博士論文

Synthesis and biological activity of new spherical polylysine oligosaccharide dendrimers

(新規球状オリゴ糖鎖デンドリマーの合成と生理活性)

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Chapter1

General Introduction

1.1 Carbohydrates

Carbohydrate is a biological molecule consisting of carbon, hydrogen and oxygen atoms. The term is generally understood in the biochemistry sense, where it is a synonym of saccharide. Saccharide is a cheapest nutrient of the three major nutrients for the body energy. It is divided into two categories in food: The one is that can be absorbed in the body for example monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Another one is that cannot be absorbed in the body for example Cellulose. Carbohydrates perform numerous

roles in living organisms. Polysaccharides serve for the storage of energy and as structural components. The 5-carbon monosaccharide ribose is an important component of coenzymes and the backbone of the genetic molecule known as RNA. The related deoxyribose is a component of DNA. Recent developments show that the diversity and complexity of carbohydrates permit these molecules to carry out a wide range of functions.

1.2 Oligosaccharides and polysaccharides

Oligosaccharide is a compound containing 2-10 simple sugars linked by glycoside bond. They are often associated with protein or lipid by covalently bound into glycoprotein or glycolipid that found on the plasma membrane of animal cells where they can play a role in cell-to-cell recognition. Oligosaccharides are widely used in food, health products, beverages, pharmaceuticals, and feed additives areas.

Polysaccharide is polymeric carbohydrate molecules composed of long chains of at least 10 monosaccharide units bound together by glycosidic linkages and on hydrolysis give the constituent monosaccharides or oligosaccharides.

Polysaccharides is widely distributed in nature, some constitute to cell wall components of animals and plant. For example Peptidoglycan and Cellulose. Some are nutrient storage of animals and plants such as Glycogen and Starch. Some have special biological activity: Heparin has anticoagulant effects and polysaccharide from cell wall of pneumococcal has antigen effects.

1.3 Sulfonated Oligosaccharides and Polysaccharides

Heparin is a kind of anticoagulant first discovered from the liver, is also present in the lung, blood vessel wall, the intestinal mucosa and other tissues. It is widely used on thromboembolism, acute myocardial infarction (AMI), cardiovascular surgery, cardiac catheterization, hemodialysis (HD) etc. Heparin is a Sulfated glycosaminoglycan constitute of Glucosamine, L-Iduronic glycosides, Acetyl glucosamine, and Glucoronide with 15K average molecular weight.[1] In the early 1960s it was demonstrated that heparin and related polyanionic substances act as herpes simplex virus inhibitors.[2]

1.4 Dextran Sulfate

Dextran is a complex, branched glucan composed of chains of varying lengths. It existed in secreted mucus of certain microorganisms during growth. Dextran having a higher molecular weight with predominantly of α-(1-6) linked D-glucose units and with different branch chain length that via α-(1-3) and a-(1-4) branch points. The different structures of Dextran are due to the different microbial species and growing conditions. Since dextran was already used as an antithrombotic agent and pure dextrans of controlled MW were readily available, it was one of the first polysaccharides to be sulfated and to produce derivatives exhibiting heparin-like activity.

However, in toxicity tests, some active preparations demonstrated dose. Therefore, it was of interest to establish structural requirements for active anticoagulants with low toxicity, an essential condition for potential clinical use.[3]

1.5 Curdlan Sulfate

The curdlan sulates is b-1,3-glucan sulfates that Prof. Yoshida et al synthesized. It was tested in classical coagulation

assays to evaluate structure activity relationships. Low sulfur content curdlan sulfonates showed a low anti-HIV activity even though they had high molecular weights. It compared with standard dextran fulfonate, suggested that curdlan sulfonates were preferred for an anti-HIV drug because of low cytotoxicity.[3]

1.6 Glycoproteins

Glycoproteins are complex carbohydrate that is an oligosaccharide chain covalently linked to polypeptide branched chains. Oligosaccharide parts of glycoproteins contain mannose, galactose, fucose, glucosamine, galactosamine, sialic acid, etc. There are two kinds of binding modes between oligosaccharides and protein: Hemiacetal hydroxyl group on sugar and hydroxyl group contained in amino acids attached to O-glycosidic bond type. Hemiacetal hydroxyl group on sugar and amide group of asparagine go to N-glycosidic bond type. Glycoproteins are fundamental to many important biological processes including immune defense, viral replication, parasitic infection, cell growth, cell-cell adhesion, degradation of blood clots, and inflammation.

1.7 Dendrimer

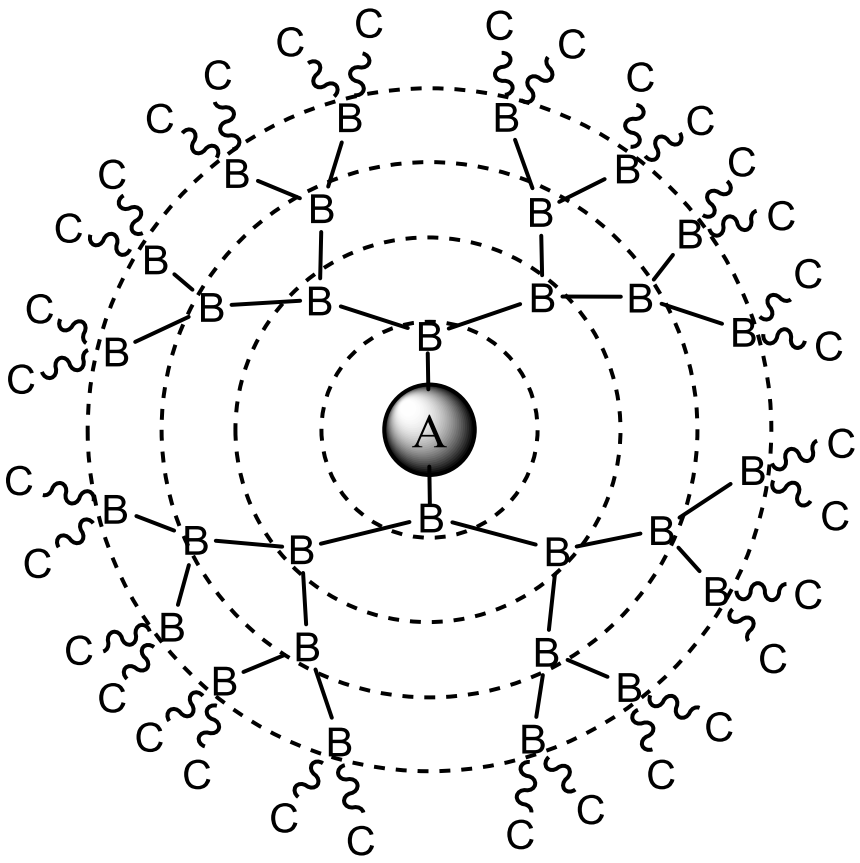

Figure1.1 The image of dendrimers
A-Core functional group, B-Scaffold unit,C-Surface functional group

Dendrimers (shown in Figure 1.1) were first introduced by Fritz VÖgtle in 1978 [4] and the synthesis of a dendrimer family was first reported by Donald Tomalia in 1985.[5] Dendrimers are noteworthy for their unique structure, such as a hyper-branched structure, exact molecular weight and size, well-controlled molecular shape and branch length/density ratio, unique monodispersity, cavity inside of molecule, and functionality of

molecule surface.[6] Dendrimers with high branch structure have three areas. The first area is the core functional groups, which can generate chemical species that show unparalleled properties. The second area is scaffold units, consisting of repeating branch cell units and the third area is the surface functional groups that are the outer shell or periphery with active groups.

With the deepening of dendrimer research, the scientists' attention has gradually shifted from the synthesis of different types of dendrimers to recover the functionalization of dendrimers and their applications.[7] Dendrimers have a variety of surface functionalization, Amongst them the end group functionalized dendrimer is the simplest, most practical, and also the most generally to be studied. Recent studies are focusing on introduction variety of functional groups to the terminal groups of the dendrimers to react. Each dendrimer comprising a plurality of end groups, so the reactions with high selectivity and activity are needed. For example, in 1993, Kawber, etc. introduced the different groups into the surface of the polyphenylene ether dendrimer, so that dendrimers surface with a dual function.[8] The most typical structure is an abundance of hydrophobic end groups of the phenyl group, the other half is hydrophilic hydroxyl group, spherical

macromolecules results amphiphilic surfactant properties, it can play in the oil-water interface amphiphilic role.

1.8 Glycodendrimer

The study found the interaction between protein and sugar in vivo, this effect can be increased between them binding strength and specificity, the so-called "multivalent effect" or "cluster effect ".[9,10] Inspired by this multivalent effect in vivo, using chemical methods to simulate this effect, the ligand molecule was designed and synthesized with multiple branches --- multivalent carbohydrate sugar molecule clusters (glycocluster) and multivalent sugar Dendrimers (glycodendrimer). Under the guidance of the above ideas, scientists began to conducted extensive research on synthesis and biological activity of multivalent carbohydrate molecules.[11] In the early eighties, Lee first reported the synthesis of compounds containing three YEE N-acetylgalactosamine branches (GalNAcAH) 3,[12] such compounds are later developed into multivalent sugar molecule clusters. In 1996, Roy synthesized sugar Dendrimers using dendrimer as a scaffold.[13]

Sugar dendrimer cluster molecule structure is that the

terminal molecular of monosaccharide or oligosaccharide linked by glycosidic linkages with non-sugar scaffold. The molecular weight of the sugar cluster molecules are generally relatively small, usually from two to five glycosylation. Sugar dendrimer molecule has two forms, one-way and spherical type, scaffold by repeating the same connection arm to form a dendrimer structure which had greater molecular weight and higher valence number than a sugar cluster molecule. Currently, the cluster multivalent sugar molecule or sugar containing dendrimer molecules are used in: (1) inhibition of virus, bacteria and toxins of adhesion, such as E. coli [14,15], hemagglutinin (HA) [16,17] as well as Shiga toxin inhibition[18,19]; (2) the treatment of liver targeting sbutances[20,21]; (3) the tumor vaccine[22,23]; (4) allogeneic transplantation[24,25].

1.9 Synthesis of Glycodendrimer

According to their characteristic of sugar scaffold, sugar cluster molecules can be divided into several forms scaffold such as the multiple branches linear molecules as the scaffold, a rigid aromatic ring as the scaffold, with sugar as the center, basing upon the sugar scaffold, cyclodextrin and other chitosan

and proteins as the scaffold. From the perspective of synthetic strategy, synthesis of dendrimer can be divided into two convergent and divergent methods. The first step of convergent synthesis is glycosyl donor reacted with multibranched molecular unit. This step can be achieved by a O[26], S[27,28], N-glycosidic[29] bond. That may also linked by other connecting branches to give multivalent sugar clusters or sugar dendrimer molecule.The advantage of the divergent method is that with the designer's intent can get specific structure multivalent sugar molecules, the central nucleus has its diversity, and can introduce different connecting baranches controblly. The disadvantage of this method is that the desired product with the continuous extension of the branch, increasing the number of synthetic steps is gradually increasing.

Divergent synthesis method is relatively simple. It involves "grafting" sugar moiety into the existing multi-branch scaffold. This method is similar to the synthesis of Neoglycoprotein that protein as a carrier, which requires sufficiently multi-branch active groups on surface (such as amino, carboxy, etc.). Those scaffolds include polyamino amines[27,28] (PAMAM), poly (propyleneimine) (DAB)[29], poly-L-lysine[30], gallium acid[31], etc. The advantage of this method is that you can directly use

some commercially available molecular scaffold, reducing the synthesis step procedure. Its disadvantages are also obvious, scaffold molecular of diversity will be limited by commercial sources.

These two synthetic strategies have advantages and disadvantages, in practical applications, to follow the structural characteristics of the target compound or be used alone or in combination.

1.10 Application of Glycodendrimer

In 1983, Lee first reported that the synthesis of galactose cluster compounds to sialic acid receptors on the liver have a powerful combination. Attention then began to be given to the "cluster effect". Polylysine dendrimers with sialic acid were found to have potent inhibitory effects against infection by influenza virus and the haemagglutinin of erythrocytes[32-34]. The activity was evaluated by interaction with selectin, indicating that the activity increased with the increase in the number of generations of dendrimers[35]. A sialic acid dendrimer of the fourth generation gave four times the inhibitory activity of the first generation of the dendrimer. A sphere type fully substituted

oligosaccharide alanine polylysine dendrimer with a sharp molecular weight distribution was synthesized by reductive amination of disaccharides and a polylysine dendrimer having alanine at the terminal[36]. Carbosilane dendrimers as a core scaffold for the preparation of glycodendrimers were synthesized from chlorosilans by a combination of alkenylation and hydroxylation. The trisaccharide moiety of globotriaosyl ceramide was produced in liquid ammonia by a one-pot reaction involving removal of benzyl groups and subsequent SN2 reaction to give carbosilane dendrimers having trisaccharide moieties[37].

Currently, multivalent sugar cluster molecule or sugar dendrimers are used in: (1) inhibition of virus, bacteria and toxins of adhesion, such as E. coli[38, 39], hemagglutinin (HA)[40, 41] as well as Shiga toxin inhibition[42, 43]; (2) the treatment of liver targeting[44, 45]; (3) the tumor vaccine[46, 47]; (4) allogeneic transplantation.[48, 49]

1.11 Objective of this study

As described above, since the dendrimer having a broad interior cavity, and a large number of functional external

distribution groups, molecular structure can be precisely designed. This experiment was designed and synthesized water-soluble sugar containing dendrimers with terminal hydroxyl groups.

Specific studies of this topic is, (1) In this paper, we report the synthesis of first, second, and third generation novel spherical polylysine dendrimers. Since oligosaccharide chains do not express physiological activity by themselves, we were expecting a cluster effect by dendrimer structure. In order to increase the flexibility of the sugar chain, C6 alkyl spacer is introduced between the core polylysine dendrimer and surface cellobiose units at the terminal groups. (2) The structure of synthesized first, second, and third-generation spherical polylysine dendrimers was analyzed by high resolution NMR, FT-IR, and MALDI TOF MS spectroscopies. (3) After the sulfation of first, second, and third-generation spherical polylysine dendrimers, we investigate the anti-HIV activity, zeta potential, and particle size of spherical polylysine dendrimers to elucidate the relationship between structures of spherical polylysine dendrimers and biological activities.

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Chapter 2

Synthesis of new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer

2.1 Introduction

Several reports have appeared on the synthesis of glycodendrimers. Polylysine dendrimers with sialic acid were found to have potent inhibitory effects against infection by influenza virus and haemagglutinin of erythrocytes.[1,2] The activity was evaluated by interaction with selectin, indicating that the activity increased with the increase in the number of generations of dendrimers. The inhibitory activity of the fourth

generation dendrimer sialic acid four times the first generation dendrimer. Completely substituted sphere-type oligosaccharide-β-alanine-polylysine dendrimer was synthesized by polylysine dendrimer having terminal alanine with reductive amination of disaccharides.[3] Preparation of carbosilane dendrimers as core holder glycodendrimer was accomplished by alkenylation and hydroxylated of chlorosilanes. Globotriaosyl ceramide trisaccharide moiety was through the one-pot reaction in liquid ammonia followed by removal of benzyl groups and subsequent SN_2 reaction to to give the carbosilane dendrimer having trisaccharide moieties.[4] We have also reported the synthesis of several new types of sulfated glycodendrimers that were found to have potent anti-HIV activity due to the cluster effects of sugar moieties.[5]

The final purpose of our researches is to elucidate the structure biological activity relationship on anti-HIV and interaction of glycodendrimers with polylysine in vivo, we wish to report a synthesis of new types of the spherical polylysine dendrimers generation 3 with oligosaccharides through a C6 methylene spacer. The structure of the glycodendrimers were determined by NMR, IR and MALDI TOF mass spectrometric analyses.

2.2 Materials

The reagents, 1, 4-Diaminobutane, α-D-Cellobiose Octaacetate and Ethyl 6-hydroxy hexanoate were purchased from Sigma Aldrich Co. LLc., Inc. Di-tert-butyl-dicarbonate, N, N′-diisopropylethylamine (DIEA) and benzo-triazol-1- yloxytris -(dimethylamino) phosphonium hexafluorophosphate (BOP reagent, Wako Pure Chemical Industries, Ltd.) Dimethylformamide (anhydrous, DMF) were purchased from Kanto Chemical Co., Inc and all of the used reagents without purification. N, N'-Bis (tert-butyloxycarbonyl) -L- lysine dicychlohexylamine salt (Boc-lysine (Boc)- COOH) and Di-Boc -lysine were prepared according to the literature [4,5]. A dialysis tube (molecular weight cut off 500~1000, 2000, 3500; Spectrum Laboratories, Inc.) were used for the purification of alkyl cellobiosiden and polylysine dendritic cellobiose.

Nuclear magnetic resonance spectroscopy ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM AEC-600 spectrometer at 600 MHz and 150 MHz, respectively, at 40℃ in D_2O or DMSO-d_6 solvent. Chemical shifts in D_2O are expressed as ppm downfield from 4,4'-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard. DEPT135 spectrum was measured by

using a program provided by JEOL. Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer by a KBr pellet method. The MALDI TOF MS spectrum was measured by a Bruker Ultraflex III instrument with 337 nm nitrogen laser. A methanol solution of a mixture of 2,5-dihydroxybenzoic acid was used as a matrix on a Nafion-coated plate [6].

2.3 Experimental Section

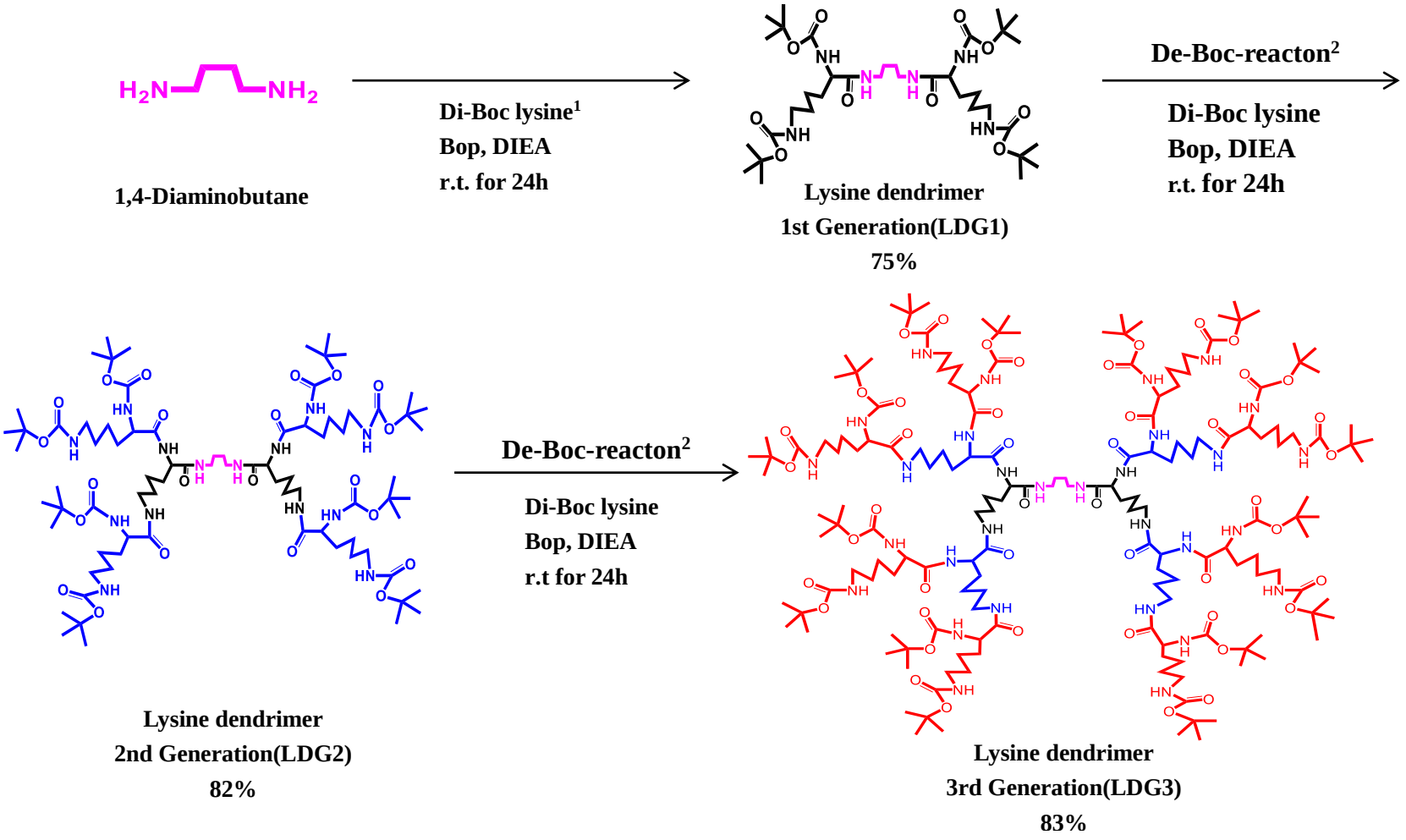
2.3.1 Synthesis of polylysine dendrimer generations 1, 2, 3

1,4-Diaminobutane (0.46 g, 5.0 mmol) and di-boc-lysine dicyclo hexylamine salt (5.38 g, 10.2 mmol) were dissolved in anhydrous DMF (50 ml) under a nitrogen atmosphere and then DIEA (1.74 ml, 10 mmol) was added to the solution. After the solution was cooled to 0℃in an ice bath, BOP reagent (4.51 g, 10.2 mmol) was added. The reaction mixture was stirred for 30 min at 0℃and then for 24 h at room temperature. The solvent was removed under in 200 ml of ethyl acetate, followed by washing successively with 30% NaCl, 5% aqueous citric acid, 5% $NaHCO_3$ solutions, and water. The solution was dried over anhydrous Na_2SO_4 and then concentrated. The resultant syrup was purified by column chromatography over silica gel using

chloroform and methanol (10:1) as a mixed eluent. The polylysine dendrimer generation 1(LDG1) (2.80 g) was obtained as a white crystal in 75% yield after recrystallization from a mixture of methanol and ethyl acetate (1:1).

The synthesized Poly (lysine) dendrimer generation 1 (LDG1) (2.0 g, 2.68 mmol) was stirred in a mixed solution of trifluoroacetic acid (TFA) and dichloromethane (1:1) at room temperature for 30 min. After the solvent was evaporated under reduced pressure, diethyl ether was added to cause precipitation. The precipitate was collected by centrifugation and washed with anhydrous diethyl ether three times and deprotected poly (lysine) dendrimer generation 1 was dried in vacuo and di-boc-lysine added to a deprotected lysine dendrimer generation 1 again, to give in 82% yield of polylysine dendrimer generation 2 (LDG2). In the similar method, we obtained polylysine dendrimer generation 3 (LDG3) in 83% yield (scheme 2.1).

Scheme2.1 Synthesis of polylysine dendrimer generations 1, 2, 3 (LODG1, 2, 3)



2.3.2 Nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectrometer (IR) measurements

Figure 2.1 shows the ^{1}H spectra of each generation of the spherical polylysine dendrimer generations 1, 2, 3 (LDG1, 2, 3). Figure 2.2 shows the IR spectra of the spherical polylysine dendrimer generations 1, 2, 3 (LDG1, 2, 3). The NH vibrations due to the amino group can be absorbed at 3325 cm^{-1}, the vibration of methylene group due to lysine and core a, b were absorbed at 2935 cm^{-1} and 2977 cm^{-1}, amino group were absorbed at 1692 cm^{-1}. We can see β, γ, δ and α, which is due to core butane were all appeared at 1.2ppm-1.7ppm. b due to core butane was appeared at 3.05ppm, the number of b-NH and α-NHε -NH peaks became more and more many.

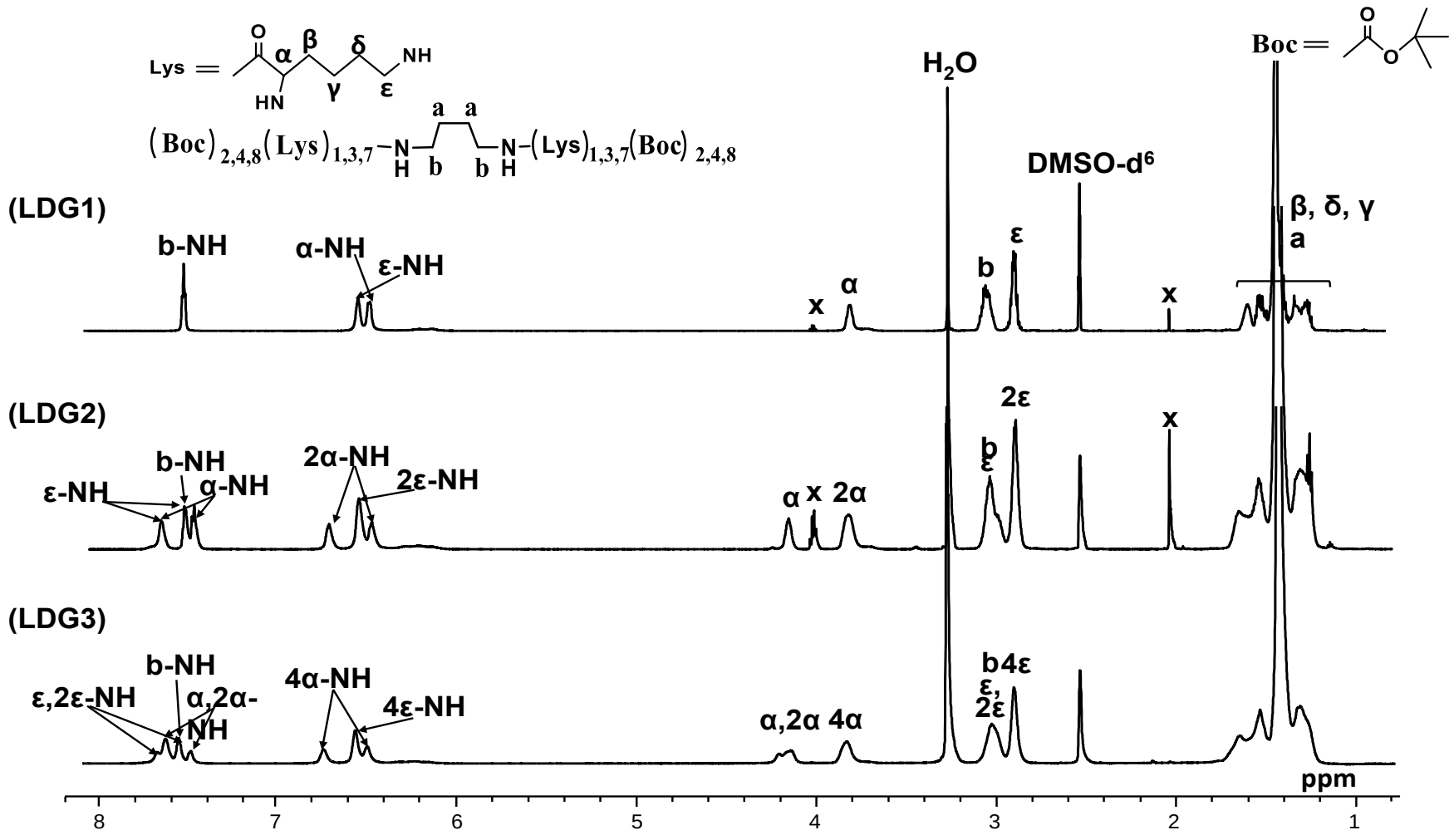


Figure 2.1 600MHz ^{1}H NMR spectra of the Lysine dendrimer generations 1,2,3, in DMSO-d_6 at 40℃(up to down)

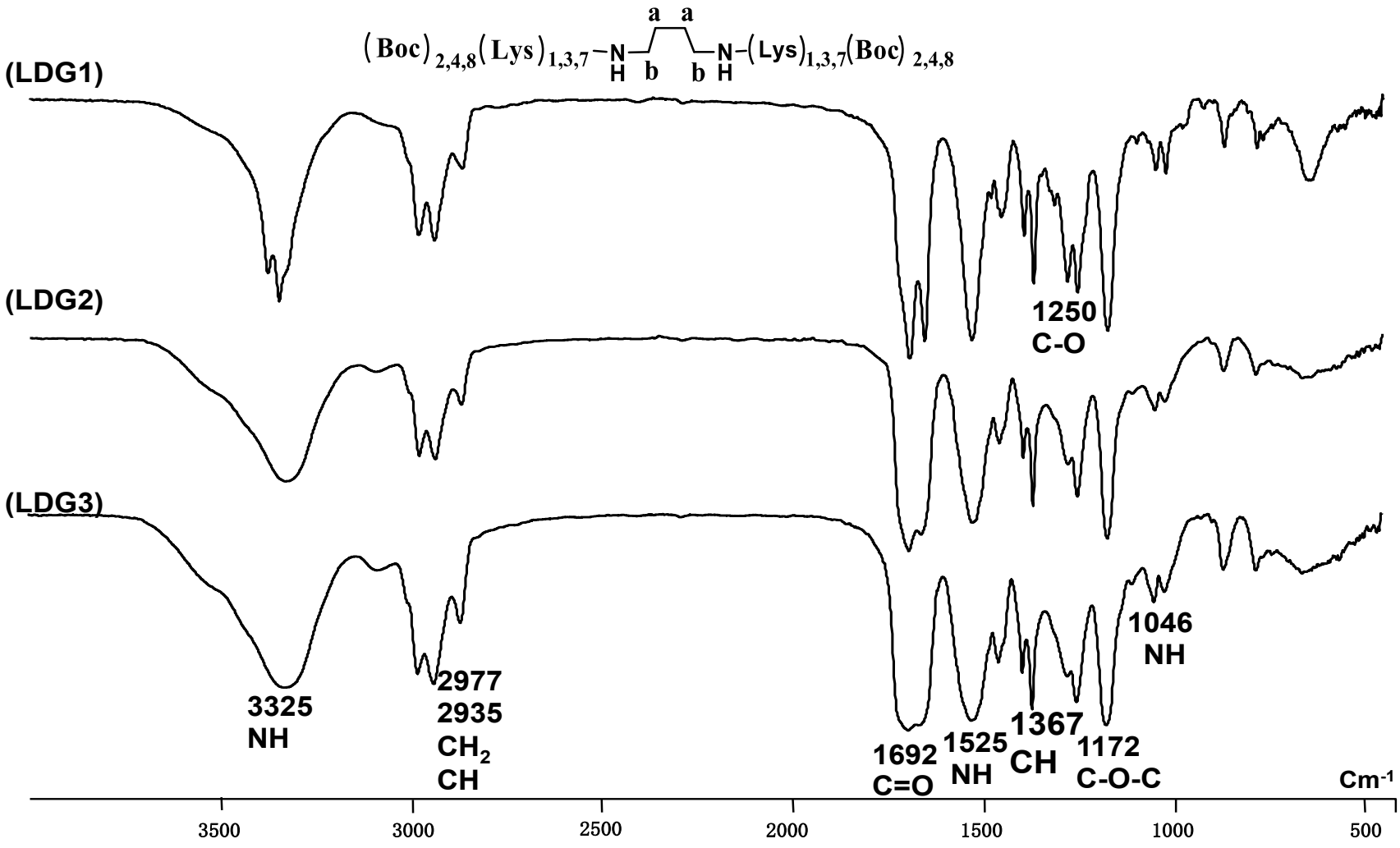


Figure 2.2 FT-IR spectra of the Lysine dendrimer generations1, 2, 3

2.3.3 Analysis of Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI TOF MS) spectra of polylysine dendrimer generations 1, 2, 3

The molecular weight of polylysine dendrimer generations 1, 2, 3 (LDG1, 2, 3) were measured by MALDI TOF MS measurement and the 2,4-DHB isomer was investigated because it has been used previously to analyse oligosaccharides. The calculated molecular weight (m/z) of polylysine dendrimer generations 1, 2, 3 were 766, 1681, 3509 (Figure 2.3), while the observed molecular weight (m/z) of polylysine dendrimer generations 1, 2, 3 were 766, 1681, 3509 m/z. The difference of the m/z between the absorptions were approximately m/z=23 and 39, which value
is approximately equal to the molecular weight of Na and K(Tab.2.1).

Sample	Calcd	Obsd
LDG1	$[744+Na]^+$	766
LDG2	$[1658+Na]^+$	1681
LDG3	$[3479+K]^+$	3509

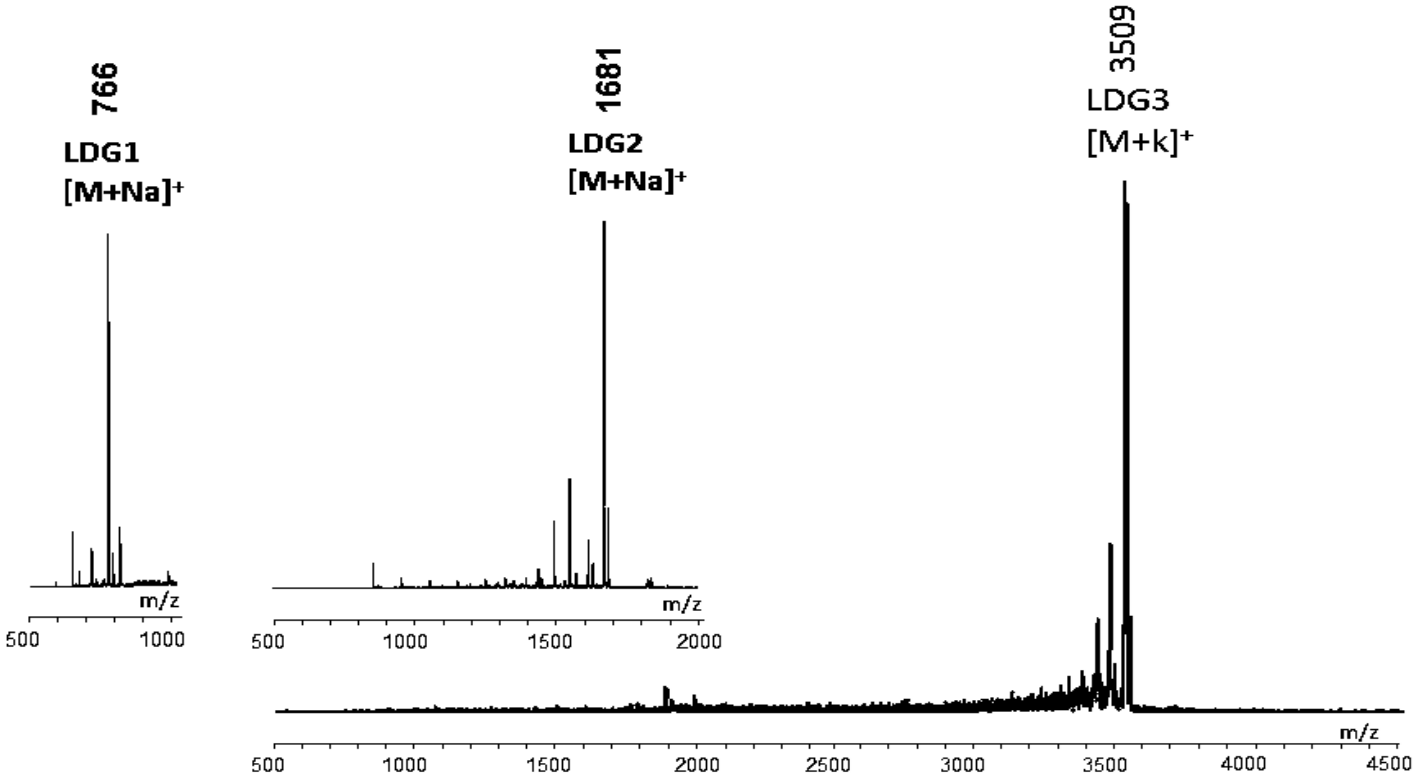
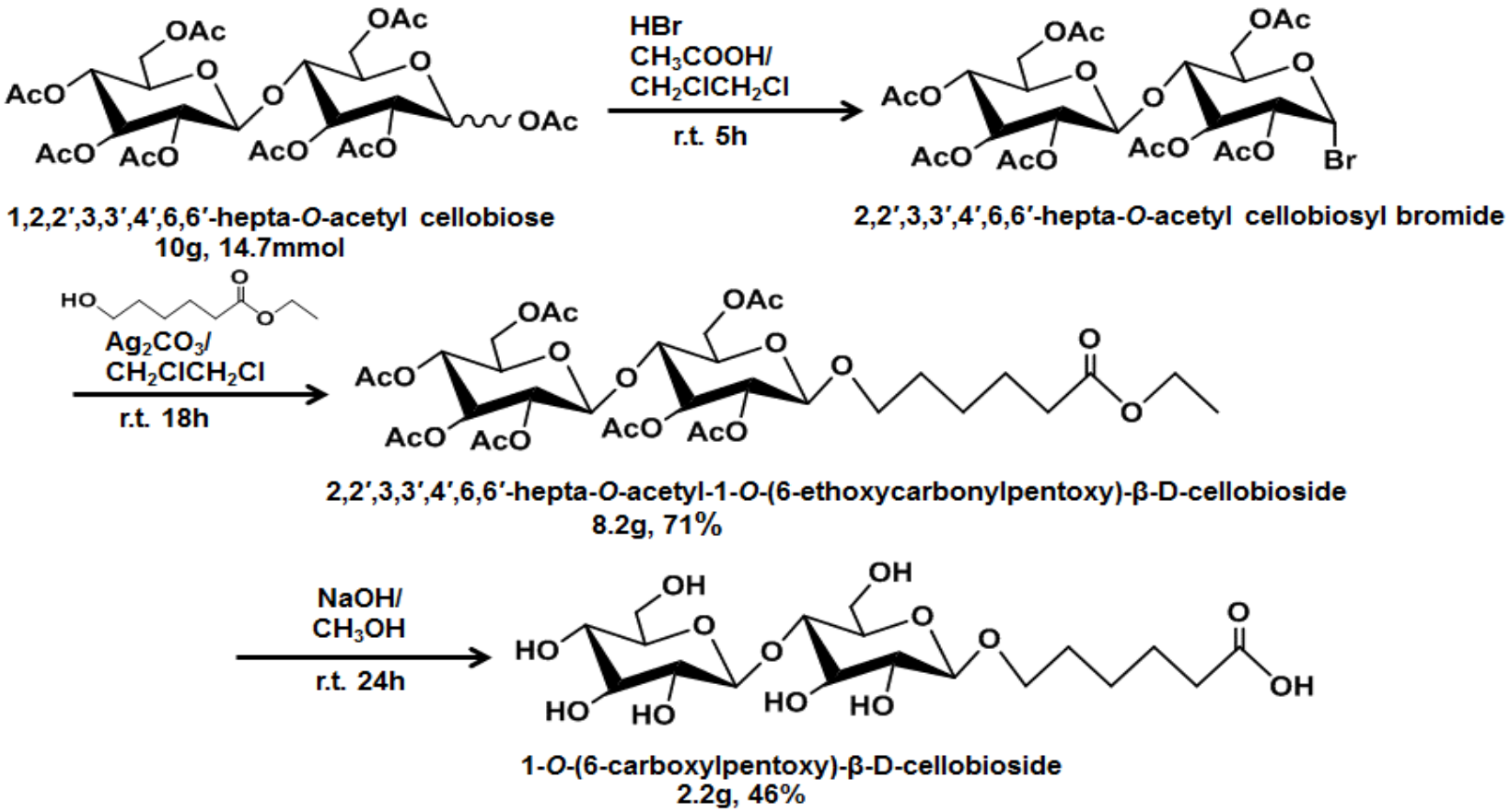


Figure 2.3 MALDI-TOF-MS spectra of the Lysine dendrimer generations1, 2, 3

2.3.4 Synthesis of alkyl cellobioside

Hydrogen bromide (32% solution in acetic acid, 40ml) was added dropwise to a solution of 1,2,2’,3,3’,4’6,6’-hepta-O-acetyl cellobiose (10g, 14.7mmol) in a mixed solvent of 1,2-dichloroethane (25ml) and acetic acid (50ml). The mixture was stirred for 5h at room temperature and then poured into ice water. The product was extracted with chloroform. The chloroform layer was successively washed with saturated $NaHCO_3$ and 30 wt% NaCl aqueous solutions, and then dried on anhydrous Na_2SO_4. After concentration, 2,2′ , 3,3′ , 4′ , 6,6′ -hepta-O-acetyl-cellobiosyl bromide was obtained as a syrup in 95% yield and used without further purification. Ethyl 1-hydroxyhexanoate (3.54 g), silver carbonate (12.4 g), and drierite (20 g) were added to the solution of hepta-O-acetyl cellobiosyl bromide in 1,2-dichloroethane (150 ml). After the mixture was stirred for 18h in the dark at room temperature, the mixture was filtered and then concentrated. The residue was purified by column chromatography over silica gel with a mixed eluent of ethyl acetate and hexane (1:1) to give 8.2 g of 2,2’,3,3’,4’,6,6’-hepta-O-acetyl-1-O-(6-ethoxycarbonylpentoxy) -β-D- cellobioside in 71% yield as a white powder.

The acetyl cellobiose unit (6.78 g, 8.71 mmol) was deacetylated with 5 wt% sodium methoxide in methanol (150 ml) by stirring for 3h at room temperature. After removal of the methanol, the residue was neutralized with an ion exchange resin (H^+) and washed with dichloromethane and water several times. The aqueous phase was evaporated and purified over silica gel chromatography with a mixed eluent of dichloromethane and methanol (2:1) to obtain 1-O-(6-carboxylpentoxy)-β-D-cellobioside (1.82 g) as a white powder in 46% yield (Scheme 2.2).



Scheme 2.2 Synthesis of 1-O-(6-carboxylpentoxy)-β-D-cellobioside

2.3.5 Nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectrometer (IR) measurements

Figure 2.4 shows the ^{13}C NMR spectra of acetyl cellobioside and after hydrolysis of acetyl cellobioside. The methyl groups at 21ppm and carbonyl groups near the 170ppm give from the acetyl groups were disappeared and the F, G duo to ethyl groups at around 60ppm and 14ppm were all disappeared.

At the same time, after hydrolysis of acetyl cellobioside, OH bonds stretching vibrations appeared at 3390 cm^{-1} and the carbonyl group due to ester group at 1747 cm^{-1} changed into 1719 cm^{-1} of carboxylic acid. It is proved that the hydrolysis reaction performed efficiently.

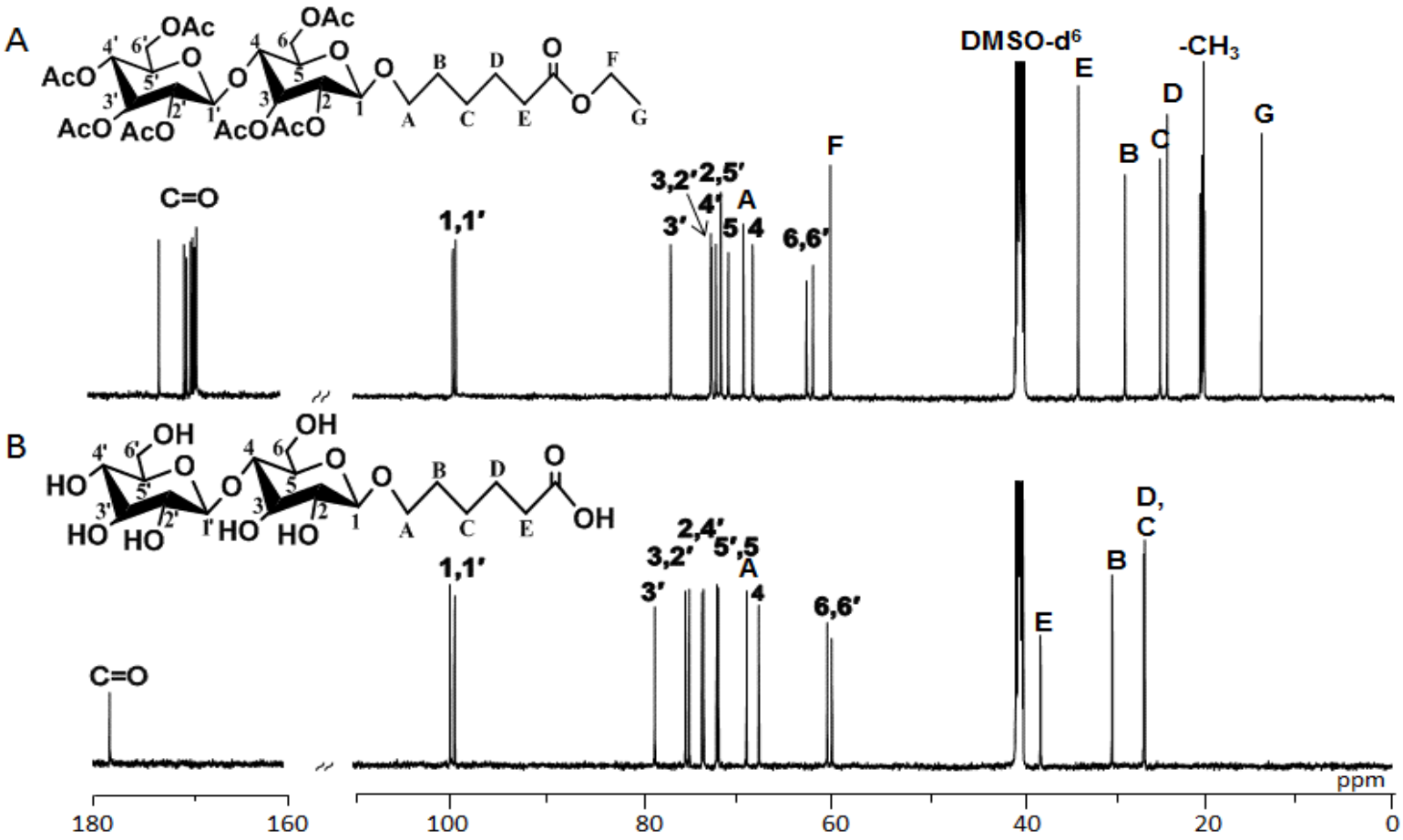


Figure 2.5 600MHz ^{13}C spectra of A: acetyl cellobioside and B: after hydrolysis of acetyl cellobioside in DMSO-d_6, at 40℃

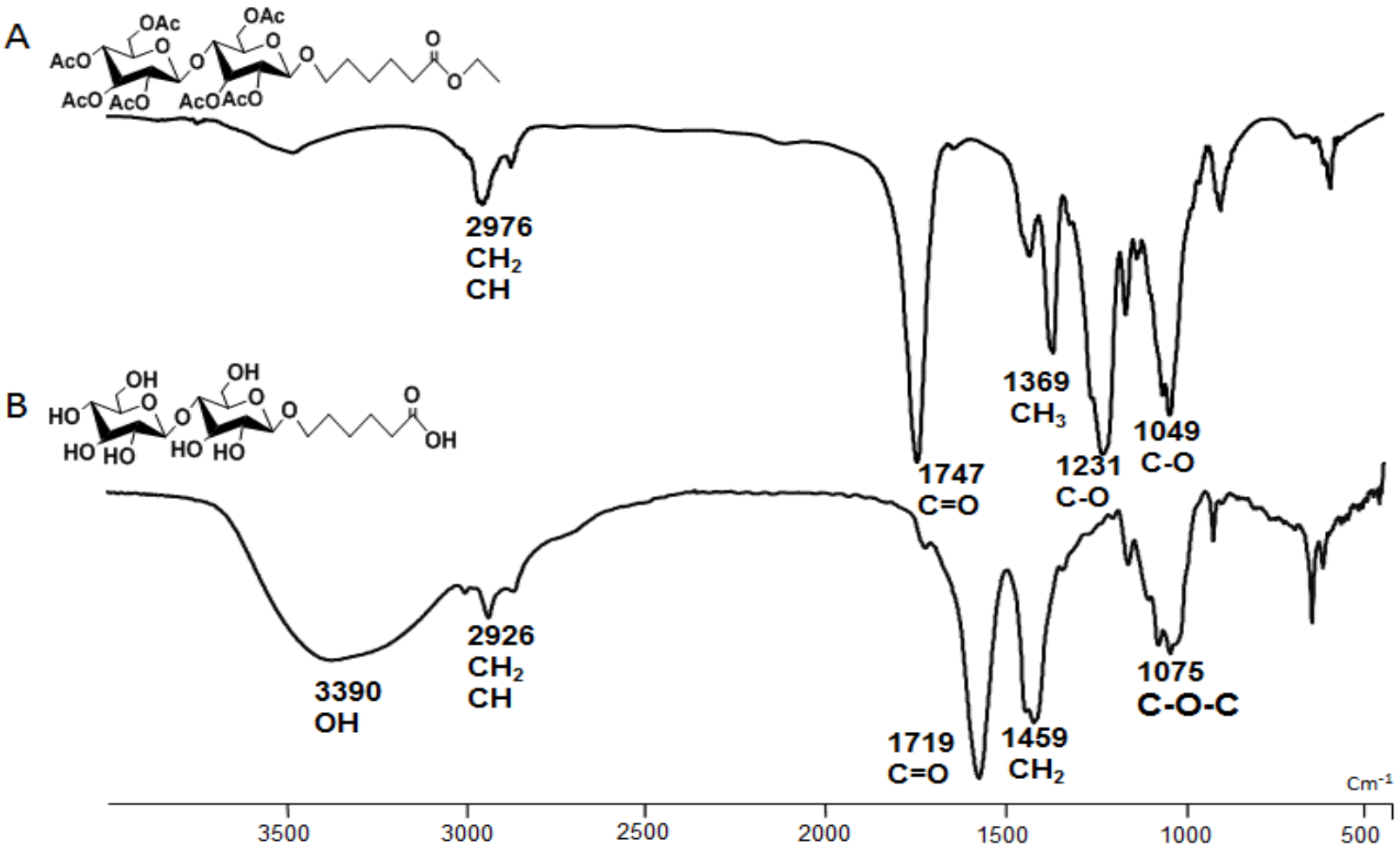
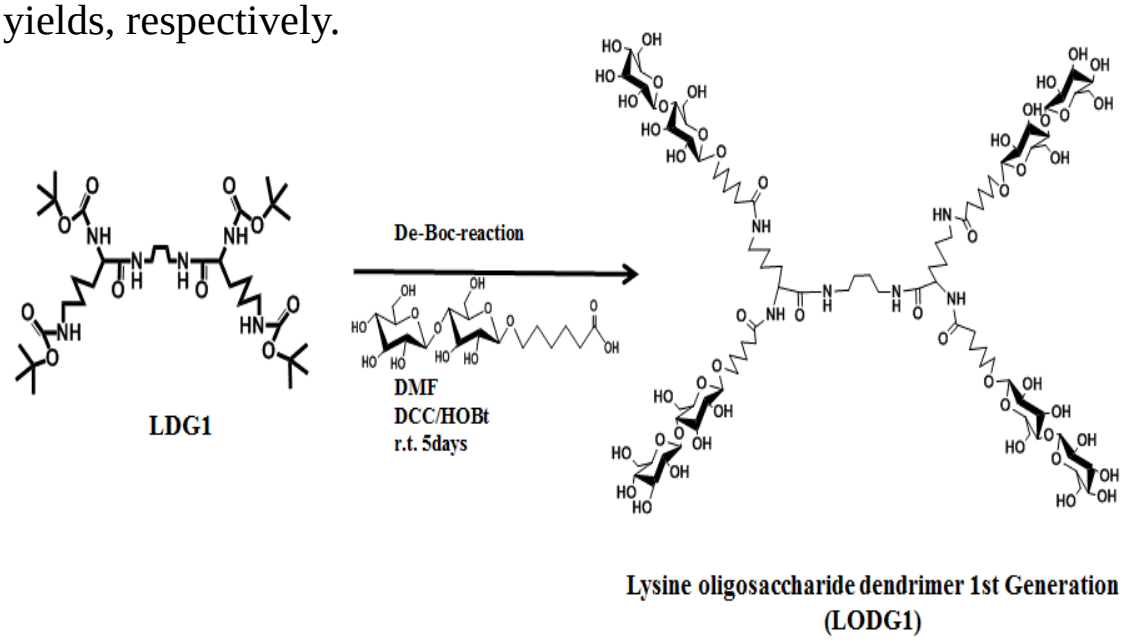


Figure 2.6 FT-IR spectra of A: acetyl cellobioside and B: after hydrolysis of acetyl cellobioside

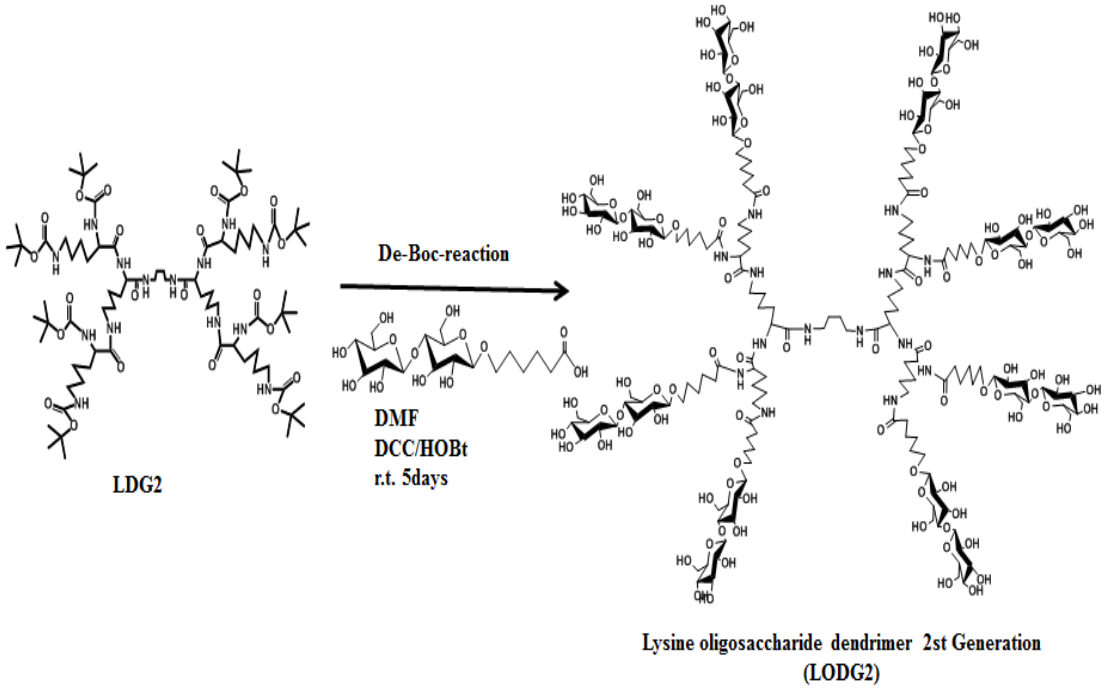
2.3.6 Synthesis of spherical polylysine oligosaccharide dendrimers generations 1, 2, 3 with C6 methylene spacer

The first generation polylysine dendrimer 1 (0.25 g, 0.33 mmol) was stirred for 30 min in a mixed solution of 2N trifluoroacetic acid (TFA) (15 ml) and dichloromethane (15 ml) at room temperature. After the solvent was evaporated under reduced pressure, diethyl ether was added to produce a precipitate, which was collected by centrifugation and washed three times with anhydrous diethyl ether. After drying under reduced pressure overnight, the deprotected first generation polylysine dendrimer was obtained. The deprotected first generation polylysine polylysine dendrimer was dissolved in anhydrous DMF (30 ml) at 0℃ and then the cellobiose unit with a free hydroxyl group and carboxylic acid, DIEA (0.35 ml, 20mmol), and BOP reagent (0.88 g, 2mmol) were added at 0℃ in an ice bath. The mixture was stirred for 48 h at room temperature and then dialyzed against deionized water for 2 days to give first generation cellobiose-C6 spacer-polylysine dendrimer (Scheme 2.3) in 48% yield after freeze-drying. Second (Scheme .24) and third (Scheme 2.5) generation cellobiose-C6 spacer-polylysine dendrimers were obtained from

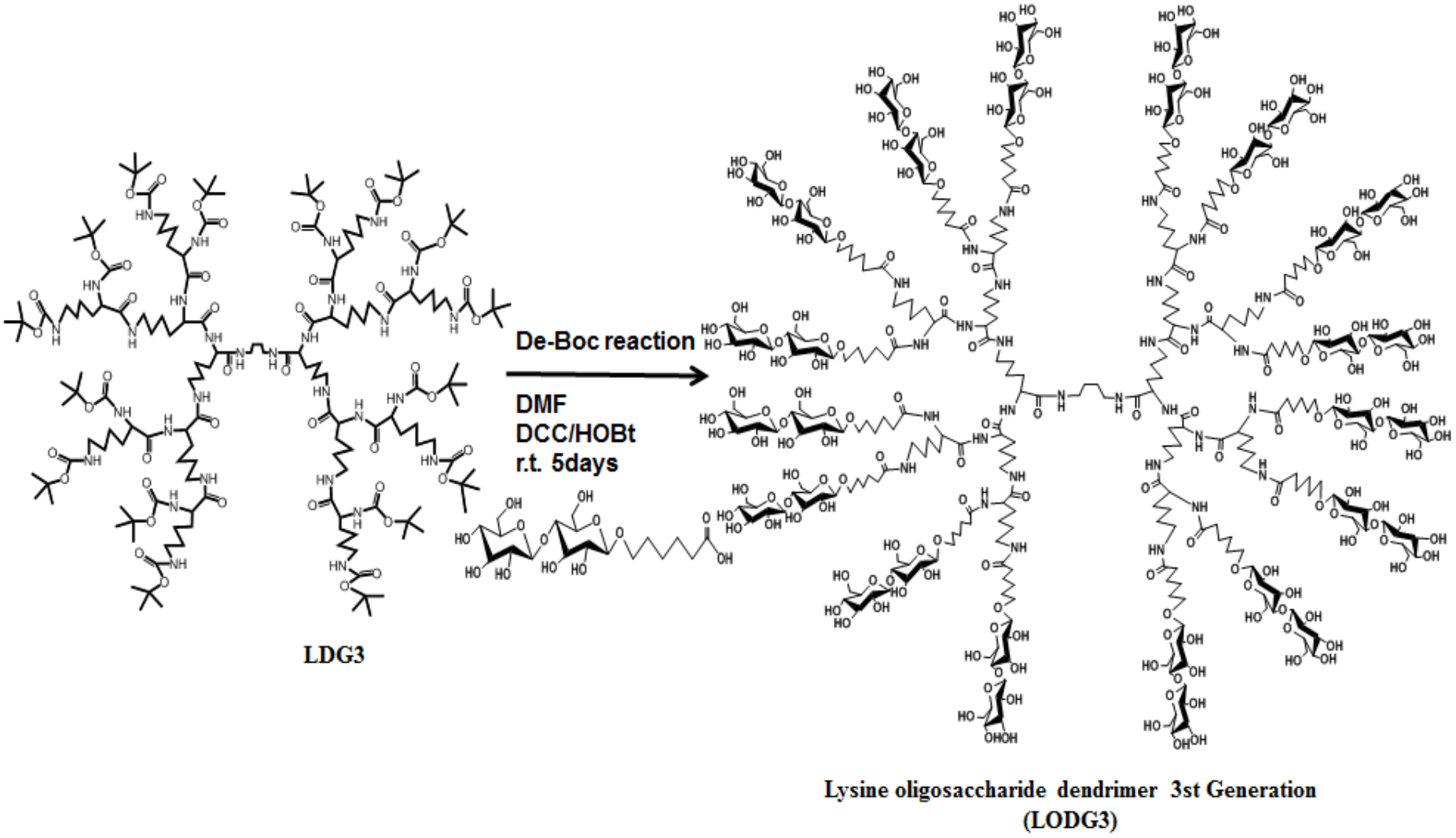
second and third generation polylysine dendrimers and by the same procedures as the synthesis of cellobiose-C6 spacer-polylysine dendrimer first generation in 53% and 55% yields, respectively.



Scheme 2.3 Synthesis of first generation of polylysine oligosaccharide dendrimer (LODG1)



Scheme 2.4 Synthesis of second generation of polylysine oligosaccharide dendrimer (LODG2)



Scheme 2.5 Synthesis of third generation of polylysine oligosaccharide dendrimer (LODG3)

2.3.7 Nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectrometer (IR) measurements

Dendrimers of the first, second and third generations ^{13}C-NMR (Figure 2.7) measured in D_2O at 40℃. The ^{13}C NMR spectra were similar to each other and the carbon signals were assigned by the 2D NMR measurements. The C1 and C1′ signals due to cellobiose appeared as singlet peaks at 107 and 106 ppm, and the amido signals due to peptide bonds of lysine residues at 182 ppm. The C2-C6 and C2′ -C6′ signals of cellobiose residues were absorbed between 63 and 83 ppm as several singlet peaks. The C6 alkyl spacer and lysine side chain signals appeared etween 24 and 43 ppm, and the α carbon of the lysine side chain and methylene signal E next to the oxygen of cellobiose appeared at 58 ppm and 40.5 ppm as singlet signals, respectively.

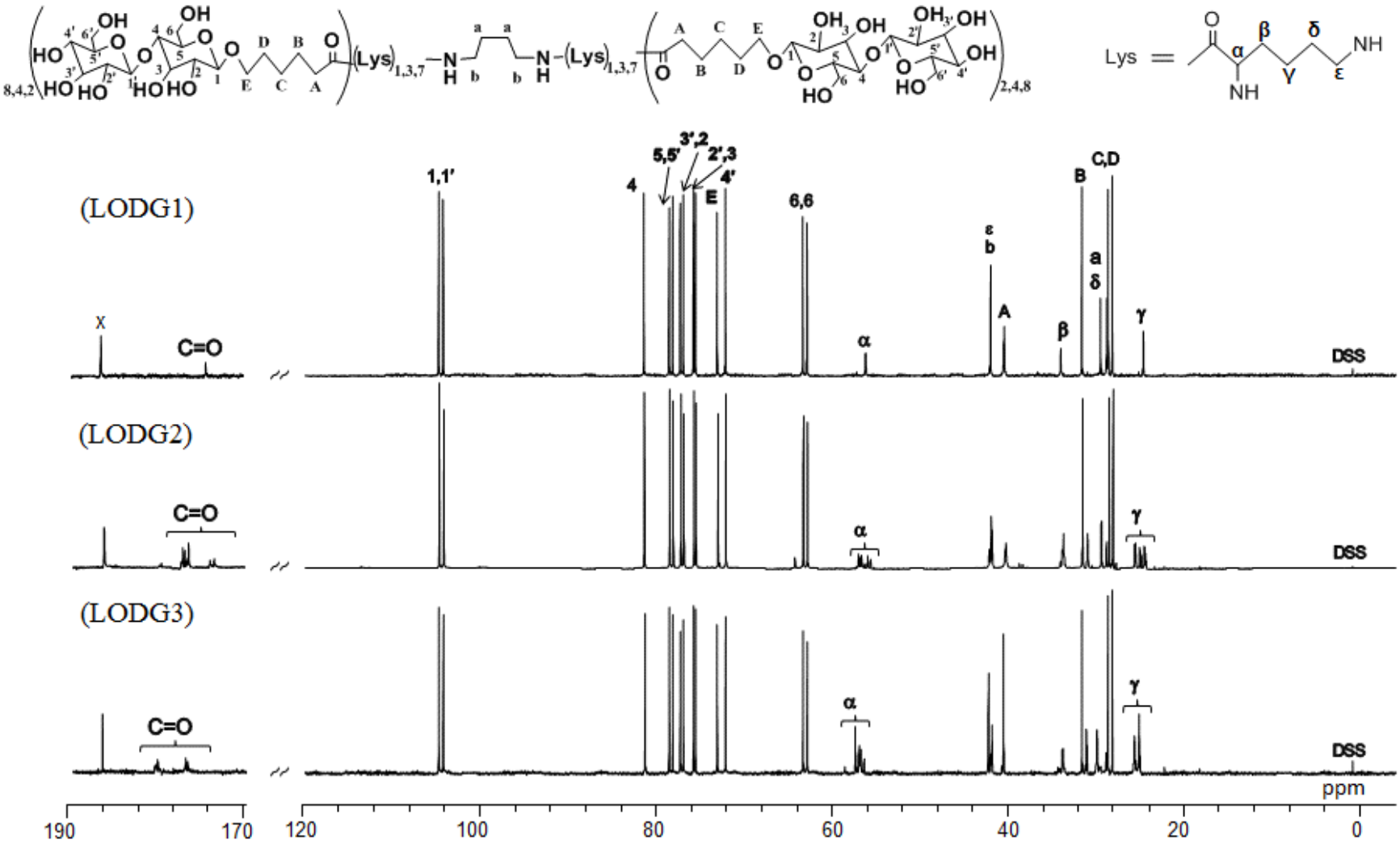


Figure 2.7 150MHz ^{13}C-NMR spectra of cellobiose-C6 spacer-polylysine dendrimers fist, second, third generations (up to down) in D_2O at 40℃. DSS (0.015 ppm) was used as an internal standard.

On the other hand, first, second and third generations measured by FT-IR (Figure 2.8). The first, second and third generations Carbonyl and NH bending vibrations due to peptide bonds appeared at 1677 cm^{-1} and 1547 cm^{-1}, respectively, and the intensities increased with increasing generations of dendrimer, due to the increasing proportion of the peptide bond. The alkane stretching vibrations due to methylene signals of the C6 methylene spacer and lysine side chain were absorbed at 2936 cm^{-1} and 2866 cm^{-1}. Large and broad absorptions appeared at 3295 cm^{-1} due to NH and OH stretching vibrations of the peptide bonds and hydroxyl groups due to the core polylysine and cellobiose, respectively. In addition, the C-O-C or C-O signal due to the ether linkage between cellobiose and the C6 methylene spacer or due to cellobiose should be absorbed at 1203 cm^{-1}. No signals due to acetyl groups appeared, suggesting that the cellobiose connected with dendrimers had free hydroxyl groups.

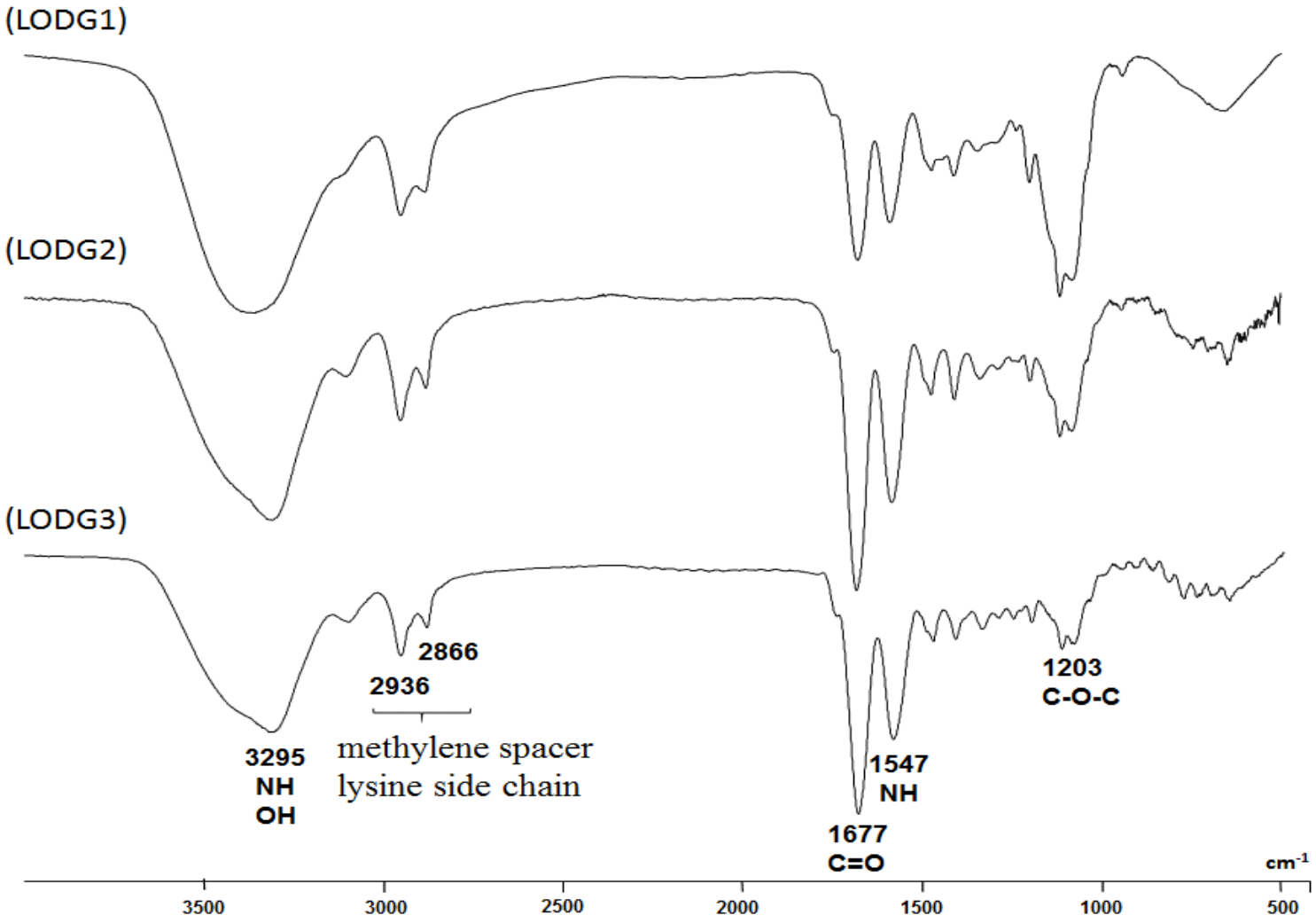


Figure 2.8 FT-IR spectra of cellobiose-C6 spacer-polylysine dendrimers.
(A) first, (B) second and (C) third generations

2.3.8 Analysis of Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI TOF MS) spectra of spherical polylysine oligosaccharide dendrimers generation 1, 2, 3 with C6 methylene spacer

Dendrimers with 2,5-dihydroxybenzoic acid as the matrix by using a Nafion-coated plate. Nafion was used for ionization of the cellobiose dendrimer to obtain the MALDI TOF MS profile because the cellobiose dendrimer was difficult to ionize.

The molecular weight of signal at m/z = 2190 Da appeared to be due to the cellobiose-C6-spacer polylysine dendrimer first generation (Figure 2.9-LODG1) with the substitution of 4 cellobiose units. The signal intensity decreased with increasing substitution of the cellobiose unit, probably due to lower ionization of the cellobiose dendrimer with high molecular weight. The difference of the molecular weight between signals decreased around 444 Da due to the molecular weight of the cellobiose unit. In similar to cellobiose-C6-spacer polylysine dendrimer first generation MALDI TOF MS spectroscopy, the molecular weight of signal at m/z=4403 Da appeared to be due to the cellobiose-C6-spacer polylysine dendrimer second generation (Figure 2.9-LODG2) with the substitution of 8

cellobiose units. The difference of calculated and observed molecular weight was suggested to gives from the molecular weights of the Na or K ion. However the molecular weight signal at m/z=2190 Da appeared to be due to the third generation (Figure 2.9-LODG3) was core poly-L-lysine. dendrimer, and other signals were in agreement with the degree of substitution of 1-14 cellobiose units, respectively, Although the average molecular weight of the cellobiose dendrimers synthesized here was not determined by MALDI TOF MS.

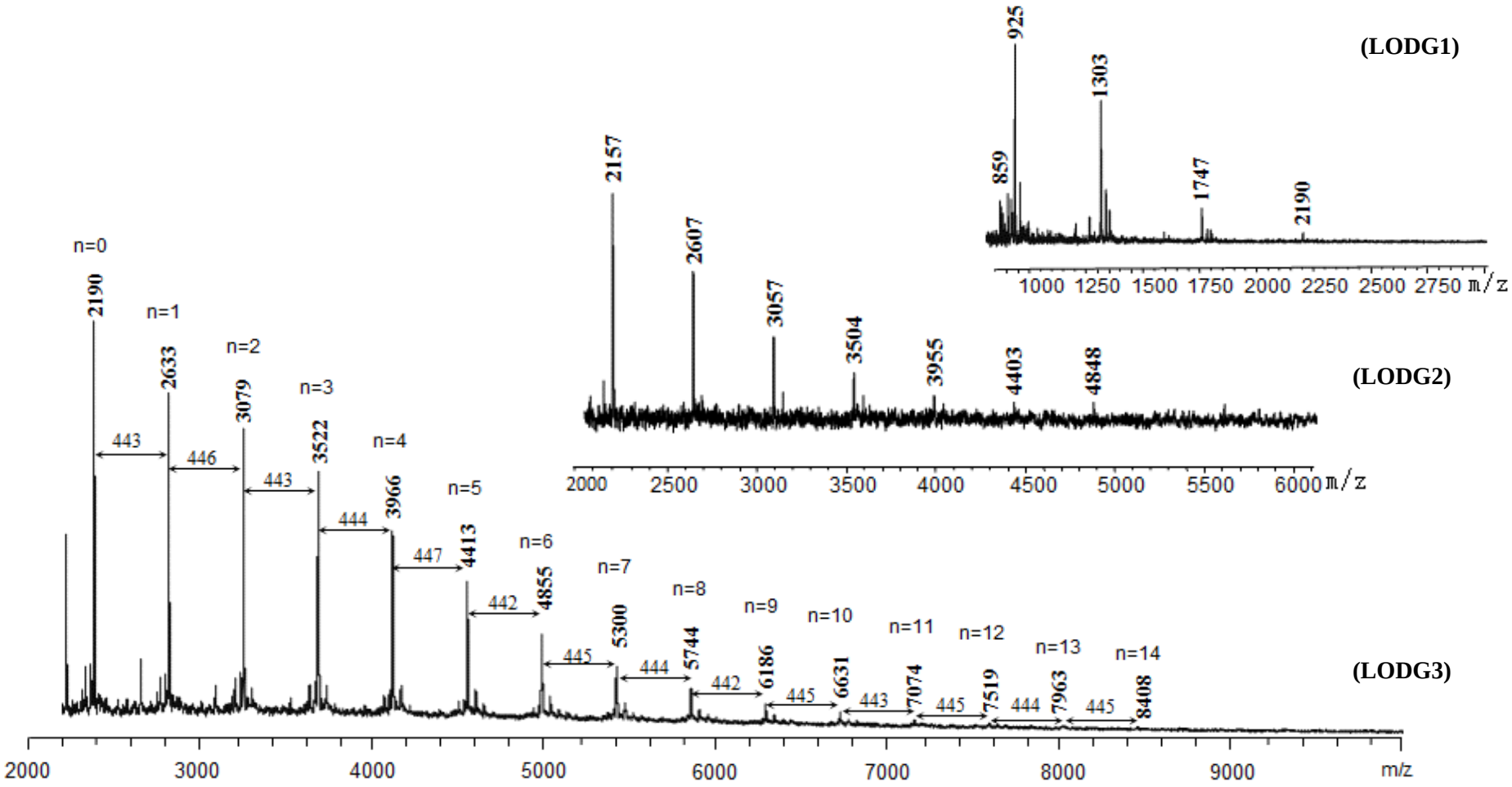


Figure 2.9 FALDI TOF MS profile of the cellobiose-C6-spacer polylysine dendrimers first (LODG1), second (LODG2) and third (LODG3) generations.

2.3.9 Integration of ^{1}H-NMR spectra of spherical polylysine oligosaccharide dendrimers generation 1, 2, 3 with C6 methylene spacer

The average degree of substitution of the cellobiose unit was calculated from the ^{1}H NMR signals (Figure 2.9.1). From the integration ratio of a-H and H1, H1′of cellobiose, we calculated the number of inserted glycoside.

2.4 Conclusion

New spherical first, second and third generation cellobiose-C6 spacer-polylysine dendrimers were synthesized, respectively, from 1,4-diaminobutane as a starting compound by the repeated condensations and deprotections by di-boc-lysine, and then condensing the cellobiose unit with the C6 methylene spacer having carboxylic acid at the end of the methylene groups with amino groups at the terminal of the polylysine core dendrimers. Cellobiose was used as a model compound of oligosaccharides. The C6 methylene spacer should effectively increase the flexibility and cluster effect of the attached oligosaccharides.

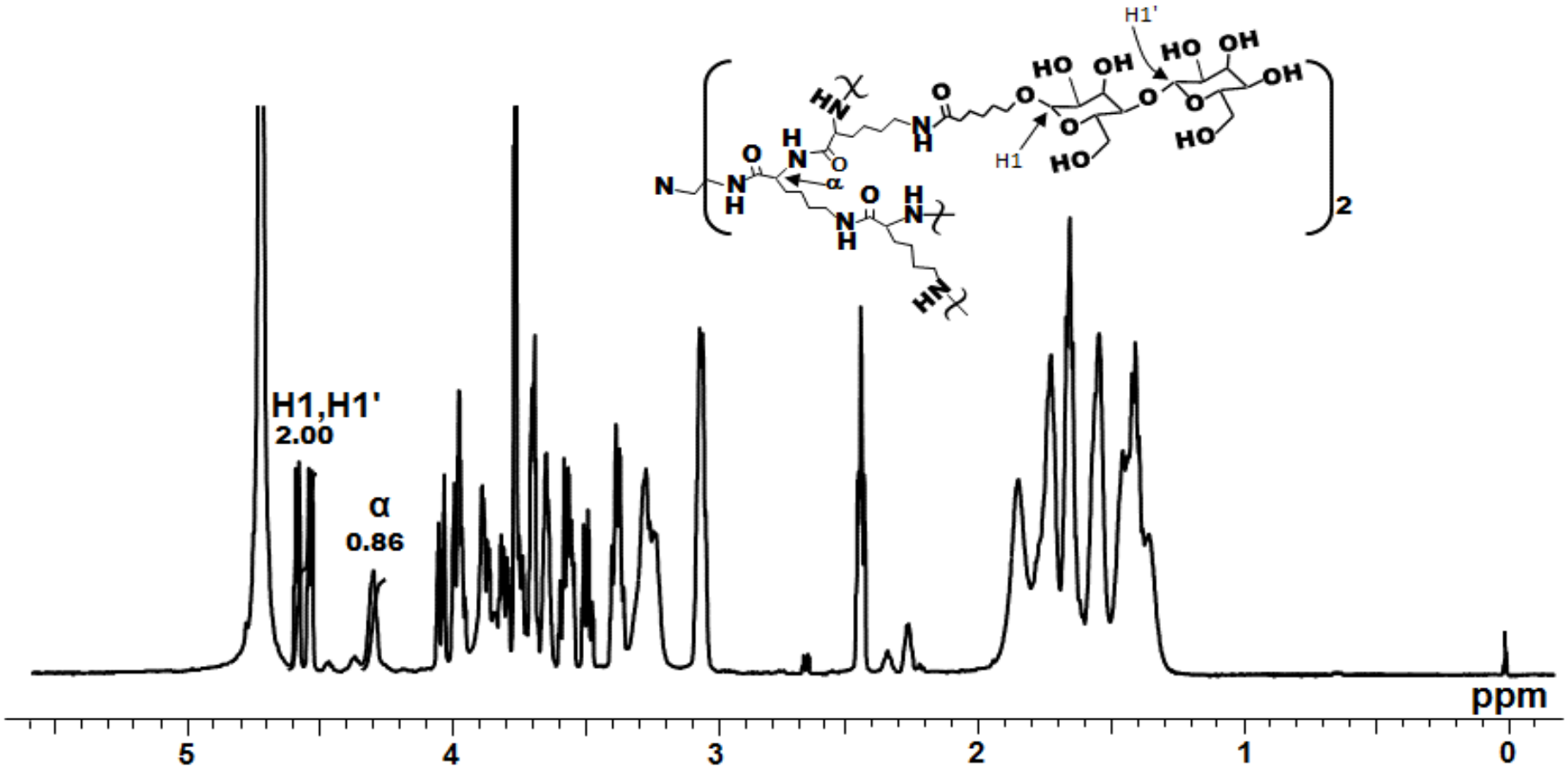


Figure 2.9.1 600MHz ^{1}H-NMR spectra of cellobiose-C6 spacer-polylysine dendrimer second generation in D_2O at 40℃. DSS (0.015 ppm) was used as an internal standard.

Cellobiose was used as a model compound of oligosaccharides. The C6 methylene spacer should effectively increase the flexibility and cluster effect of the attached oligosaccharides. Cellobiose was attached to the polylysine dendrimers without decomposition though the C6 methylene spacer. Characterization of the structure of the dendrimers by NMR, IR and MALDI TOF MS measurements, indicated that 4, 6.8 and 11 units (Table 2.1) of the cellobiose unit were introduced in the first, second and third generation of the dendrimers with 4, 8, 16 amino groups at the terminals, respectively.

Table 2.1 Calculation and observed number of sugar chain

Sample	No. of sugar residues	
	Cal.	Obs.
LODG1	4	4
LODG2	8	6.8
LODG3	16	11

Reference

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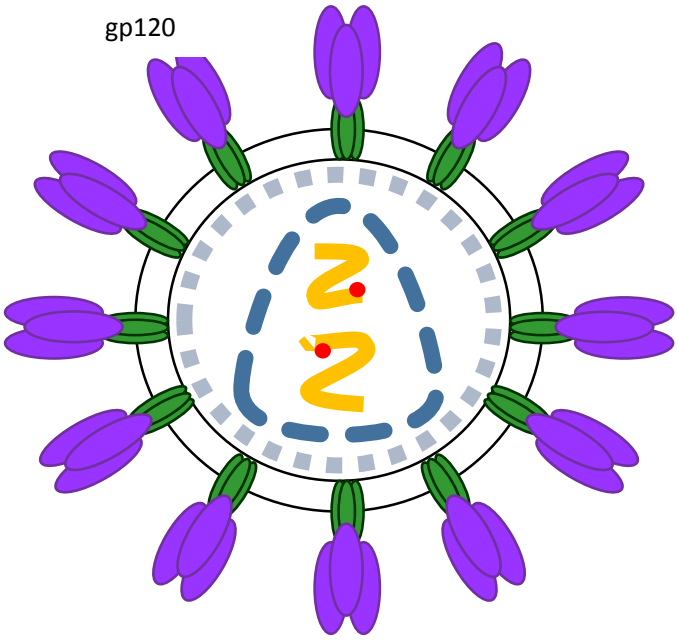
Chapter 3

Biological activity of new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer

3.1 Introduction

AIDS (Acquired immune deficiency syndrome or acquired immunodeficiency syndrome) is a fatal disease caused by a virus called HIV (Human Immunodeficiency Virus, shown in 3.1). Since the first cases discovered in 1981, AIDS spread



3.1 Image of HIV virus structure

rapidly worldwide and became a serious threat to human life. As of 2012, approximately 35.3 million people are living with HIV globaly. [1]

Through extensive research, the mechanism by which HIV invades host cells is now basically clear. HIV virus through its envelope glycoprotein gpl20 combined to CD4 receptor on the target cell specifically. That leading the gp120 to conformational changes to bind with the target cells of CCR5 or CXCR4, thus contributing to its hydrophobic fusional peptide portion into the host cell membrane, causing fusion phenomenon of the virus envelope with the host cell membrane, to achieve viral RNA invasion to the host cell. [2, 3]

Binding of sulfated polysaccharide and glycoprotein gp120 is the specific reaction. Ionic interactions of sulfated polyanions with oppositely charged cell surface components, including CD4, have been assumed to be the inhibitory mechanism.[4]

We have continuously worked on the elucidation of the relationship between structures of polysaccharides and biological activities.[5] Curdlan sulfate, which was obtained by sulfation of curdlan, a naturally occurring polysaccharide with a linear (1→3)-β glucopyranosidic structure was found to completely inhibit the infection of MT-4 cells by HIV in a concentration as low as 3.3μg /ml and had low cytotoxicity at a concentration as high as 1000μg /ml.[6] In addition, curdlan sulfate inhibited the infection of LLC-MK2 cells by Dengue virus at a 50% effective concentration as low as 0.1μg /ml.[7]

We have also reported the synthesis of several new types of sulfated glycodendrimers that were found to have potent anti-HIV activity due to the cluster effects of sugar moieties.[8, 9] A polylysine dendrimer with cellobiose at the terminal was synthesized by a third generation core lysine dendrimer and cellobiose connected through a C12 spacer that was synthesized by a peptide condensation between 6-amino-1-hexanol and adipic acid. The spacer was connected to cellobiose by a glycosylation bond. After sulfation, the sulfated cellobiose dendrimer had potent anti-HIV activity of the EC_{50} = 3.2μg /ml for the 50% inhibitory concentration of virus replication and low cytotoxicity as high as 1000μg /ml, which is the 50% cytotoxic

concentration (CC_{50}) for MT-4 cells.[10] In addition, an amphiphilic sulfated cellobiose dendrimer synthesized from the polylysine dendrimer with a stearyl amide group at the core of the lysine dendrimer and cellobiose at the terminal was found to have high anti-HIV activity at an EC_{50} as low as 6.7μg /ml and a CC_{50} as high as 1000μg /ml.[11] The EC_{50} was almost the same as that of a clinical AIDS drug, DDC, with EC_{50} = 3.3μg/ml. The hydrophobic stearyl group should be immobilized on the hydrophobic surfaces by hydrophobic interaction. The anti-HIV activity was improved by the cluster effect of the sulfated cellobiose due to the dendritic structure. These amphiphilic dendrimers are expected to create a new biological material with the hydrophilic surface functionality of clustered sulfated oligosaccharides.[12]

3.2 Experimental Section

3.2.1 Materials

Infrared spectra were taken on a Perkin Elmer Spectrum One FT-IR spectrometer using a KBr pellet method. Specific rotation was measured by using a JASCO DIP-140 digital polarimeter in H_2O at 25℃ in a water-jacketed 10 ml quartz cell. The surface

plasmon resonance (SPR) spectrum was taken on a Biacore X100 instrument at 25℃ using a CM5 sensor chip. Nuclear magnetic resonance spectroscopy ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM AEC-600 spectrometer at 600 MHz and 150 MHz, respectively, at 40℃ in D_2O or DMSO-d_6 solvent. Chemical shifts in D_2O are expressed as ppm downfield from 4,4'-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal standard. Poly-*L*-lysine with a molecular weight of 1000-5000 was purchased from Sigma-Aldrich, Co. A SA sensor chip, an amine coupling kit, HBS-EP+ 10x buffer (including 0.1M 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), 30mM EDTA, and 0.5 v/v% polyoxyethylene (20) sorbitan monolaurate surfactant (surfactant P20)), and 50mM NaOH solution were supplied by GE Healthcare Japan, Co. Ltd. The HBS-EP+ 10x buffer was diluted 10 times with Milli-Q water. The average particle size and ζ-potential of the polylysine oligosaccharide dendrimer generations 1, 2, 3 and sulfated polylysine oligosaccharide dendrimer generations1, 2, 3 were evaluated using an ELSZ-1000 zeta-potential and particle size analyzer (Otsuka Electronics Co., Ltd.)

3.2.2 Preparation of sulfated new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer

Synthesis of spherical polylysine oligosaccharide dendriemers with C6 methylene spacer was prepared by the method described in literature.[13] Spherical polylysine oligosaccharide dendriemer generation 3 as an example, dissolved in DMSO (8 ml) and then SO_3- pyridine (SO_3-Py) complex (0.31 g, 1.89 mmol) was added and stirred at 38℃. After 3 h, the mixture was cooled to room temperature and then neutralized with 10% NaOH solution and dialyzed in a dialysis tube (3500 molecular weight cut-off) for 2 day, and then freeze-dried to give 67%, 62.6 mg of sulfated spherical polylysine oligosaccharide dendriemer generation 3 and it have as high as 2.1 degree of sulfation. Information of sulfated spherical polylysine oligosaccharide dendriemer generation 1 and 2 shown in Table 3.1.

Table 3.1 Sulfation result of samples

Sample	Yield (%)	Mw* (×10³)	$[a]_D^{25}$	Elemental analysis (%)				DS*
				C	H	N	S	
SLODG1	67	3.3	-6.1	20.79	2.98	0.60	16.56	1.8
SLODG2	89	7.0	-6.9	26.65	3.48	2.65	15.91	2.1
SLODG3	93	12.8	-7.2	32.38	4.66	6.32	13.29	2.1

Mw*: Calculated molecular weight.
DS*: Degree of sulfation

3.2.3 Nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectrometer (IR) measurements

Figure 3.2 shows the ^{13}C NMR spectra of sulfated spherical polylysine oligosaccharide dendriemer generations 1, 2, 3 and spherical polylysine oligosaccharide dendriemer generation 3, respectively. The absorption of poly lysine oligosaccharide dendriemer generation 3 C1,1′appearing at 105 ppm, and after sulfation, the C1,1′ absorption appearing at 102 ppm and the C6,6′ absorption appearing at 63 ppm and moved to 74 ppm.

Figure 3.3 shows the FT-IR spectra of sulfated spherical polylysine oligosaccharide dendriemer generations 1, 2, 3 and spherical polylysine oligosaccharide dendriemer generation 3,

respectively. We can see, the stretching vibration of OH absorptions appeared around 3299 cm^{-1}, after sulfation, absorption of SO_3 appeared around 1243 cm^{-1}.

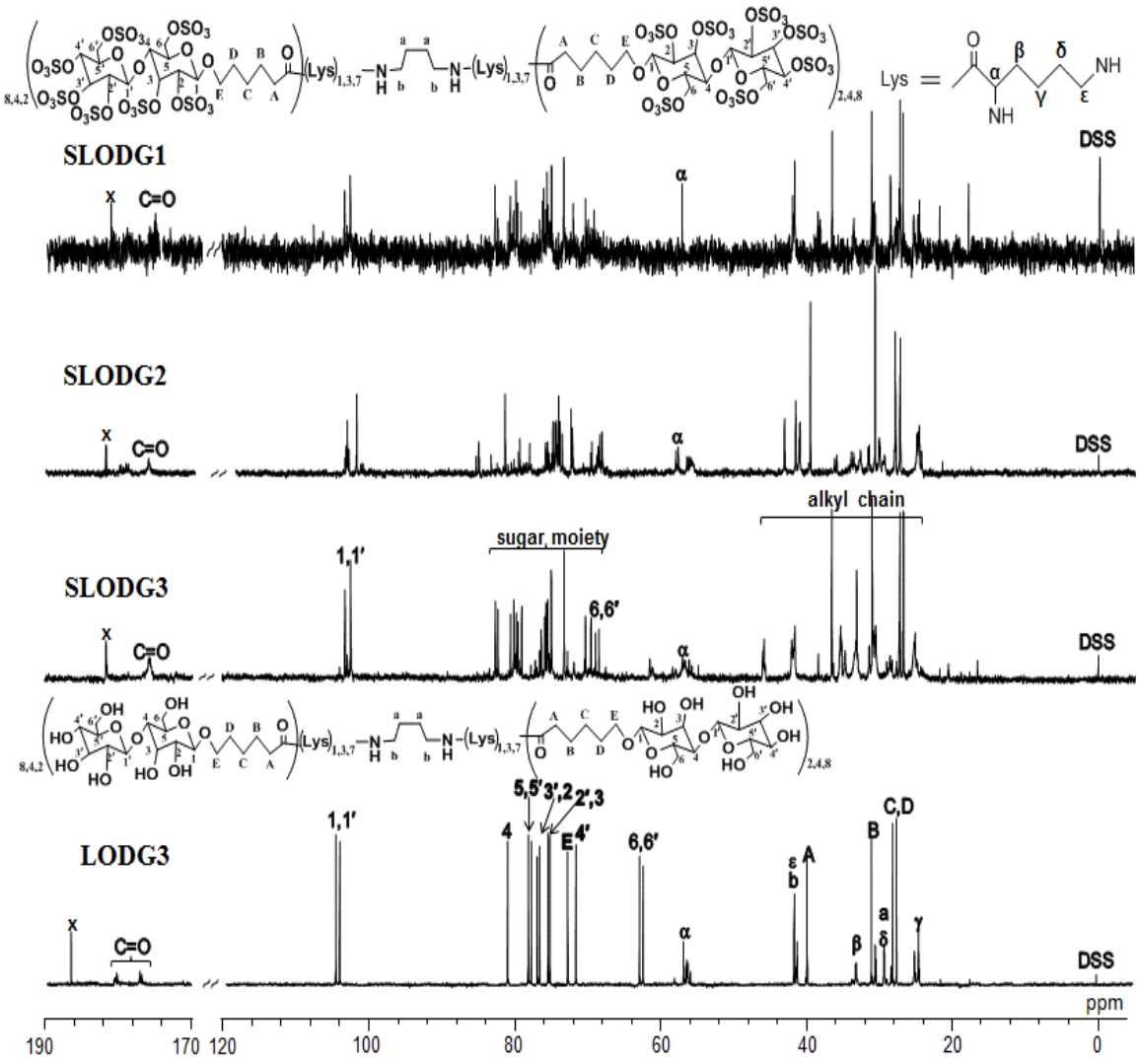


Figure 3.2 ^{13}C NMR spectra of sulfated spherical polylysine oligosaccharide dendriemer generations 1(SLODG1), 2(SLODG2), 3(SLODG3) and spherical polylysine oligosaccharide dendriemer generation 3(LODG3)

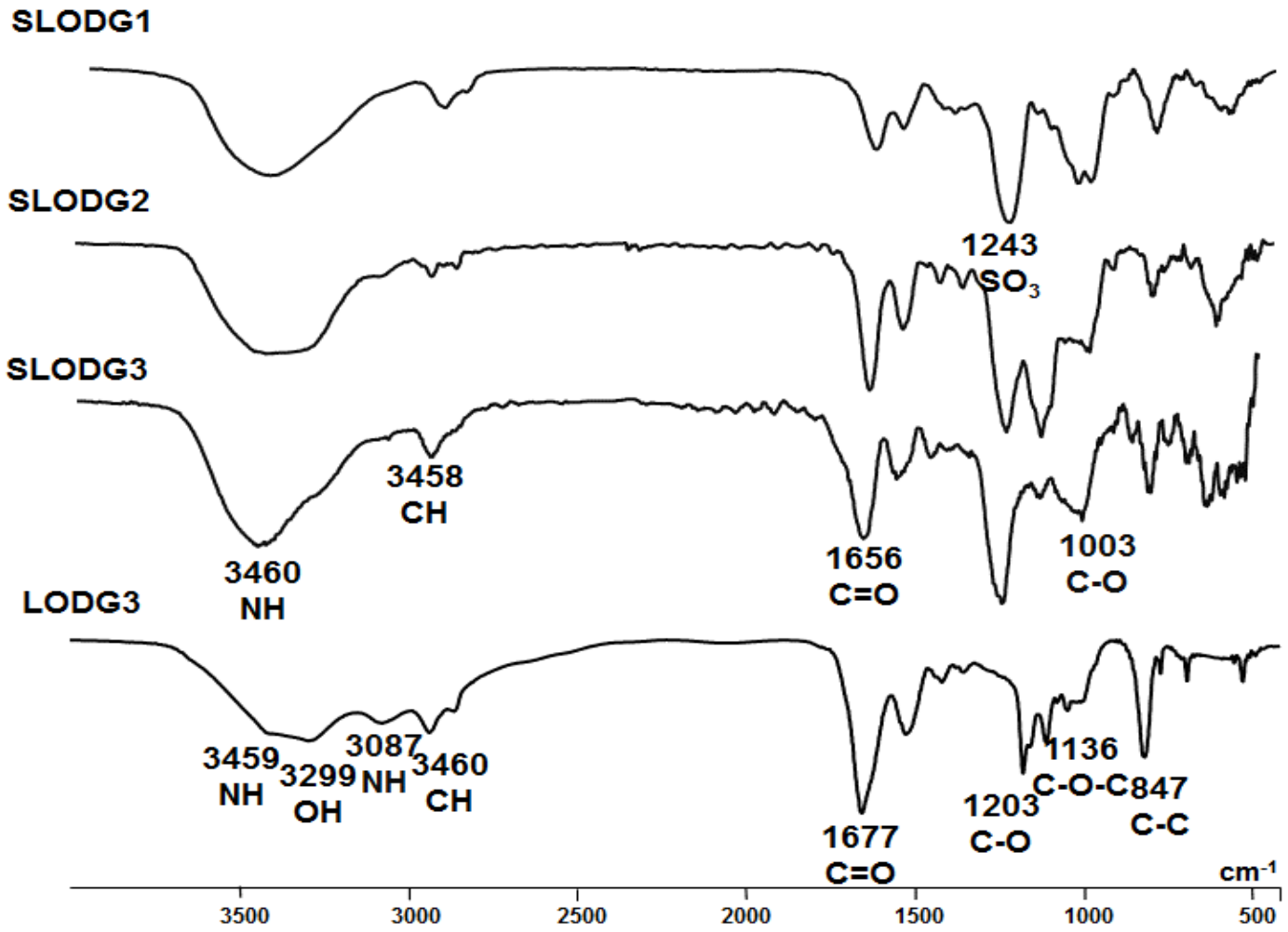


Figure 3.3 FT-IR spectra of sulfated spherical polylysine oligosaccharide dendriemer generations 1(SLODG1), 2(SLODG2), 3(SLODG3) and spherical polylysine oligosaccharide dendriemer generation 3(LODG3)

3.3 Anti-HIV assay of new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer

The viability of both HIV and mock-infected cells was assessed spectrophotometrically via the in situ reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The procedure was optimized as to make optimal use of multichannel pipettes, microprocessor-controlled dispensing and optical density reading. The absorbance ratio of the mock-infected control to the HIV-infected samples was about 20. This allowed an accurate determination of the 50% effective doses, namely effect concentration EC50 of decreasing HIV-induced cytopathic. The cytotoxicity concentration CC_{50} was determined as inhibiting cytotoxicity on 50% of MT-4 cells. Anti-HIV activities test was measured by the cooperation of Nakajima* professor of St. Marianna University. MTT method for determining anti-HIV activity as follows: MT-4 cells (with lymphotropic virus type I Human T4-positive cells) were placed in 96 small plates, in the presence of a series of different concentrations of the test substance, making them infected with HIV-1. Meanwhile, the use of the normal M11 called cell cytotoxicity was measured subject substance. The HIV infection

MT-4 cells and MT-4 cells were stay in 37℃, under the CO_2, incubated for 5 days. Then the cells mixed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (MTT), measured by a photometer of alive MT-4 cell concentration stained with MTT, you can get the survival rate[14].

Professor Hideki Nakajima of St. Marianna University of Medical Center for AIDS Research that made sulfated sugar-containing starch and continuous synthetic sulfated polysaccharide starch AZT anti-HIV activity, and HIV active 2',3'-Dideoxycytidine (ddC), sulfated dextran (DS), Sulfated curdlan (CRDS) and azide thymidine (AZT) were contrast.

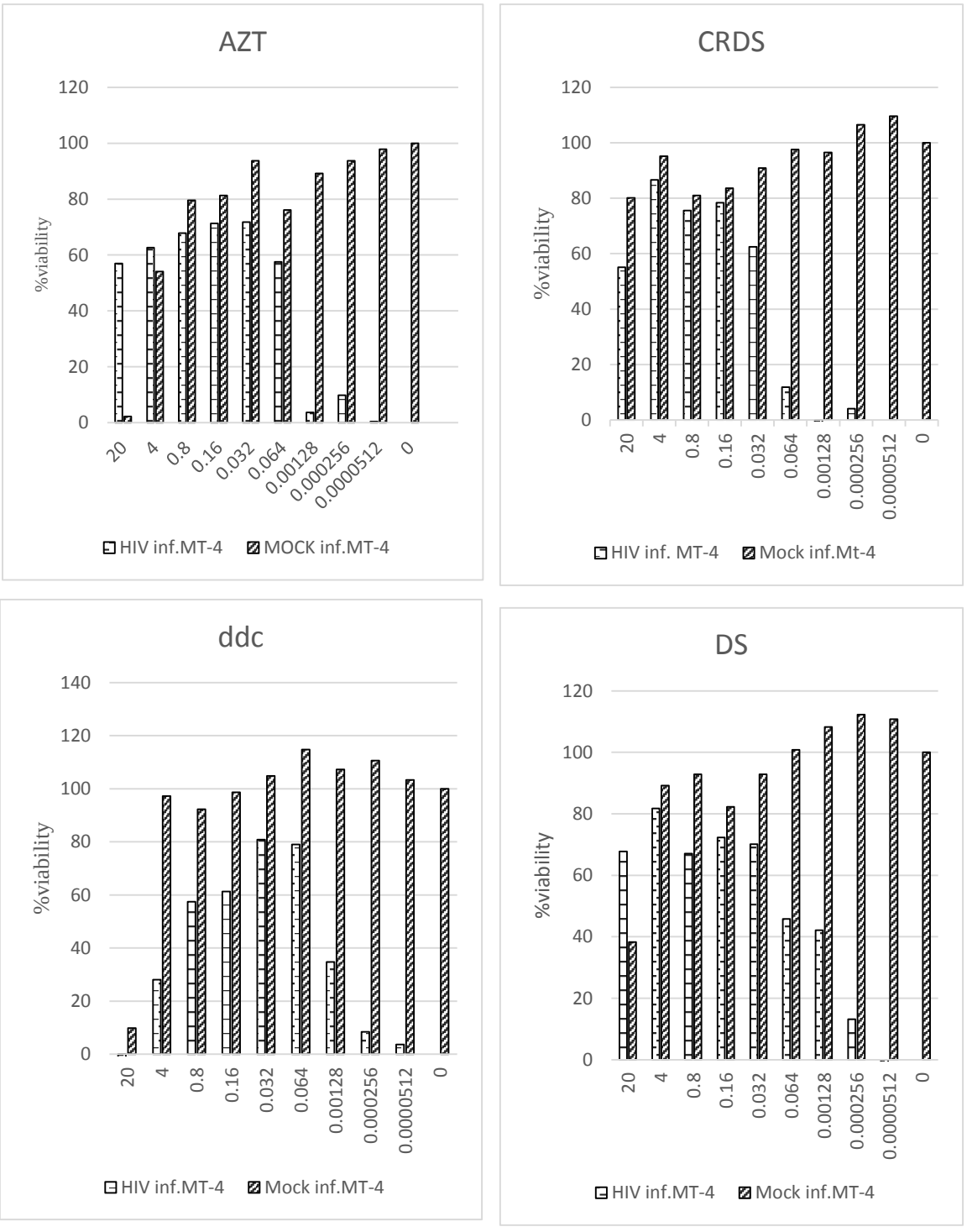
The test results are shown in Table 3.2 and Figure 3.4, SI for the effective coefficient (SI = CC_{50} / EC_{50}).

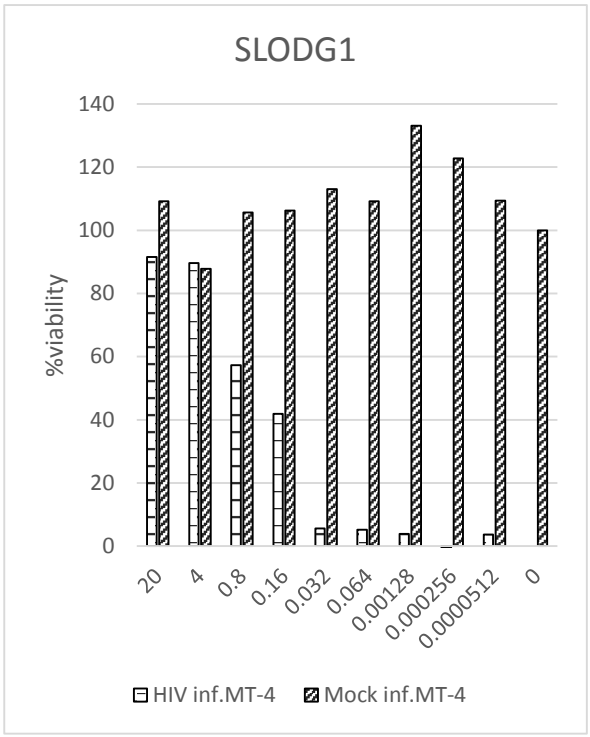
Table 3.2 Measured results of anti-HIV activity

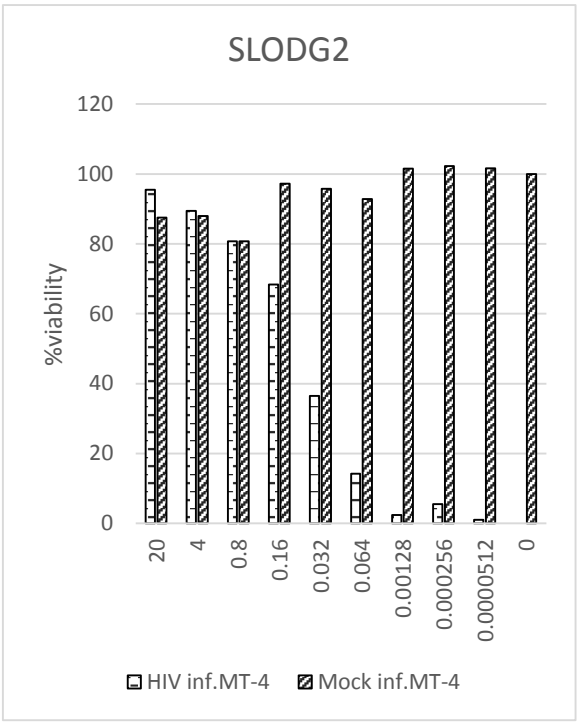
Sample	EC_{50} μg/ml	CC_{50} μg/ml	SI
SLODG1	3.73	>200	>54
SLODG2	0.63	>200	>130
SLODG3	1.54	>200	>316
DS	0.42	=691	=1631
CRDS	0.22	>1000	>4644
AZT(uM)	=113.34	=0.005	=22176
ddC(uM)	=2386.83	=0.56	=4278

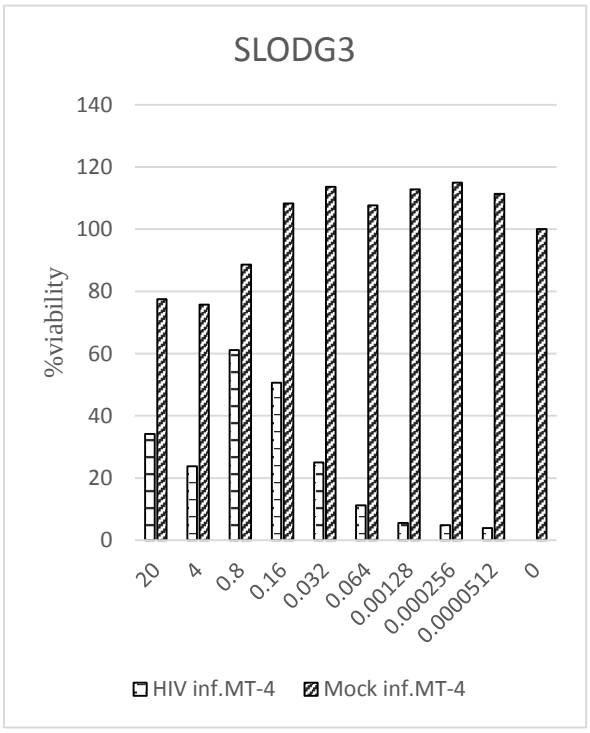
Operator：St. Marianna *Univ.*, Kanemoto Taisei ,Terakubo Shigemi
Date：4th，March/2015

Figure 3.4 The relation between subsist ratio of cell and concentration of determined substance









AZT: azide thymidine

CRDS: Sulfated curdlan

ddC: 2',3'-Dideoxycytidine

DS: Sulfated dextran

SLODG1: Sulfated polylysine oligosaccharide dendrimer generation 1

SLODG2: Sulfated polylysine oligosaccharide dendrimer generation 2

SLODG3: Sulfated polylysine oligosaccharide dendrimer generation 3

Table3.3 Data of Determined Activity

AZT		CRDS		ddC		DS		SLODG1		SLODG2		SLODG3	
C(μg/ml)	ER(%)	C(μg/ml)	ER(%)	C(μg/ml)	ER(%)	C(μg/ml)	ER(%)	C(μg/ml)	ER(%)	C(μg/ml)	ER(%)	C(μg/ml)	ER(%)
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.0000512	0.36	0.00256	4.05	0.0128	3.67	0.00256	-3.36	0.000512	3.64	0.000512	0.98	0.000512	3.93
0.000256	9.78	0.0128	-2.11	0.064	8.41	0.0128	13.14	0.00256	-0.51	0.00256	5.55	0.00256	4.79
0.00128	3.62	0.064	11.81	0.32	34.70	0.064	42.12	0.0128	3.84	0.0128	2.40	0.0128	5.56
0.0064	57.54	0.32	62.47	1.6	78.98	0.32	45.76	0.064	5.25	0.064	14.25	0.064	11.21
0.032	71.81	1.6	78.33	8	80.86	1.6	70.08	0.32	5.56	0.32	36.46	0.32	25.06
0.16	71.30	8	73.66	40	61.27	8	72.32	1.6	41.92	1.6	68.34	1.6	50.64
0.8	67.83	40	75.59	200	57.42	40	67.01	8	57.27	8	80.72	8	61.16
4	62.61	200	86.61	1000	28.09	200	81.73	40	89.60	40	89.42	40	23.78
20	56.96	1000	55.07	5000	-10.38	1000	67.75	200	91.52	200	95.50	200	34.13

C:Concentration ER: Efficient rate

Table3.4 Data of Determining Toxicity

AZT		CRDS		ddC		DS		SLODG1		SLODG2		SLODG3	
C(μg/ml)	SR(%)	C(μg/ml)	SR(%)	C(μg/ml)	SR(%)	C(μg/ml)	SR(%)	C(μg/ml)	SR(%)	C(μg/ml)	SR(%)	C(μg/ml)	SR(%)
0	100	0	100	0	100	0	100	0	100	0	100	0	100
0.00128	97.88	0.00256	109.63	0.0128	103.35	0.00256	110.80	0.000512	109.35	0.000512	101.60	0.000512	111.30
0.0064	93.70	0.0128	106.47	0.064	110.59	0.0128	112.28	0.00256	122.76	0.00256	102.28	0.00256	114.93
0.032	89.16	0.064	96.48	0.32	107.24	0.064	108.28	0.0128	133.13	0.0128	101.54	0.0128	112.77
0.16	76.14	0.32	97.56	1.6	114,79	0.32	100.81	0.064	109.20	0.064	92.85	0.064	107.61
0.8	93.76	1.6	90.87	8	104.80	1.6	92.83	0.32	113.02	0.32	95.75	0.32	113.61
4	81.28	8	83.61	40	98.63	8	82.25	1.6	106.24	1.6	97.23	1.6	108.23
20	79.59	40	80.95	200	92.30	40	92.83	8	105.61	8	80.76	8	88.63
100	54.03	200	95.11	1000	97.26	200	89.20	40	87.84	40	87.98	40	75.79
500	2.24	1000	80.09	5000	9.83	1000	38.31	200	109.20	200	87.48	200	77.46

C:Concentration SR: Survival rate

Obtained from the data in Table 3.3 and 3.4, at very low concentrations of SLODG1, SLODG2 and SLODG3 (less than 0.064ug / ml), the HIV-infected MT-4 cells have low efficiency (less than 15%), there are 91.52% and 95.50% efficiency at 200 ug / ml of SLODG1 and SLODG2, while at 8ug / ml SLODG3 have 61.16% efficiency. Over a wide concentration range SLODG1, SLODG2 and SLODG3 have a good inhibition for the HIV virus. When the concentration increases further, efficiency begins to drop, indicating that when the concentration is too large, samples have a certain degree of toxicity.

The toxicity of the samples is determined by measuring MT-4 cells of survival rate for the substances (or mortality), in a wide concentration range have a very low mortality. No significant effect on the concentration of toxic. In short, SLODG1, SLODG2 and SLODG3 are low in toxicity, high selectivity of compounds that having a potential treatment of AIDS. However, the in vivo anti-HIV activity test and resistance to other pharmacological experiments should be further study.

3.4 Interaction of sulfated new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer with poly-L-lysine

3.4.1 SPR measurement

Excitation of surface plasmons is based on total internal reflection when an incident beam of p-polarized light strikes an electrically conducting gold layer at the interface of a glass sensor with high RI (Refractive Index) and an external medium (gas or liquid) with low RI. At a given angle, the excitation of surface plasmons takes place resulting in a reduced intensity of the reflected light. A slight change at the interface (e.g. a change in refractive index or formation of a nanoscale film thickness) will lead to a change in SPR signal, allowing precise measurements of thin film properties as well as surface molecular interactions in real-time(Shown in Figure3.5).

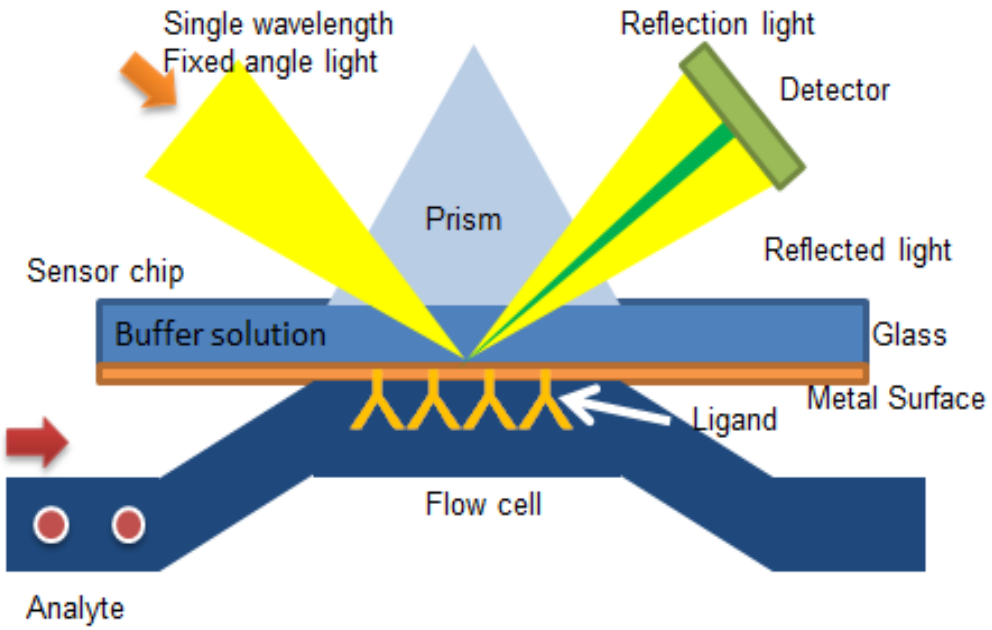


Figure3.5 Surface plasmon resonance (SPR)

Following in the content, polylysine was used as a model compound of epidermal protein of the virus. The affinity of sulfated new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer to poly-L-lysine as a model compound of proteins and peptides was examined to elucidate the interaction mechanism of the biological activities using surface plasmon resonance (SPR) measurement.[15] Polylysine in 10 mM sodium acetate buffer (pH 5.5) was immobilized on the CM5 sensor chip according to the Biacore protocols (Fischer, 2010). The concentration was 5 mg/ml and the flow rate was 10 µl/min for 7 minutes. The values for the immobilized polylysine was 1000 response unit (RU). A reference cel without polylysine was used. Sulfated spherical polylysine

oligosaccharide dendriemer generation 1, 2, 3 (5μg/ml) were injected for 3 min (30 μl/min) over the polylysine immobilized sensor chip, and then the solution was injected for 12 min to determine the association and dissociation rate constants. The Sulfated spherical polylysine HBS-EP$^+$ running buffer ne oligosaccharide dendriemer generation 1, 2, 3 (5μg/ml) were prepared in the HBS-EP$^+$ buffer, and diluted to various concentrations with the HBS-EP$^+$ buffer, then injected over the sensor chip at a flow rate of 30 μl/min for 3min for association, and then 12dissociation was carried out with injection of only the HBS-EP$^+$ buffer for 12 min. For regeneration of the the CM5 sensor chip, NaOH (50 mM) solution was used.

Table 3.5 Kinetic results of Dextrane sulfate, SLODG1, SLODG2 and SLODG3

Sample	Mw* ($\times 10^3$)	k_a [1/Ms]	k_d [1/s]	K_D [M]
SLODG1	3.3	6.62×10^3	4.37×10^{-3}	6.60×10^{-8}
SLODG2	7.0	2.45×10^4	4.55×10^{-4}	1.86×10^{-8}
SLODG3	12.8	5.77×10^4	2.37×10^{-4}	4.10×10^{-9}
Dextran sulfate	8.5	1.03×10^5	5.29×10^{-4}	5.14×10^{-9}

a) Samples was injected 90μl for 180 sec at a flow rate of 30 μ l/min of a HBS-EP running buffer at 25 ℃ and then the running buffer was further flowed for 600 sec. The concentration was 5.0, 2.5, 1.25, 0.625, and 0.3125 μ g/ml, respectively.

b) *ka*; Association-rate, *kd*; dissociation-rate constants, and dissociation constant KD = *kd*/*ka*

We found that SLODG1interacted weakly with poly-L-lysine. The RU values increased with increasing branches number of spherical polylysine oligosaccharide dendrimer, namely the number of cellobioside. The SLODG3 with the more cellobioside number of n=11 gave the higher association response of about 320 RU (Figure 3.6) compared to the lower number of cellobioside with n= 6.8 and n= 4 (Figures) at the concentration of 500 μg/ml, indicating that the interaction was dependent on the number of sulfated cellobiside or branches number of dendrimers.

After sulfation, sulfated SLODG1, SLODG2, SLODG3 Had higher association (*ka*) and lower dissociation (*kd*) rate constants, indicating that sulfated had a high affinity to poly-L-lysine by the electrostatic interaction of the negative charges of sulfate groups and positive charges of amino groups. Therefore, the SPR measurement was found to support the elucidation of biological activities.

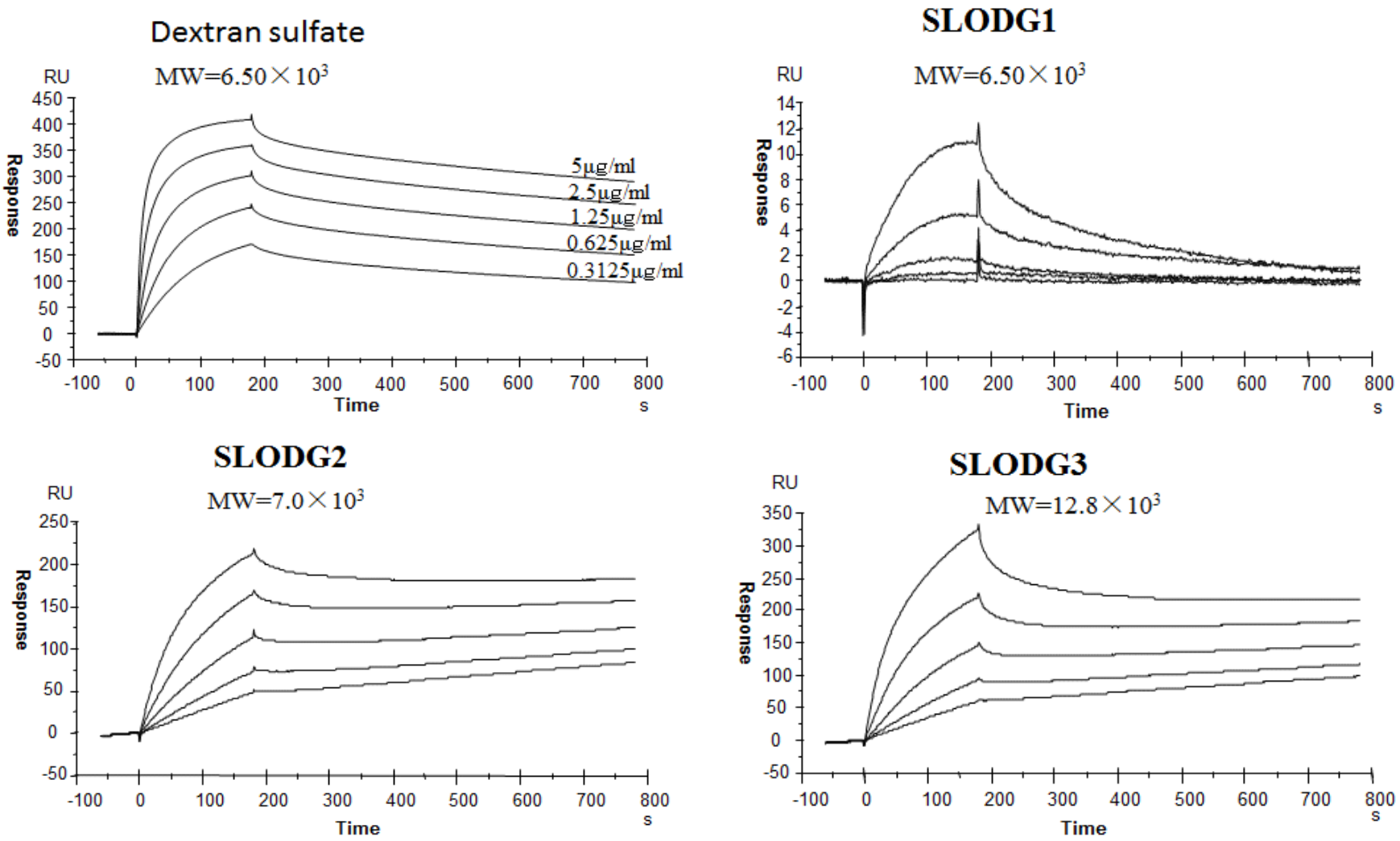


Figure 3.6 Binding curves of Dextran sulfate,SLODG1, SLODG2 and SLODG3, immobilized poly-L-lysine. the concentrations were 5, 2.5,1.25, 0.625, and 0.3125μg/ml.

3.4.2 Partical size and Zeta-potential analysis

Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler Shift when the light hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle. It is possible to compute the sphere size distribution and give a description of the particle's motion in the medium (Figure 3.7).

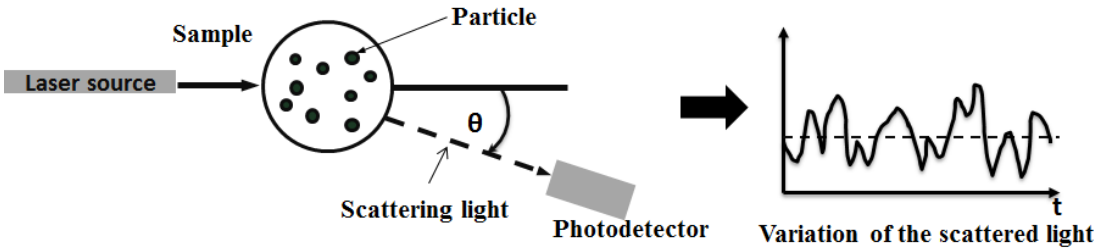


Figure 3.7 The hypothetical dynamic light scattering of samples

From the Table 3.6, we can see the particle size deta before and after sulfation of spherical polylysine oligosaccharide dendrimers generations 1, 2, 3.

Table 3.6 The particle size data

Sample name	Before sulfation Cal.BL(nm)	Obs.BL(nm)	Sample name	After sulfation Cal. BL(nm)	Obs.BL(nm)
LODG1	6.8	1.5±0.0	SLODG1	11.0	21.5±3.4
LODG2	17.2	22.8±7.0	SLODG2	25.6	65.8±0.0
LODG3	34.6	46.4±5.9	SLODG3	51.4	86.8±10.2

Before sulfation, the particle size of spherical polylysine oligosaccharide dendrimers generations 1, 2, 3 (LODG1, LODG2 and LODG3) were 6.8, 17.2 and 34.6nm and after sulfation theirs particle size became 21.5±3.4, 65.8±0.0, 86.8±10.2nm. Mixed with the same concentration and volume of Poly-L-lysine, particle size of sulfated spherical polylysine oligosaccharide dendrimers generations 1, 2, 3 became 164.3±22.3, 248.5±13.2, 324.2±54.0 nm (Table 3.7).

Table 3.7 The particle size of the mixtures of SLODG with polylysine.

Sample name	Poly-L-lysine Absent	Present
	Particle size (nm)	Particle size (nm)
SLODG1	21.5±3.4	164.3±22.3
SLODG2	65.8±0.0	248.5±13.2
SLODG3	86.8±10.2	324.2±54.0
Dextran sulfate	11.7±2.1	30.6±4.1

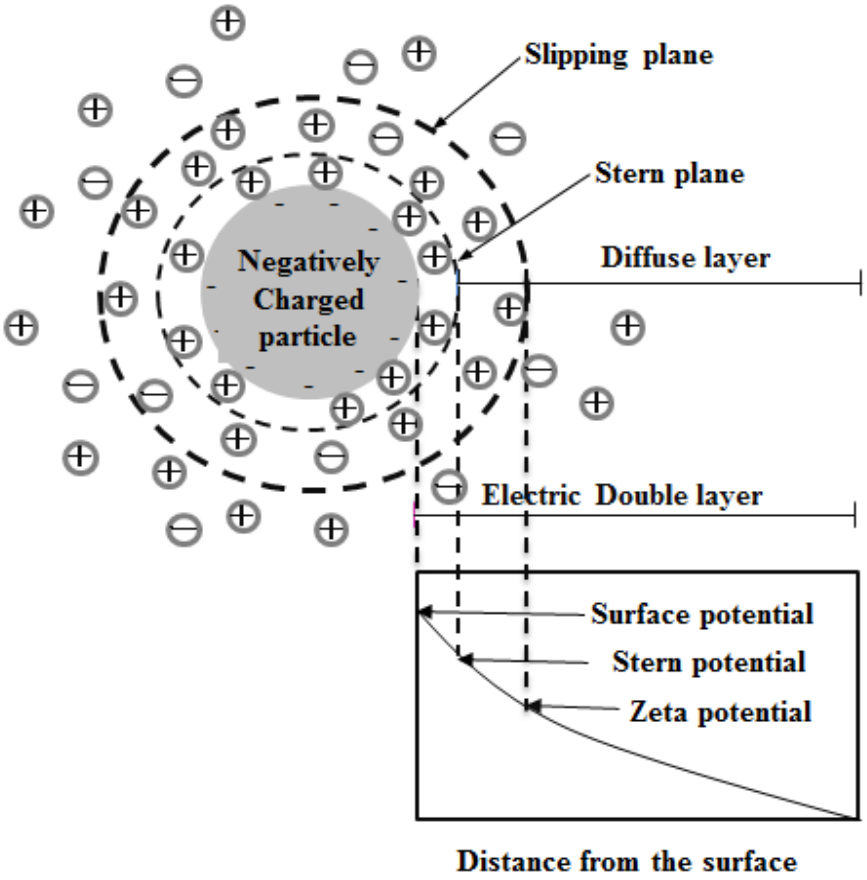


Figure 3.8 Scheme of electrochemical double layer

Zeta potential (Figure 3.8) is an important indicator of the stability of colloidal and suspended solids. Zeta potential is determined by the surface charge of the particles and the surrounding environmental conditions common solution. For example, with negatively charged particles: due to negative charge particles attract some positively charged ions (referred to as a counter ion, that with opposite electrical surface charge with particles) in close proximity to the surface of the particles formed the close connection with the distribution, that is "Stern

layer". With increasing distance from the surface with charged particles, positively charged counter-ions are still attracted by the negative charge particles, but at the same time there is the exclusion within close layer of positively charged ions, the dynamic equilibrium resulting in the formation of counter ions "diffusion layer".

In the near vicinity of particle on the diffusion layer, counter ion concentration is high, but decreased with increasing distance, until the main counter-ion concentration to form a normal balance. Stern layer and the diffusion layer together is called a "double-layer".[16,17]

In general, the surfaces of the particles with a positive charge, its zeta potential is positive; the contrary, when the surfaces of the particles with a negative charge, its zeta potential value is negative. We investigated samples at 25℃, dissolved in HBS-EP$^+$ buffer (PH6.8), the results shown in Table 3.8.

Table 3.8 was shown Zeta Potential results of polylysine dendrimers generations 1, 2, 3 and sulfated polylysine oligosaccharide dendrimers generations 1, 2, 3 at 25℃, in HBS-EP$^+$ buffer (PH6.8)

Table 3.8 Zeta Potential results of polylysine dendrimers generations 1, 2, 3 and sulfated polylysine oligosaccharide dendrimers generations 1, 2, 3 at 25℃, in HBS-EP$^+$ buffer (PH6.8)

Sample	ζ(mv)	Sample	ζ (mv)
LODG1	2.77	SLODG1	-27.47
LODG2	5.24	SLODG2	-25.55
LODG3	20.02	SLODG3	-0.72

After sulfation, the zeta potential of samples changed from 2.77, 5.24, 20.02 to -27.47, -25.55 and -0.72. When the poly-L-lysine was mixed to sample (at same volume ratio), the result of zeta potential measurement as follows (Table 3.9). After mixed with poly-L-lysine, the zeta potential of mixed solutions were become 0.01, 0.23, 0.56 mv.

Table 3.9 Zeta Potential measurement of mixed poly-L-lysine and sulfated polylysine oligosaccharide dendrimers generations 1, 2, 3 at 25℃, in HBS-EP$^+$ buffer (PH6.8)

Sample	Poly-L-lysine	
	Absent	Present
	ζ (mv)	ζ (mv)
SLODG1	-27.47	0.01
SLODG2	-25.55	0.23
SLODG3	-0.72	0.56

3.5 Results and discussion

Since we synthesized new spherical polylysine oligosaccharide dendrimers with C6 methylene spacer the well-defined structures and molecular weights, specific biological activities such as anti-HIV was examined to elucidate structure-activity relationships. Anti-HIV activity was evaluated by the 50% inhibitory concentration (EC_{50}) of sulfated spherical polylysine oligosaccharide dendrimers with C6 methylene spacer generations 1, 2 ,3 on the infection of HIV to MT-4 cell. As the degree of sulfation 1.8, 2.1 and 2.1, the anti-HIV activity were 3.73, 0.63 and 1.54mg/mL, respectively.

The cytotoxicity of the sulfated spherical polylysine oligosaccharide dendrimers with C6 methylene spacer generations 1, 2, 3 were low, since the CC_{50}, 50% cytotoxic concentration of sulfated samples on MT-4 cell, was more than 200 mg/mL. In order to approach to the mechanism how structure affect the biological activities, Surface plasmon resonance (SPR), particle size and zeta potential analyzer etc. was utilized, and the relation between the structural details and interaction was well discussed. As model of compounds of glycoproteins that polysaccharides interact with, poly-L-lysine

was immobilized on SPR sensor chip CM5 and dynamic constant including association rate constant *ka*, dissociation rate constant *kd*, and dissociation constant *KD* was calculated by using 1 to 1 binding fitting model. In the dynamic study, poly-L-lysine as an all-positive charged polypeptide model was used to be evaluated the activity of sulfated polylysine oligosaccharide dendrimers generations 1, 2, 3. It was found that the interaction increased with increasing branch number of dendrimers. This implies high branch number of the sulfated polylysine oligosaccharide dendrimers were expected for long-term effectives and were possible candidates as an anti-HIV medicine in the future.

We supposed that the electrostatic interaction of negatively charged sulfated groups of spherical polylysine oligosaccharide dendrimers with C6 methylene spacer generations 1, 2, 3 and positively charged amino groups of the HIV virus surface glycoproteins or between poly-L-lysine play an important role in the biological activities. More detailed investigations on the relationship between the activities and the structure of polymers are in progress.

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Mar 2015 **YingLi**