS) and zeta (z) potential were performed at 25°C on an OTSUKA ELECTRONICS ELSZ-1000ZS zeta potential and particle size analyzer in deionized water solution at the concentrations of 1 mg/ml or 0.5 mg/ml of sulfated polysaccharides and poly-L-lysine, respectively, and the data were analyzed by the maker provided software.

## 2.2 Experimental part

### *2.2.1 Synthesis of 1,6-Anhydro-2,3,4-tri-O-benzyl-β-D-glucopyranose*[1]




Scheme 2.1. Synthesis of 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranose

NaH (3 g, 74.1 mmol) was added to a solution of 1,6-anhydro-*β*-D-glucopyranose (4 g, 24.7 mmol) in anhydrous DMF (50 ml) with stirring at room temperature. The suspension was stirred for 30 min then cooled in an ice bath. Benzyl bromide was added to the suspension dropwise, and after 10min the ice bath was removed. The reaction mixture was being stirred for 18 h, and then an excess of methanol was added dropwise to the solution. The mixture was concentrated in vacuum at 55℃ and diluted with chloroform and water. The separated chloroform layer was washed with water several times, dried with $Na_2SO_4$, and concentrated in vacuum. The residue was purified by silica gel chromatography (1:2 EtOAc-Hexane) twice to give colorless viscous. Recrystallization from EtOH gave 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranose (6.4 g) in 60% yield as a colorless crystal:$[\alpha]_D$= -33.9° (c 1.0, $CHCl_3$,25).

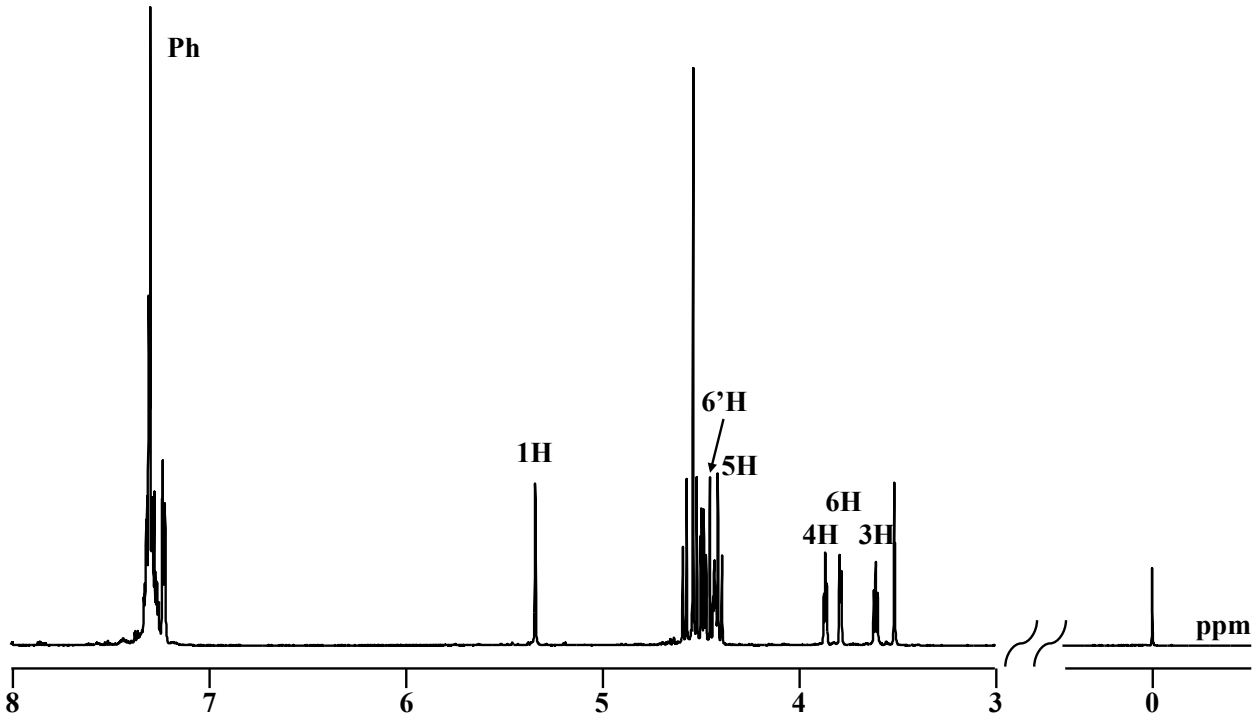



Figure 2.1. $^{1}$H-NMR spectra of 1,6-Anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranose

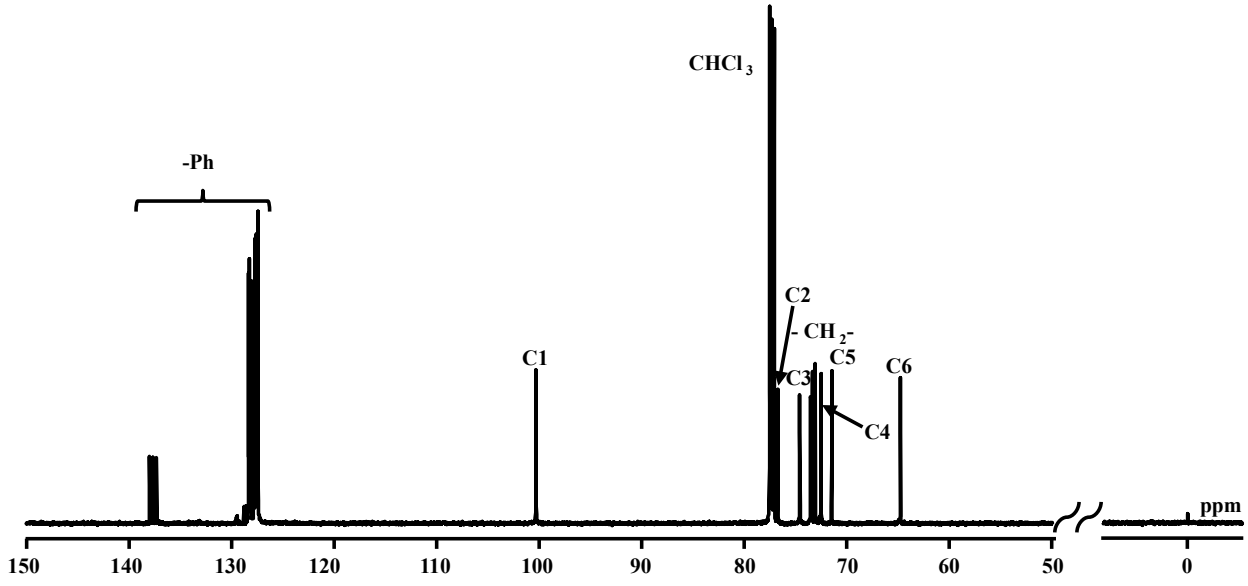
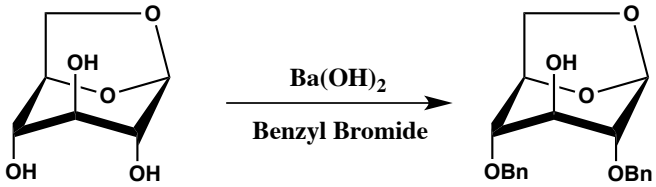


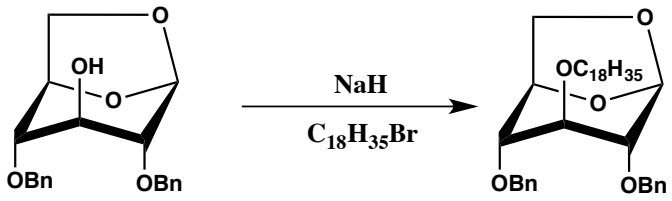

Figure 2.2. $^{13}$C-NMR spectra of 1,6-Anhydro-2,3,4-tri-*O*-benzyl-β-D-glucopyranose

### *2.2.2 1,6-Anhydro-2,4-di-O-benzyl-3-O-octadecyl-β-D-glucopyranose*[2]




Scheme 2.2. Synthesis of 1,6-anhydro-2, 4-di-*O*-benzyl-β-D-glucopyranose

Barium hydroxide $8H_2O$ (7.5 g) was added to the 1,6-anhydro-*β*-D-glucopyranose (2 g, 12.3mg) in DMF (60 ml) stirred 30 min, and then added benzyl bromide (7.5 ml) dropwise stirred at room temperature for 3h. When the reaction was over, excess of methanol was added to destroy excess benzyl bromide. After 30 min the reaction mixture was diluted with chloroform, filtered, and washed with water. Evaporation of the solvents left a syrup that was purified on a silica gel column chromatography ( hexane - ethyl acetate 2:1) to give 3.34 g (84.11 %) of : $[\alpha]_D^{25}$= -31.18° (c 1.0, $CHCl_3$, 25).




Scheme 2.3. Synthesis of 3-*O*-octadecyl-1,6-anhydro-2, 4-di-*O*-benzyl-β-D-glucopyranose

1,6-anhydro-2,4-di-*O*-benzyl-*β*-D-glucopyranose (2 g) in DMF (37.5 ml) was added with sodium hydride (1.5 g) which treated by hexane previously and stirring at 50℃ for 90 min. 1-Bromoactadecane (3.25 g) in DMF (25 ml) was added, and the mixture was heated at 70℃ for 60 min. The solution was treated with chloroform (50

ml) and water (50 ml) and then the $CHCl_3$ layer was washed with water 3 times and 5% of sodium hydrogen carbonate solution several times and dried over anhydrous sodium sulfate and concentrated. The product was purified by silica gel chromatography with benzene-ether (8:1 (v/v)) as eluent. A colorless crystalline material was obtained in a 89.6% yield: 3.02g, $[\alpha]_D$= -23.5° (c 1.0, $CHCl_3$,25);

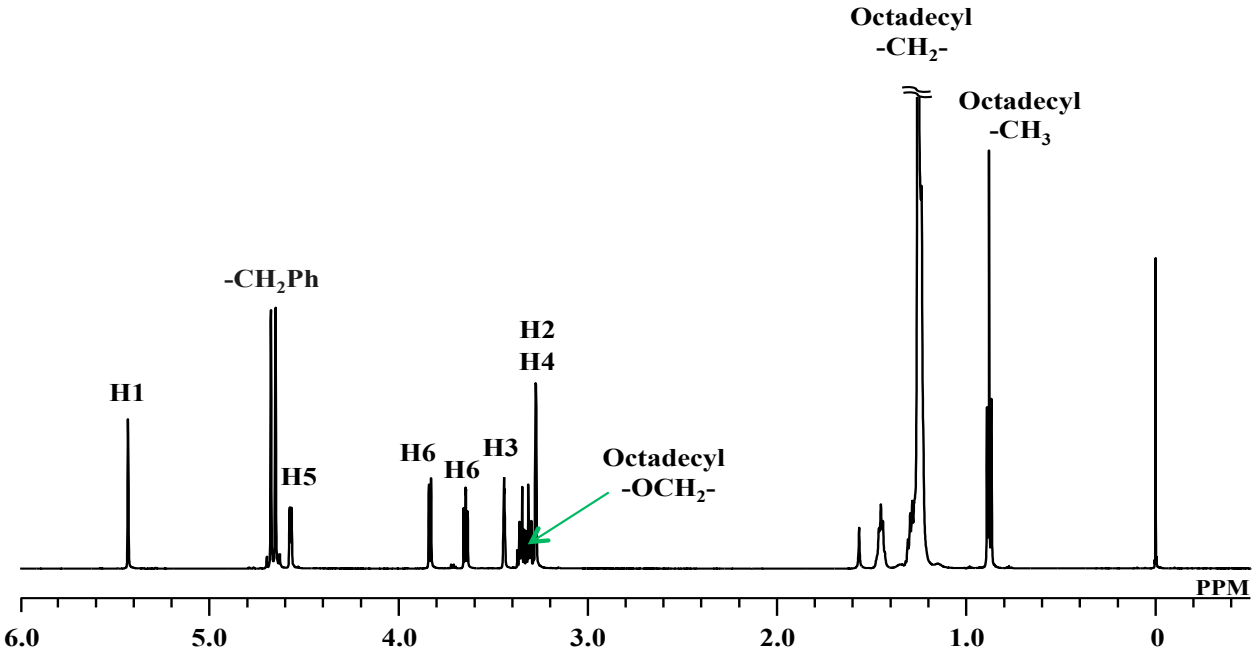



Figure 2.3. $^{1}$H-NMR spectra of 3-*O*-octadecyl-1,6-anhydro-2, 4-di-*O*-benzyl-b-D-glucopyranose

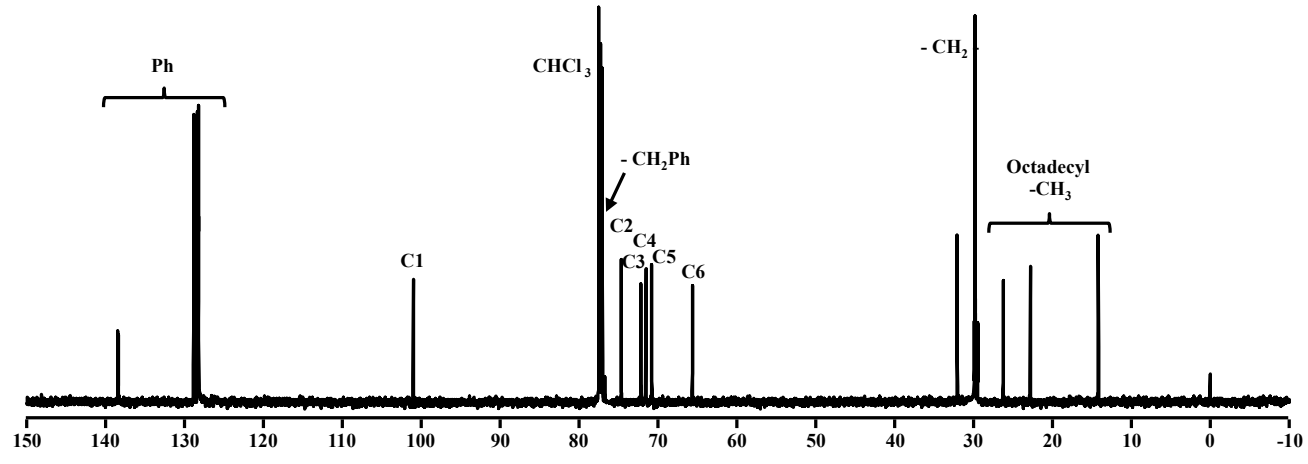
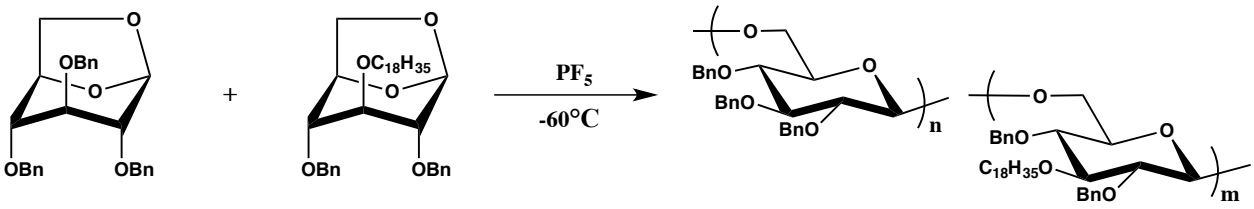



Figure 2.4. $^{13}$C-NMR spectra of 3-*O*-octadecyl-1,6-anhydro-2, 4-di-*O*-benzyl-b-D-glucopyranose

### *2.2.3 Polymerization*




Scheme 2.4. Synthesis of copoly (LGTBE-LGDBE3-18)

Typical procedure for the polymerization and copolymerization are as follows. The monomers 1,6-Anhydro-2,3,4-tri-*O*-benzyl-*β*-D- glucopyranose and 1,6-Anhydro-2,4-di-*O*- benzyl-3-*O*-octadecyl-*β*- D-glucopyranose were thoroughly dried in a poly -merization ampoule by evacuating it overnight and then dissolving in dry $CH_2Cl_2$ and phosphorus pentafluoride ($PF_5$) as a catalyst were transferred into the polymerization ampoule and copolymeri -zation was carried out under high vacuum. Methanol was added to the reaction mixture to terminate the polymerization. The resulting polymers were precipitated by $CHCl_3$-methanol several times and then freeze-dried from benzene to give purified polymers.

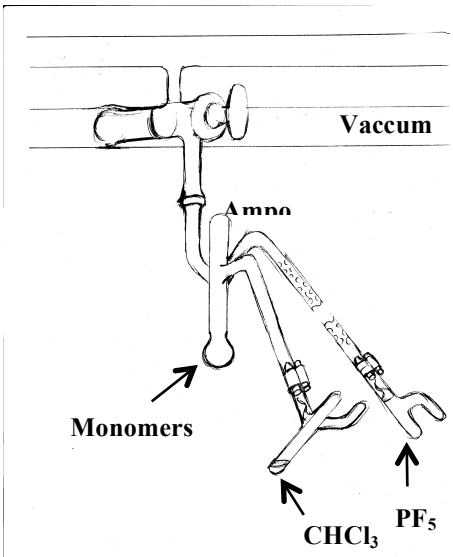



Figure 2.5. Glassware of ring-opening polymerization method

Table 2.1. Ring-opening copolymerization of 2, 3, 4-tri-*O*-benzyl (LGTBE) with 2, 4-di-*O*-benzyl-3-*O*-octadecyl 1, 6-anhydro-β-D-glucopyranose LGDBE3-18[a]

| No | LGTBE | | LGDBE3-18 | | Polymer | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Time | Yield | $\overline{M}n$[b] | LGDBE3-18 unit | $[a]_D^{25}$ |
| | g | mol% | g | mol% | h | % | x$10^3$ | in polymer[c] | deg |
| 1 | 0.5 | 100 | 0 | 0 | 0.5 | 82.5 | 59.1 | 0 | +114.1 |
| 2 | 0.475 | 96.3 | 0.025 | 3.7 | 4 | 91.9 | 83.5 | 1.3 | +111.2 |
| 3 | 0.45 | 95 | 0.05 | 5 | 4 | 90.6 | 117.6 | 3.2 | +111.6 |
| 4 | 0.425 | 90 | 0.075 | 10 | 4 | 89.7 | 65.7 | 4.7 | +107.8 |
| 5 | 0.325 | 70 | 0.175 | 30 | 4 | 91.9 | 112.5 | 22.2 | +101.2 |
| 6 | 0.08 | 50 | 0.12 | 50 | 4 | 92.7 | 136.5 | 46.9 | +92.5 |
| 7 | 0.05 | 30 | 0.15 | 70 | 4 | 94.3 | 128.2 | 72.9 | +91.4 |
| 8 | 0 | 0 | 0.2 | 100 | 4 | 91.8 | 163.1 | 100 | +86.9 |

a) Total monomer weight: 0.2 and 0.5g, Solvent: $CH_2Cl_2$; 0.4 ml, Catalyst: $PF_5$; 5 mol%, Temperature: -60℃.
b) Determined by GPC.
c) Calculated from $^1$H NMR spectrum (mol%)

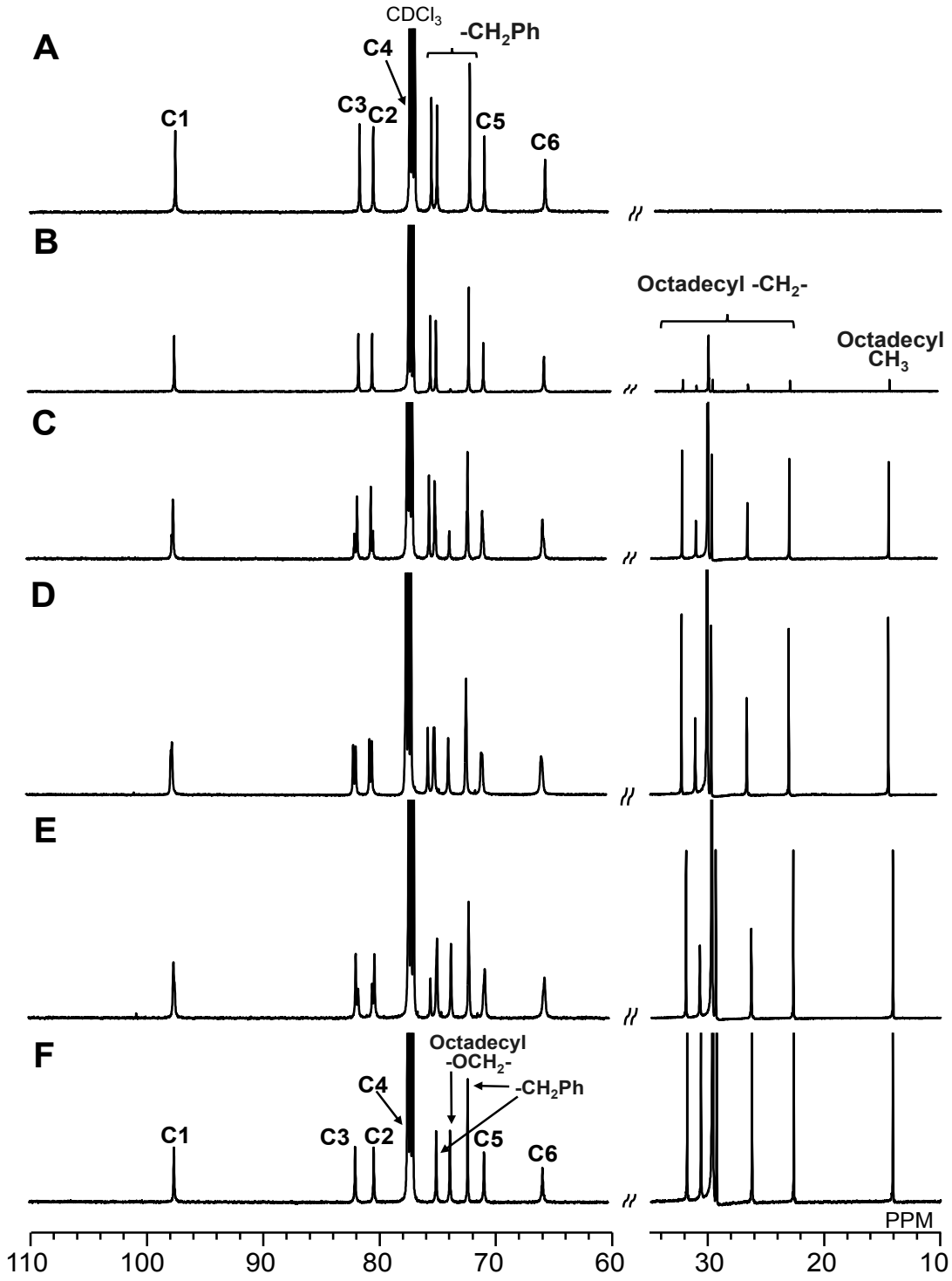
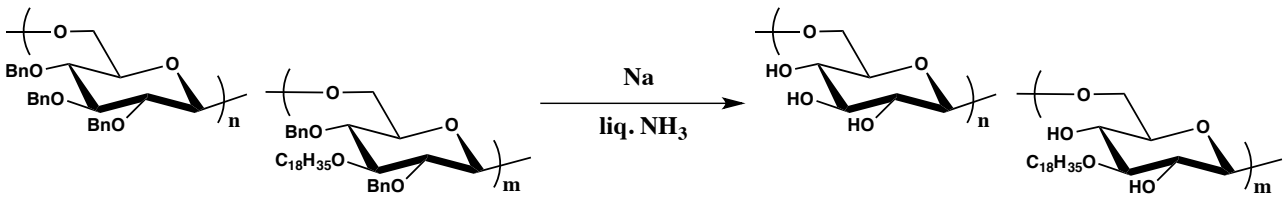



Figure 2.6. $^{13}C$ NMR spectra of copoly(LGTBE-LGDBE3-18)s in $CDCl_3$ at room temperature. (A) Poly(LGTBE), (B)-(E) copoly(LGTBE-LGDBE3-18)s with the proportion of LGDBE3-18 unit of 1.3, 22.2, 46.9 and 72.9 mol%, respectively, and (F) poly(LGDBE3-18), which polymers are corresponded in Table 1.

### *2.2.4 Deprotection of Benzyl Groups in Copolymers*




Scheme 2.5. Deprotection of benzylated 3-*O*-octadecyl (1→6)-$\alpha$-D-glucopyranans

The copolymer (0.315 g, No. 1 in Table 2.2) dissolved in 2 mL of dry dimethoxyethane was added dropwise to 100 mL of liquid ammonia at -78°C under nitrogen. The small pieces of freshly cut sodium metal 0.8 g were added in several portions until the solution become dark blue. After 2 h of stirring at the same temperature, ammonium chloride was added until the deep blue color disappeared and then small amount of MeOH 10 ml was added to inactivate the sodium that remained. After evaporation of the liquid ammonia at room temperature, water 50 mL was poured into the flask. The aqueous solution was washed with $CH_2Cl_2$ to remove organic impurities and then dialyzed for 2 d with deionized water. The aqueous solution was concentrated to 20 mL, and freeze-dried under vacuum to give debenzylated copolysaccharide (0.12 g) in 64.9% yield (No. 1 in Table 2.2). The proportion of 3-*O*-octadecyl glucopyranose unit was 2.8 mol%. This copolysaccha -ride was difficult to dissolve in water and partially soluble in DMSO. [3]

Table 2.2. Debenzylation of 2, 3, 4-tri-*O*-benzyl (LGTBE) with 2, 4-di-*O*-benzyl-3-*O*-octadecyl 1, 6-anhydro-*β*-D-glucopyranose (LGDBE3-18)[a]

| No | Benzylated polymer | | | | | Free polymer | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Proportion | | wt | $\bar{M}_n^b$ | $[a]_D^{25}$ | wt | Yield | $\bar{M}_n^c$ | LGDBE3-18 unit |
| | LGTBE | LGDBE-18 | g | $\times 10^3$ | deg | g | (%) | $\times 10^3$ | mol%[e] |
| 1 | 98.7 | 1.3 | 0.315 | 83.5 | +111.2 | 0.115 | 64.9 | nd[d] | 2.8 |
| 2 | 96.8 | 3.2 | 0.4 | 117.6 | +111.6 | 0.116 | 51.7 | nd | 4.7 |
| 3 | 77.8 | 22.2 | 0.367 | 112.5 | +101.2 | 0.177 | 85.9 | nd | 15.7 |

*a*) Condition: Na; 0.4 g, solvent: Liq. $NH_3$; 50 mL, Time: 90 min, Temp: -78°C

*b*) Calculated by $CHCl_3$ GPC

*c*) Calculated by aqueous GPC

*d*) Insoluble in water

*e*) Calculated from $^1$H NMR spectrum in DMSO-$d_6$

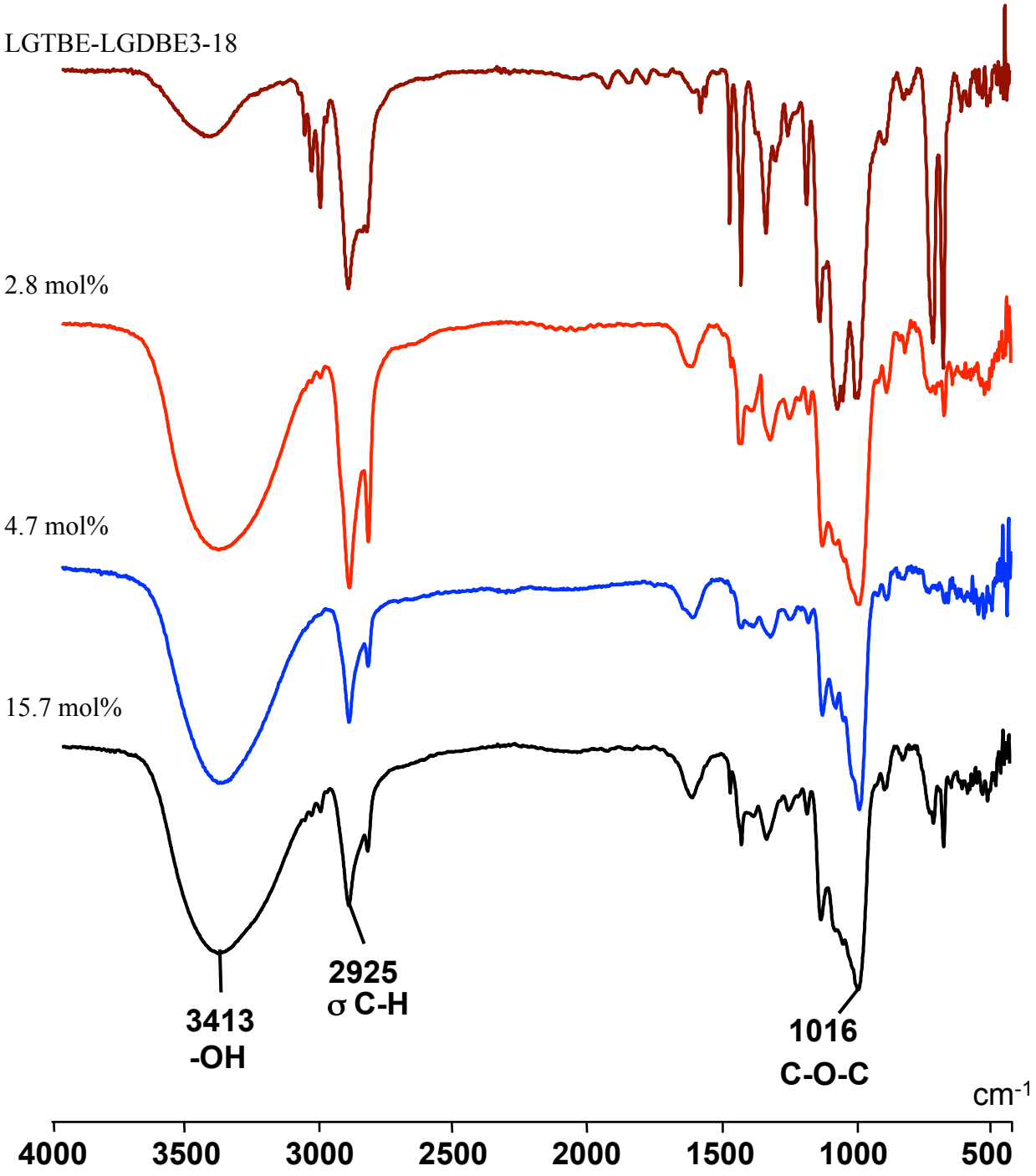



Figure 2.7. FT-IR spectra of copoly (LGTBE-LGDBE3-18) and (1→6)-α-D-glucopyranans with 3-*O*-octadecyl gourps in the proportion of 2.8, 4.7 and 15.7% in $CHCl_3$

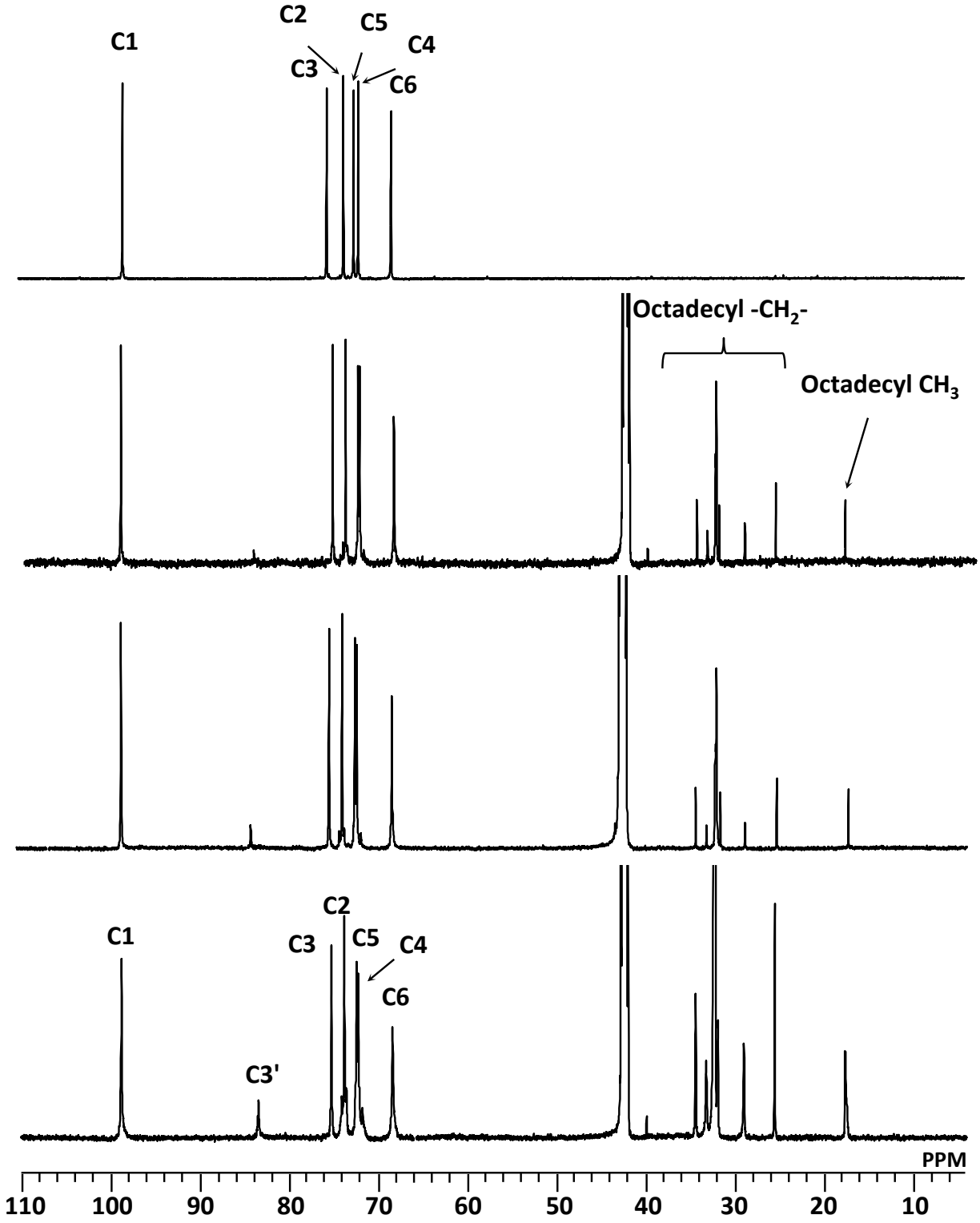


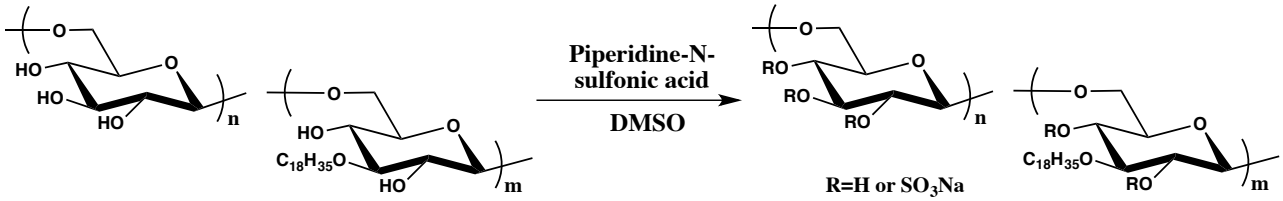

Figure 2.8. 150MHz $^{13}C$ NMR spectra of (1→6)-$\alpha$-D-glucopyranan in $D_2O$ and (1→6)-$\alpha$-D-glucopyranans with 3-*O*-octadecyl groups in the proportion of 2.8, 4.7 and 15.7 mol% in DMSO-$d_6$ at 40℃.

### *2.2.5 Sulfation of 3-O-octadecy (1→6)-α-D-glucopyranans*

*Piperidine N-Sulfonic Acid* [4]

A mixture of 9.4 g of piperidine and 75 ml of $CHCl_3$ was cooled at -50℃, and it was added to 25 ml of $CHCl_3$ solution containing 4.3 g of $ClSO_3H$. The reaction temperature of the mixture was controlled not to exceed 0 and the mixture was stirred for 1h. After the distillation of $CHCl_3$ from the mixture, the residue was dissolved in 60 ml of 10% $Na_2CO_3$, extracted with ethyl ether for several times to remove piperidine. The water layer was separated and adjusted to pH=1 with Dowex 50 ($H^+$). The solution was evaporated at 30-40℃ till needle like crystal formed. After the removal of precipitates, the clear solution was passed through a column of Dowex 50 ($H^+$) again to obtain the solution of free piperidine N-sulfonic acid. The solution was evaporated to dryness and then recrystallized from hot water to afford long needles of mp 190-191.5℃ . The yield was 3.37 g (55%).

*Sulfation of 3-O-octadecy (1→6)-α-D-glucopyranans*




Scheme 2.6. Sulfation of 3-O-octadecy (1→6)-α-D-glucopyranans

Debenzylation polymer was treated with piperidine-N-sulfonic acid (0.8 g) in anhydrous $Me_2SO$ (8 ml) at 85℃ under $N_2$ atmosphere. After stirring for 1.5h, the

mixture was cooled to room temperature, neutralized with saturated 5% NaOH aqueous solution, and then dialyzed for 24 h with deionized water. The dialysate was concentrated to 20 ml and freeze-dried to give 145 mg of sulfated glucopyranan with 3-O-octadecyl group in the proportion of 2.8 mol% and the $\overline{M}n=5.1\times10^3$ (umber 1 in Table 2.3). The sulfur concentration was 12.2% and the degree of sulfation 1.03.

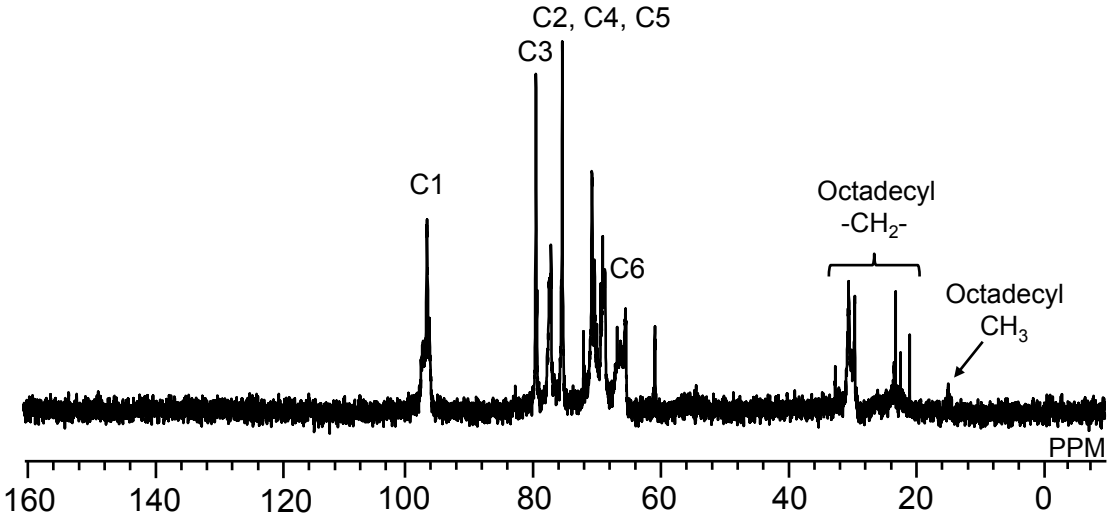



Figure 2.9. $^{13}C$ NMR spectrum of sulfated (1→6)-$\alpha$-D-glucopyranan with 3-*O*-octadecyl groups in the proportion of 2.8 mol% in $D_2O$ at 40℃. The degree of sulfation was 1.03 and $\overline{M}n=5.1\times10^3$.

Table 2.3. Sulfation and anti-HIV activity of 3-*O*-octadecy (1→6)-$\alpha$-D-glucopyranans[a]

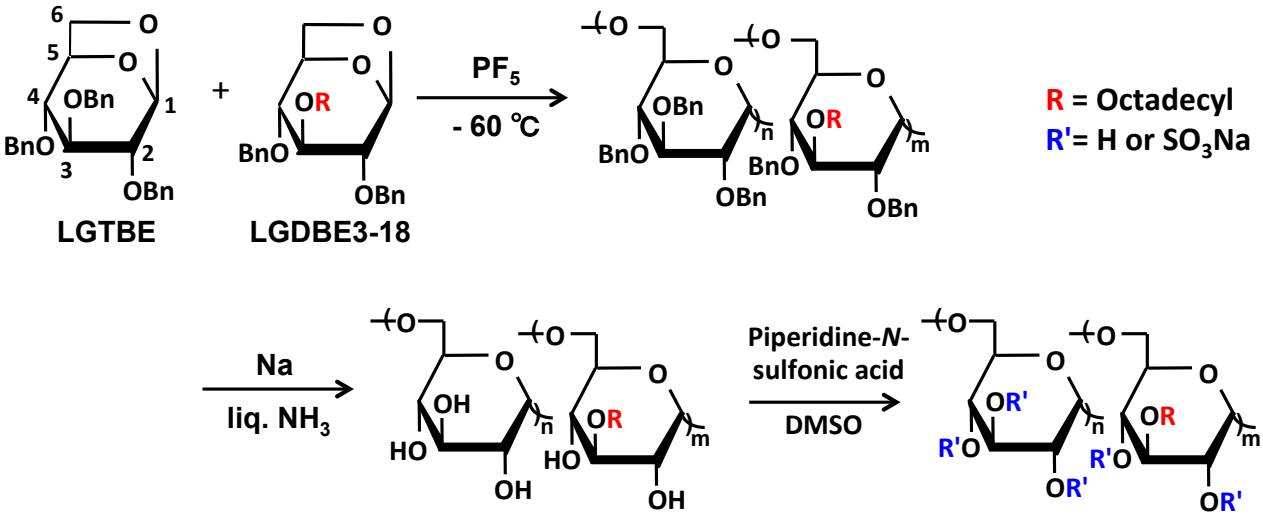
| No | Free 3-O-octadecyl glucopyranan | | PSA | Temp | Time | Yield | Sulfated 3-*O*-octadecyl glucopyranan | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Proportion of 3-*O*-octadecy group | | | | | | Mn | DP[b] | Elemental analysis | | | |
| | | | | | | | | | C | H | S | DS[c] |
| | mg | mol% | g | ℃ | h | mg | $\times 10^3$ | | | % | | |
| 1 | 115 | 2.8 | 1.50 | 85 | 1.5 | 145 | 5.1 | 25.2 | 26.5 | 4.5 | 12.2 | 1.03 |
| 2 | 120 | 2.8 | 1.06 | 85 | 1.5 | 145 | 4.3 | 30.6 | 30.6 | 4.4 | 10.3 | 0.75 |
| 3 | 80 | 2.8 | 0.49 | 85 | 1.5 | 130 | 5.1 | 26.1 | 26.1 | 4.3 | 11.8 | 1.02 |
| 4 | 75 | 4.7 | 0.69 | 85 | 1.5 | 103.5 | 2.5 | 13.5 | 34.9 | 4.9 | 8.6 | 0.55 |
| 5 | 60.5 | 6.7 | 0.58 | 85 | 1 | 95.4 | --[f] | --[f] | 37.6 | 4.7 | 9.5 | 0.57 |
| 6 | 155.4 | 15.7 | 0.92 | 85 | 1 | 262.5 | 5.5 | 29.7 | 47.5 | 6.1 | 8.4 | 0.52 |
| | Dextran sulfate | | | | | | 8.5 | | | | 18.4 | 2.1 |
| | Curdlan sulfate | | | | | | 79.0 | | | | 14.1 | 1.4 |

a) Sulfation was carried out with piperidine-*N*-sulfonic acid (PSA) in DMSO.
b) Degree of polymerization.
c) Degree of sulfation.
d) 50% Effective concentration on HIV.
e) 50% Cytotoxic concentration on MT4 cell.
f) Not determined. It was difficult to dissolve in water.

## 2.3 Result and Discussion

### *2.3.1. Ring-opening copolymerization of 2, 3, 4-tri-O-benzyl (LGTBE) with 2, 4-di-O-benzyl 3-O-octadecyl 1, 6-anhydro-b-D-glucopyranose (LGDBE3-18) monomers*

The polymerization of the 3-*O*-octadecyl glucose monomer LGDBE3-18 with glucose monomer was already reported by Kobayashi,[5] indicating that the monomer was polymerized with $PF_5$ (5 mol %) as catalyst, unter the -60℃ to give the corresponding (1→6)-α-D-glucopyranosidic polymers with high molecular weights. However, the exact structure of the homopolymers and copolymers has not been reported. Therefore, we examined the copolymerization of LGTBE with LGDBE3-18 and then the structure of the resulting copolymer was investigated by high resolution NMR measurements.




Scheme 2.7. Synthesis route of sulfated copolysaccharides.

Scheme 2.7 shows the synthesis route of sulfated copolysaccharides. The two monomers in the various feeds were copolymerized with $PF_5$ as catalyst under

pressure below $10^{-5}$ mmHg at -60 °C to give the corresponding copolymers benzylated 3-*O*-octadecy (1→6)-*a*-D-glucopyranans in good yields. After debenzylation with sodium in liquid ammonia at -78°C under nitrogen the benzyl groups were recovered by hydroxyl groups to give various proportions of 3-*O*-octadecy (1→6)-*a*-D-glucopyranan. Debenzylation polymer was treated with piperidine-N-sulfonic acid in anhydrous $Me_2SO$ at 85℃ under $N_2$ atmosphere, the sulfated glucopyranans with a long octadecyl group at the C3 position were obtained. The results of the copolymerization are summarized in Table 2.1. The LGDBE3-18 monomer was readily polymerized with $PF_5$ in 4 h at -60°C to give the corresponding polymer in 91.8% yield, whose number-average molecular weight ($\bar{M}n$) measured by GPC with $CHCl_3$ as an eluent was high, $\bar{M}n = 163.1 \times 10^3$, as shown in No. 8.

When the copolymerization with LGTBE and LGDBE3-18 monomers was carried out with $PF_5$ in the monomer proportions ranging from 3.7 mol% to 70 mol%, the corresponding copolymers were obtained in more than 90% yields. The molecular weights of the copolymers were also high, giving from $\bar{M}n = 65.7 \times 10^3$ to $136.5 \times 10^3$. Specific rotation values were high and positive for poly(LGTBE) and decreased gradually from +114.1° to +91.4° (No. 7) with an increasing proportion of LGDBE3-18 monomeric units in the feed; poly(LGDBE3-18) gave +86.9° (c1, $CHCl_3$). In the ring-opening polymerization of 1, 6-anhydro sugars, 1, 6-scission occurred with a cationic catalyst to form pyranosidic polymers with a (1→6) linkage. Taking into account the high and positive specific rotations, the copolymers that resulted should have (1→6)-α stereoregularity.

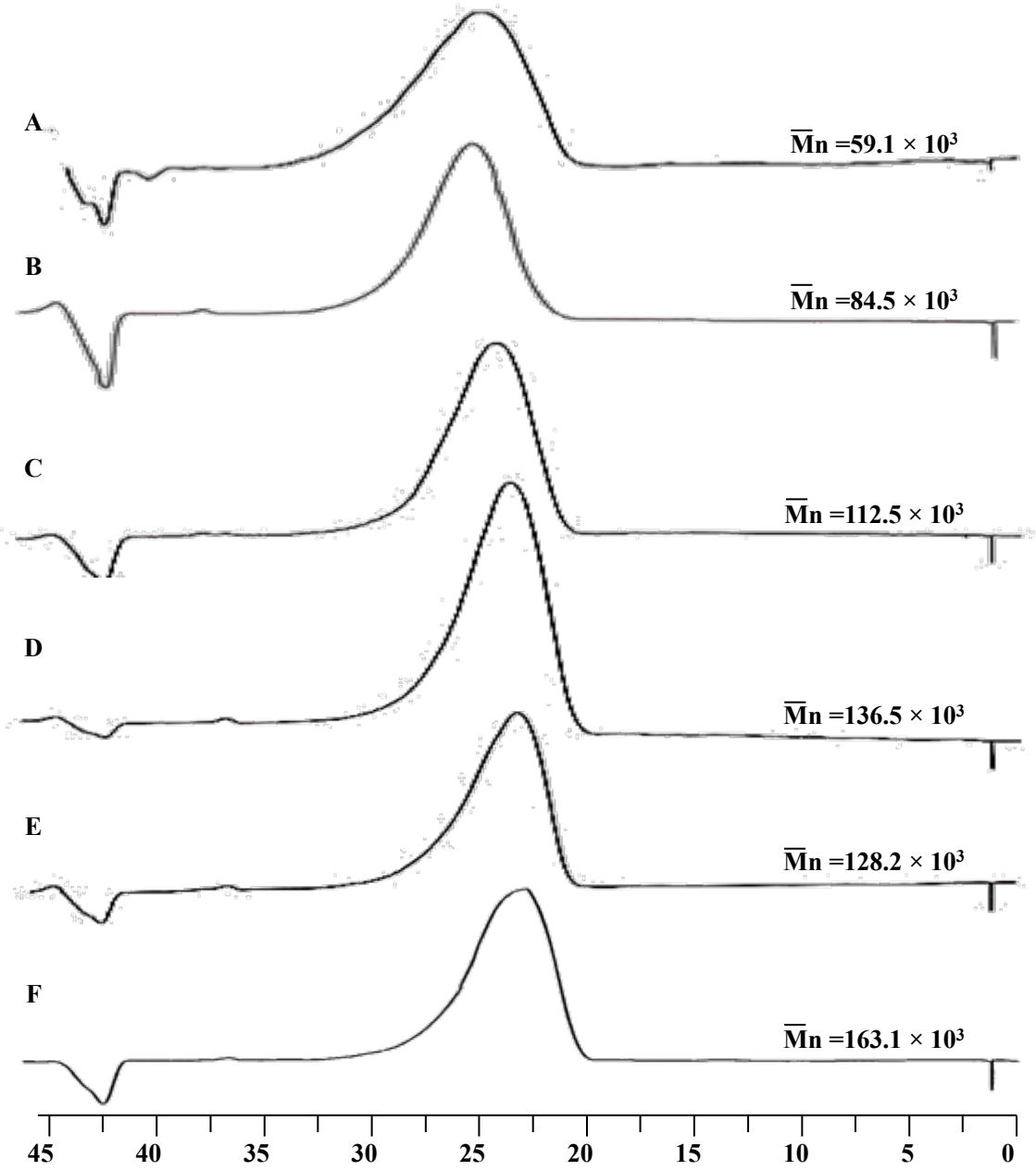



Figure 2.10. Chloroform GPC of benzylated alkyl (1→6)-α-D-glucopyranans, Content of each monomer in feeds (A) LGTBE:LGDBE-18=100:0, (B)=96.3:3.7, (C)=70:30, (D) =50:50, (E)=30:70, (F)=0:100

The proportion of LGDBE3-18 units in the copolymers depended on the monomer feed and ranged from 1.3 mol% to 72.9 mol%, which were calculated from the integration values in the $^{1}$H NMR spectra at 3.32 ppm and 3.22 ppm due to the H2 proton in LGTBE and LGDBE3-18 residues, respectively.

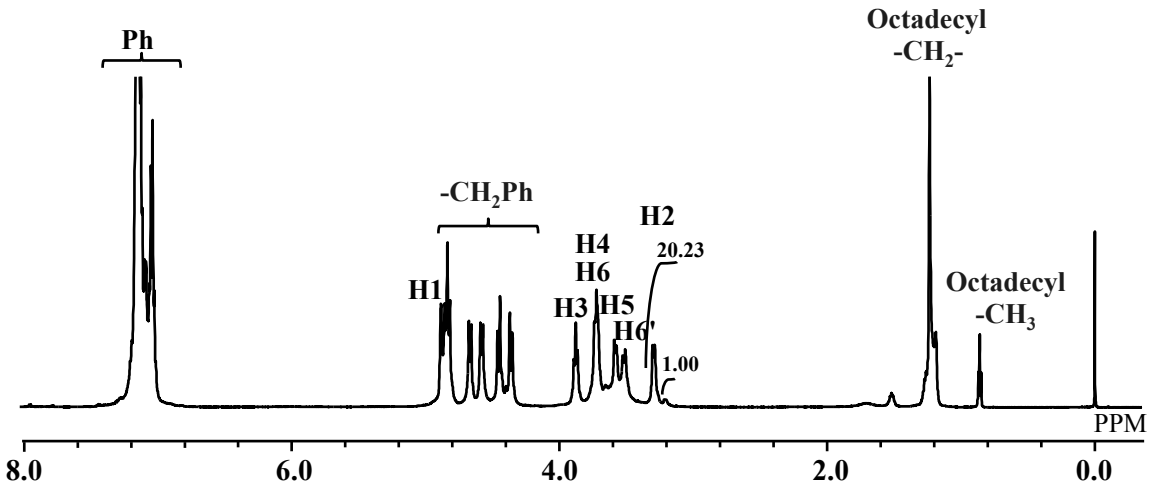



Figure 2.11. $^{1}$H-NMR spectrum of copoly(LGTBE-LGDBE3-18) in $CDCl_3$ at room temperature with $\bar{M}n$ 65.7 × $10^3$ and $[a]_D^{25}$= +107.8° .

Figure 2.11 shows the $^{1}$H NMR spectrum of the copolymer of LGTBE and LGDBE3-18 units in the proportion of 90:10 mol% in feed. From the integration value at 3.25 ppm to 3.35 ppm, the proportion of LGDBE3-18 unit in the copolymer was calculated to be 4.7 mol% (No. 4 in Table 2.1). Figure 2.6 represents the $^{13}$C NMR spectra of the copolymers in $CDCl_3$ at 25°C. Figures 2A and 2F are spectra of homopolymers of LGTBE and LGDBE3-18 monomers, respectively. The C1 carbon signals appeared at 97.9 ppm in both 2A and 2F spectra as singlet signals, revealing that the polymers have high stereoregularity. The terminal methyl signal of octadecyl group appeared at 13 ppm and methylene signals also appeared between 22 and 32 ppm. A methylene signal next to oxygen at the C3 position of LGDBE3-18 residue was found to be shifted to a lower magnetic field at 74 ppm. For the copolymers having the 3-*O*-octadecyl group shown in Figures 2B to 2E, the carbon signals due to glucopyranose units appeared in almost the same positions as those of homopolymers. With the increase of the proportion of LGDBE3-18 units, the intensity of octadecyl carbon signals and the protective benzyl signal at the C3 position of the LGTBE unit gradually increased and decreased, respectively. The results of the NMR and specific rotations in Table 2.1 indicate that the homo and copolymers had high

stereoregularity and were composed of (1→6)-α linked glucopyranosidic units.

### *2.3.2. Deprotection of benzyl groups in copolymers*

Removal of benzyl groups from the benzylated copolymers with 3-*O*-octadecyl groups in proportions below 22.2 mol% was carried out with sodium in liquid ammonia to give OH-free glucopyranans with a 3-*O*-octadecyl group. The results are shown in Table 2.2. The OH-free glucopyranans were insoluble in water and partially soluble in DMSO, so it was impossible to measure their molecular weights and specific rotations. However, the molecular weights of the OH-free glucopyranans were estimated to be relatively high. After debenzylation, the characteristic IR absorptions of large hydroxyl and octadecyl groups appeared at 3413 $cm^{-1}$ and 2925 $cm^{-1}$, respectively, indicating that the substituted octadecyl group at the C3 position still remained. However, the proportion decreased somewhat before debenzylation as measured by NMR, probably because the debenzylation was performed by strong alkaline conditions. Figure 2.8 exhibits the $^{13}C$ NMR spectra of the OH-free glucopyranans with 3-*O*-octadecyl group in the proportion of 2.8 to 15.7 mol% in DMSO-$d_6$ at 40°C. The methylene signals due to the protective benzyl group between 70 ppm and 80 ppm disappeared, suggesting that debenzylation proceeded to recover hydroxyl groups without elimination of the octadecyl group, because the 3-*O*-octadecyl signals appeared between 17 ppm and 33 ppm. The C3' signal due to the 3-*O*-octadecylated glucopyranosidic residue appeared clearly at 82.9 ppm, and increased gradually in intensity with the increase in the proportion of the 3-*O*-octadecyl group in the glucopyranans. The proportion of the octadecyl group was calculated from the integration values at 3.72 ppm and 0.81 ppm due to the H2 signal of the glucose residue and the terminal $CH_3$ signal of 3-*O*-octadecyl group, respectively, in the $^1H$ NMR spectrum.

### *2.3.3. Sulfation of 3-O-octadecyl glucopyranans*

We reported the anti-HIV activity of sulfated (1→6)-α-D-glucopyranan prepared by the ring-opening polymerization of LGTBE monomer and then debenzylation. [6][7][8] Therefore, in this thesis, sulfation of the 3-*O*-octadecylated sulfated (1→6)-α-D-glucopyranans was performed. Sulfation of glucopyranans with the 3-*O*-octadecyl group in proportions below 15.7 mol% was performed by piperidine-*N*-sulfonic acid in DMSO at 85°C to give sulfated (1→6)-α-D-gluco -pyranans with a 3-*O*-octadecyl group. The results are listed in Table 2.3. After sulfation, the sulfated glucopyranans were soluble in water, and their molecular weights were $2.5 \times 10^3$ - $5.5 \times 10^3$ except No.5 in Table 2.3. The solubility of the No. 5 sulfated glucopyranan in water was low probably because the degree of sulfation was low and the molecular weight should be relatively high. Figure 2.9 shows the $^{13}C$ NMR spectrum of the sulfated glucopyranan with 3-*O*-octadecyl group in the proportion of 2.8 mol% and the molecular weight of $\overline{M}n$ $5.1 \times 10^3$ (No. 1 in Table 2.3).

After sulfation, signals broadened and shifted to a lower or higher magnetic field, suggesting that the sulfate group was introduced into the hydroxyl groups of the glucopyranosidic units. The C1, C3, and C5 signals due to glucopyranosidic residue appeared at 97 ppm, 79 ppm, and 66 ppm, respectively. The C2, C4, and C5 signals appeared between 69 ppm and 77 ppm. The terminal methyl signal due to octadecyl group appeared at 14.5 ppm and other signals were absorbed between 22 and 32 ppm. The methylene signal next to oxygen ($-OCH_2-$) at the C3 position should appear around 70 ppm, a signal overlapped by other signals. The degree of sulfation was calculated to be 1.03 from the ratio of carbon to sulfur in the elemental analysis as shown in Table 2.3. In the FT-IR spectra, the large absorption of hydroxyl groups was still remained after sulfation because the sulfate groups were introduced into the

hydroxyl groups in the proportion below 1/3. The large absorption due to sulfate group appeared at 1261 $cm^{-1}$.

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# *Chapter 3*

# *Biological activity of Sulfation of 3-O-octadecyl glucopyranans*

## 3.1 Introduction

### *3.1.1 HIV*

The human immunodeficiency virus' diameter About 120 nanometers and shaped spherical. The membrane of virus is a lipid envelope embedded with protein gp120 and gp41. The gp41 is transmembrane protein binding with surface protein by non-covalent binding effect. The interior composed by spherical matrix of the protein p17 and half-formed conical capsid of

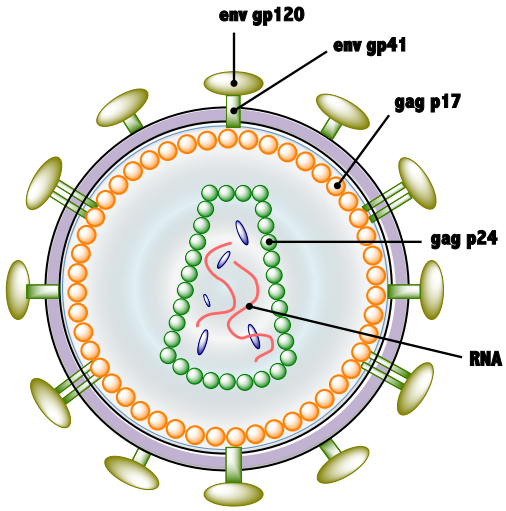


Figure 3.1. The structure of HIV

protein p24. Capsids contained virus' RNA genome, enzymes (Reverse transcriptase, integrase, protease) and other components derived from host cells. 1

HIV gains access to several human cell types, most notably CD4 T cells, macrophages, and dendritic cells. Be known to have two mechanisms of HIV entry into host cells: fusion and uptake. HIV virus invade target cells by glycoprotein to adsorb membrane surface.[1][2] In the mid-1980, people found that the CD4 cell is a receptor cell for HIV virus,[3] and in 1996 found that CXCR4 and CCR5 (chemokine receptor) are the coreceptor for HIV.[4]

HIV virus invade target cells is a very complex process. The first step is a combination of gp120 and CD4 molecules and chemokine receptors. This binding induces a conformational change in gp41. The change leads gp41 freed from the gp120 on non-covalent binding thereby expose the 7 amino acid residues repeat. The exposure of fusion peptide and the coil structure induce a fusion intermediates. And then cause fusion peptide insert to the target cell membrane. The structure change pull the virus and cell close enough to make the membrane fusion possible.[5]

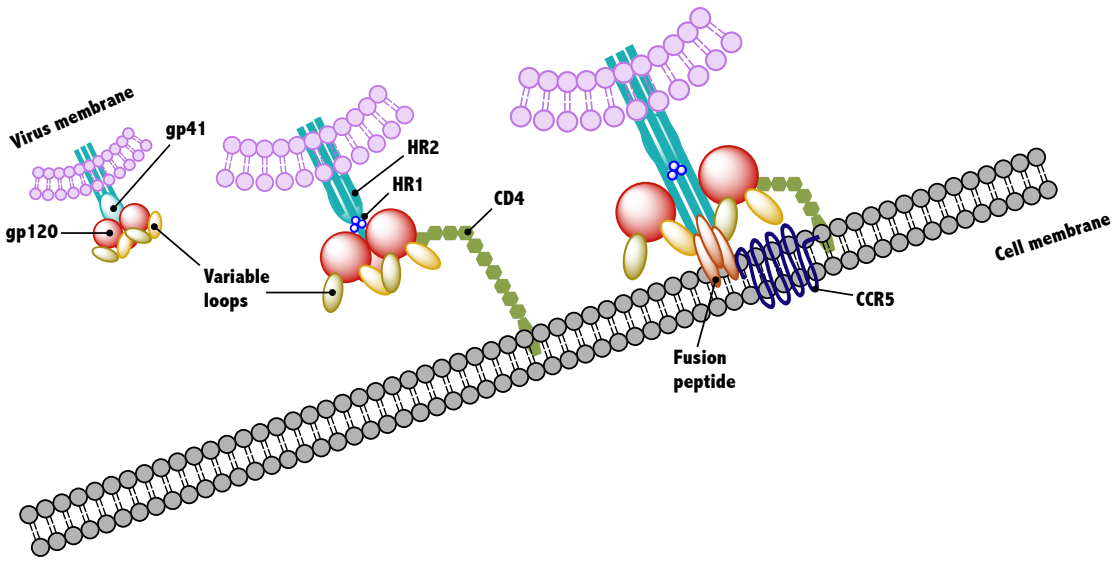


Figure 3.2. The mechanism of HIV infected cell.

Researcher recognized that sulfated polysaccharides having potent in vitro anti-HIV activity since 1980s. Several naturally occurring sulfated polysaccharides, such as heparin sulfate and dextran sulfate exhibit to inhibit the binding of HIV to CD4-positive cells in vitro in the microgram per milliliter or micromolar range.[6][7] The mechanism of sulfated polysaccharides to inhibit HIV was considered that there are regions in gp 120 containing multiple basic amino acids: V3 loop, the C-terminal region[8][9] These regions interact with polyanions (HS and DS) to prevent binding to complementary antibodies.[10]

### *3.1.2 Surface Plasmon Resonance*

SPR is a physical optical phenomenon. One of the necessary conditions for the formation of surface plasmon resonance is the presence of metal and dielectric surfaces. The plasma is a relatively high density of free positive and negative charges of gas composition, which have equal numbers of positive and negative charged particles. The valence electron on metal surface as a moving electron gas under the uniform positive charged background, which is actually a plasma. When the electromagnetic interference occurs on the metal, the electron density distribution inside the metal will becomes nonuniform. In the Coulomb force and

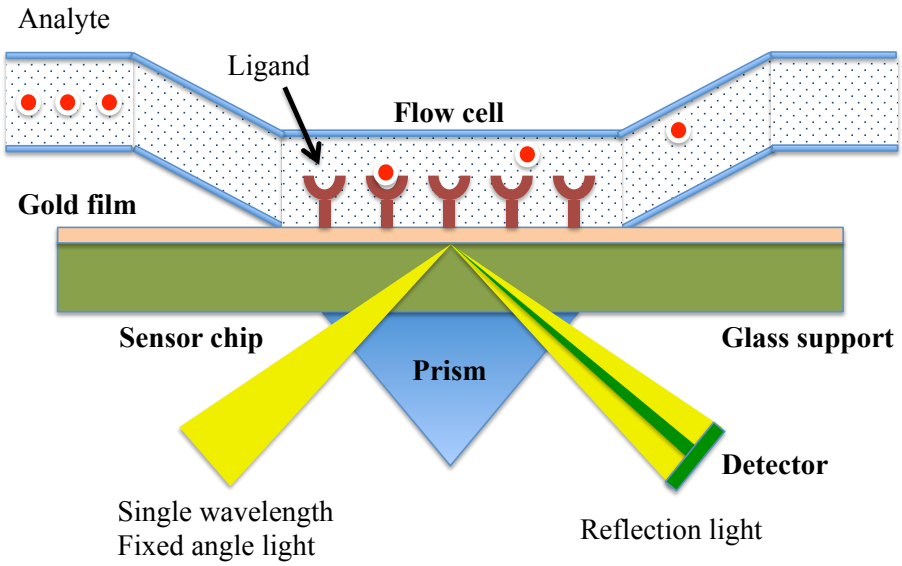


Figure 3.3. Surface plasmon resonance (SPR)

repulsion, electrons repeatedly shocked in the entire area, and performed in the form of waves. The light total reflection phenomenon occurs to the prism and on the metal film surface, evanescent wave into the optically thinner medium.

However, cause some plasma waves in the medium that led to the resonance. When the evanescent wave and surface plasma wave resonance, reflecting the detected light intensity will be greatly weakened. Most of the energy of the incident light is absorbed by the surface plasma wave, the transference of energy from photons to the surface of the plasma that led to the energy of the reflected light sharp decline. The reflected light intensity at an angle greatly weakened, the angle of reflected light is completely gone called SPR angle. SPR angle changing from the gold surfaces refractive index changes, and the refractive index change is proportional to the molecular weight bound on the gold surface. The specific signal interaction between biological molecules can be gotten by dynamic changes in biological processes of SPR angle.

Therefore the SPR can be used to detect the combination of sulfated polysaccharide with specific protein of HIV surface to investigate the extent of the anti HIV activity. In addition to SPR, the particle size and ζ potential can be reacted from the other side to react the combination of sulfated polysaccharide with proteins of HIV surface.

### *3.1.3 Dynamic light scattering measurement*

Use laser as light source, the light is monochromatic light with a certain wavelength. The wavelength of incident light will be changed if the monochromatic beam hits the spherical moving particles of Brownian motion. Spatial distribution (angle) of diffraction and scattering light only related to particle size. Scattered light is detected by the sensor and then converted into an electric signal. The particle size

is calculated by the computers based scattering signals. The interaction between sulfated polysaccharide and proteins by the electrostatic interaction could be detected directly by the changes of particle size after mixing.

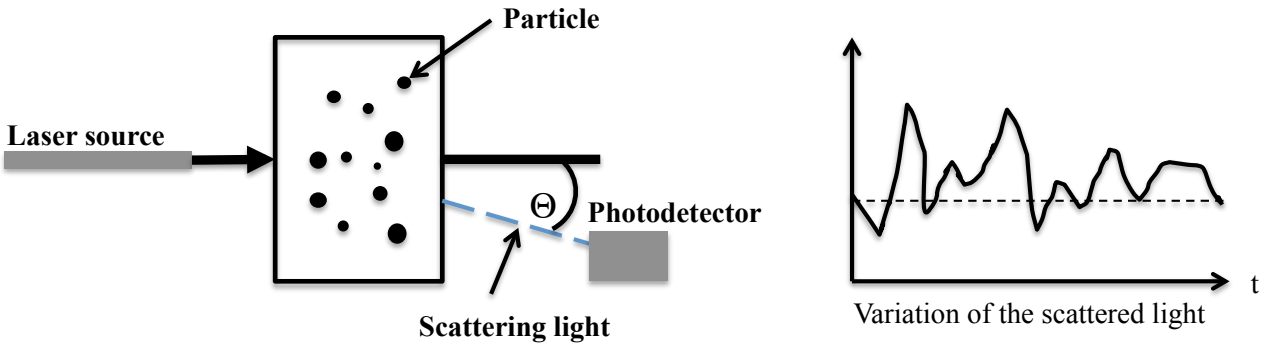


Figure 3.4. The hypothetical dynamic light scattering of samples

### *3.1.4 Zeta potential*

There are electric charges on the dispersed particles surface that attract counter ions around it. These counter ions distributed to diffusion state in two-phase interface to form diffused double layer (Stern plane and Diffuse layer). The stern plane is defined as a layer of ion adsorbed on the surface of the electrode. Stationary layer include Stern plane and inner parts of slipping plane that the potential of a point in a fluid that is far from the interface is called a Zeta potential. The Zeta potential is potential difference between the continuous phase and the stationary layer. [11][12]

Zeta potential is an important indicator of the stability of colloidal and suspended solids. We investigate the changes of the zeta potential of sulfated 3-*O*-Octadecy (1→6)-α-D-glucopyranans and Dextran added poly-L-lysine to elucidate the interaction of sulfated 3-*O*-Octadecy (1→6)-α-D-glucopyranans with poly-L-lysine and Dextran with poly-L-lysine.

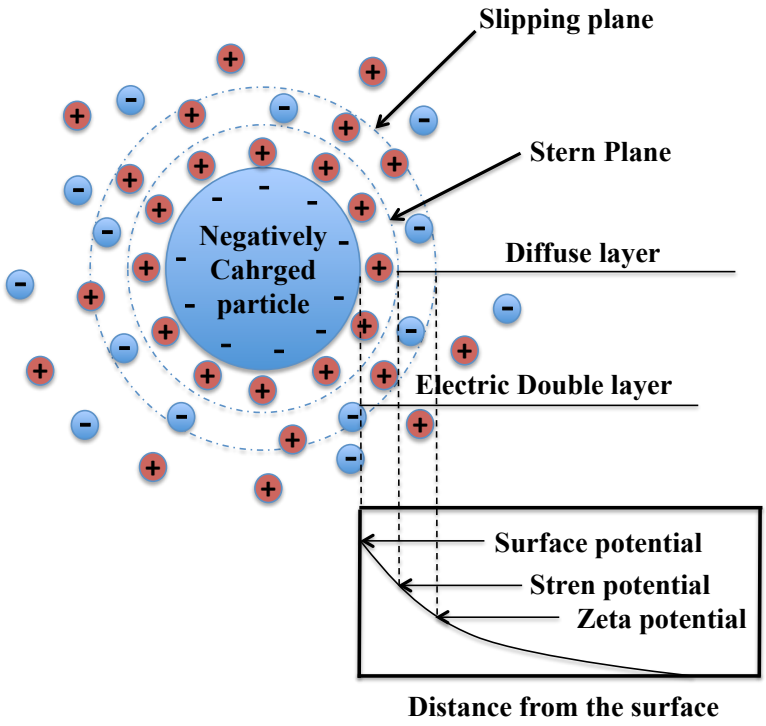


Figure 3.5. Scheme of electrochemical double layer

## 3.2 Biological activity of Sulfated 3-O-octadecyl(1→6)-α-D-glucopyranans

### *3.2.1 Anti-HIV assay of sulfated 3-O-octadecy (1→6)-α-D-glucopyranans*

Cells and viruses: MT-4 cells, a human T-cell line bearing human immune-odeficiency virus type 1.

Aliquots of $3 \times 10^4$ MT-4 cells per ml were infected with HIV-1at a multiplicity of infection (MOI) of 0.01. The HIV-infected or mock-infected MT-4 cells were placed in 96-well culture plates with various concentrations of the compounds and incubated at 37°C under $CO_2$. After 5 days, cell viability was quantified by MTT assay. The 50% cytotoxic concentration (CC50), 50% effective concentration (EC50), and selectivity index (SI = CC50/EC50) were then calculated from the cell viability for each concentration of the compound.

Table 3.1: DLS measurements of 3-*O*-octadecyl (1→6)-α-D-glucopyranans in the present or absent of poly-L-lysine

| No | Proportion of 3-*O*-octadecyl group mol% | $\overline{M}_n$ | DS[b] | $EC_{50}$ μg/ml | $CC_{50}$ μg/ml | SI |
|---|---|---|---|---|---|---|
| 1 | 2.8 | 5.1 | 1.03 | 0.05 | >162 | 3524 |
| 2 | 2.8 | 4.3 | 0.75 | 0.09 | >163 | 1763 |
| 3 | 2.8 | 5.1 | 1.02 | 0.04 | >200 | 4943 |
| 4 | 4.7 | 2.5 | 0.55 | 1.25 | >147 | 118 |
| 5 | 6.7 | -- | 0.57 | 20.34 | >200 | 10 |
| 6 | 15.7 | 5.5 | 0.52 | 6.50 | >200 | 27 |
| Dextran sulfate | | 8.5 | 2.1 | 0.05 | >621 | 12363 |
| Cardlan sulfate | | | | 0.18 | >1000 | 5523 |
| AZT(uM) | | | | 0.015 | >233 | 15882 |
| ddC(uM) | | | | 1.2 | >2145 | 1789 |

Operator: St. Marianna Univ., Kanemoto Taisei, Terakubo Shigemi

Date: 16th, July/2014

### *3.2.2 Interaction of Sulfated 3-O-Octadecy (1→6)-α-D-glucopyranans with poly-L- lysine*

#### *3.2.2.1 Surface Plasmon Resonance measurement*

Commercially available poly-L-lysine (ligand) with the molecular weight of 1000-5000 was used as a model compound of epidermal protein of the virus. Elucidating interaction between sulfated octadecyl glucopyranan and poly-L-lysine was determined by a Biacore X100 SPR instrument at 25℃ and used dextran sulfate with potent anti-HIV activity ($EC_{50}$ = 0.05 mg/ml) as a standard sulfated poly -saccharide and immobilized on the gold coating CM5 sensor chip by an amine coupling method to give 2100 response units (RU), which is an immobilization rate. The sulfated copolysaccharides were dissolved in a HBS-EP manufacturer-supplied running buffer (0.1 M HEPES, 1.5 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P 20, pH7.4) and the solution was passed over the surface of the poly-L-lysine immobilized sensor chip at the flow rate of 30 mL/min for 180 sec for association.

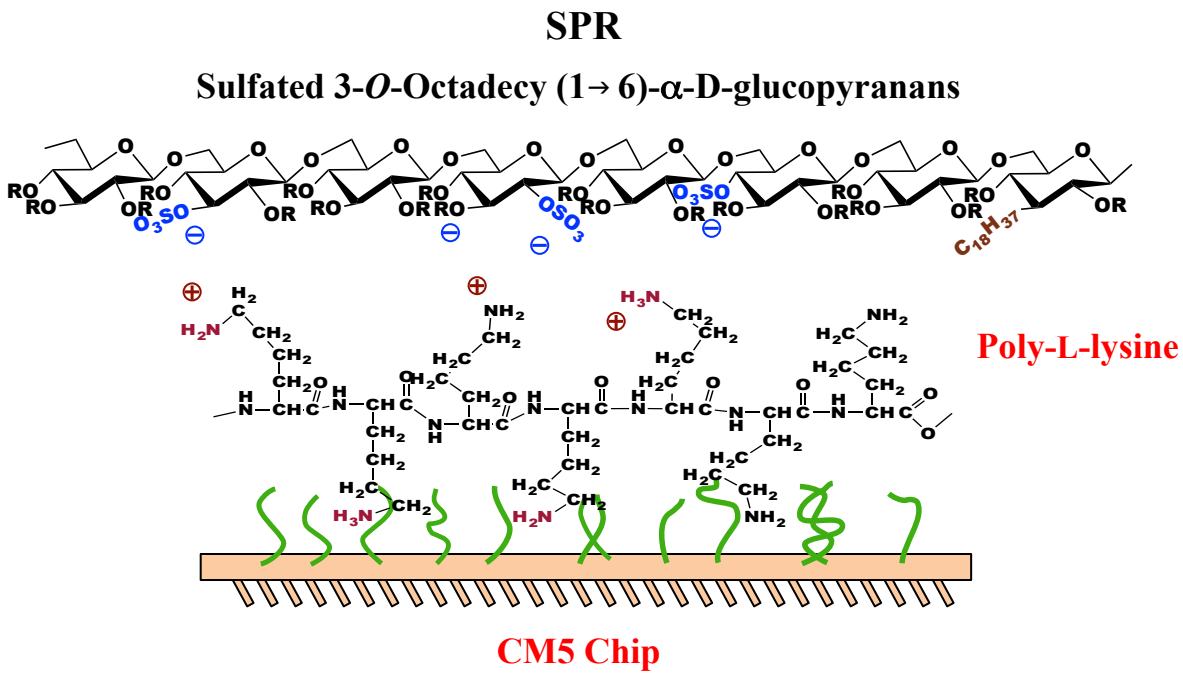


Figure 3.6. Image of interaction between sulfated polysaccharide with poly-L-lysine

Sulfated 3-*O*-Octadecy (1→6)-α-D-glucopyranans were injected 90 µl with concentrations of 500, 250, 125, 62.5, and 31.3 mg/ml, respectively. The running buffer was continuously flowed for a further 600 sec at the same rate for dissociation. The association ($k_a$) and dissociation rate ($k_d$) constants were determined by the 1:1 binding model using Biacore- supplied soft wear provided by GE Healthcare UK Ltd. The dissociation constant ($K_D$) was calculated from the $k_a$ and $k_d$.

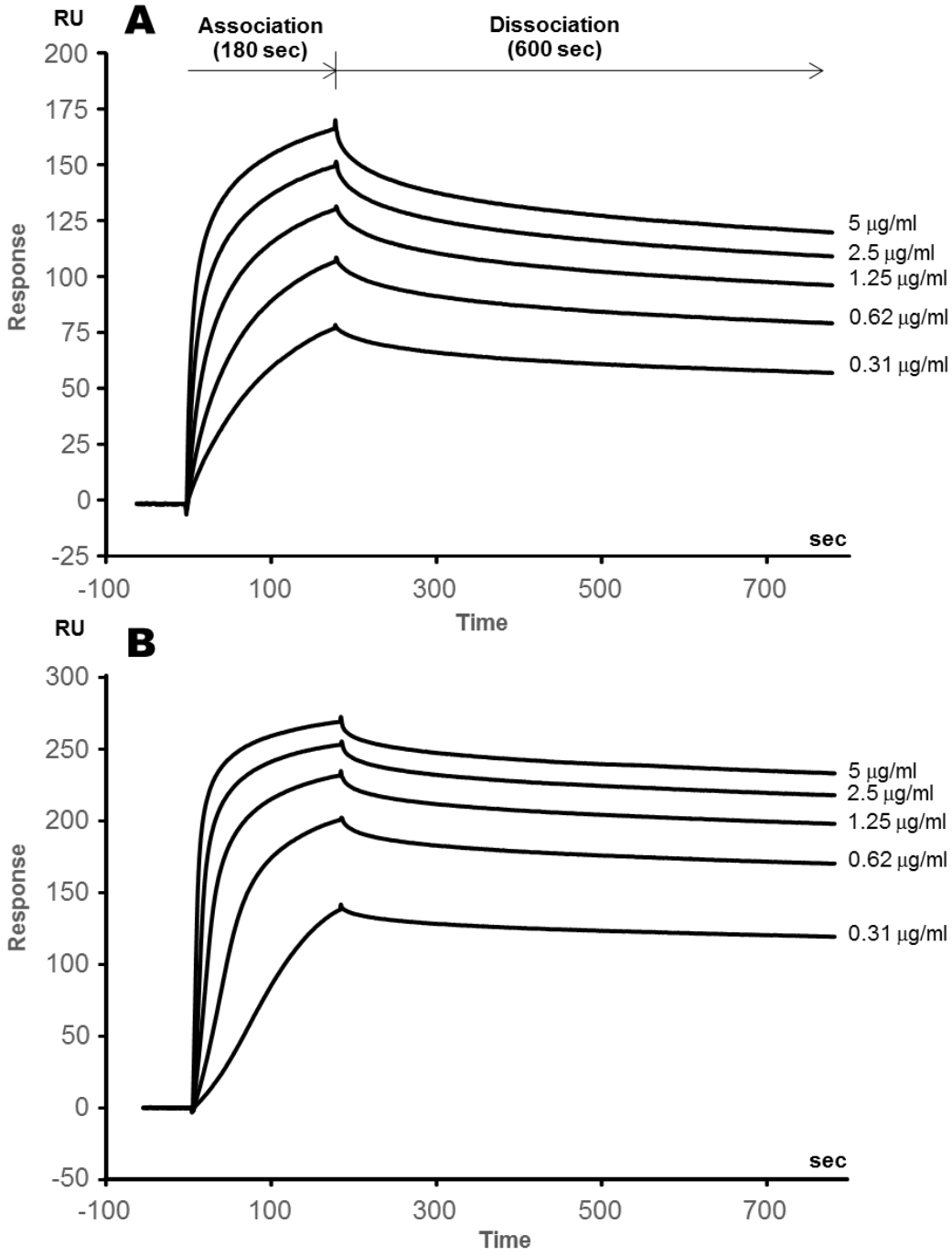


Figure 3.7. SPR binding affinity of (A) sulfated (1→6)-α-D-glucopyranan with 3-*O*-octadecyl groups in the proportion of 2.8 mol% and with $\overline{M}_n = 5.1 \times 10^3$ and (B) dextran sulfate with $\overline{M}_n = 8.5 \times 10^3$ to poly-L-lysine, respectively. The apparent kinetic constants were $k_a = 8.1 \times 10^4$ 1/Ms, $k_d = 3.7 \times 10^{-4}$ 1/s, and $K_D = 4.6 \times 10^{-9}$ M for sulfated 3-*O*-octadecyl (1→6)-α-D-glucopyranan (A) and $k_a = 29.0 \times 10^4$ 1/Ms, $k_d = 1.89 \times 10^{-4}$ 1/s, and $K_D = 0.65 \times 10^{-9}$ M for standard dextran sulfate.

Table 3.2. SPR measurements of 3-*O*-octadecyl (1→6)-*α*-D-glucopyranans in the present or absent of poly-L-lysine

| No | Proportion of 3-*O*-octadecyl group mol% | $\overline{M}_n$ | DS[b] | Kinetic result | | |
|---|---|---|---|---|---|---|
| | | | | $k_a$ 1/Ms | $k_d$ 1/s | $K_D$ M |
| 1 | 2.8 | 5.1 | 1.03 | $8.1\times10^{4}$ | $3.7\times10^{-4}$ | $4.6\times10^{-9}$ |
| 2 | 2.8 | 4.3 | 0.75 | $4.9\times10^{4}$ | $3.3\times10^{-4}$ | $6.8\times10^{-9}$ |
| 3 | 2.8 | 5.1 | 1.02 | $8.0\times10^{4}$ | $3.1\times10^{-4}$ | $3.9\times10^{-9}$ |
| 4 | 4.7 | 2.5 | 0.55 | $6.8\times10^{4}$ | $1.0\times10^{-3}$ | $1.6\times10^{-8}$ |
| 5 | 6.7 | -- | 0.57 | $6.6\times10^{4}$ | $1.1\times10^{-3}$ | $1.5\times10^{-8}$ |
| 6 | 15.7 | 5.5 | 0.52 | $4.3\times10^{4}$ | $4.2\times10^{-3}$ | $1.0\times10^{-8}$ |
| Dextran sulfate | | 8.5 | 2.1 | $29.0\times10^{4}$ | $1.9\times10^{-4}$ | $0.7\times10^{-9}$ |

a) Commercially available poly-L-lysine (1 mg/ml or 0.5 mg/ml) with the molecular weight of 1000-5000 was used.
b) Degree of sulfation was calculated from the results of the elemental analysis.
c) Soluble part in phosphate buffer (pH=7.4) was measured.

### *3.2.2.2 Particle size and Zeta potential analysis*

The particle size and zeta potential of the sulfated 3-*O*-Octadecyl (1→6)-α-D-glucopyranans in the presence or absence of poly-L-lysine were determined at 25°C on an Otsuka Electronics ELSZ-1000ZS particle size and zeta potential analyzer in phosphate buffer solution (pH=7.4). Dextran sulfate with $\overline{M}n=8.5x10^{3}$ was used to standard, sulfated 3-*O*-Octadecyl (1→6)-α-D-glucopyranans with $\overline{M}n$: 5.1, 4.3, $2.5\times10^{3}$ and Commercially available poly-L-lysine with the molecular weight of 1000-5000 were made concentration of 1 mg/ml or 0.5 mg/ml by phosphate buffer. All samples were subjected to ultrasonication for 5 min before measuring. The sample of poly-L-lysine present was prepared for mixing the equivalent of sulfated 3-*O*-Octadecyl (1→6)-α-D-glucopyranan and poly-L-lysine.

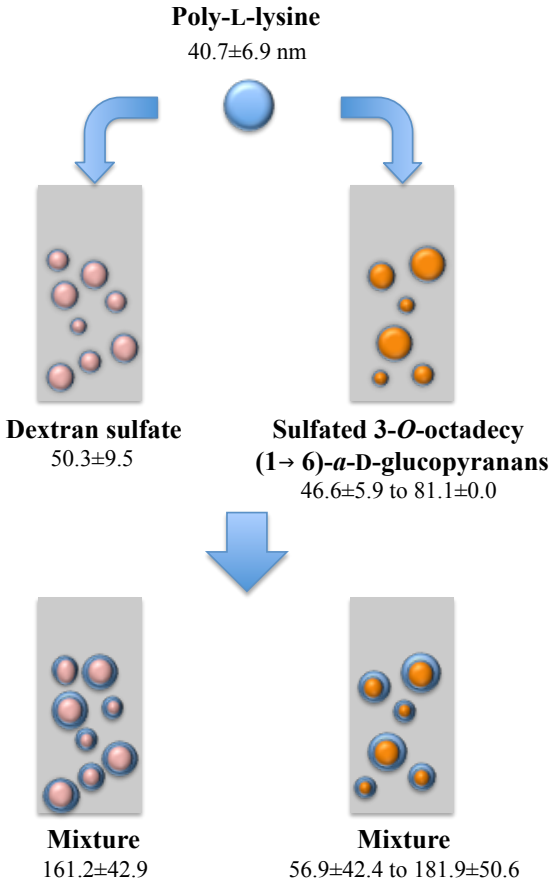


Figure 3.8. Image of combination of sulfated 3-*O*-octadecy (1→6)-*α*-D-glucopyranans and dextruan sulfate with poly-L-lysine

Table 3.3. DLS and zeta potential measurements of sulfated 3-*O*-octadecy (1→6)-*α*-D-glucopyranans in the present or absent of poly-L-lysine

| No | $\bar{M}n$ | DS[b] | Proportion of 3-*O*-octadecy group mol% | Poly-L-lysine[a] Present Particle size nm | Present ζ mV | Absent Particle size nm | Absent ζ mV |
|---|---|---|---|---|---|---|---|
| 1 | 5.1 | 1.03 | 2.8 | 181.9±50.6 | -24.44 | 69.6±19.8 | -30.61 |
| 2 | 4.3 | 0.75 | 2.8 | 147.6±45.8 | -18.10 | 81.1±0.0 | -18.43 |
| 3 | 5.1 | 1.02 | 2.8 | 129.4±31.9 | -15.91 | 63.1±12.3 | -18.27 |
| 4 | 2.5 | 0.55 | 4.7 | 91.3±35.1 | -16.15 | 47.4±6.6 | -18.0 |
| 5[e] | -- | 0.57 | 6.7 | 56.9±42.4 | -5.14 | 46.6±5.9 | -9.95 |
| 6 | 5.5 | 0.52 | 15.7 | 64.2±20.5 | -14.23 | 63.0±22.8 | -20.09 |
| Dextran sulfate | 8.5 | 2.1 | | 161.2±42.9 | -11.87 | 50.3±9.5 | -4.67 |

a) Commercially available poly-L-lysine (1 mg/ml or 0.5 mg/ml) with the molecular weight of 1000-5000 was used.
b) Degree of sulfation was calculated from the results of the elemental analysis.
e) Soluble part in phosphate buffer (pH=7.4) was measured.
f) Standard dextran sulfate with $\bar{M}n=8.5\times10^3$ was used.

### *3.2.3 Interaction of Sulfated 3-O-Octadecy (1→6)-α-D-glucopyranans with Liposome*

#### *3.2.3.1 Preparation of Liposome*

POPC (70 mg) was dissolved with chloroform (10 ml) in 20 ml flask and then distilled the chloroform and further vacuum evaporate for 2h. After drying, to add the PBS buffer 10 ml, shaken and fully mixed. The suspension was frozen by liquid $N_2$ for 5 times. The thawy suspension was passed 500nm filter membrane by syringe, and then to prepare liposome by Avanti Polar Lipids, Inc provided Mini-extruder. The filtered solution was passed the double layer membrane of 100 nm and 50 nm sequentially 19 ~ 21 times. Eventually the suspension became to colorless solution which can be saved for 2 months at 4°C.

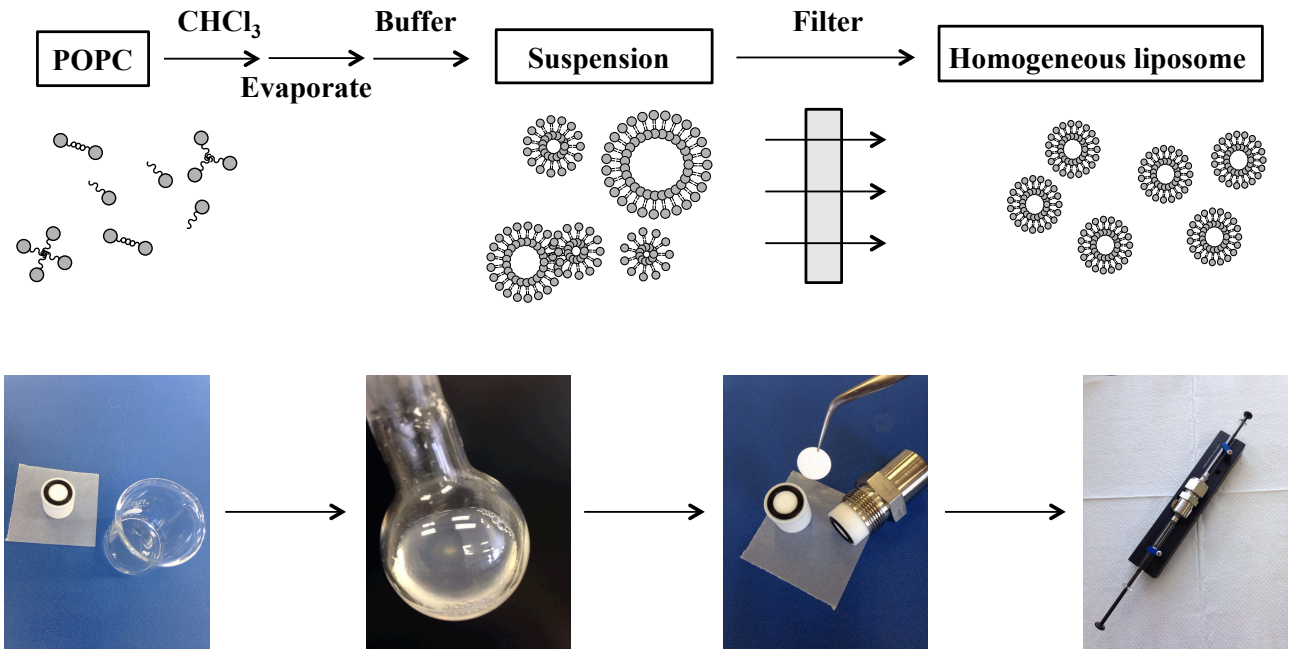


Figure 3.9. Process of preparation of liposome

#### *3.2.3.2 Particle size and Zeta potential analysis*

The particle size and zeta potential of the sulfated 3-*O*-Octadecyl(1→6)-α-D-glucopyranan in the presence or absence of liposome were determined at 25°C on an Otsuka Electronics ELSZ-1000ZS particle size and zeta potential analyzer in phosphate buffer solution (pH=7.4). Dextran sulfate with $\overline{M}n=8.5x10^3$ was used to standard, sulfated 3-*O*-Octadecyl (1→6)-α-D-glucopyranan with $\overline{M}n$: $5.1\times10^3$ and prepared liposome were made concentration of 1 mg/ml by phosphate buffer. All samples were subjected to ultrasonication for 5 min before measuring. Liposome present sample was prepared for mixing the equivalent of sulfated 3-*O*-Octadecyl (1→6)-α-D-glucopyranan and liposome.

Table 3.4. DLS measurements of sulfated 3-*O*-octadecy (1→6)-*α*-D-glucopyranans in the present or absent of liposomes

| | Liposome 58.4±20.0 (nm) | | Liposome 107.3±27.9 (nm) | |
|---|---|---|---|---|
| | Absent | Present | Absent | Present |
| Dextran sulfate[a] | 50.3±9.5 | 57.2±11.2 | 50.3±9.5 | 105.1±26.7 |
| Octadecyl glucopyranan | 67.2±13.9 | 104.3±25.1 | 67.2±13.9 | 267.8±54.0 |

a) Dextran sulfate and sulfated 3-*O*-Octadecyl (1→6)-α-D-glucopyranan were concentration of 1 mg/ml respectively.

b) The liposome passed by 50nm filter is 58.4±20.0nm and the liposome passed by 100nm filter is 107.3±27.9nm.

Table 3.4. Zeta potential measurements of sulfated 3-*O*-octadecy (1→6)-*α*-D-glucopyranans in the present or absent of liposomes

| | Liposome 58.4±20.0 nm 0.12 mV | | Liposome 107.3±27.9 nm 0.07 mV | |
|---|---|---|---|---|
| | Absent | Present | Absent | Present |
| Dextran sulfate[a] | -31.78 mV | -10.4 mV | -31.78 mV | -8.64 mV |
| S. Octadecyl glucopyranan | -29.29 mV | -33.46 mV | -29.29 mV | -34.86 mV |

## 3.3 Result and discussion

### *3.3.1 Anti-HIV assay of sulfated 3-O-octadecy (1→6)-α-D-glucopyranans*

The results of anti-HIV activity of sulfated 3-*O*-octadecyl glucopyranans are listed in Table 3.1, indicating that the dextran and curdlan sulfates with a high degree of sulfation used as a standard had potent anti-HIV activity. A sulfated 3-*O*-octadecyl glucopyranan with a lower degree of sulfation (No. 5) and low solubility in water had lower anti-HIV activity. However, a water-soluble sulfated 3-*O*-octadecyl glucopyranan with $\overline{M}n = 5.1 \times 10^3$ had high anti-HIV activity at concentrations as low as 0.05 mg/ml (No. 1), even though the sulfated glucopyranan had a low molecular weight. Other sulfated 3-*O*-octadecyl glucopyranans had high anti-HIV activity. Previously, we reported the effect of the relationship between the molecular weight and the long alkyl group on the anti-HIV activity of sulfated ribofuranans.[13] A sulfated ribofuranan with $\overline{M}n = 6 \times 10^3$ showed low anti-HIV activity with an $EC_{50}$ = 68.6 mg/ml. After introduction of an octadecyl group, the anti-HIV activity increased to $EC_{50}$ = 0.6 mg/ml and 2.5 mg/ml for sulfated octadecyl ribofuranans with $\overline{M}n = 6 \times 10^3$ and $\overline{M}n = 3 \times 10^3$, respectively. These results suggest that the enhancement of the activity was attributed to a hydrophobic effect due to the long alkyl group. That is, the environment around HIV might be changed to hydrophobic from a hydrophilic condition and then the hydrophobicity around HIV might reduce its infectivity in MT-4 cells.[14][15] Therefore, we considered that sulfated 3-*O*-octadecyl glucopyranans with lower molecular weights had high anti-HIV activity. A sulfated 3-*O*-octadecyl glucopyranan with the low molecular weight of $\overline{M}n = 2.5 \times 10^3$ had, in fact, high anti-HIV activity at concentrations as low as 1.25 mg/ml (No. 6 in Table 3.1).

### *3.3.2. Interaction of sulfated 3-O-octadecyl glucopyranans with poly-L-lysine*

The interaction between sulfated 3-*O*-octadecyl glucopyranan having $\overline{M}n = 5.1 \times$

$10^3$ and DS = 1.03 with poly-L-lysine as a model protein was quantitatively analyzed by using SPR. Figure 3.7 shows the binding curves of the sulfated 3-*O*-octadecyl glucopyranan in the concentration range of 0.31-5.0 mg/mL on a commercially available poly-L-lysine with the molecular weight of $M_W$ = 1000-5000, which was immobilized on the CM5 sensor chip at the concentration of 2100 RU. The interaction was compared to that of dextran sulfate, a standard sulfated polysaccharide with potent anti-HIV activity as shown in Table 3.1. Table 3.2 summarizes the apparent kinetic results of the sulfated 3-*O*-octadecyl glucopyranans with poly-L-lysine calculated from the 1:1 binding model. The sulfated 3-*O*-octadecyl glucopyranans had high association rate constants, $k_a$ = 8.1 × $10^4$ 1/Ms - 4.3 × $10^4$ 1/Ms, low dissociation rate constants, $k_d$ = 3.7 × $10^{-4}$ 1/s - 0.1 × $10^{-4}$ 1/s, and low dissociation constants $K_D$ = 4.6 × $10^{-9}$ M - 0.1 × $10^{-9}$ M, indicating that the binding constants were almost the same orders of those of the standard dextran sulfate, $k_a$ = 29.0 × $10^4$ 1/Ms, $k_d$ = 1.89 × $10^{-4}$ 1/s, and $K_D$ = 0.65 × $10^{-9}$ M, respectively. It was found that the sulfated glucopyranan with a 3-*O*-octadecyl group in the proportion of 2.8 mol% was strongly bound to poly-L-lysine. Table 3.3 also represents the particle size and zeta (ζ) potential of the sulfated 3-*O*-octadecyl glucopyranans in the absence and presence of poly-L-lysine. The particle size of the sulfated 3-*O*-octadecyl glucopyranans increased with addition of poly-L-lysine, indicating that the sulfated 3-*O*-octadecyl glucopyranans were aggregated with poly-L-lysine. The absolute value of the ζ potential of the sulfated 3-*O*-octadecyl glucopyranans decreased after addition of poly-L-lysine probably because the sulfated 3-*O*-octadecyl glucopyranans with poly-L-lysine were in an aggregate state. These results of SPR and DLS suggest that the interaction was attributable to the electrostatic interaction between the negatively charged sulfate groups of the sulfated 3-*O*-octadecyl glucopyranan and the positively charged amino groups of poly-L-lysine.

HIV virus adsorption and binding a host cell, is generally thought to be due to the high affinity of the HIV gp120 with the CD4 molecule on the cell surface. But actually, CD4 on the cell surface affinity with HIV gp120 is weak and most of host cells expression is few. This shows that in addition to the CD4 molecule, there are other cell surface molecules plays an important role in the adsorption and binding of the virus and the cell surface.[16] In this interaction, the long octadecyl group was independent and the participation of the long alkyl chain on anti-HIV activity was still unclear. However, we previously reported that introduction of a long alkyl chain into the sulfated ribofuranan with low molecular weights and oligosaccharides, respectively, increased the anti-HIV activity as high as that of curdlan sulfate and dextran sulfate with higher molecular weights and high anti-HIV activity.[13][17][18] Therefore, we have continuously investigated the synergistic role of the long alkyl chain on anti-HIV activity.

The polymerization of the 3-*O*-octadecyl glucose monomer LGDBE3-18 for the purpose of development of polysaccharide-coated liposomes was already reported by Kobayashi in 1986. On this study, we synthesized the 3-*O*-octadecyl glucopyranan once again, and sulfated it. And then investigated it's anti-HIV activity in vitro, and the binding effect with poly-L-lysine.

### *3.3.2. Interaction of sulfated 3-O-octadecyl glucopyranans with liposome*

To elucidate the binding effect of alkyl chain with viral membrane surface, we were prepared two uniform size liposomes of 50nm and 100nm, respectively. To compare to no alkyl chain dextran sulfate, it was found that there are no changes in the particle size after adding dextran sulfate to 50nm liposome. However, dextran sulfate size of 50nm added to 100nm size of liposome the mixed particles are less than or equal to the original liposomes particle size. This shows that, since dextran

sulfate only have hydrophilic groups, no hydrophobic groups, so that liposomes have no interaction with each other, that resulting in an overall average particle size of the mixture is less than or closed to the maximum particle size before mixing. After sulfated 3-*O*-octadecyl glucopyranan mixed with 50nm, 100nm size of liposomes, the mixed particle size increased to about 100nm, 270nm, respectively. It can be estimated roughly that 50nm size limposome and sulfated 3-*O*-octadecyl glucopyranan may combined with ratio of 1:1. However, when the 100nm sized liposomes and sulfated 3-*O*-octadecyl glucopyranan mixed together, there may be a combination 2~3 sulfated 3-*O*-octadecyl glucopyranan on one liposome.

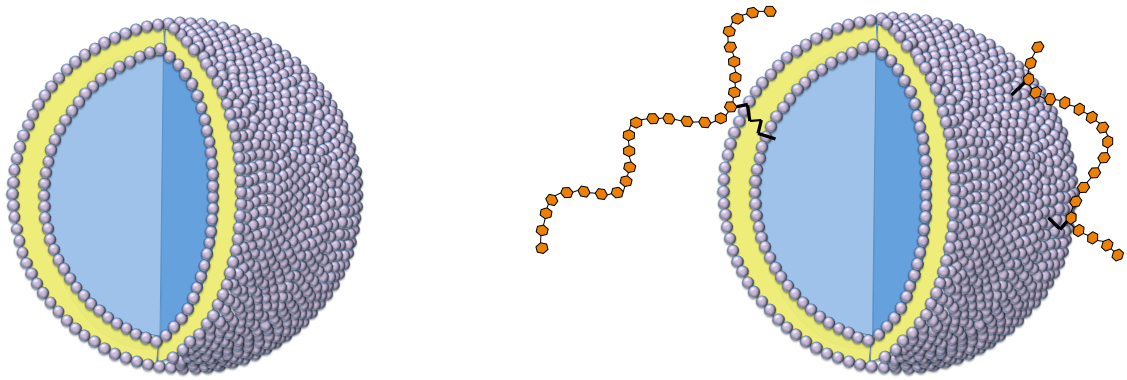

Figure 3.10 The image of the liposome before and after interacting with sulfated 3-*O*-octadecyl glucopyranan

The results of zeta potential experiment shows that after adding negative potential dextrane sulfate to liposomes (50 nm, 100 nm) which almost no potential, the mixing potential were -10.4 mV and -8.4 mV, respectively. That is the mixed potential value is the average of two independent particles potential. However, the potential of -29.29 mV sulfated 3-*O*-octadecyl glucopyranan mixed to liposomes (50 nm, 100 nm), the mixed potential were changed to -33.4 mV and -34.68 mV.

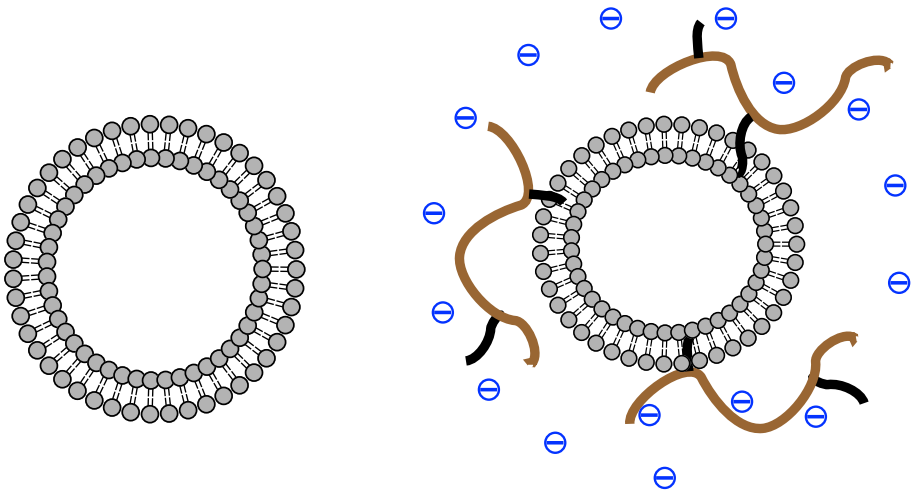

Figure 3.11 Change of zeta potential of liposome before and after mixing with sulfated 3-*O*-octadecyl glucopyranan

It means that the liposomes are not independent particles after mixing, but they combined with sulfated 3-*O*-octadecyl glucopyranan and coated by negative charges. Although the proportion of the combination can not be directly explained by the changes of electric potential, but it can be determined that the two kind of particles really interacted by some force to forming a combined particle. Adjusting for the results and the compounds group, it would presumed that the alkyl chain of sulfated 3-*O*-octadecyl glucopyranan interacted liposomes by hydrophobic interaction which may be an another mechanism of the anti-HIV activity.

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# *Chapter 4*

# *Conclusion*

In conclusion, sulfated 3-*O*-octadecyl glucopyranans were prepared by the ring-opening copolymerization of benzylated 1, 6-anhydro glucose and 3-*O*-octadecylated 1, 6-anhydro glucose monomers and subsequent debenzylation to recover hydroxyl groups and sulfation. The structure of the 3-*O*-octadecylated glucopyranans was determined by using high resolution NMR measurements. It was preliminarily found that sulfated 3-*O*-octadecyl glucopyranans with low molecular weights had high anti-HIV activity and fast association and slow dissociation constants on poly-L-lysine as a model compound of proteins by using SPR measurement. The octadecyl group could be independent of the binding of the sulfated glucopyranans to poly-L-lysine; however, the DLS and zeta potential experiments proved that the sulfated 3-*O*-octadecyl glucopyranans combined with

liposomes in a certain proportion by hydrophobic interaction, it could produce hydrophobicity around HIV to enhance the activity.

We continue to investigate the anti-HIV mechanism of sulfated polysaccharides with long alkyl groups by SPR to quantitative analyze the interaction between the combination degree of the sulfated3-*O*-octadecyl glucopyranans and liposome, and to develop a new biomaterial that has entrapment functionality for viruses such as HIV, influenza, and Dengue by utilizing the amphiphilic interaction of hydrophilic sulfated polysaccharides and hydrophobic long alkyl groups.

## *Acknowledgment*

I would like to take this opportunity to express my sense of gratitude and respectful regards to my supervisor and mentor Dr. Yoshida Takashi, Professor, Department of Bio-Environment Chemistry, Kitami Institute of Technology for his untiring help and encouragement and stimulating guidance through the course of this work.

I shall be failing in my duties if I do not express my heartfelt respects to Pro. Toshiyuki Uryu, Pro. Kazuyuki Hattori, Pro. Taisei Kanamoto for their former work and help with Bio-assay.

I am grateful to Dr. Mitsuhiro Okimoto, Dr. Masashi Komata, Dr. Han and all faculty members, Doctor degree and Master degree students for their enthusiastic co-operation and constant encouragement.

Besides, I wish to thanks all staffs of International communication center and of Student support office, Kitami Institute of Technology and citizen in Kitami for their kind cooperation and help on my work and life in Kitami.

I am also deeply appreciative of and thankful to my parents, Mr. Togtag Bai, Mrs. Haihua Huang and my sister Miss. Shili Bai whom I adore most for their love and sacrifices, forming the very platform of my life. Special thanks should go to my wife Mrs. Ying Li for her constant companion for ten years longer and her support and endless love.

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