Doctoral Thesis

Interaction mechanism between sulfated polysaccharides and HIV oligopeptides analyzed by SPR

(SPRによる硫酸化糖鎖とHIVオリゴペプチド間の

相互作用メカニズムの解析)

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A thesis submitted to Graduate School of Engineering in partial fulfillment of the requirements for the degree of Doctor of Engineering

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ABREVIATIONS and ACRONYM

AA	Amino Acid
Boc	T-Butyloxycarbonyl
CH2Cl2	Dichloromethane
DIC	N,N′-Diisopropylcarbodiimide
DLS	Dynamic Light Scattering
DMF	Dimethylformaldehyde
DMSO	Dimethyl Sulfoxide
DS	Degree of Sulfation
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EMS	Enhanced MS
ESI	Electrospray Ionization
Fmoc	9-fluorenylmethyloxycarbonyl
GPC	Gel permeation chromatography
Gp120	Glycoprotein 120
HA	Hemagglutinin
HF	Hydrofluoric Acid
HIV	Human Immunodeficiency Virus
HOBT	1-Hydroxybenzotriazole
LC/MS	Liquid Chromatography Mass Spectrometry
MALDI	Matrix Assisted Laser Desorption Ionization
MeOH	Methanol
Mn	Molecule Mass
MS	Mass Spectrometry
MW-SPPS	Microwave Assisted Solid Phase Peptide Synthesis
NaOH	Sodium hydroxide
NHS	N-Hydroxyl succinimide
NMR	Nuclear Magnetic Resonance
RP-HPLC	Reverse phase High Performance Liquid Chromatography
SPR	Surface Plasmon Resonance
TFA	Trifluoroacetic acid
TIC	Total Ion Chromatography
TOF	Time of Flight
XIC	Extracted Ion Chromatography

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GENERAL ABSTRACT

Sulfated polysaccharides have specific antiviral activities, which biological mechanism is assumed to the electrostatic interaction between (+)-charged virus surface glycoproteins and (-)-charged sulfate groups. For the elucidation of the mechanism, several oligopeptides referenced by the sequence of Human Immunodeficiency Virus glycoprotein 120 (HIV gp120) and hemagglutinin (HA) of influenza A and B were synthesized by a peptide synthesizer and the interaction with structurally distinct sulfated polysaccharides such as curdlan sulfate and dextran sulfate was analyzed by SPR.

In this study, three oligosaccharides were synthesized from the sequence of the V3 loop, C-terminus, and CD4 binding domain in the HIV gp120. Oligopeptide A from the V3 loop comprises 20 amino acids with seven positively charged lysine and arginine in the sequence. The basic amino acids were relatively dispersed along the sequence compared with that of oligopeptide B. Likewise, oligopeptide B from the C–terminus comprises seven lysine and arginine, also oligopeptide of Influenza A/Yamagata HA and Influenza A/Brisbane HA comprises 23 amino acids with eight positively charged lysine and arginine in the sequence. Oligopeptide C from the CD4 binding domain and Influenza B /Hong Kong from the HA comprises one lysine and next to the biotin. The biotinylated peptides were synthesized by a microwave assisted solid phase peptide synthesizer using Fmoc protected amino acids. The peptides were purified by RP-HPLC and identified the structure by using MALDI TOF MS. Peptides A and B from HIV gp120 were found to have interacted strongly with dextran and curdlan sulfates, however, the peptide C without positively charged amino acids showed no interaction. These results suggest that the interaction was due to the electrostatic interaction between negatively charged sulfate groups and positively charged amino groups of the

peptides. The results of influenza HAs, influenza A (Yamagata and Brisbane) and B (Hong Kong) viruses, are also presented.

Curdlan and dextran sulfates were found to increase the interaction with increasing the molecular weights and degree of sulfation (DS), which were found to be important factors for the antiviral activity of sulfated polysaccharides. Based on the above, suggesting the antivirus mechanism of sulfated polysaccharides to be the electrostatic interaction of negatively charged sulfated polysaccharides and virus surface glycoprotein at the positively charged amino acid regions.

Keywords: Sulfated polysaccharide, HIV gp120 oligopeptide, Influenza virus hemagglutinin, Interaction, SPR

CHAPTER 1.

General Introduction

1.1 Biologically activity of sulfated polysaccharides

Naturally occurring and synthetic sulfated polysaccharides have biological activities such as anticoagulant, antioxidant, immunomodulatory, anti-tumor, anti-inflammatory, antiviral, anti-allergic and antithrombin activities [1]. In 1958, Gerber first time reported that sulfated polysaccharides extracted from sea alga had anti-influenza virus activity [2]. In 1987, Yamamoto and Nakashima et al reported the anti-HIV activity of sulfated polysaccharides also extracted from sea algae [3].

Marine algae are the most important source of naturally occurring sulfated polysaccharides. Sulfated polysaccharides from algae possess important pharmacological activities such as anticoagulant, antithrombotic, immune-inflammatory, antioxidant, antilipidemic, antiviral activities [4]. Also, our laboratory reported the structural analysis of polysaccharides in Chinese lacquer by NMR spectroscopy and have reported those specific biological activities such as antitumor, anti-HIV, anticoagulation [5, 6].

Since these report, many researchers reporting have biological activity of sulfated polysaccharides, such as heparin, carrageenan, dextran sulfate, glucosaminoglycan, pentosane polysulfate, mannan polysulfate for antivirus activities. In 1990, Uryu et al reported biological activity of lentinan, aribinosyl curdlan, galactosyl curdlan, curdlan, and those sulfated derivatives. The without sulfated polysaccharides have potent antitumor but do not show ant-HIV activity. Sulfated polysaccharides have potent anti-HIV activity, but do not show antitumor activity [7].

Nature occurring many polysaccharides having important specific biological

activities. But, that polysaccharides have a complex structure. It is very difficult to study the relationship between structure and biologically activities. So, our laboratory study in the synthesis of several types of polysaccharides by ring-opening polymerization of anhydro-sugar derivatives and those relationship between structure and biological activities [8]. Recently, our laboratory also reported sulfated natural galactomannans and synthetic galactomannans having specific anticoagulant and antiviral activity [9,10].

1.2 Human Immunodeficiency Virus (HIV), anti-HIV mechanism of sulfated polysaccharides

1.2.1 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) is a subgroup of retrovirus, that causes the acquired immunodeficiency syndrome (AIDS). HIV is occurs as two types of HIV-1 and HIV-2. Both types are transmitted through direct contact with HIV-infected body fluids, such as blood, semen, and vaginal fluids, or from a mother who has HIV to her child during pregnancy, labor and delivery, or breast feeding. HIV-1 is responsible for the majority of HIV infections worldwide. HIV-1 was found to be significantly more infectious than HIV-2[11].

1.2.2 Anti-HIV mechanism of sulfated polysaccharides

HIV entry to host cell, the first phase is HIV envelope protein must bind to the host cell membrane of various cell attachment factors [12]. As shown in figure 1.

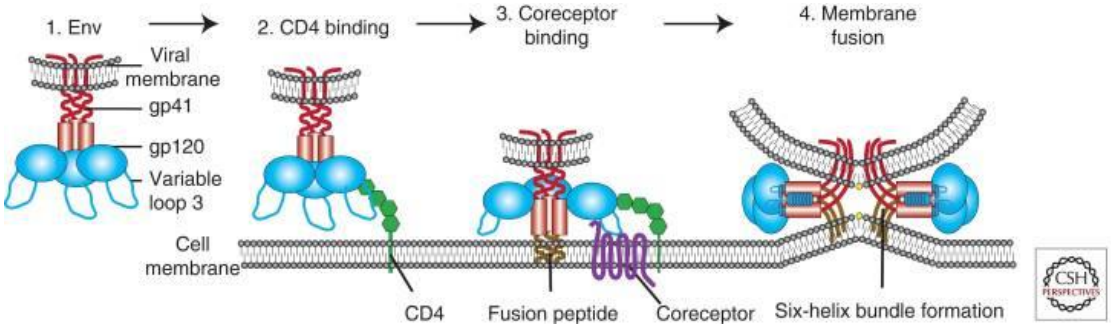


Figure1. Overview of HIV entry to host cell.

(1) Structure of HIV envelope glycoprotein. (2) first attaches to the host cell, binding CD4. (3)This causes conformational changes in envelope, allowing coreceptor binding, which is mediated in part by the V3 loop of envelope. (4)This initiates the membrane fusion process as the fusion peptide of gp41 inserts into the target membrane, followed by six-helix bundle formation and complete membrane fusion. [12]

In 1987, Yamamoto and Nakashima et al first reported the anti-HIV activity of sulfated polysaccharides also extracted from sea algae [3]. Also, at the same time, Yoshida and Uryu reported synthetic sulfated polysaccharides such as dextran sulfate, ribofuranan sulfate, xylofuranon sulfate having anti-HIV activity [13]. In 1990, Yoshida reported carried out the sulfation of curdlan, that curdlan sulfate has anti-HIV activity [14]. In 1993, also Yoshida reported synthetic and natural dextran sulfates having anti-AIDS activity, was assayed in vitro by using a MT-4 cell line [15]. Since these report, many researchers reported natural sulfated polysaccharides and synthetic sulfated polysaccharides having anti-HIV activity. Sulfated polysaccharides isolated from various algae having anti-HIV activities such as carrageenans, galactan sulfate [16, 17]. Muschin and Budragchaa reported sulfated natural galactomannans and synthetic galactomannans having specific anticoagulant and antiviral activities [9,10].

The anti-HIV mechanism of sulfated polysaccharides could be considered the electrostatic interaction between negatively charged sulfate groups in sulfated polysaccharides and positively charged amino acids in the HIV surface glycoprotein gp120 (HIV gp120) by considering the blood anticoagulant mechanism of heparin. Uryu and Yoshida reported that the anti-HIV activity of sulfated polysaccharides could be estimated as the electrostatic interaction with the positively charged amino acid accumulated region at the C-terminus of the HIV gp120, Thr–Lys–Ala–Lys–Arg–Arg–Val–Val–Gln–Arg–Glu–Lys–Arg by a high-resolution NMR [18]. Kozbor suggesting the V3 domain was the most probable site for the interaction with curdlan sulfate because of having six positively charged amino acids, R304, K305, R306, R308, R311, and R315[19].

1.3. Influenza virus, anti-influenza virus mechanism of sulfated polysaccharides

1.3.1 Influenza virus

Influenza virus belongs to a family of RNA virus termed as Orthomyxoviridae. There are four genera of this family: types A, B, C and D. However, only genera A and B are clinically relevant for humans. While influenza B infections occur only in humans, influenza A viruses can also infect pig and horse. Influenza virus as an enveloped virus. Influenza viruses are assumed to be transmitted predominantly by aerosol infection. In addition, transmission can occur by direct contact with virus-contaminated surfaces and subsequent mouth-nose contact. After infection the viruses replicate in the nasal and laryngeal mucosae. This replication also affects the lower airways as the infection progresses [20]. Influenza virus is an enveloped virus with a genome made up of negative sense, single-stranded, segmented RNA. The Influenza A viruses have eight segments that encode for the 11 viral genes: hemagglutinin (HA), neuraminidase (NA),

matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1 – F2 (PB1-F2) [21].

1.3.2 Anti-influenza virus mechanism of sulfated polysaccharides

Hemagglutinin (HA) is a homo trimer that forms spikes on the viral lipid membrane. It is responsible for binding the virus to cells with sialic acid on the membranes, such as cells in the upper respiratory tract or erythrocytes [22]. The sialic acid is an essential component on the host cell surface for influenza A virus infection. Influenza A viruses do not bind to or infect cells treated with neuraminidase, which catalyzes removal of sialic acid residues [23]. In 1958, Gerber first time reported that sulfated polysaccharides extracted from sea alga had anti-influenza virus activity [2]. Also, many researchers reported naturally occurring polysaccharides and synthetic sulfated polysaccharides having anti-influenza virus activity [24].

1.4 Microwave assisted solid phase peptide synthesis (MW-SPPS)

Peptide synthesis is several amino acids are linked by amide bonds. Peptides are chemically synthesized by the condensation reaction of the carboxyl group of one amino acid to the amino group of another [25]. In 1963, Bruce Merrifield pioneered introduced the synthesis of a tetrapeptide was assembled by solid phase peptide synthesis method [26]. Since then, all aspects of SPPS have been further developed and refined. SPPS is defined by the set of Nα-protecting groups, side-chain protecting groups, coupling reagents, resins, and linkers [27].

The two most widely used Nα-protecting groups in SPPS are the fluoren-9-

ylmethyloxycarbonyl (Fmoc) and the tert-butoxycarbonyl (Boc), each defining an overall strategy for SPPS. The Boc strategy, initially introduced by Merrifield, the using trifluoroacetic acid (TFA) or a similar acid for removal of the Boc groups, while often used on hydrofluoric acid (HF) for cleavage of the assembled peptide from the support. The Fmoc group can be removed under mild conditions with secondary amines, typically 1:4 piperidine–DMF. Fmoc and Boc strategies have successfully been applied together with microwave-irradiation to increase reaction rates and crude peptide purity. However, many researchers reported to peptide synthesis by microwave-assisted SPPS, variants of the Fmoc strategy has been used [27].

SPPS is generally very compatible with elevated temperature, while especially in the coupling step. During the 1990s the use of microwave heating in organic synthesis evolved as a new parameter for improving both the reaction speed, yield and several special microwave instruments for organic synthesis became commercially available. Moreover, the data showed that the solid support did not degrade under the microwave-assisted synthesis.

In 2006 Brandt et al. reported the use of a Biotage Initiator for manual peptide synthesis, which was later developed further to perform semi-automated synthesis with manual addition of amino acids and automated washing and Nα-deprotection. MW-SPPS usually used reactor solvent is DMF and NMP. Many researchers reported for optimization condition of MW-SPPS such as coupling reagents, temperature and time [27-32].

Our study survey showed that the most common coupling reagents and additives used in MW-SPPS. The classical examples of in situ coupling reagents are N, N'-dicyclohexylcarbodiimide (DCC) and the related N, N'-diisopropylcarbodiimide. The generality of carbodiimide-mediated couplings is extended significantly by the use of

either 1-hydroxybenzotriazole (HOBT) or 1-hydroxy-7-azabenzotriazole (HOAT) [31]. Cysteine, histidine and aspartic acid were containing in the peptide sequence, maybe have occurred racemization and epimerization. So, that to the lowering microwave coupling temperature and addition of HOBT to the deprotection solutions for limiting epimerization and racemization [33].

1.5 Surface plasmon resonance

Surface plasmon resonance (SPR) has become an important optical biosensor technology in the areas of biochemistry, biology, and medical sciences because of its real-time, label-free, and noninvasive nature. In 1902, Wood first observed pattern of "anomalous" dark and light bands in the reflected light, when he shone polarized light on mirror on a diffraction grating on its surface. In 1983, Leidberg et al first demonstrated for application of SPR based sensors to biomolecular interaction monitoring. In 1990, Pharmacia Biosensor AB launched the first commercial SPR product, the Biacore instrument. Biacore instruments are becoming more and refined. The measurement of surface plasmon resonance (SPR) is one of the superior methods to analyze the interactions between biomolecules quantitatively [34].

Reference

[1] L.S. Costa, G.P. Fidelis, S.L. Cordeiro, R.M. Oliveira, D.A. Sabry, R.B.G. Câmara, L.T.D.B. Nobre, M.S.S.P. Costa, J. Almeida-Lima, E.H.C. Farias, E.L. Leite, H.A.O. Rocha, Biological activities of sulfated polysaccharides from tropical seaweeds, Biomed. Pharmacother. 64 (2010) 21–28.

[2] Gerber, P., Dutcher, J. D., Adams, E. V., & Sherman, J. H. Protective Effect of Seaweed Extracts for Chicken Embryos Infected with Influenza B or Mumps

virus.Proceedings of the Society for Experimental Biology and Medicine, 99(1958)590-593

[3] H.Nakashima, Y. Kido, N. Kobayashi, Y. Motoki, Purification and Characterization of an Avian Myeloblastosis and Human Immunodeficiency Virus Reverse Transcriptase Inhibitor , Sulfated Polysaccharides Extracted from Sea Algae, 31 (1987) 1524–1528

[4] G.Jiao, G. Yu, J. Zhang, H.S. Ewart, Chemical Structures and Bioactivities of Sulfated Polysaccharides from Marine Algae, (2011) 196–223.

[5] Rong Lu, Kazuyuki Hattori, Zuyong Xia, Takashi Yoshida, Jianhong Yang, Lina Zhang, Yumin Du, Tetsuo Miyakoshi, Toshiyuki Uryu, Structural Analysis of Polysaccharides in Chinese Lacquer by NMR Spectroscopy, Sen'I Gakkaishi,55(1999) 47-56.

[6] R. Lu, T. Yoshida, H. Nakashima, M. Premanathan, R. Aragaki, T. Mimura, Y. Kaneko, N. Yamamoto, T. Miyakoshi, T. Uryu, Specific biological activities of Chinese lacquer polysaccharides, 43 (2000) 47–54.

[7] Kaneko, Y, Yamamoto, N., and Uryu, T, Biological actions of polysaccharides and those derivatives against cancer and AIDS, Immuno. Prosp. Infect. Dis, (1990) 109-119.

[8] T. Yoshida, Synthesis of polysaccharides having specific biological activities, Prog. Polym. Sci. 26 (2001) 379–441.

[9] T. Muschin, D. Budragchaa, T. Kanamoto, H. Nakashima, International Journal of Biological Macromolecules Chemically sulfated natural galactomannans with specific antiviral and anticoagulant activities, Int. J. Biol. Macromol. 89 (2016) 415–420.

[10] D. Budragchaa, S. Bai, T. Kanamoto, H. Nakashima, Synthetic galactomannans with potent anti-HIV activity, 130 (2015) 233–242.

[11] P.B. Gilbert, I.W. McKeague, G. Eisen, C. Mullins, A. Guéye-NDiaye, S. Mboup, P.J. Kanki, Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal, Stat. Med. 22 (2003) 573–593.

[12] C.B. Wilen, J.C. Tilton, R.W. Doms, HIV: Cell binding and entry, Cold Spring Harb. Perspect. Med. 2 (2012) 1–14.

[13] H. Nakashima, O. Yoshida, T. Tochikura , T. Yoshida, T. Mimura, Y. Kido, Y. Motoki,Y. Kaneko, T. Uryu, N. Yamamoto, Sulfation of polysaccharides generates potent and selective inhibitors in vitro. Jpn. J. Cancer Res. (Gan) 78 (1987) 1164–1168.

[14] T. Yoshida, K. Hatanaka, T. Uryu, Y. Kaneko, N. Yasuda, T. Mimura, O. Yoshida, N. Yamamoto, Synthesis and structural analysis of curdlan sulfate with potent anti–AIDS virus activity. Macromolecules 23 (1990) 3717–3722.

[15] T. Yoshida, H. Nakashima, N. Yamamoto, T. Uryu, Anti-AIDS virus activity in vitro of dextran sulfates by sulfation of synthetic and natural dextrans. Polymer J. 25 (1993)1069−1077.

[16] Yamada, A. Ogamo, T. Saito, J. Watanabe, H. Uchiyama, and Y. Nakagawa, “Preparation and anti-HIV activity of low-molecular-weight carrageenans and their sulfated derivatives, Carbohydrate Polymers, 32(1997)51–55.

[17] M. Witvrouw, J. A. Este, M. Q. Mateu et al., “Activity of a sulfated polysaccharide extracted from the red seaweed Aghardhiella tenera against human immunodeficiency virus and other enveloped viruses,” Antiviral Chemistry and Chemotherapy, 5(1994)297–303.

[18] T. Uryu, N. Ikushima, K. Katsuraya, T. Shoji, N. Takahashi, T. Yoshida, K. Kanno, T. Murakami, H. Nakashima, N. Yamamoto, Sulfated alkyl oligosaccharides with potent inhibitory effects on human immunodeficiency virus infection, Biochem. Pharmacol. 43 (1992) 2385–2392.

[19] P. P. Jagodzinski, J. Wustner, D. Kmieclak, T. J. Wasik, A. Fertala, A. L. Sieron, M. Takahashi, T. Tsuji, T. Mimura, M. S. Fung, M. K. Gorny, M.Kloczewiak, Y. Kaneko, D. Kozbor, Role of the V2, V3, and CD4–binding domains of gp120 in curdlan sulfate neutralization sensitivity of HIV–1 during infection of T lymphocytes. Virology 226 (1996) 217–227.

[20] Arbeitskreis Blut, Untergruppe, Influenza Virus., Transfus. Med. Hemother. 36 (2009) 32–39.

[21] T. Samji, Influenza A : Understanding the Viral Life Cycle, 82 (2009) 153–159.

[22] R.J. Russell, P.S. Kerry, D.J. Stevens, D.A. Steinhauer, S.R. Martin, S.J. Gamblin, J.J. Skehel, Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 17736–17741.

[23] K.A. Brogden, The sweet spot: defining virus–sialic acid interactions Jennifer, Nat Rev Microbiol. 3 (2005) 238–250.

[24] A. Ahmadi, S.Z. Moghadamtousi, S. Abubakar, K. Zandi, Antiviral Potential of Algae Polysaccharides Isolated from Marine Sources : A Review, 2015 (2015).

[25] M. Amblard, J. Fehrentz, J. Martinez, G. Subra, Methods and Protocols of Modern Solid Phase Peptide Synthesis, 33 (2006)

[26] Merrifield RB(1963). "Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide".J. Am. Chem. Soc.85(14): 2149–2154.

[27] S.L. Pedersen, A.P. Tofteng, L. Malik, K.J. Jensen, Chem Soc Rev Microwave heating in solid-phase peptide synthesis, (2012) 1826–1844.

[28] R. Microwave-as, S. Phase, P. Synthesi, Rapid Microwave-Assisted Solid Phase Peptide Synthesis, (2002) 1592–1596.

[29] C.O. Kappe, Controlled Microwave Heating in Modern Organic Synthesis Angewandte, (2004) 6250–6284.

[30] B. Bacsa, B. Desai, G. Abor, Rapid solid-phase peptide synthesis using thermal and, (2006).

[31] M. Bradley, M.A. Fara, J. Jose, Microwave-assisted coupling with DIC / HOBt for the synthesis of difficult peptoids and fluorescently labelled peptides — a gentle heat goes a long way, 47 (2006) 1011–1014.

[32] V. Mäde, S. Els-heindl, A.G. Beck-sickinger, Automated solid-phase peptide synthesis to obtain therapeutic peptides, (2014) 1197–1212.

[33] S.A. Palasek, Z.J. Cox, J.M. Collins, Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis, (2007) 143–148.

[34] R. B. M. Schasfoort, A. J. Tudos, Ed., Handbook of surface plasmon resonance. RSC Publishing, The Royal Society of Chemistry (2008), Cambridge, UK.

CHAPTER 2.

Interaction mechanism between sulfated polysaccharides and HIV oligopeptides analyzed by SPR

ABSTRACT

This study aims to quantitatively investigate the interaction between sulfated polysaccharides with potent anti-HIV activity, dextran and curdlan sulfates with negatively charged sulfate groups, and poly-L-lysine as a model protein and oligopeptides from a HIV surface glycoprotein gp120 with positively charged amino acids using surface plasmon resonance (SPR) and dynamic light scattering (DLS) to elucidate the anti-HIV mechanism of sulfated polysaccharides. The apparent association- (k_a) and dissociation rate (k_d) constants of dextran and curdlan sulfates against poly-L-lysine were k_a=6.92×10^4–2.17×10^6 1/Ms and k_d=4.29×10^{-5}– 2.22×10^{-4} 1/s; these kinetic constants were dependent on the molecular weights and degree of sulfation of sulfated polysaccharides. For interaction, the three oligopeptides from the HIV gp120 were peptide A ^{297}TRPNNNTRKRIRIQRGPGRA^{316} with several lysine (K) and arginine (R) in the V3 loop region, peptide B ^{493}PLGVAPTKAKRRVVQREKR^{511} with several K and R in the C-terminus region, and oligopeptide C ^{362}KQSSGGDPEIVTHSFNCGG^{380} with little basic amino acids in the CD4 binding domain. Sulfated polysaccharides exhibited strong interaction against oligopeptides A and B, (k_a=5.48×10^4–2.96×10^6 1/Ms and k_d=2.25×10^{-4}–1.27×10^{-3} 1/s), no interaction was noted against oligopeptide C. Moreover, the particle size and zeta potential by DLS indicated the interaction between sulfated polysaccharides and oligopeptides A and B, suggesting the anti-HIV mechanism of sulfated polysaccharides to be the electrostatic interaction of negatively charged sulfated polysaccharides and

HIV at the positively charged amino acid regions.

2.1. Introduction

Since our first report on the anti-HIV activity of sulfated synthetic polysaccharides [1], we have investigated the anti-HIV activity of sulfated polysaccharides obtained by sulfation of both natural and synthetic polysaccharides [2, 3].

The anti-HIV mechanism of sulfated polysaccharides could be considered the electrostatic interaction between negatively charged sulfate groups in sulfated polysaccharides and positively charged amino acids in the HIV surface glycoprotein gp120 (HIV gp120) by considering the blood anticoagulant mechanism of heparin[4], a sulfated natural polysaccharide with potent antithrombotic activity that induced the electrostatic interaction with antithrombin III [5, 6]. Uryu and Yoshida reported that the anti-HIV activity of sulfated polysaccharides could be estimated as the electrostatic interaction with the positively charged amino acid accumulated region at the C-terminus of the HIV gp120, Thr–Lys–Ala–Lys–Arg–Arg–Val–Val–Gln–Arg–Glu–Lys–Arg[4]. To elucidate the interaction, Uryu and Yoshida used a high-resolution NMR [4]. When the mixture of curdlan sulfate and oligopeptide was prepared in the proportion of 0.27, a gel formation was observed and the ^{1}H-NMR spectra revealed the electrostatic interactions under acidic to weakly acidic conditions [7, 8]. Kozbor proposed the interaction of curdlan sulfate with monomeric and oligomeric gp120 mutants, which was analyzed by using a molecular modeling Chem−X software[9], suggesting that the V3 domain was the most probable site for the interaction with curdlan sulfate because of having six positively charged amino acids, R304, K305, R306, R308, R311, and R315.

The measurement of surface plasmon resonance (SPR) is one of the superior methods to analyze the interactions between biomolecules quantitatively [10]. A study

investigated the binding affinity of heparin-proteins, such as lactoferrin, antithrombin III, avidin, and thrombin, by SPR measurements [11]. Biotinylated heparin derivatives immobilized on a Biacore streptavidin sensor chip SA had a high binding affinity, probably because the binding of proteins occurred at the intrachain bare amines or carboxyl groups of uronic acids in heparin. In addition, the authors measured the anticoagulant activity of immobilized heparin on the sensor chip SA against antithrombin III by the SPR. The results were comparable to the chromogenic assay and the SPR method was found to be preferred for time, cost, and easy procedures [12]. In addition, we also reported that a naturally occurring acidic polysaccharide, lacquer polysaccharide with glucuronic acids at the terminals of the complex branches, exhibits specific biological activities including the promotion of blood coagulant and antitumor activities [13]. Moreover, the interaction with poly-L-lysine as a model protein was measured using SPR to elucidate the biological activities. The interaction was dependent on the molecular weights and was found to be comparable to the specific biological activities of lacquer polysaccharides [14]. Furthermore, sulfated natural and synthetic galactomannans were reported to have potent anti-HIV and anti-dengue virus activities, the antiviral activities of which were analyzed by SPR measurements [15, 16].

2.2. Experimental

2.2.1 Materials

We purchased Biacore CM5 and SA sensor chips, an amine coupling kit containing N-hydroxyl succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 1.0 M ethanolamine hydrochloride solution (pH 8.5), and HBS−EP buffer solution for SPR measurements from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Poly-L-lysine hydrobromide

(M_W=1000−5000), dextran sulfates, and poly(sodium 4-styrenesulfonate) were obtained from Sigma−Aldrich, Japan. Dextran and heparin sodium salt were purchased from Nacalai Tesque (Tokyo, Japan). Sulfurtrioxide−pyridine complex (SO_3−Py) was purchased from TCI (Tokyo, Japan). Other dextran sulfates were purchased by sulfation of dextran with the SO_3−Py complex as reported previously [17]. Of note, curdlan sulfate was kindly gifted by Ajinomoto Co. Ltd. Furthermore, for the synthesis of oligopeptides, Fmoc-amino acids were purchased from Watanabe Chemical IND., LTD (Hiroshima, Japan). Other for the peptide synthesis reagents were purchased Tokyo Chemical IND., LTD (Tokyo, Japan) and Sigma-Aldrich.

2.2.2 Measurement

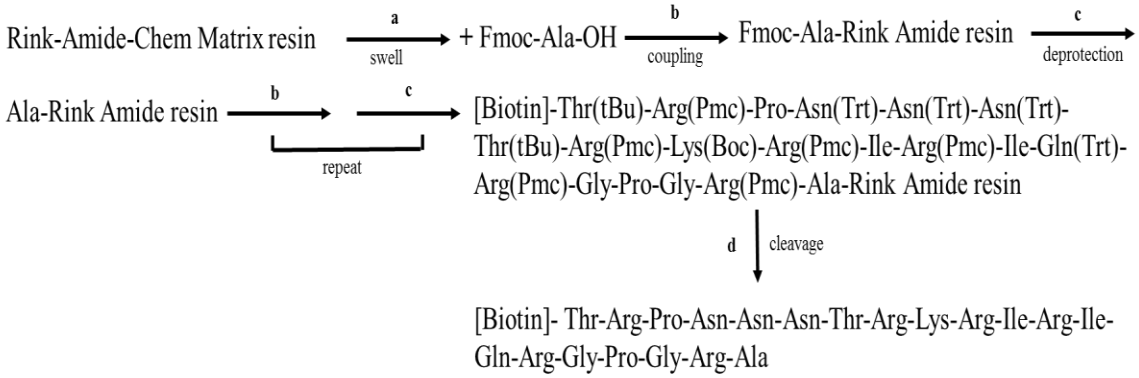
We used a Biacore X100 to measure the SPR spectrum using a CM5 sensor chip immobilized poly-L-lysine and an SA sensor chip for biotinylated oligopeptides, respectively. A DLS and zeta potential were measured at 25°C by an Otsuka Electronics ELSZ−1000ZS zeta potential and particle size analyzer in the HBS-EP buffer solution (pH7.4) at the concentrations of 1 or 0.5 mg/ml of dextran and curdlan sulfates and poly-L-lysine. We determined the average molecular weights of sulfated polysaccharides using an aqueous-phase GPC equipped with TOSOH TSK-gel columns (7.6 mm×600 mm) of G2500PW_{XL}, G3000PW_{XL}, and G4000PW_{XL} eluted with 66.7 mmol phosphate buffer solutions at 40°C, and detected the signals using an RI detector. In addition, standard pullulans were used to evaluate the molecular weights. The specific rotation was measured by a JASCO DIP−140 digital polarimeter in H_2O at 25°C in a quartz cell. Furthermore, oligopeptides were synthesized using a Biotage Initiator+ SP Wave peptide synthesizer.

2.2.3. Synthesis of oligopeptides referenced by HIV gp120 sequence

The HIV gp120 has 511 amino acids and is composed of several domains. In this study, three oligosaccharides were synthesized from the sequence of the V3 loop, C-terminus, and CD4 binding domain in the HIV gp120. Because we previously estimated that the interaction between sulfated polysaccharides and the HIV gp120 occurred at the positively charged regions at the C-terminus in the HIVgp120 [4]. Kozbor assumed that the V3 loop could be a binding domain with curdlan sulfate [9]. Oligopeptide A from the V3 loop comprises 20 amino acids with seven positively charged lysine and arginine in the sequence. The basic amino acids were relatively dispersed along the sequence compared with that of oligopeptide B. Likewise, oligopeptide B from the C–terminus comprises seven lysine and arginine, which were continuously connected behind the sequence. Oligopeptide C from the CD4 binding domain comprises one lysine next to the biotin. The three oligopeptides were synthesized by a microwave assisted solid phase peptide synthesizer using Fmoc-amino acids, and, finally, the N-terminus of the oligopeptides was biotinylated for immobilization on the sensor chip SA.

Peptide synthesis was using Rink Amide-Chem Matrix resin 96.3 mg (0.51 mmol/g, 0.5 mmol) was placed in a peptide synthesis vessel, swollen in DMF 2 hours at the room temperature. After washing by DMF, a solution of 4 equiv. Fmoc-AA-OH, 4 equiv HOBT, and 4 equiv DIC were dissolved in DMF added to the peptide synthesis vessel. Then the mixture was coupling under microwave irradiation for 5 min and washed by DMF three times. Deprotection was by 25% piperidine in DMF for 3 min and 10 min at under room temperature, respectively. Kaiser test was applied to examine if there was free amino group existence. Then crude peptide was yielded after cleaving of the peptide with 10 mL Reagent R (TFA/Thioanisole/ EDT/ Anisole 90:5:3:2, V/V) for 120

min at room temperature [18]. The synthetic route was shown in scheme 1. Peptide was precipitated by addition cold ethyl ether; after centrifugation, the ether was removed, and this process was repeated three times. We purified the synthesized oligopeptides by a RP-HPLC. We confirmed the structures using MALDI TOF MS (Bruker Ultra Flex III) and LC/MS/MS (AB Science Inc. API 4000QTrap), suggesting that the calculated molecular weights agreed the observed values. These three oligopeptides were used for the interaction between dextran and curdlan sulfates with potent anti-HIV activity.



Scheme 1. Synthetic route of oligopeptide A of HIV gp 120 .

Reagents and conditions: (a) DMF, room temperature, 2 hour swelling; (b) Fmoc-AA-OH, DIC, HOBT, 75^{o}C temperature, microwave irradiation; (c) 25% piperidine/DMF (V/V), room temperature; (d) Reagent R, room temperature, 2 hour.

2.2.4. Immobilization of poly-L-lysine and biotinylated oligopeptides

The immobilization of poly-L-lysine and HIV oligopeptides was conducted according to the Biacore-provided a protocol. Poly-L-lysine was immobilized on the CM5 sensor chip by the amine coupling method. Briefly, a new CM5 sensor chip was set in the Biacore X100 instrument and then activated by the mixed solution of NHS and EDC. Next, poly-L-lysine (1000μg/ml) was dissolved in acetate buffer (pH5.5) and the solution (70 μL) was injected to the Biacore flow cell; the flow rate was 10 μL/min. Finally, the unreacted NHS-activated moieties were blocked by the injection of 1.0-M

ethanolamine solution (pH 8.5). The proportion of immobilized poly-L-lysine on the CM5 sensor chip was around 2000 response unit (RU) by the Biacore X100 instrument.

Then, biotinylated oligopeptides were immobilized on the sensor chip SA using affinity of biotin and streptavidin. Notably, the proportion of HIV oligosaccharides on the sensor chip SA was around 945−1131 RU.

2.2.5 Interaction of dextran and curdlan sulfates with poly-L-lysine and oligopeptides

The interaction between dextran sulfate and poly-L-lysine was executed by a multicycle analysis method based on the Biacore-provided a protocol. The flow rate of buffers was adjusted to 30 μL/min. In addition the concentration of dextran sulfate was adjusted to 0.313, 0.625, 1.25, 2.50, and 5.00 μg/ml using the HBS−EP running buffer solution. After three blank runs with the running buffer, the dextran sulfate solution was injected in the flow cell for 120 s; then, the running buffer (HBS−EP) was flowed for 480 s to measure the association- (k_a) and dissociation (k_d) rate constants. After 600 s, the sensor chip was washed and regenerated with 50-mM NaOH solution (45 s) and then the running buffer HBS−EP flowed for 180 s. The k_a and k_d were calculated by the Biacore-provided BIA evaluation software and 1:1 binding fitting model. The interaction between sulfated polysaccharides and HIV oligopeptides was measured by the same procedures as described above.

2.2.6 Particle size and zeta potential of poly-L-lysine and oligopeptides with sulfated polysaccharides

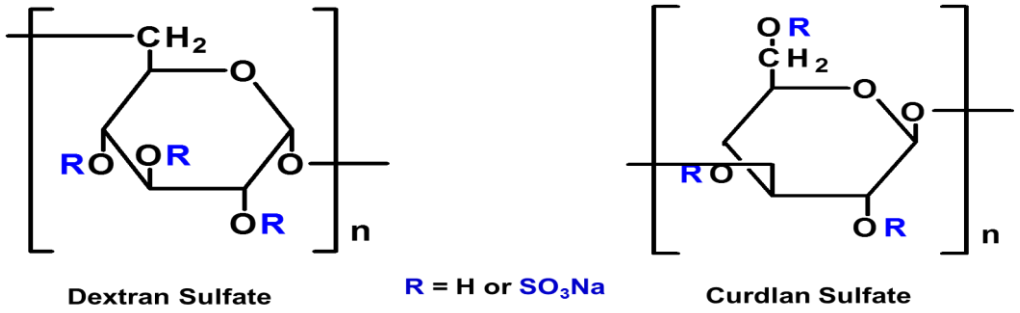
The dynamic light scattering (DLS) and zeta (ʒ) potential were measured at 25°C in HBS-EP buffer solution (pH=7.4) by an Otsuka Electronics ELSZ-1000ZS

particle size and zeta potential analyzer. The ultrasonicated the buffer solution of sulfated polysaccharides (0.5 or 1 mg/ml) with poly-L-lysine (0.5 or 1 mg/ml) respectively, then the sample was measured.

2.3. Results and discussion

2.3.1. Dextran and curdlan sulfates with different molecular weights and degree sulfation

We used dextran and curdlan sulfates as a model sulfated polysaccharides having a potent anti-HIV activity to elucidate the anti-HIV mechanism. In addition, three commercially available dextran sulfates with the number average molecular weights ($\overline{M}_n$) and the degree of sulfation (DS) ($\overline{M}_n$=1.31x10^4, 1.65x10^4, and 2.3x10^4, and 0.59, 0.69, and 0.69) were used for measuring SPR. Other dextran sulfates, as shown in Table 2, were synthesized by sulfation of dextran as reported previously [17], and dextran sulfates were classified into three groups based on the molecular weights. The $\overline{M}_n$<4.72x10^4 was low, $\overline{M}_n$>6.02x10^4 was middle, and $\overline{M}_n$>15.9x10^4 was high molecular weights. The molecular weight distribution was not so wide, ($\overline{M}_w/\overline{M}_n$=1.15−2.84), and the DS was between 0.2 and 1.59. The apparent kinetic constants of dextran sulfates were measured by using SPR. Curdlan sulfate with $\overline{M}_n$= 6.84x10^4, $\overline{M}_w/\overline{M}_n$=1.41, and DS=1.29 was used for the interaction with oligopeptides from the HIV gp120. Furthermore, poly-L-lysine as a model peptide of the HIV gp120 and three HIV oligopeptides synthesized were used for measuring the interaction with sulfated polysaccharides.



Scheme 2. Chemical structure of Dextran sulfate and Curdlan sulfate

2.3.2 Synthesis of oligopeptides referenced by the HIV gp120 sequence

In this study, three oligopeptides were synthesized from the sequence of the V3 loop, C-terminus, and CD4 binding domain in the HIV gp120, as shown in Table 1, The three oligopeptides were synthesized by a microwave assisted solid-phase peptide synthesizer using Fmoc-amino acids, and, finally, the N-terminus of the oligopeptides was biotinylated for immobilization on the sensor chip SA. We purified the synthesized oligopeptides by a RP-HPLC. Oligopeptide A from the V3 loop comprises 20 amino acids with seven positively charged lysine and arginine in the sequence. The basic amino acids were relatively dispersed along the sequence compared with that of oligopeptide B. Likewise, oligopeptide B from the C–terminus comprises seven lysine and arginine, which were continuously connected behind the sequence.

Table 1. Biotinylated oligopeptides from HIV gp120

Name	Position	Sequence[a]	AA[b]	Molecular weight[c]		Remark
				Calcd	Obsd	
Peptide A	V3 loop	[Biotin]-^{297}TRPNNNTRKRIRIQRGPGRA^{316}	20	2587.35	2586.75	Lys/Arg rich
Peptide B	C-terminus	[Biotin]-^{493}PLGVAPTKAKRRVVQREKR^{511}	19	2415.34	2414.95	Lys/Arg rich
Peptide C	CD4 binding domain	[Biotin]-^{362}KQSSGGDPEIVTHSFNCGG^{380}	19	2145.85	2145.25	Lys/Arg poor

a) Positively charged amino acid was marked by underline.

b) Number of amino acid.

c) The structure of peptides was confirmed by using MALDI TOF MS.

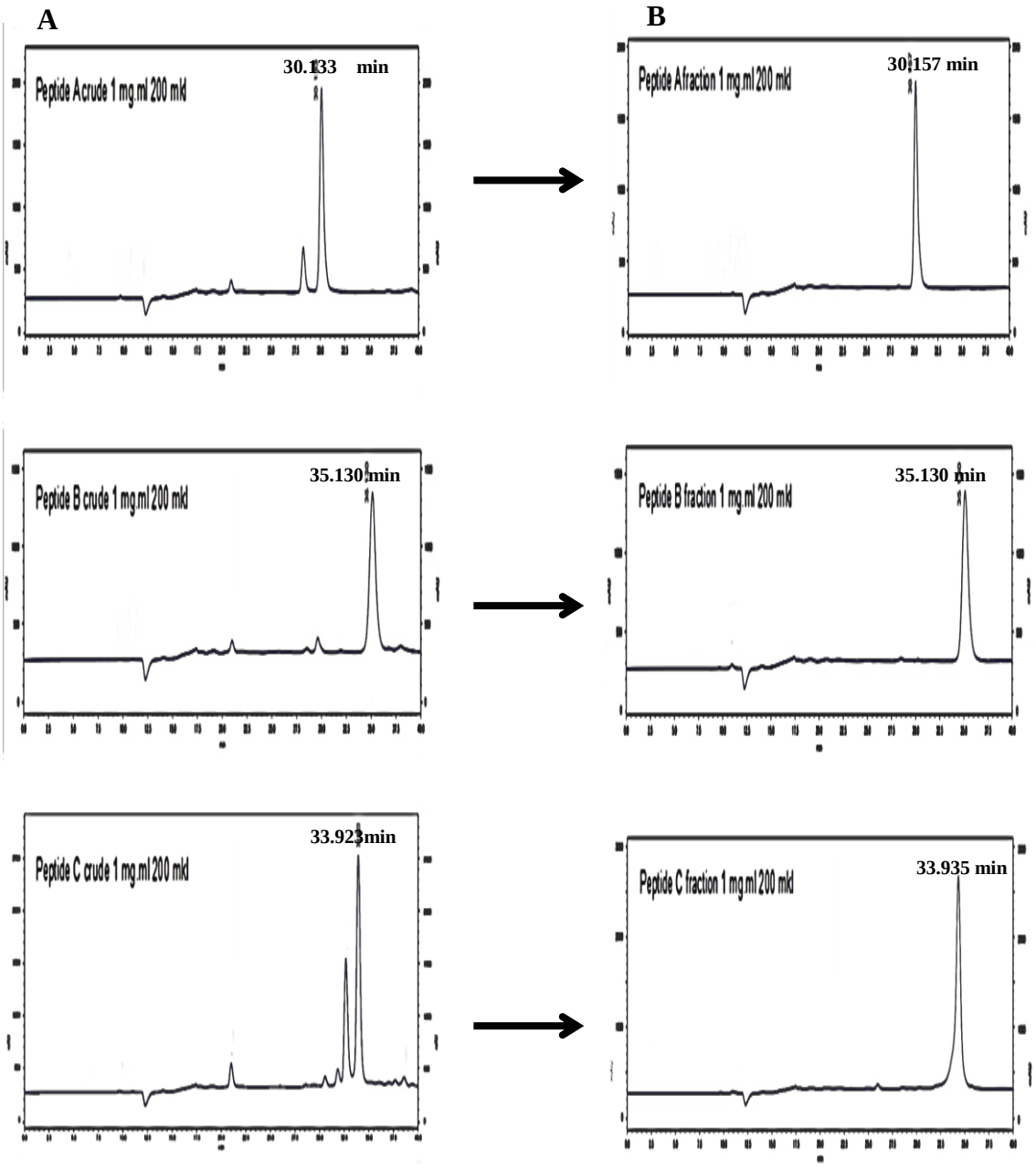


Figure 2. RP-HPLC profiles of purification of synthetic crude oligopeptides of HIV gp120.

(A) Crude oligopeptides, (B) Purified oligopeptides

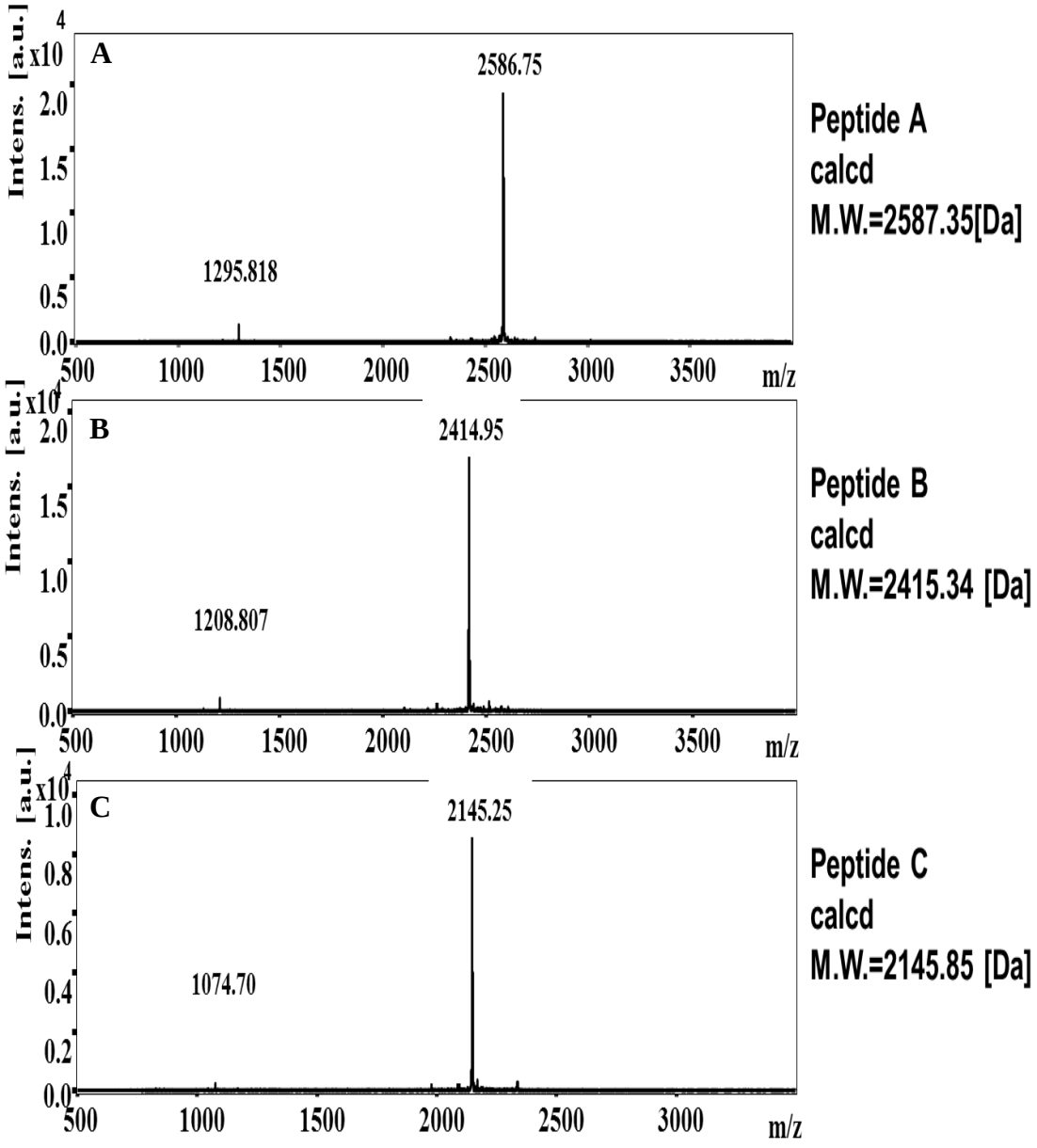


Figure 3. MALDI TOF MS profiles of oligopeptides of HIV gp 120 (A) Oligopeptide A, (B) Oligopeptide B, (C) Olgiopeptide C

2.3.3. Interaction of dextran sulfates with poly-L-lysine.

Using SPR measurements, we measured the interaction of poly-L-lysine having positively charged amino acids and dextran sulfates with various molecular weights and the DS. Sulfated polysaccharides, for example, dextran sulfate, has negatively charged sulfate groups.

Table 2. Interaction between dextran sulfates and poly-L-lysine[a, b]

	Dextran sulfate					Apparent kinetic constant[c]		
No	$\overline{M}_n$[d] ×10^{-4}	$\overline{M}_w/\overline{M}_n$	$[\alpha]_D^{25}$	S %	DS[e]	k_a 1/Ms	k_d 1/s	K_D nM
1	1.31	1.15	+87.0	18.6	0.59	6.66×10^{5}	2.22×10^{-4}	3.33×10^{-10}
2	1.65	1.31	+79.9	20.8	0.69	2.34×10^{5}	1.97×10^{-4}	8.42×10^{-10}
3	3.11	1.76	+147.9	7.59	0.20	6.92×10^{4}	1.72×10^{-4}	2.48×10^{-9}
4	4.72	1.96	+121.8	21.4	0.73	5.71×10^{5}	1.48×10^{-4}	2.59×10^{-10}
5	6.02	1.78	+103.8	29.4	1.20	9.08×10^{5}	1.44×10^{-4}	1.59×10^{-10}
6	6.58	1.72	+93.5	34.1	1.59	1.72×10^{6}	1.37×10^{-4}	7.97×10^{-11}
7	6.23	1.94	+154.3	9.94	0.27	8.95×10^{4}	1.46×10^{-4}	1.56×10^{-9}
8	7.47	2.07	+142.0	13.7	0.40	6.59×10^{5}	2.00×10^{-4}	3.03×10^{-10}
9	9.51	2.02	+124.5	22.5	0.78	2.17×10^{6}	1.85×10^{-4}	8.53×10^{-11}
10	10.6	1.92	+122.6	28.1	1.11	1.90×10^{6}	1.67×10^{-4}	8.79×10^{-11}
11	15.9	2.60	+152.2	9.03	0.24	3.68×10^{5}	9.70×10^{-5}	2.64×10^{-10}
12	24.2	2.71	+137.5	16.5	0.51	6.12×10^{5}	6.46×10^{-5}	1.06×10^{-10}
13	32.5	2.84	+108.6	31.0	1.33	1.33×10^{6}	6.40×10^{-5}	4.81×10^{-11}
14	36.9	2.34	+109.0	30.2	1.26	1.26×10^{6}	4.29×10^{-5}	3.40×10^{-11}

a)Dextran sulfate was obtained by sulfation of dextran with piperidine−N−sulfuric acid.

b)Dextransulfates were classified into three groups by low (Nos. 1−4), middle (Nos.5−10), and high

(Nos.11−14) molecular weights

c)Apparent association-rate (k_a), dissociation-rate (k_d), and dissociation (K_D) constants were measured by a Biacore X100 SPR apparatus and used 1:1 binding fitting model for the calculation.

Table 2 shows the apparent k_a and k_d of dextran sulfates with poly-L-lysine as a model peptide of the HIV gp120 immobilized on the sensor chip CM5 using an amine coupling method. The apparent kinetic constants, k_a and k_d, were evaluated by the 1:1 binding model per the Biacore provided a protocol. Regarding the correlation between the molecular weights and the kinetic constants, it was found that the larger the molecular weights, the stronger the interaction. For the low-molecular-weight dextran sulfates with lower DS as shown in nos. 3, 7, and 11, the k_a and k_d were relatively lower and higher values, (k_a=6.92x10^4–3.68x10^{5}1/Ms and k_d=1.72x10^{-4}–9.70x10^{-5}1/s), compared with dextran sulfates with higher DS. When the DS increased to 0.73 as shown in no. 4, the k_a and k_d were found to exhibit relatively higher and lower values, (k_a=5.71x10^{5}1/Ms and k_d=1.48x10^{-4} 1/s), suggesting that the interaction increased. In nos. 5, 6, 10, 13, and 14, dextran sulfates with higher DS exhibited a strong interaction with poly-L-lysine, namely, these dextran sulfates exhibited higher k_a and lower k_d. Conversely, the high-molecular- weight dextran sulfates with the high DS (nos. 13 and 14) exhibited the highest k_a, (k_a=1.33x10^{6}1/Ms and k_a=1.26x10^{6}1/Ms), and the lowest k_d, (k_d=6.40x10^{-5} 1/s and k_d=4.29x10^{-5} 1/s), suggesting that the molecular weights and the DS of sulfated polysaccharides plays a vital role in the electrostatic interaction of poly-L-lysine; the interaction exhibited the same tendency of potent anti-HIV activity of sulfated polysaccharides [19].

Fig. 4 provides the plots of the data as shown in Table 2. While Fig. 4A presents the correlation between the k_a and k_d, Fig. 4B shows the correlation between the DS and these kinetic constants of dextran sulfate.

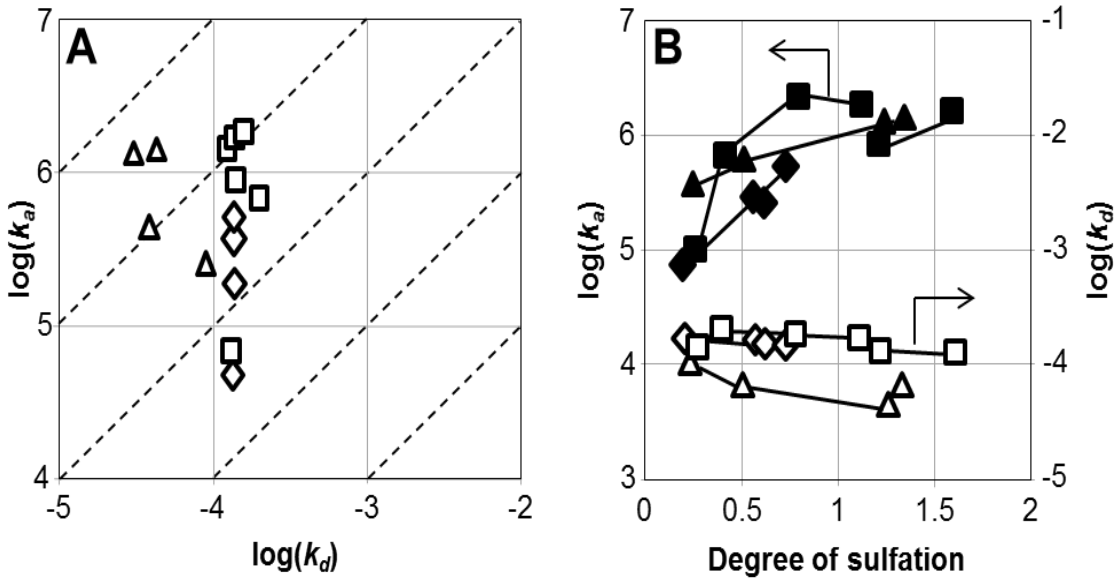


Figure 4. Interaction between dextran sulfates and poly-L-lysine.

(A) Relationship of association- (k_a) and dissociation rate (k_d) constants of dextran sulfates with poly-L-lysine.

(B) Relationship of the DS of dextran sulfates and kinetic constants, k_a and k_d.

The molecular weight of dextran sulfate is as follows; △▲: High-molecular-weight of M_n=15.9×10^4–36.9×10^4, □■: Medium-molecular-weight of M_n=6.02×10^4–10.6×10^4, and ◇◆: Low-molecular-weight of M_n=1.31×10^4–4.72×10^4, respectively.

In Fig. 4A, the k_d without high-molecular-weight (open rhombus and square) dextran sulfates had almost the same order of 10^{-4} 1/s and the high-molecular-weight dextran sulfates had lower k_d in the order of 10^{-5} 1/s. The k_a increased with increasing the molecular weights and, in particular, dextran sulfate with the high DS exhibited higher k_a.Fig. 4B presents the correlation between the k_a(closed symbols) and k_d (open symbols) constants against the DS of dextran sulfate. The k_a increased with increasing the DS of dextran sulfate, whereas the k_d decreased with increasing the DS, suggesting that the DS was an essential factor for the potent anti-HIV activity of sulfated polysaccharides. These SPR results corroborated the results of the potent anti-HIV activity of sulfated polysaccharides [19]. Table 3 shows the interaction between sulfated polysaccharides and poly-L-lysine, in which dextran without sulfation exhibited no

electrostatic interaction with poly-L-lysine.

Table 3. Interaction between dextran and sulfated polysaccharides and poly-L-lysine

	Polysaccharide					Apparent kinetic constant[a]		
No	$\overline{M}_n$[b]	$\overline{M}_w/\overline{M}_n$	$[\alpha]_D^{25}$	S	DS[c]	k_a	k_d	K_D
	$\times 10^{-4}$			%		1/Ms	1/s	nM
Dextran								
1	2.46	1.76	+173.8	–	–	–	–	–
2	6.08	1.64	+173.1	–	–	–	–	–
3	17.1	1.54	+175.0	–	–	–	–	–
Curdlan sulfate								
4	6.84	1.41	-0.5	30.5	1.29	1.22×10^{6}	1.82×10^{-4}	1.49×10^{-10}
Heparin								
5	4.18	1.21	+51.9	26.3	1.02	6.49×10^{6}	2.79×10^{-4}	4.30×10^{-11}
Polystyrene sulfate								
6	2.49	1.69	–	50.0	–	2.58×10^{5}	1.28×10^{-4}	4.96×10^{-11}

a) Apparent association-rate (k_a), dissociation-rate (k_d), and dissociation (K_D) constants were measured bya Biacore X100 SPR apparatus and used 1:1 binding fitting model for the calculation.

b) Number average molecular weight $\overline{M}_n$ was determined by GPC.

c) Degree of sulfation (DS) was calculated from the elementary analysis.

For curdlan sulfate with potent anti–HIV activity and heparin with blood anticoagulant activity, the k_a and k_d had high and low values in the order of 10^6 and 10^{-4}, respectively, suggesting that these sulfated polysaccharides had the strong electrostatic interaction with poly-L-lysine; these results were consisted with the results

of their potent biological activities. Conversely, polystyrene sulfate, a sulfated polymer, also had a strong interaction. Thus, the electrostatic interaction was found to be an essential factor for the anti-HIV activity of sulfated polysaccharides.

2.3.4 Interaction of oligopeptides with dextran and curdlan sulfates

We quantitatively analyzed the interaction of oligopeptides from the HIV gp120 with dextran and curdlan sulfates using the SPR measurement to elucidate the anti-HIV mechanism of sulfated polysaccharides. Table 4 presents the results of the interaction of oligosaccharides A, B, and C immobilized on the sensor chip SA and dextran and curdlan sulfates.

Table 4. Interaction between sulfated polysaccharides and HIV gp120 oligopeptides[a, b]

	Dextran sulfate						Apparent kinetic constant[c]		
No	$\overline{M}_n$[d]	$\overline{M}_w/\overline{M}_n$	$[\alpha]_D^{25}$	S	DS[e]		k_a	k_d	K_D
	×10^{-4}				%		1/Ms	1/s	nM
Dextran sulfate									
1	1.31	1.15	+87.0	18.6	0.59	Peptide A	5.48×10^{4}	6.24×10^{-3}	1.14×10^{-7}
2						Peptide B	1.43×10^{5}	6.90×10^{-4}	4.84×10^{-9}
3						Peptide C	–	–	–
4	1.65	1.31	+79.9	20.8	0.69	Peptide A	2.92×10^{5}	1.27×10^{-3}	4.35×10^{-9}
5						Peptide B	3.73×10^{5}	3.84×10^{-4}	1.03×10^{-9}
6						Peptide C	–	–	–
7	2.30	1.20	+86.3	20.6	0.69	Peptide A	2.39×10^{5}	2.52×10^{-4}	1.06×10^{-9}
8						Peptide B	8.69×10^{5}	2.72×10^{-4}	3.13×10^{-10}

9						Peptide C	–	–	–
Curdlan sulfate									
10	6.84	1.41	-0.5	30.5	1.29	Peptide A	5.69×10^{5}	2.25×10^{-4}	3.95×10^{-10}
11						Peptide B	2.96×10^{6}	1.74×10^{-4}	5.90×10^{-11}
12						Peptide C	–	–	–

a)Oligopeptides were synthesized by sequences of HIV gp120 regions, V3 loop, C-terminus, and CD4-binding domain, respectively.

Peptide A: [Biotin]-^{297}TRPNNNTRKRIRIQRGPGRA^{316},

Peptide B: [Biotin]-^{493}PLGVAPTKAKRRVVQREKR^{511},

Peptide C: [Biotin]-^{362}KQSSGGDPEIVTHSFNCGG^{380}.

The underline shows basic amino acid.

b)Dextran and curdlan sulfates were synthesized by sulfation of dextran and curdlan, respectively, with piperidine−N−sulfuric acid.

c)Apparent association-rate (k_a), dissociation-rate (k_d), and dissociation (K_D) constants were measured bya Biacore X100 SPR apparatus and used 1:1 binding fitting model for the calculation.

d)Number average molecular weight $\overline{M}_n$ was determined by GPC.

e)Degree of sulfation (DS) was calculated from the elementary analysis.

Dextran sulfates in Table 4 had almost the same DS. From the k_a and k_d, the interaction of dextran sulfates against oligopeptide B was marginally higher than that against oligopeptide A, suggesting that oligopeptides B having a continuous connection of basic amino acids in the sequence exhibited higher interaction. Conversely, the higher the molecular weights of dextran sulfates, the higher the interaction;however, the difference of the interaction was not high. Previously, we reported that sulfated polysaccharides could have a strong interaction with positively charged amino acids residues in the C-terminus of the HIV gp120, the sequence from 499 to 511, TKAKRRVVQREKR [4]. The SPR results in Table 4 corroborated our previous estimates on the anti-HIV mechanism of sulfated polysaccharides. Dextran sulfates exhibited no interaction with oligopeptide C in the CD4 binding site of HIV, because

oligopeptide C comprised few basic amino acids. Hence, the anti-HIV activity of sulfated polysaccharides should be induced by the electrostatic interaction. Fig 5 and 6 shows the SPR profiles of curdlan sulfate and dextran sulfates with oligopeptides A and B immobilized on the sensor chip SA, respectively.

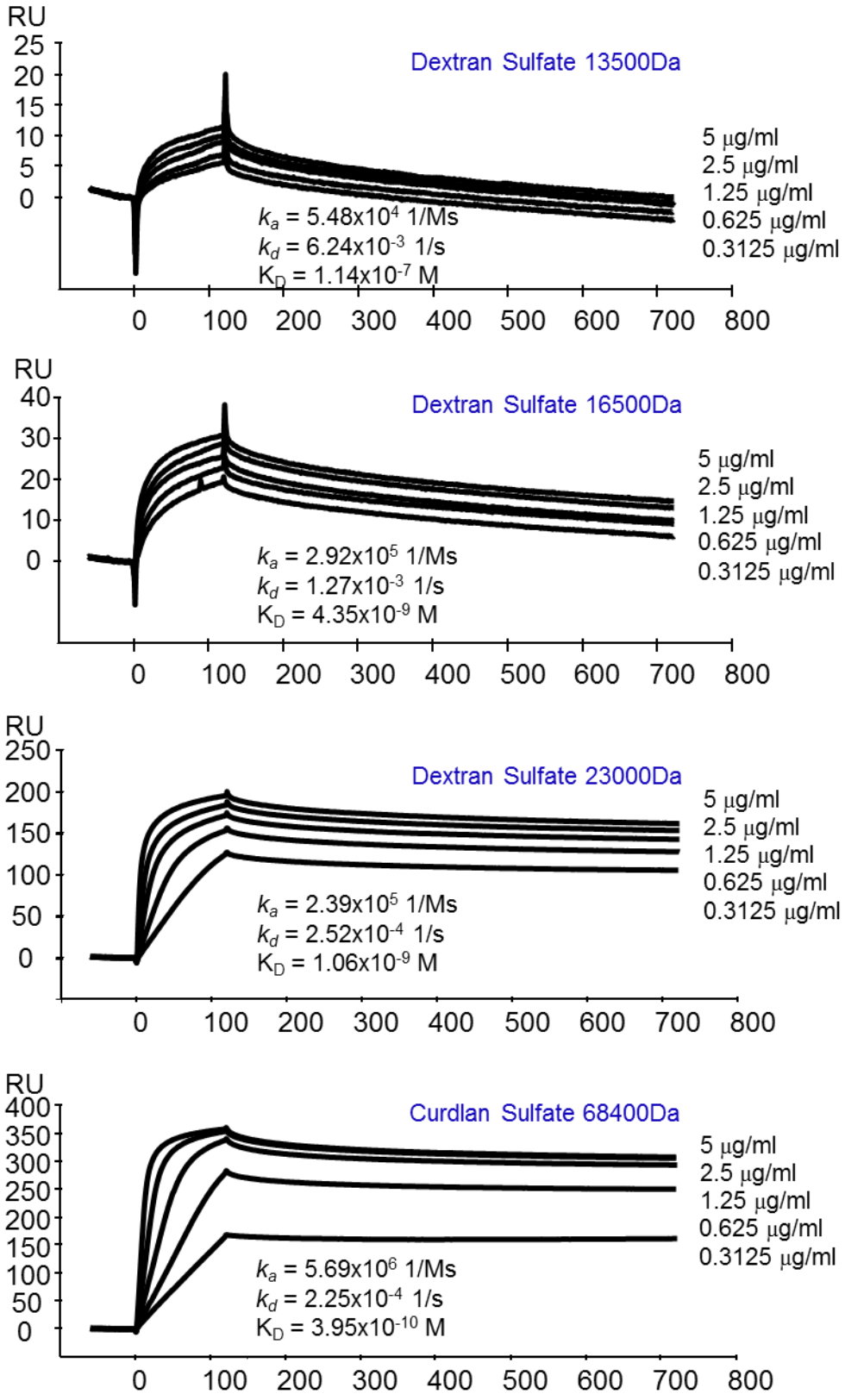


Figure 5. SPR measurement of sulfated polysaccharides with oligopeptide A from HIV gp120. Oligopeptide A with 20AA from V3 loop has several Lys and Arg with positively charged amino acid, [Biotin]-^{297}TRPNNNTRKRIRIQRGPGRA^{316}.

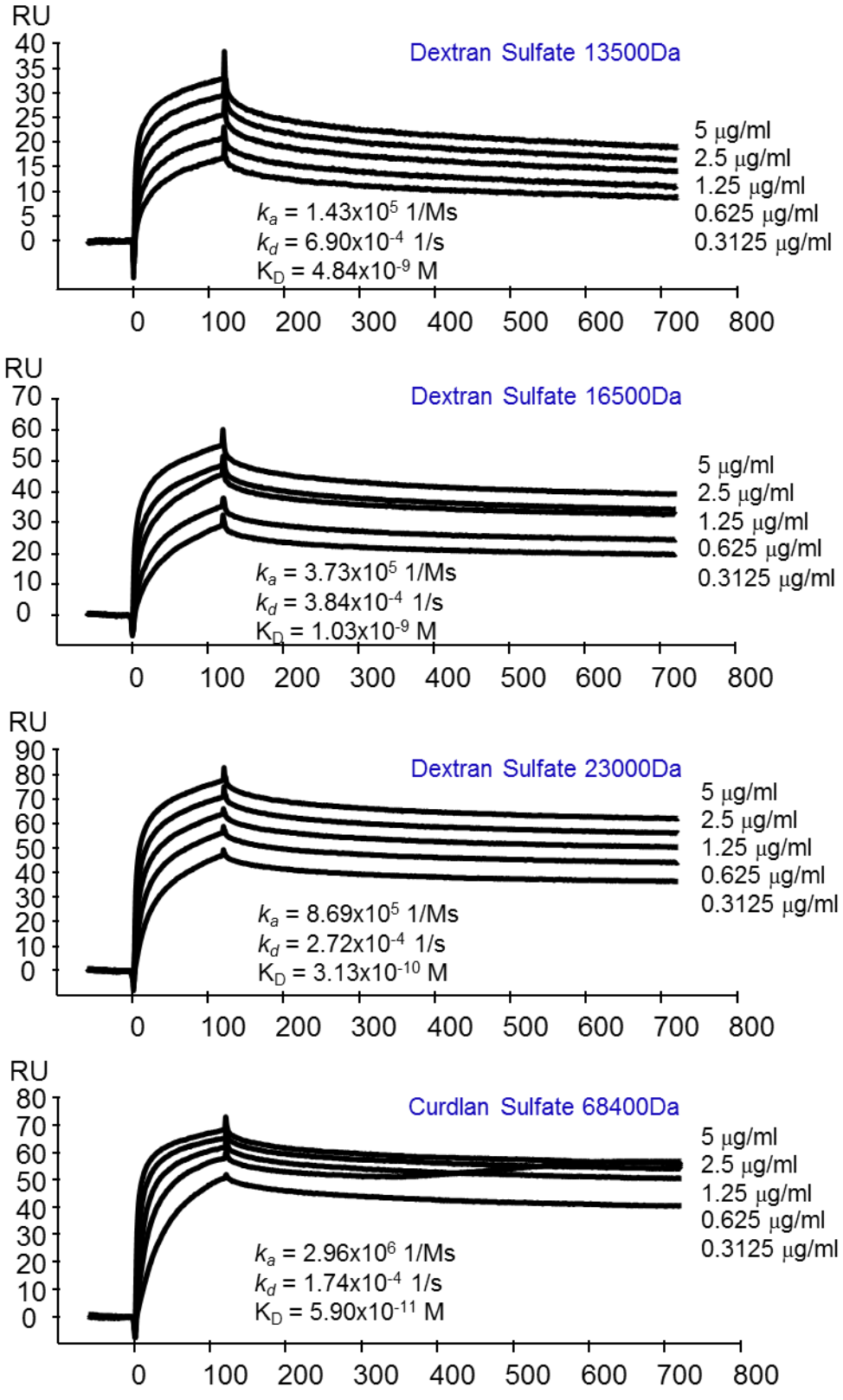


Figure.6 SPR measurement of sulfated polysaccharides with oligopeptide B from HIV gp120. Oligopeptide B with 19AA from C-terminus has several Lys and Arg with positively charged amino acid, [Biotin]-^{493}PLGVAPTKAKRRVVQREKR^{511}.

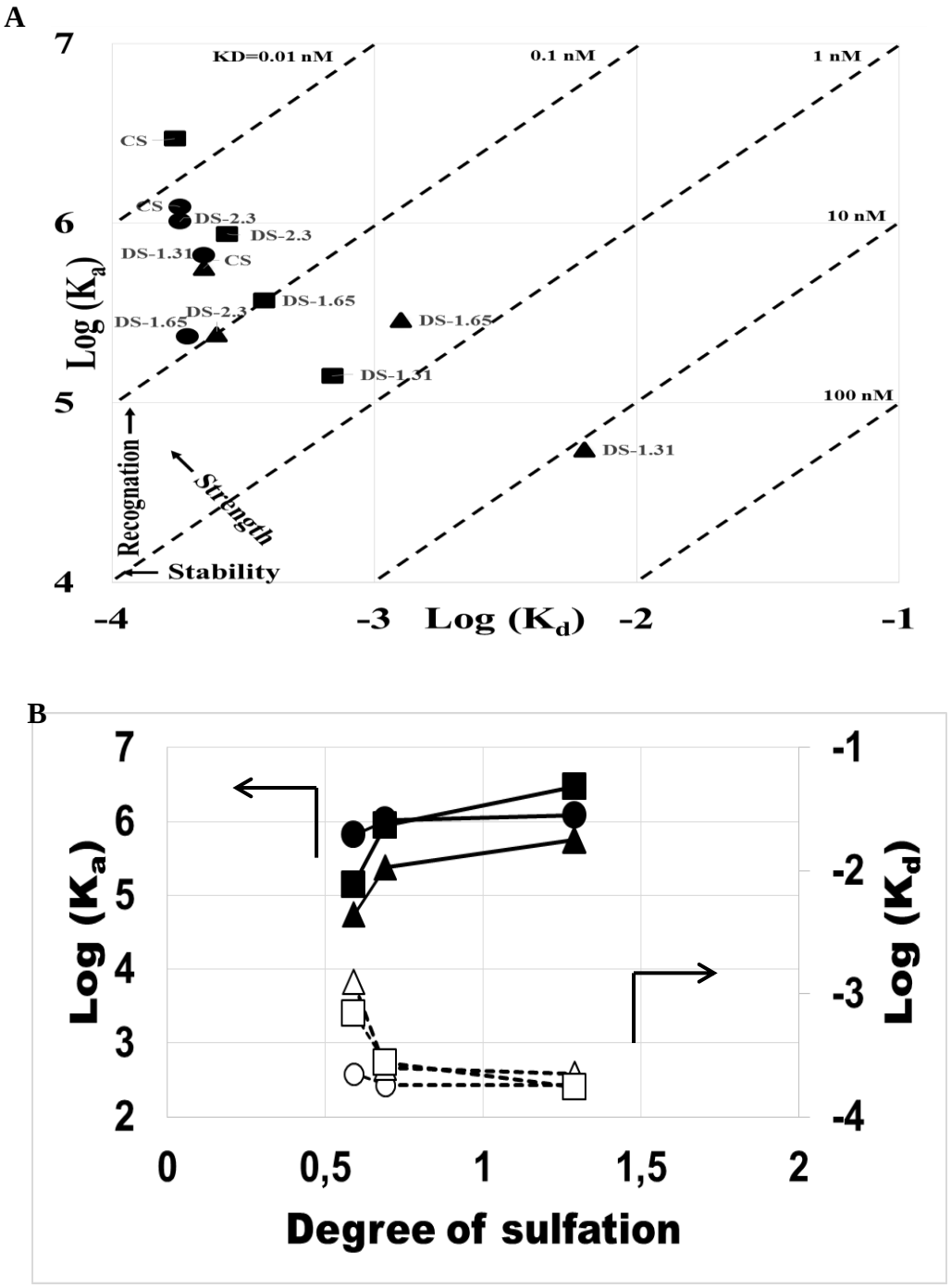


Figure 7.Interaction between curdlan sulfate and dextran sulfates with oligopeptides of HIV gp120.

(A) Relationship of association- (k_a) and dissociation rate (k_d) constants of curdlan sulfate and dextran sulfates with oligopeptides.

(B) Relationship of the DS of sulfated polysaccharides and k_a and k_d. were kinetic constants, The oligopeptides is as follows; △▲: Oligopeptide A, □■: Oligopeptide B and ○●: Poly-L-lysine, respectively.

Curdlan sulfate and dextran sulfates were diluted from 5 to 0.325 μg/mL and injected over oligopeptides A, B, and C immobilized sensor chip SA, to measure the k_a. After 120s, the solution of curdlan sulfate was replaced with the HBS−EP buffer solution to measure k_d of the curdlan sulfate−oligopeptides complex. Of note, the results are also shown in Table 4. Both RU in Fig 5 and 6 was decreased gradually with decreasing the concentration of curdlan sulfate and the k_a and k_d were $k_a=5.69\times10^5$1/Ms and $k_d=2.25\times10^{-4}$1/s for oligopeptide A and $k_a=2.96\times10^6$1/Ms and $k_d=1.74\times10^{-4}$1/s for oligopeptide B, respectively. We observed that curdlan sulfate with higher molecular weight and the DS strongly interacted with oligopeptides from the HIV gp120 and the interaction with oligopeptide B from the C-terminus of the HIV gp120 was marginally higher than that of oligopeptide A from the V3 loop. The SPR results corresponded to the potent anti-HIV activity of curdlan sulfates [4, 18], suggesting that sulfated polysaccharides interacted electrostatically with the V3 loop domain from the C-terminus of the HIV gp120 to prevent the HIV infection.

2.3.5 Particle size and zeta potential of poly-L-lysine with dextran and curdlan sulfates

In this study the interaction was further investigated by measuring the particle size and zeta potential of dextran and curdlan sulfates with poly-L-lysine (Mw=1000−5000) using a DLS. Table 5 shows the particle size of dextran sulfate ($\overline{M}_n=1.31\times10^4$, DS=0.59) in HBS−EP buffer was 50.2±6.9 nm. After the addition of poly-L-lysine with the particle size of 39.9±9.0 nm, the particle size of dextran sulfate increased to 92.8±17.7 nm because of the electrostatic interaction. The particle size of dextran ($\overline{M}_n= 2.46\times10^4$) without sulfate groups was unchanged before (37.8±8.7 nm) and after (43.2±9.9 nm) addition of poly-L-lysine. The zeta potential of dextran sulfate ($\overline{M}_n=1.31\times10^4$,

DS=0.59) was negative (-41.3 mV). After the addition of poly-L-lysine with the zeta potential of 0.2 mV, the zeta potential of dextran sulfate marginally increased to -35.5 mV by the interaction with the positively charged poly-L-lysine. Curdlan sulfate had almost the same tendency as dextran sulfate. The particle size increased from 38.8±7.7 nm to 265.6±12.2 nm. The zeta potential of curdlan sulfate was -32.6 mV. After the addition of poly-L-lysine, the zeta potential increased to -19.1 mV. These findings revealed that the electrostatic interaction occurred between negatively charged sulfated polysaccharides and positively charged poly-L-lysine.

Table 5. Particle size and zeta potential of poly-L-lysine with sulfated polysaccharides

Sulfated polysaccharides	Mn $\times 10^4$	DS	Poly-L-Lysine[a]			
			Absent		Present	
			Particle size (nm)	ζ (mV)	Particle size (nm)	ζ (mV)
Dextran Sulfate	1.31	0.59	50.2±6.9	-41.3	92.8±17.7	-35.5
Curdlan Sulfate	6.84	1.29	38.8±7.7	-32.6	265.6±12.2	-19.1

[a] Commercially available poly-L-lysine (0.5 mg/ml) with the molecular weight of 1000-5000 was used. **DSL=39.9±9.0 nm, ζ =0.2 mV**

2.4. Conclusion

In this study, dextran and curdlan sulfates exhibited a strong interaction with poly-L-lysine and the interaction increased with increasing their molecular weights and the DS. In addition, oligopeptides A and B from the V3 loop and C-terminus of the HIV gp120 interacted strongly with dextran and curdlan sulfates, and the interaction of oligopeptide B was marginally higher than that of oligopeptide A. Although the continuous connections of basic amino acids worked effectively to interact with sulfated polysaccharides, the difference of the interaction between oligopeptides was limited. Of note, oligopeptide C with dextran and curdlan sulfates exhibited no interaction, because oligopeptide C had no basic amino acids in the sequence. The interaction correlated with the molecular weights and the DS of sulfated polysaccharides by the measurements with poly-L-lysine having positively charged amino groups. The SPR and DLS measurements clearly reveal that the potent anti–HIV activity of sulfated polysaccharides induced the electrostatic interaction between the negatively charged sulfate groups and positively charged amino acid regions in the HIV gp120. This study suggest that the anti-HIV activity of sulfated polysaccharides could be induced by the electrostatic interaction between the negatively charged sulfated polysaccharides and positively charged HIV gp120 at the V3 loop domain from the C-terminus.

Reference

[1] H.Nakashima, O.Yoshida, T. Tochikura , T.Yoshida, T. Mimura, Y.Kido, Y. Motoki, Y. Kaneko, T.Uryu, N. Yamamoto, Sulfation of polysaccharides generates potent and selective inhibitors in vitro. Jpn. J. Cancer Res. (Gan) 78(1987) 1164–1168.

[2] T. Uryu, Polysaccharides in Models of biopolymers by ring−opening

polymerization. S.Penczek, Ed., CRC Press, (1990) pp. 133–233, Boca Raton, Florida.

[3] T. Yoshida, Synthesis of polysaccharides having specific biological activities. Prog. Polym. Sci. 26(2001) 379–441.

[4] T.Uryu, N.Ikushima, K.Katsuraya, T.Shoji, N.Takahashi, T.Yoshida, K.Kanno, T.Murakami, H.Nakashima, N.Yamamoto, Sulfated alkyl oligosaccharides with potentinhibitory effects on human immunodeficiency virus infection. Biochem. Pharmacol. 43(1992) 2385−2392.

[5] D. A.Lane,I.Bjork, U.Lindahl, Ed. Heparin and related polysaccharides. Advances inexperimental medicine and biology vol. 313 (1992) Plenum press, New York.

[6] U. Lindahl, G. B ackstrom, L. Thunberg, The antithrombin−binding sequence in heparin. J. Biol. Chem. 258 (1983) 9826−9830.

[7] K. J. Jeon, K. Katsuraya, Y. Kaneko, T. Mimura, T. Uryu, Studies on interaction mechanism of sulfated polysaccharides as an AIDS drug by NMR. Macromolecules 30 (1997) 1997–2001.

[8] K. J. Jeon, K. Katsuraya, T. Inazu, Y. Kaneko, T. Mimura, T. Uryu, NMR Spectroscopic detection of interaction between a HIV protein sequence and a highly anti-HIV active curdlan sulfate. J. Am. Chem. Soc. 122 (2000) 12536–12541.

[9] P. P. Jagodzinski, J. Wustner, D. Kmieclak, T. J. Wasik, A. Fertala, A. L. Sieron, M. Takahashi, T. Tsuji, T. Mimura, M. S. Fung, M. K. Gorny, M. Kloczewiak, Y. Kaneko,D. Kozbor, Role of the V2, V3, and CD4–binding domains of gp120 in curdlan sulfate neutralization sensitivity of HIV–1 during infection of T lymphocytes. Virology 226(1996) 217–227.

[10] R. B. M. Schasfoort, A. J. Tudos, Ed., Handbook of surface plasmon resonance. RSC Publishing, The Royal Society of Chemistry (2008), Cambridge, UK.

[11] R. I. W. Osmond, W. C. Kett, S. E. Kett, D. R. Commbe, Protein−heparin interactions measured by Biacore 2000 are affected by the method of heparin

immobilization. Anal. Biochem. 310 (2002) 199–207.

[12] J. Zhao, X. Liu, A. Malhotra, Q. Li, F. Zhang, R. J. Linhardt, Novel method for measurement of heparin anticoagulant activity using SPR. Anal. Biochem. 526 (2017) 39–42.

[13] R. Lu,K.Hattori, T.Yoshida, H.Nakashima,M.Premanathan, R.Aragaki,T.Mimura,Y.Kaneko,N.Yamamoto,T.Miyakoshi, T.Uryu, Specific biological activities of Chinese lacqure polysaccharides. Carbohydr. Polym. 43 (2000) 47−54.

[14]Y.Bai,T.Yoshida, Separation of lacquer polysaccharides and interaction withpoly-L-lysine, Carbohydr. Polym.98 (2013) 270–275.

[15] M. Tegshi,D. Budragchaa, T. Kanamoto, H. Nakashima,K. Ichiyama, N. Yamamoto,S. Han,T. Yoshida,Synthesis of sulfated natural galactomannans with specific antiviral activities.Int. J. Biol. Macromol. 89(2016) 415−420.

[16] D. Budragchaa,S. Bai,T. Kanamoto, H. Nakashima, S. Han,T. Yoshida, Synthetic galactomannans with potent anti-HIV activity. Carbohydr. Polym. 130 (2015) 233−242.

[17] T. Yoshida, H. Nakashima, N. Yamamoto, T. Uryu, Anti-AIDS virus activity in vitro of dextransulfates by sulfation of synthetic and natural dextrans. Polymer J. 25 (1993)1069−1077.

[18] S.L. Pedersen, A.P. Tofteng, L. Malik, K.J. Jensen, Chem Soc Rev Microwave heating in solid-phase peptide synthesis, (2012)

[19] T.Yoshida, K. Hatanaka, T. Uryu, Y. Kaneko, N.Yasuda, T. Mimura, O. Yoshida, N. Yamamoto,Synthesis and structural analysis of curdlan sulfate with potent anti–AIDS virus activity. Macromolecules 23 (1990) 3717–3722.

CHAPTER 3.

Interaction mechanism between sulfated polysaccharides and oligopeptides of Influenza virus hemagglutinin analyzed by SPR

ABSTRACT

The naturally and synthetic sulfated polysaccharides also have potent anti-influenza virus [1]. Chapter 1 reported interaction of sulfated polysaccharides and HIV surface glycoprotein 120 were the anti-HIV mechanisms. Influenza virus also have surface glycoprotein. Influenza virus surface glycoprotein hemagglutinin is binding to the host cell receptors. This study aims to quantitatively investigate the interaction between sulfated polysaccharides with potent anti-influenza activity, dextran and curdlan sulfates with negatively charged sulfate groups, and oligopeptides from an influenza virus surface glycoprotein hemagglutinin with positively charged amino acids using surface plasmon resonance (SPR) and dynamic light scattering (DLS) to elucidate the anti-influenza virus mechanism of sulfated polysaccharides. For interaction, the three oligopeptides from the influenza virus were oligopeptide of influenza virus A/Yamagata ^{221}SHYSRRFTPEIAKRPKVRGQEGR^{243} with several lysine (K) and arginine (R) in the HA, oligopeptide of influenza A/Brisbane ^{221}SHYSRKFTPEIAKRPKVRDQEGR^{243} with several K and R in the HA, and oligopeptide of influenza B/Hong Kong ^{221}SNPQKFTSSANGVTTHYVSQIGG^{243} with little basic amino acids in the HA. Sulfated polysaccharides exhibited strong interaction against oligopeptides influenza A, (k_a=1.02×10^5–1.81×10^6 1/Ms and k_d=1.53×10^{-4}–4.56×10^{-4} 1/s), no interaction was against oligopeptide influenza B/Hong Kong, that result suggesting the anti-influenza mechanism of sulfated polysaccharides to be the electrostatic interaction of negatively charged sulfated polysaccharides and

Influenza virus HA at the positively charged amino acid regions. These result consistent with the anti-HIV results of sulfated polysaccharides.

3.1. Introduction

The sulfated polysaccharides have specific biological activities such as blood anticoagulant and antiviral activities. In 1958, Gerber reported for the first time that sulfated polysaccharides extracted from sea alga had anti-influenza virus activity [1]. In 1987, Yamamoto et al reported the anti-HIV activity of sulfated polysaccharides also extracted from sea alga [2].

In 1987, our laboratory reported synthetic sulfated polysaccharides, such as dextran sulfate, ribofuranan sulfate, and xylofuranan sulfate had potent anti-HIV activity [3]. In 1990, also our laboratory reported curdlan sulfate was have potent anti-HIV activity [4].

Recently many researchers reported sulfated polysaccharides from sea algae having anti-influenza virus, anti-dengue virus 2, anti-herpes simplex virus-2 activities [5-7]. Uryu and Yoshida reported that the anti-HIV activity of sulfated polysaccharides could be estimated as the electrostatic interaction with the positively charged amino acid accumulated region at the C-terminus of the HIV gp120, to elucidate the interaction by a high-resolution NMR [8]. Influenza virus is infected into the cells by a target of sialic acid on the surface of cells [9]. Nature occurring many polysaccharides having specific biological activities. But, it is very difficult to study the relationship between structure and that's biological activities. So, our laboratory study synthesis and polymerization of anhydrous sugars, these the specific biological activities [10]. These results suggest that sulfated polysaccharides had the inhibition on the stage of virus absorption and entry to host cells. The anti-influenza mechanism is assumed to the electrostatic interaction between negatively charged sulfate groups of sulfated

polysaccharides and a positively charged surface glycoprotein HA of the influenza A viruses [11].

3.2. Experimental

3.2.1 Materials

We purchased Biacore SA sensor chip and HBS−EP buffer solution for SPR measurements from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Dextran sulfates were obtained from Sigma−Aldrich, Japan. Curdlan sulfate was kindly gifted by Ajinomoto Co. Ltd. Furthermore, for the synthesis of oligopeptides, Fmoc-amino acids were purchased from Watanabe Chemical IND., LTD (Hiroshima, Japan). Other for the peptide synthesis reagents were purchased Tokyo Chemical IND., LTD (Tokyo, Japan) and Sigma Aldrich.

3.2.2 Measurement

We used a Biacore X100 to measure the SPR spectrum using a SA sensor chip immobilized for biotinylated oligopeptides. A DLS and zeta potential were measured at 25°C by an Otsuka Electronics ELSZ−1000ZS zeta potential and particle size analyzer in the phosphate buffer solution (pH7.4) at the concentrations of 1 or 0.5 mg/ml of dextran and curdlan sulfates with poly-L-lysine and oligopeptides. We determined the average molecular weights of sulfated polysaccharides using an aqueous-phase GPC equipped with TOSOH TSK-gel columns (7.6 mm×600 mm) of G2500PW$_{XL}$, G3000PW$_{XL}$, and G4000PW$_{XL}$ eluted with 66.7 mmol phosphate buffer solutions at 40°C, and detected the signals using an RI detector. In addition, standard pullulans were used to evaluate the molecular weights. The specific rotation was measured by a JASCO DIP−140 digital polarimeter in H_2O at 25°C in a quartz cell.

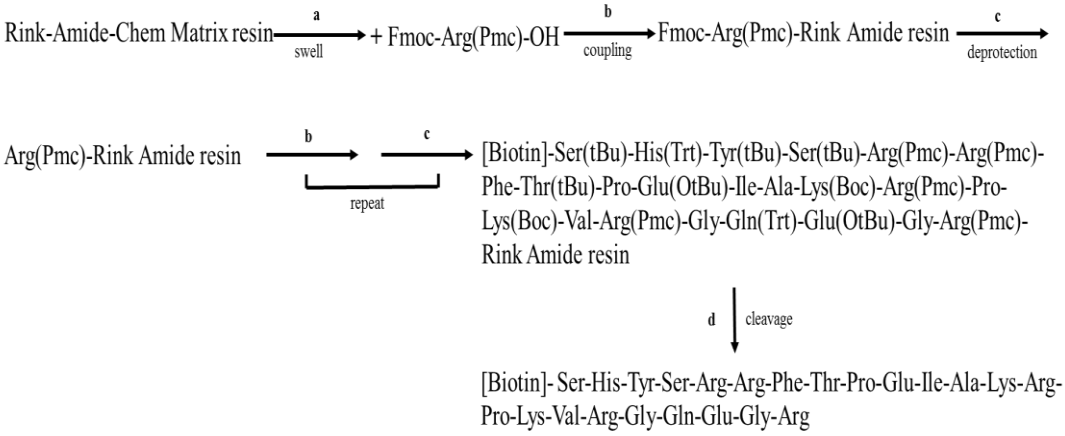
Furthermore, oligopeptides were synthesized using a Biotage Initiator+ SP Wave peptide synthesizer.

3.2.3. Synthesis of oligopeptides referenced by Influenza virus hemagglutinin

In this study, three oligopeptides were synthesized from the HA sequence of the three strain Influenza virus. Oligopeptide of Influenza A/Yamagata from the HA comprises 23 amino acids with eight positively charged lysine and arginine in the sequence. Likewise, oligopeptide of Influenza A/Brisbane from the HA comprises eight positively charged lysine and arginine, while the percentage of arginine and lysine were to the different. Oligopeptide of Influenza B/Hong Kong from the HA comprises one lysine and one histidine next to the biotin. The three oligopeptides were synthesized by a microwave assisted solid-phase peptide synthesizer using Fmoc-amino acids, and, finally, the N-terminus of the oligopeptides was biotinylated for immobilization on the sensor chip SA.

Rink Amide-Chem Matrix resin 96.3 mg (0.51 mmol/g, 0.5 mmol) was placed in a peptide synthesis vessel, swollen in DMF 2 hours at the room temperature. After washing by DMF for three times, a solution of 4 equiv. Fmoc-AA-OH, 4 equiv HOBT, and 4 equiv DIC in 1.6 mL in DMF was added to the vessel. Then the mixture was coupling at under microwave irradiation for 5 min and washed with DMF three times. Deprotection was with 25% piperidine in DMF for 3 min and 10 min at under room temperature. Kaiser test was applied to examine if there was free amino group existence. Then crude peptide was yielded after cleaving of the peptide with 10 mL Reagent R (TFA/Thioanisole/ EDT/ Anisole 90:5:3:2, V/V) for 120 min at room temperature [12]. Synthetic route of oligopeptides was shown in scheme 3. Peptide was precipitated by addition cold ethyl ether; after centrifugation, the ether was removed, and this process

was repeated three times. We purified the synthesized oligopeptides by a RP-HPLC. We confirmed the structures using MALDI TOF MS (Bruker Ultra Flex III) and LC/MS/MS (AB Science Inc. API 4000QTrap), suggesting that the calculated molecular weights agreed the observed values. These three oligopeptides were used for the interaction between dextran and curdlan sulfates with potent anti-influenza virus activity.



Scheme 3. Synthetic route of oligopeptide sequence of Influenza A/Yamagata HA.
Reagents and conditions: (a) DMF, room temperature, 2 hours swelling; (b) Fmoc-AA-OH, DIC, HOBT, 75^{o}C temperature, microwave irradiation; (c) 25% piperidine/DMF (V/V), room temperature; (d) Reagent R, room temperature, 2 hours.

3.2.4. Immobilization of biotinylated oligopeptides

The immobilization of oligopeptides of Influenza virus was conducted according to the Biacore-provided a protocol. Biotinylated oligopeptides were immobilized on the sensor chip SA using affinity of biotin and streptavidin. Briefly, a new SA sensor chip was set in the Biacore X100 instrument and then activated by the mixed solution of 50 mM NaOH/ 1M NaCl and 2-propanol. Next, biotinylated oligopeptides (1000 μg/ml) was dissolved in HBS-EP buffer and the solution (90 μL) was injected to the Biacore flow cell; the flow rate was 10μL/min. Finally, the washed for sensor chip SA by the

50 mM NaOH/1M NaCl. The proportion of immobilized oligopeptides of Influenza virus HA on the sensor chip SA was around 945−1131 RU by the Biacore X100 instrument.

3.2.5 Interaction of dextran and curdlan sulfates with oligopeptides

The interaction between sulfated polysaccharides and biotinylated oligopeptides was executed by a multicycle analysis method based on the Biacore-provided a protocol. The flow rate of buffers was adjusted to 30 µL/min. In addition the concentration of dextran sulfate was adjusted to 0.313, 0.625, 1.25, 2.50, and 5.00 µg/ml using the HBS−EP running buffer solution. After three blank runs with the running buffer, the dextran sulfate solution was injected in the flow cell for 120 s; then, the running buffer (HBS−EP) was flowed for 480 s to measure the association- (k_a) and dissociation (k_d) rate constants. After 600 s, the sensor chip was washed and regenerated with 50-mM NaOH solution (45 s) and then the running buffer HBS−EP flowed for 180 s. The k_a and k_d were calculated by the Biacore-provided BIA evaluation software and 1:1 binding fitting model.

3.3. Results and discussion

3.3.1 Synthesis of oligopeptides referenced by the influenza virus HA sequence

In this study, three oligopeptides were synthesized from the sequence of HA of the three strain Influenza virus. As shown in Table 6. Oligopeptide of Influenza A/Yamagata from theHA comprises 23 amino acids with eight positively charged lysine and arginine in the sequence. Likewise, oligopeptide of Influenza A/Brisbane from the HA comprises eight positively charged lysine and arginine, while the percentage of arginine and lysine were to the different. Oligopeptide of Influenza B /Hong Kong from

theHA comprises one lysine and one histidine next to the biotin. The three oligopeptides were synthesized by a microwave assisted solid-phase peptide synthesizer using Fmoc-amino acids, and, finally, the N-terminus of the oligopeptides was biotinylated for immobilization on the sensor chip SA. We purified the synthesized oligopeptides by a RP-HPLC. We confirmed the structures using MALDI TOF MS (Bruker Ultra Flex III) and LC/MS/MS (AB Science Inc. API 4000QTrap), suggesting that the calculated molecular weights agreed the observed values

Table 6. Biotinylated oligopeptides from Influenza virus hemagglutinin

Name	Position	Sequence[a]	AA[b]	Molecular weight[c]		Remark
				Calcd	Obsd	
A / Yamagata	HA	[Biotin]-^{221}S<u>H</u>YS<u>RR</u>FTPEIA<u>KR</u>P<u>K</u>V<u>R</u>GQEG<u>R</u>^{243}	23	2981.74	2981.73	Lys/Arg rich
A / Brisbane	HA	[Biotin]-^{221}S<u>H</u>YS<u>RK</u>FTPEIA<u>KR</u>P<u>K</u>V<u>R</u>DQEG<u>R</u>^{243}	23	3012.03	3011.28	Lys/Arg rich
B /Hong Kong	HA	[Biotin]-^{221}SNPQ<u>K</u>FTSSANGVTT<u>H</u>YVSQIGG^{243}	23	2606.37	2606.03	Lys/Arg poor

a) Positively charged amino acid was marked by underline.

b) Number of amino acid.

c) The structure of peptides was confirmed by using MALDI TOF MS.

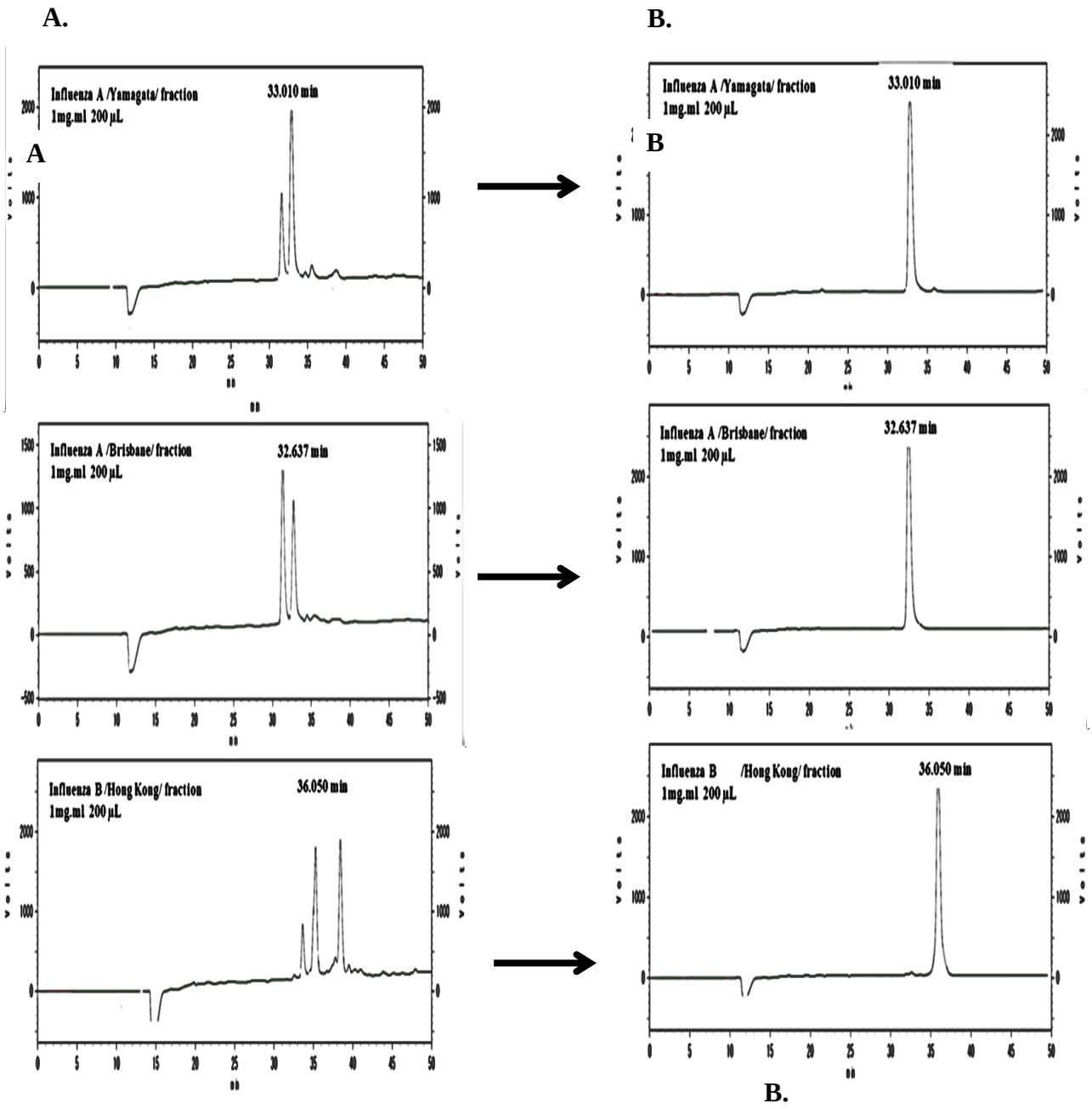


Figure 8. RP-HPLC profiles of purification of synthetic crude oligopeptides of Influenza virus HA

(A) Crude oligopeptides, (B) Purified oligopeptides

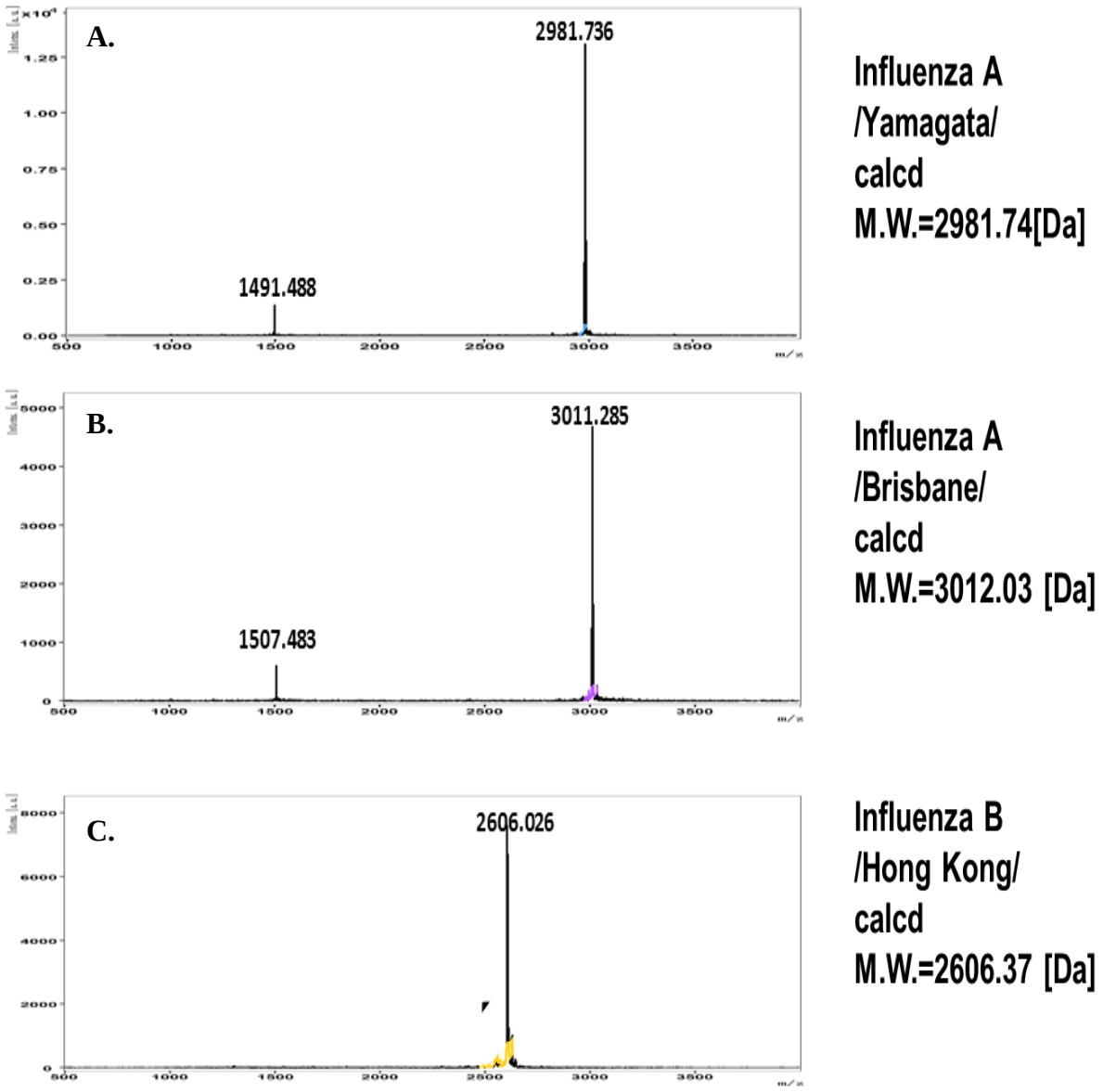


Figure 9. MALDI TOF MS profiles of (A) Oligopeptides of influenza A/Yamagata HA, (B) Oligopeptides of influenza A/Brisbane HA, (C) Oligopeptides of influenza B/Hong Kong HA

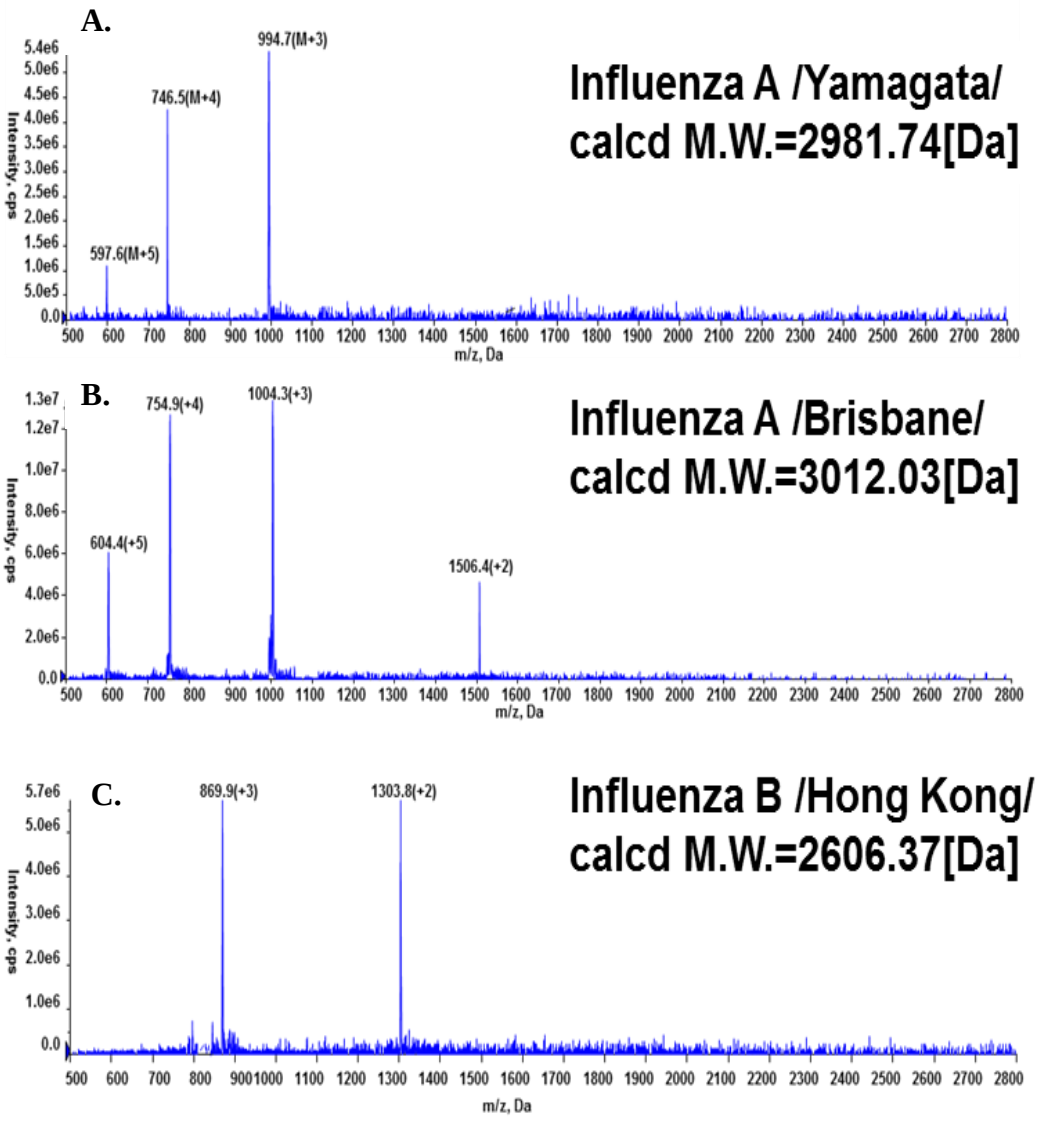


Figure 10. LC/MS/MS profiles of (A) Oligopeptides of influenza A/Yamagata HA, (B) Oligopeptides of influenza A/Brisbane HA, (C) Oligopeptides of influenza B/Hong Kong HA

3.3.2. Interaction of oligopeptides with dextran and curdlan sulfates

We quantitatively analyzed the interaction of oligopeptides from the Influenza virus HA with dextran and curdlan sulfates using the SPR measurement to elucidate the anti-influenza virus mechanism of sulfated polysaccharides. Table 7 presents the results of the interaction of oligopeptides immobilized on the sensor chip SA with dextran and curdlan sulfates.

Table 7. Interaction between sulfated polysaccharides and Influenza virus HA oligopeptides[a, b]

No	$\overline{M}_n$[d]	$\overline{M}_w/\overline{M}_n$	$[\alpha]_D^{25}$	S	DS[e]	Apparent kinetic constant[c]		
						k_a	k_d	K_D
	$\times 10^{-4}$			%		1/Ms	1/s	nM
Dextran sulfate								
1	1.31	1.15	+87.0	18.6	0.59	Inf A /Yama/	1.29×10^{5}	4.56×10^{-4}	3.53×10^{-9}
2						Inf A /Bris/	1.02×10^{5}	3.44×10^{-4}	3.36×10^{-9}
3						Inf B /HK/	−	−	−
4	1.65	1.31	+79.9	20.8	0.69	Inf A /Yama/	2.43×10^{5}	3.38×10^{-4}	1.39×10^{-9}
5						Inf A /Bris/	2.39×10^{5}	2.75×10^{-4}	1.15×10^{-9}
6						Inf B /HK/	−	−	−
7	2.30	1.20	+86.3	20.6	0.69	Inf A /Yama/	5.39×10^{5}	2.41×10^{-4}	4.47×10^{-10}
8						Inf A /Bris/	5.36×10^{5}	2.06×10^{-4}	3.85×10^{-10}
9						Inf B /HK/	−	−	−
Curdlan sulfate									
10	6.84	1.41	-0.5	30.5	1.29	Inf A /Yama/	1.81×10^{6}	2.06×10^{-4}	1.14×10^{-10}
11						Inf A /Bris/	1.59×10^{6}	1.53×10^{-4}	9.62×10^{-11}
12						Inf B /HK/	−	−	−

a) Oligopeptides were synthesized by sequences of Influenza virus hemagglutinin regions.
Inf A /Yama/: [Biotin] - ^{221}SHYSRRFTPEIAKRPKVRGQEGR^{243},
Inf A /Bris/: [Biotin] - ^{221}SHYSRKFTPEIAKRPKVRDQEGR^{243},
Inf B /HK/: [Biotin] - ^{221}SNPQKFTSSANGVTTHYVSQIGG^{243}
The underline shows basic amino acid.

b) Dextran and curdlan sulfateswere synthesized by sulfation of dextran and curdlan, respectively, with piperidine−N−sulfuric acid.

c) Apparent association-rate (k_a), dissociation-rate (k_d), and dissociation (K_D) constants were measured by a Biacore X100 SPR apparatus and used 1:1 binding fitting model for the calculation.

d) Number average molecular weight $\overline{M}_n$ was determined by GPC.

e) Degree of sulfation (DS) was calculated from the elementary analysis.

From the k_a and k_d, the interaction of dextran sulfates and curdlan sulfate against oligopeptides of Influenza virus A was higher than that against oligopeptide Influenza virus B, suggesting that amount of basic amino acids in the sequence exhibited higher interaction. Furthermore, the higher the molecular weights of dextran sulfates, the higher the interaction; however, the difference of the interaction was not high.

Sulfated polysaccharides was diluted from 5 to 0.325μg/mL and injected over oligopeptides of Influenza virus A and B HA immobilized on the sensor chip SA, respectively. Then was measured interaction. Of note, the results are also shown in Table 7. We observed that curdlan sulfate and dextran sulfate with higher molecular weight and the DS strongly interacted with oligopeptides from the influenza virus HA. The SPR results corresponded to the potent anti-influenza virus activity of curdlan sulfates, suggesting that sulfated polysaccharides interacted electrostatically with the HA of Influenza virus to prevent the influenza virus infection.

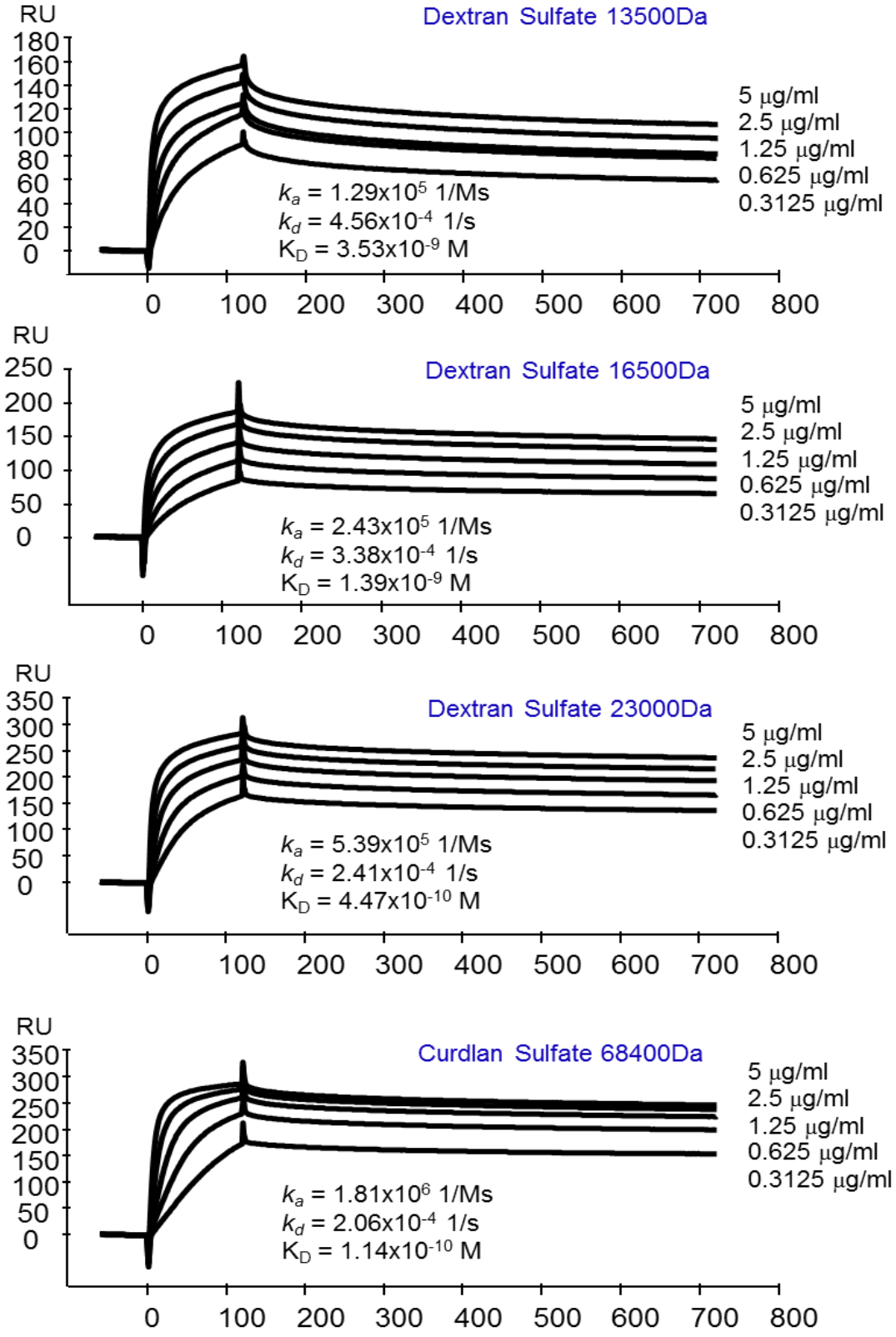


Figure11. SPR measurement of sulfated polysaccharides with oligopeptide from Influenza A /Yamagata HA. Oligopeptide with 23AA from HA has several positively charged amino acid, [Biotin]-^{221}SHYSRRFTPEIAKRPKVRGQEGR^{243}.

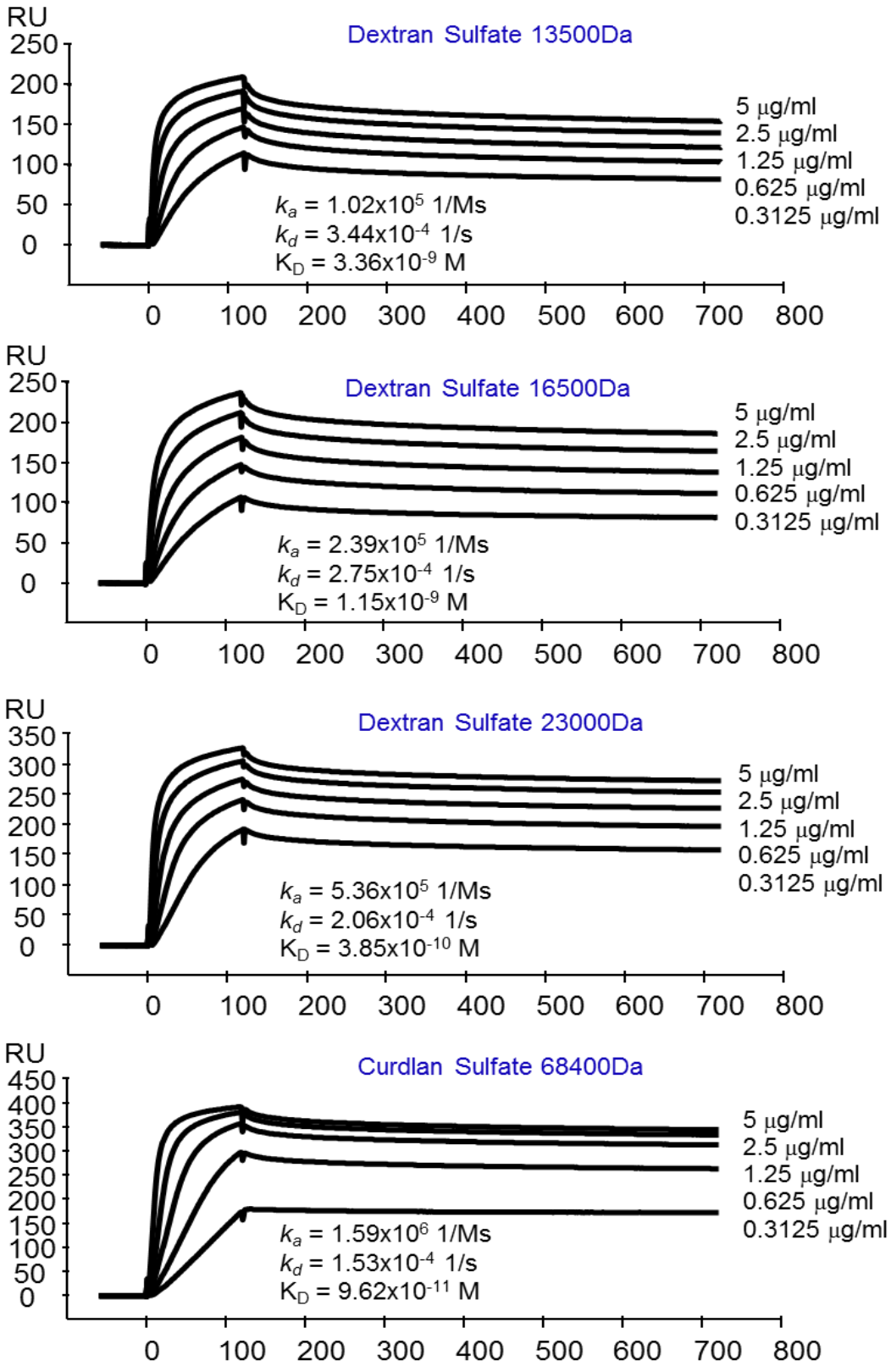


Figure12. SPR measurement of sulfated polysaccharides with oligopeptide from Influenza A /Brisbane HA. Oligopeptide with 23AA from HA has several positively charged amino acid, [Biotin]-^{221}**SHYSRKFTPEIAKRPKVRDQEGR**^{243}.

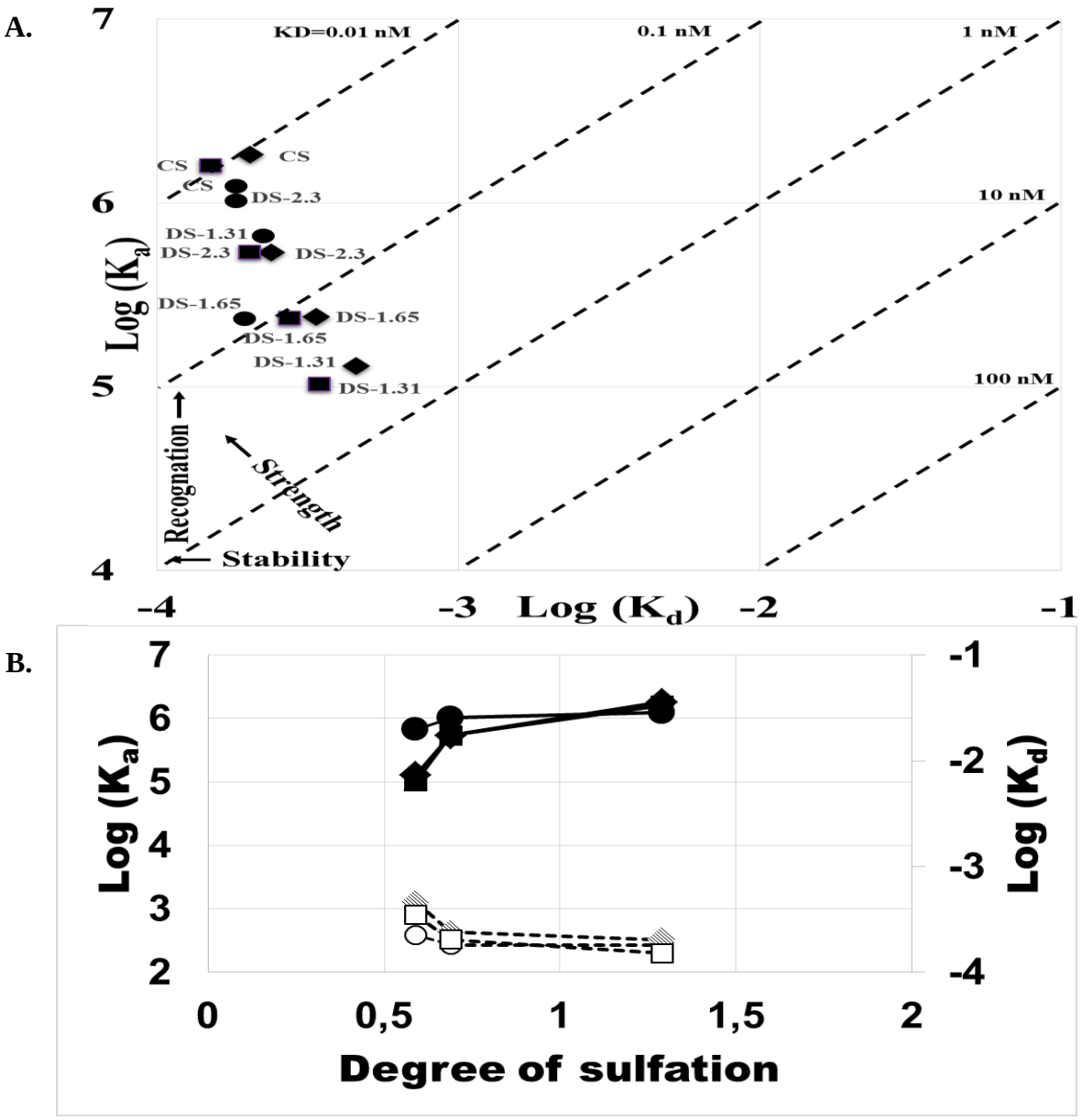


Figure 13. Interaction between curdlan sulfate and dextran sulfates with oligopeptides of Influenza virus HA

(A) Relationship of association- (k_a) and dissociation rate (k_d) constants of curdlan sulfate and dextran sulfates with oligopeptides.

(B) Relationship of the DS of sulfated polysaccharides and k_a and k_d were kinetic constants,

The oligopeptides is as follows; ◇◆: Oligopeptide of Influenza A/Yamagata HA,

□■: Oligopeptide of Influenza A/Brisbane HA and ○●: Poly-L-lysine, respectively.

3.4. Conclusion

In this study, dextran and curdlan sulfates exhibited a strong interaction with oligopeptides of influenza A HA and the interaction increased with increasing their molecular weights and the DS. Of note, oligopeptide of Influenza B HA with dextran and curdlan sulfates exhibited no interaction, because oligopeptide had no basic amino acids in the sequence. The interaction correlated with the molecular weights and the DS of sulfated polysaccharides by the measurements with positively charged amino groups. The SPR and DLS measurements clearly reveal that the potent anti–influenza activity of sulfated polysaccharides induced the electrostatic interaction between the negatively charged sulfate groups and positively charged amino acid regions in the oligopeptides. This study suggest that the anti-influenza virus activity of sulfated polysaccharides could be induced by the electrostatic interaction between the negatively charged sulfated polysaccharides and positively charged influenza virus HA.

Reference

[1] Gerber, P., Dutcher, J. D., Adams, E. V., & Sherman, J. H. Protective Effect of Seaweed Extracts for Chicken Embryos Infected with Influenza B or Mumps Virus.Proceedings of the Society for Experimental Biology and Medicine, 99(1958)590-593

[2] H.Nakashima, Y. Kido, N. Kobayashi, Y. Motoki, Purification and Characterization of an Avian Myeloblastosis and Human Immunodeficiency Virus Reverse Transcriptase Inhibitor , Sulfated Polysaccharides Extracted from Sea Algae, 31 (1987) 1524–1528

[3] H.Nakashima, O.Yoshida, T. Tochikura , T.Yoshida, T. Mimura, Y.Kido, Y. Motoki, Y. Kaneko, T.Uryu, N. Yamamoto, Sulfation of polysaccharides generates potent and selective inhibitors in vitro. Jpn. J. Cancer Res. (Gan) 78(1987)

[4] T.Yoshida, K. Hatanaka, T. Uryu, Y. Kaneko, N.Yasuda, T. Mimura, O. Yoshida, N.

Yamamoto,Synthesis and structural analysis of curdlan sulfate with potent anti–AIDS virus activity. Macromolecules 23 (1990) 3717–3722.

[5] Y. Jin, Y. Zhang, C. Wan, H. Wang, L. Hou, J. Chang, K. Fan, X. Xie, Immunomodulatory Activity and Protective Effects of Polysaccharide from Eupatorium adenophorum Leaf Extract on Highly Pathogenic H5N1 Influenza Infection, (2013)

[6] M.D. Noseda, M.E.R. Duarte, E.B. Damonte, The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell, 66 (2005) 103–110

[7] F.O. Gura, K.H. Ayashi, J.L. Ee, K.K. Anekiyo, T.H. Ayashi, Evaluation of an Edible Blue-Green Alga , Aphanothece sacrum , for Its Inhibitory Effect on Replication of Herpes Simplex Virus Type 2 and Influenza Virus Type A, 74 (2010) 1687–1690

[8] T.Uryu, N.Ikushima, K.Katsuraya, T.Shoji, N.Takahashi, T.Yoshida, K.Kanno, T.Murakami, H.Nakashima, N.Yamamoto, Sulfated alkyl oligosaccharides with potentinhibitory effects on human immunodeficiency virus infection. Biochem. Pharmacol. 43(1992) 2385−2392.

[9] K.A. Brogden, The sweet spot: defining virus–sialic acid interactions Jennifer, Nat Rev Microbiol. 3 (2005) 238–250.

[10] T. Yoshida, Synthesis of polysaccharides having specific biological activities. Prog. Polym. Sci. 26(2001) 379–441.

[11] T. Muschin, S. Han, H. Ishimura, T. Yoshida, Sulfated Polysaccharides as Specific Biologically Active Materials, Trends Glycosci. Glycotechnol. 23 (2011) 292–305

[12] S.L. Pedersen, A.P. Tofteng, L. Malik, K.J. Jensen, Chem Soc Rev Microwave heating in solid phase peptide synthesis, (2012) 1826–1844.

SUMMARY

1. Oligopeptides were synthesized with reference from sequences such as HIVgp120 and influenza virus HA by a MW-SPPS. Peptides A and B from HIV were found to be interacted strongly with dextran and curdlan sulfates, however, the peptide C without positively charged amino acids showed no interaction. These results suggest that the interaction was due to the electrostatic interaction between negatively charged sulfate groups and positively charged amino groups of the peptides. The results of influenza HAs, influenza A (Yamagata and Brisbane) and B (Hong Kong) viruses, are also presented.
2. Curdlan and dextran sulfates were found to increase the interaction with increasing the molecular weights and degree of sulfation, which were found to be important factors for the antiviral activity of sulfated polysaccharides.
3. Based on the above, it was considered that sulfated polysaccharides interact with viral envelope proteins to express antiviral properties. The specific antiviral mechanism is assumed the electrostatic interaction between positivity charged viral surface glycoprotein and negatively charged sulfate groups of sulfated polysaccharides.
4. We will prepare long oligopeptides from HIV and the interaction with sulfated polysaccharides is further studied in detail to reveal the anti-HIV mechanism of sulfated polysaccharides.

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