Construction of a chondroitin sulfate library with defined structures and analysis of molecular interactions

Nobuo Sugiura1*, Tatsumasa Shioiri1, Mie Chiba1, Takashi Sato3, Hisashi Narimatsu3, Koji Kimata2, and Hideto Watanabe1

From the 1Institute for Molecular Science of Medicine and 2Research Complex for the Medicine Frontiers, Aichi Medical University, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, and 3Research Center for Medical Glycoscience, Advanced Industrial Science and Technology, Tsukuba 305-8568, Japan

Running title: Chemo-enzymatic synthesis of chondroitin sulfates

*To whom correspondence should be addressed: Nobuo Sugiura, Institute for Molecular Science of Medicine, Aichi Medical University, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan. Tel: 81-561-62-3311; Fax: 81-561-63-3532; E-mail: nsugiura@aichi-med-u.ac.jp

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Background: Chondroitin sulfate (CS) is a linear polysaccharide, composed of repeating disaccharide units and modified with sulfate groups at various positions.

Results: A CS library was constructed with defined structures using CS polymerase and sulfotransferases.

Conclusions: The CS library provided details of interactions with CS-binding molecules.

Significance: Chemo-enzymatic synthesis provides a useful tool for studying the biological functions of CS.

Summary
Chondroitin sulfate (CS) is a linear acidic polysaccharide, composed of repeating disaccharide units of glucuronic acid and N-acetyl-D-galactosamine and modified with sulfate residues at different positions, that play various roles in development and disease. Here, we chemo-enzymatically synthesized various CS species with defined lengths and defined sulfate compositions, from chondroitin hexasaccharide conjugated with hexamethylenediamine at the reducing ends, using bacterial chondroitin polymerase and recombinant CS sulfotransferases including chondroitin-4-sulfotransferase 1 (C4ST-1), chondroitin-6-sulfotransferase 1 (C6ST-1), N-acetylgalactosamine 4-sulfate 6-sulfotransferase (GalNAc4S-6ST), and uronosyl 2-sulfotransferase (UA2ST).

Sequential modifications of CS with a series of CS sulfotransferases revealed their distinct features, including their substrate specificities. Reactions with chondroitin polymerase generated non-sulfated chondroitin, and those with C4ST-1 and C6ST-1 generated uniformly sulfated CS containing >95% 4S and 6S units, respectively. GalNAc4S-6ST and UA2ST generated highly sulfated CS possessing ~90% corresponding disulfated disaccharide units. Sequential reactions with UA2ST and GalNAc4S-6ST generated further highly sulfated CS containing a mixed structure of disulfated units. Surprisingly, sequential reactions with GalNAc4S-6ST and UA2ST generated a novel CS molecule containing ~29% trisulfated disaccharide units.

Enzyme-linked immunosorbent assay and surface plasmon resonance analysis using the CS library and natural CS products modified with biotin at the reducing ends, revealed details of the interactions of CS species with anti-CS antibodies, and with CS-binding molecules such as midkine and pleiotrophin. Chemo-enzymatic synthesis enables the generation of CS chains of the desired lengths, compositions, and distinct structures, and the resulting library will be a useful tool for studies...
of CS functions.

INTRODUCTION
Chondroitin sulfate (CS) is one of a glycosaminoglycan, that is a linear acidic polysaccharide composed of repeating disaccharide units (-4 D-glucuronic acid (GlcUA) β 1-3 N-acetyl-D-galactosamine (GalNAc) β 1-)ₙ, and modified with sulfate groups at various positions on the sugar residues (1). The average molecular weight (Mr) of CS is 10~100k consisting of 40~400 saccharide residues as a mature molecule (Table 1). The major disaccharide structures (Fig. 1 A) of CS are: a non-sulfated unit (0S, GlcUA-GalNAc), a monosulfated unit at the C-4 position of the GalNAc residue (4S, GlcUA-GalNAc(4S)), a monosulfated unit at the C-6 position of the GalNAc residue (6S, GlcUA-GalNAc(6S)), a disulfated unit at the C-4 and C-6 positions of the GalNAc residue (disE, GlcUA-GalNAc(4S6S)), a disulfated unit at the C-2 position of GlcUA and the C-6 position of GalNAc residues (diSD, GlcUA(2S)-GalNAc(6S)), and a trisulfated disaccharide unit (triS, GlcUA(2S)-GalNAc(4S6S)). Some GlcUA residues are epimerized into L-iduronic acid (IdoUA), and the chain containing IdoUA residues is designated as dermatan sulfate (DS). The disaccharide structures of DS are: a non-sulfated unit (IdoA/GlcUA-GalNAc), a monosulfated unit at C-4 position of the GalNAc residue (IdoUA/GlcUA-GalNAc(4S)), and a disulfated unit at the C-2 position of IdoUA/GlcUA and the C-4 position of GalNAc residues (disB, IdoUA(2S)/GlcUA(2S)-GalNAc(4S)). The disaccharide compositions of the CS/DS chains are usually analyzed using enzymes called chondroitin lyases, which specifically digest these chains via a β-elimination reaction, and generate an unsaturated 4,5-bond on the GlcUA/IdoUA residues.

CS chains are synthesized onto a linkage tetrasaccharide, GlcUA-Gal-Gal-Xyl, covalently bound to the serine residues of a core protein, by the alternating addition of monosaccharide units of GalNAc and GlcUA by CS synthases (2). During polymerization, the chain undergoes sulfation at various positions, mediated by a variety of sulfotransferases (3). Chondroitin 4-sulfotransferase (C4ST) family transfers sulfate to the 0S unit of chondroitin and generates CS containing the 4S unit (4). Chondroitin 6-sulfotransferase (C6ST) transfers sulfate to the 0S unit and generates the 6S unit (5). N-Acetylgalactosamine 4-sulfate 6-sulfotransferase (GalNAc4S-6ST) catalyzes further sulfation from 4S unit to generate diSE unit (6). Uronosyl 2-sulfotransferase (UA2ST) transfers sulfate to an uronic acid residue at the 2-O- position and generates diSD and diSB units from 6S and IdoUA-GalNAc(4S) units, respectively (6). Enzymes that generate a triS unit have not been identified yet. These diverse and complex structures of CS chains are synthesized with these enzyme complexes in the Golgi apparatus.

In animals, CS chains are present as a part of a proteoglycan molecule, and play various biological roles in development, organ morphogenesis, inflammation, and infection, by interacting with cytokines and growth factors, and regulating their signal transduction (1). For example, both midkine (MK) and pleiotrophin (PT) bind CS and promote neurite outgrowth (7). These functions are mainly ascribed to the structural diversity of oligosaccharide regions containing sulfate group modifications. Anti-CS monoclonal antibodies have been used in studies of CS chains. They are: MO225, thought to strongly recognize diSD unit-rich CS chains (8); CS-56, thought to recognize native CS chains containing 6S units (9); LY111, thought to recognize intact CS chains containing 4S units (10); and 2H6, thought to recognize the native CSA chain (11). These antibodies are useful for CS investigations, but their detailed epitope structures have not been determined yet, except for WF6, which shows high affinity to two different sequences of sulfation, but sharing some common structural envelope. Minimum binding sequences were octasaccharides, suggesting 4 adjacent sulfated disaccharides were involved in defining binding sites (12).

In this study, we chemo-enzymatically synthesized CS saccharides with defined lengths and structures, using the chondroitin polymerase K4CP and various recombinant sulfotransferases, and constructed a CS library. We then analyzed interactions between the immobilized
glycosaminoglycan conjugates and glycosaminoglycan-binding molecules using an enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) analysis. Our study clearly demonstrates the specificity of CS structure and molecular interactions of CS, and provides insight into its potential applications.

MATERIALS AND METHODS

Materials
Chondroitin (CH) produced by desulfation of CS from shark cartilage, chondroitin sulfate A (CSA) from whale cartilage, CS C from shark cartilage, CSD from shark fin cartilage, CS E from squid cartilage, dermatan sulfate (DS) from pig skin, partially depolymerized hyaluronan from chick comb, anti-CS monoclonal antibodies (MO225, CS56, LY111, and 2H6), chondroitinase ABC, and chondroitinase AC II were obtained from Seikagaku Corp. (Tokyo, Japan). Chondroitin hexasaccharide (CH6) was prepared as described previously (13). Human recombinant MK was a gift from Prof. Kadomatsu (Nagoya Univ., Nagoya, Japan). Human recombinant PT was from Calbiochem (Torrey Pines, CA). Chondroitin polymerase from E. coli strain K4 (K4CP) was prepared as described previously (14, 15). One unit of K4CP enzyme activity is defined as the amount that catalyzes the transfer of 1 pmol of GlcUA and 1 pmol of GalNAc alternately per min. Adenosine 3′-phosphate 5′-phosphosulphate tetrasodium salt (PAPS) was a gift from Dr. Ishige (Yamasa Corp., Choshi, Japan). Uridine 5′-diphospho-α-D-N-acetylgalactosamine disodium salt (UDP-GalNAc) and uridine 5′-diphospho-α-D-glucuronate trisodium salt (UDP-GlcUA) were from Yamasa Corp. The Superdex Peptide HR10/300, Superdex 30 HR16/600, Superose 12 HR 10/300, and mono Q 4.6/100 PE columns were from GE Healthcare (Piscataway, NJ). Anti-FLAG antibody gel, and anti-FLAG M2 antibody were from Sigma (St. Louis, MO). Streptavidin coated 96-well microplates were from Thermo Fisher Scientific (Waltham, MA). The 3,3′,5,5′-tetramethylenbenzidin (TMB) peroxidase substrate (SureBlue™) was from KPL (Gaithersburg, MD).

Construction of recombinant chondroitin sulfotransferases
DNA fragments that encode the putative catalytic domains of human C4ST-1 (amino acids 38–352) (4), human C6ST-1 (45–479) (5), GalNAc4S-6ST (102–561) (6), and UA2ST (73–406) (16) were amplified by PCR using a human cDNA library as the template and primers as listed in the Supplemental Table. The DNA fragments were subcloned into the pENTER/D-TOPO vector (Invitrogen, Carlsbad, CA) to construct pENTER/D-TOPO-C4ST-1, pENTER/D-TOPO-C6ST-1, pENTER/D-TOPO-GalNAc4S-6ST, and pENTER/D-TOPO-UA2ST. Following this, the mammalian expression plasmids pFLAG-CMV-3-DEST-C4ST1, pFLAG-CMV-3-DEST-C6ST-1, pFLAG-CMV-3-DEST-GalNAc4S-6ST, and pFLAG-CMV-3-DEST-UA2ST were prepared using the Gateway system (Invitrogen) with the pFLAG-CMV-3-DEST vector. FLAG-tagged DNA fragments encoding these sulfotransferases were amplified by PCR with the destination plasmids as templates, using the respective primers listed in the Supplemental Table 1. The PCR products were subcloned into the pIRESpuro vector (Clontech, Mountain View, CA). These plasmids were designated as pIRESpuro-C4ST-1, pIRESpuro-C6ST-1, pIRESpuro-GalNAc4S-6ST, and pIRESpuro-UA2ST.

Expression and purification of recombinant chondroitin sulfotransferases
HEK293T cells were transfected with the pIRESpuro expression plasmids using Lipofectamine 2000 (Invitrogen). Stable clones were selected with 5–10 µg/ml of puromycin in Dulbecco’s modified Eagle’s medium (Life Technologies, Carlsbad, CA) containing 10% (v/v) fetal bovine serum (HyClone Laboratories Logan, UT), and tested for the synthesis and secretion of the recombinant proteins by measuring enzyme activities and western blotting using an anti-FLAG M2 antibody (Sigma). The enzymes (C4ST-1, C6ST-1, GalNAc4S-6ST, and UA2ST) secreted into the conditioned media were purified by affinity chromatography using an anti-FLAG-agarose gel (Sigma), and eluted with 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 20% glycerol, and 200 µg/ml FLAG.
peptide (Sigma). The recombinant enzyme solutions obtained were stored at -80°C.

Sulfotransferase assay
Sulfotransferase activities were assayed by the radioisotope labeling method using [35S]PAPS (Perkin Elmer, Boston, MA) as described previously (17). The standard reaction mixture in a final volume of 50 µl consisted of 50 mM sodium potassium phosphate, pH 6.8, containing 2 mM dithiothreitol (DTT), [35S]PAPS (1 nmol, 0.1 μCi), CH (20 μg, for C4ST-1 and C6ST-1) or CSA (20 μg, for GalNAc4S-6ST and UA2ST), and the enzyme. The enzymatic reaction was carried out at 37°C for 60 min and stopped by heating in a boiling water bath for 1 min. The radiolabeled products were isolated by gel filtration using a Superdex Peptide HR10/300 column and quantified by liquid scintillation counting. One unit of enzyme is defined as the amount required for catalyzing the transfer of 1 pmol of sulfate per min.

Preparation of CS-hexamethylenediamine (HMDA) conjugates
CS oligo- and polysaccharides (CH6, CH, CSA, CSC, CSD, CSE, and DS) were conjugated with HMDA at the reducing end by the reductive amination method. CS (1 µmol) solution in 1.8 M HCl containing 100 µmol of HMDA (100 µl) was heated at 65°C for 2 h, followed by the addition of sodium cyanoborohydrate (150 µmol), and further heating at 65°C for 16 h. The HMDA-conjugated saccharides were purified by gel filtration chromatography on a Superdex 30 column using 0.2 M ammonium acetate as an eluent and obtained with freeze-drying repeated three times. The structure of purified CH6-HMDA was confirmed using a MALDI-TOF MS spectrometer (Autoflex; Bruker, Bremen, Germany) (18). The disaccharide compositions of CS-HMDA were measured using fluorometric post-column HPLC after digestion with chondroitinase ABC or AC II, as described below.

Preparation of sCS-HMDA library
A mixture of CH6-HMDA (0.2 µmol), UDP-GlcUA (6 µmol), UDP-GalNAc (6 µmol), and K4CP (3.0 units) in 50 mM Tris-HCl, pH 7.2, containing 0.15M NaCl, and 0.2 mM MnCl2 was incubated at 30°C for 24 h, and then chromatographed on the Superdex 30 column using 0.2 M ammonium acetate as the eluent. The elution was monitored by UV absorbance at 225 nm. The average molecular weight (Mr) of the synthesized chondroitin polysaccharide HMDA conjugate (sCH-HMDA) was estimated using analytical gel filtration chromatography on a Superose 12 HR10/300 column using short chains of hyaluronan as molecular weight standards (11). Individual sCS-HMDA conjugates were prepared as follows. For sCSA-HMDA, a solution of 50 nmol sCH-HMDA in 3 ml of 50 mM Tris-HCl, pH 6.8, containing 2 mM DTT, 11.5 µmol PAPS, and 2.0 kU C4ST-1 was incubated at 37°C for 24 h. For sCSC-HMDA, a solution of 30 nmol sCH-HMDA in 2 ml of the same buffer containing 2.3 µmol PAPS and 400 U C6ST-1 was incubated at 37°C for 24 h. For sCSAC-HMDA, a solution of 30 nmol sCH-HMDA in 2 ml of the same buffer containing 6.9 µmol PAPS, 720 U C4ST-1, and 520 U C6ST-1 was incubated at 37°C for 24 h. Each sulfated chondroitin product was purified using Superdex 30 column chromatography. Using these sulfated CS species, we further prepared sCS-HMDA as shown in Figure 1 (B). For sCSE-HMDA, a solution of 20 nmol sCSA-HMDA in 1 ml of the same buffer containing 4.6 µmol PAPS and 480 U GalNAc4S-6ST was incubated at 37°C for 24 h. Then, 2.3 µmol PAPS and 240 U GalNAc4S-6ST were added to the solution, and incubated further for 24 h. The product (sCSE-HMDA) was similarly purified using Superdex 30 column chromatography. For sCSAD-HMDA, a solution of 14 nmol sCSAC-HMDA in 1 ml of the same buffer containing 3.2 µmol PAPS and 340 U UA2ST was incubated at 37°C for 24 h. Following this, 1.6 µmol PAPS and 170 U UA2ST were added to the solution, and incubated further for 24 h. For sCSDE-HMDA, a solution of 10 nmol sCSAD-HMDA in 0.3 ml of the same buffer containing 2.3 µmol PAPS, and 160 U GalNAc4S-6ST was incubated at 37°C for 24 h. Following this, 1.2 µmol PAPS and 80 U GalNAc4S-6ST were added to the solution, and incubated further for 24 h. For sCtrlS-HMDA, CSE-HMDA (20 nmol) was dissolved in 1 ml of the same buffer, along with 4.6 µmol PAPS, and
480 U UA2ST. The mixture was incubated at 37°C for 24 h, and then 2.3 µmol PAPS and 240 U UA2ST were added to the solution, and incubated further for 24 h. The product (sCtriS-HMDA) was purified using Superdex 30 column chromatography.

**Biotinylation of CS-HMDA conjugates and ELISA**

CS-HMDA species were modified using a sulfo-NHS-activated biotin reagent (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) at the amine group of the HMDA residue at the reducing end of the saccharide chains, and resultant CS-biotin derivatives were immobilized onto streptavidin-coated microplates for ELISA system and on sensor chips for SPR analysis. Solutions of the CS-HMDA conjugates (5 nmol) in 50 mM phosphate buffer, pH 7.4, containing 50 nmol of sulfo-NHS-LC-Biotin were incubated at room temperature for 1.5 h, then further incubated at room temperature for 10 min after 100 nmol of ethanolamine was added to the solutions. The CS-biotin products were purified using Superdex Peptide column chromatography.

Interactions between the CS-biotin conjugates and anti-CS monoclonal antibodies were analyzed by ELISA system (19). Briefly, solutions (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µg/ml) of the CS-biotin conjugates (CH-, CSA-, CSC-, CSD-, CSE-, sCH-, sCSA-, sCSC-, sCSAC-, sCSAD-, sCSE-, sCSDE-, and sCtriS-biotin) in PBS (50 µl/well) were applied to streptavidin-coated 96-well microplates, and incubated at room temperature for 1 h. After washing with PBS (150 µl/well), PBS containing 1% BSA was applied to the plate (50 µl/well, at room temperature for 1 h) for blocking. After washing three times with PBS containing 0.005% (w/v) Tween 20 (PBST), the specific monoclonal antibody (MO225, CS56, LY111, or 2H6) diluted 1:250 with PBST was added to the plate (50 µl/well). After 1 h at room temperature, the plate was washed 3 times with PBST (150 µl/well), and horseradish peroxidase-conjugated goat anti-mouse IgM (1:500 dilution with PBST) was added as the second antibody (50 µl/well). After 1 h at room temperature, unbound antibodies were removed by washing as above, and TMB peroxidase substrate solution (50 µl/well) was applied for 5 min at room temperature; then, 1M HCl (50 µl/well) was added to stop the enzyme reaction. The enzyme activity in the well was quantified by measuring the absorbance at 450 nm using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA) to determine the amount of bound anti-CS antibodies.

**SPR analysis**

Interactions between CS-biotin conjugates and the CS-binding molecules MK and PT were analyzed using an SPR biosensor (BIACore 1000; GE Healthcare). A streptavidin-conjugated sensor chip (SA chip) was used to immobilize the biotin-conjugated CS chains (CH-, CSA-, CSC-, CSD-, CSE-, sCH-, sCSA-, sCSC-, sCSAC-, sCSAD-, sCSE-, sCSDE-, and sCtriS-biotin). The CS-biotin solutions (10 µg/ml) in 10 mM phosphate buffer, pH 7.2, containing 0.005% (w/v) Tween 20 (70 µl) were injected at a flow rate of 5 µl/min to immobilize the CS chains, and then the sensor chip was washed with PBST containing 2M NaCl to remove unbound material. RU values for immobilization of CS-biotin species are shown in Supplemental Table 2.

Binding assays were performed at 25°C at a constant flow rate of 20 µl/min during both the association and dissociation phases. MK and PT solutions (50, 80, 150, and 250 nM) in 10 mM HEPES buffer, pH 7.4, containing 0.15M NaCl, 3 mM EDTA, and 0.005% Tween 20 (HBS-EP, 60 µl) were injected into the flow cells at a flow rate of 20 µl/min (association), and then HBS-EP without the cytokines was flowed at the same flow rate for 180 s (dissociation). After each run, regeneration of the sensor chip surface was accomplished by an injection of 20 µl of HBS-EP containing 2M NaCl. Changes in resonance units were recorded, and the values for rate constants were determined by nonlinear regression analysis using BIAevaluation 4.1 software (GE Healthcare). Association rate constants (ka) were calculated from the linear portions of the sensorgrams during the early association phase. Dissociation rate constants (kd) were calculated from the early portion of the dissociation phase, which occurs after the completion of sample injection during the washout period. The apparent equilibrium dissociation constants (Kd) were calculated as the ratios of kd/ka.
Compositional analysis of CS derivatives

The synthetic CS derivatives were digested with chondroitinase ABC or ACII (10 mU) at 37°C for 1 h. Unsaturated disaccharide products were analyzed using fluorometric post-column HPLC system as reported previously (20).

Laser light scattering

The synthetic CH polymers were separated on a TSK-Gel G4000 pwXL column (Tosoh, Tokyo, Japan) eluted with 0.2M NaCl at 0.5 ml/min, and analyzed using a laser light scattering photometer ((DAWN DSP; Wyatt Technology, Santa Barbara, CA) to determine their absolute average molecular weights (21,22).

Results

Preparation and characterization of CS derivatives

Initially, we conjugated the CH6 oligosaccharide and various CS polysaccharides (CH, CSA, CSC, CSD, CSE, and DS) with HMDA at the reducing ends of the saccharides, as described under “Experimental Procedures.” The CS-HMDA conjugates have a free amino group at the reducing end, which is useful to conjugate another active group such as biotin and to immobilize the CS chains on activated solid materials. The average molecular weights (Mr), disaccharide compositions, and sulfation degrees (SD, number of sulfate groups per disaccharide unit) of the CS-HMDA conjugates are summarized in Table 1.

The conjugation reactions did not alter the molecular weights or sulfation patterns of the CS chains. The chain size of chemically desulfated CH was smaller than the original CSC, probably due to the partial cleavage of the glycosyl bonds (23). CH contained 90.3% 0S, 9.1% 6S, and 0.6% 4S. CSA from whale cartilage contained 77.9% 4S, 20.7% 6S, and small amounts of 0S, diSE, and diSD units. CSC from shark cartilage contained 66.9% 6S, 22.4% 4S, 8.8% diSD, and small amounts of diSE and 0S units, and its SD was 1.09. Interestingly, CSD from shark fin cartilage contained only 16.5% diSD, and its major units were 6S (42.8%) and 4S (37.9%). CSE from squid cartilage exhibited a longer chain (Mr 100k) and contained 63.8% diSE, 26.3% 4S, 7.3% 6S, and 2.6% 0S units. DS from pig skin contained 93.6% 4S units and 5.8% diSB units, and 70 ~ 80% of the uronic acid residues of DS were IdoUA residues.

Following this, we enzymatically elongated the carbohydrate chain of CH6-HMDA, using K4CP and two donor substrates (UDP-GlcUA and UDP-GalNAc). The product, sCH-HMDA consisted entirely of 0S units, whereas CH chemically desulfated chondroitin contained a few sulfated disaccharide units. The average chain size of sCH-HMDA was 9.4kDa (23 disaccharide units; Supplemental Fig. 1) when a mixture of 0.2 µmol CH6-HMDA, 6 µmol UDP-GlcUA and 6 µmol UDP-GalNAc, and 3 units K4CP was incubated at 30°C for 24 h as described under “Experimental Procedures.” The chain size could be elongated up to 350kDa by manipulating the conditions of the enzymatic reactions, such as the concentrations of the donor and acceptor substrates, the amount of enzyme, and the reaction time (Supplemental Fig. 2).

Construction of a CS library with defined compositions

We prepared various recombinant chondroitin sulfotransferases from stable clones of HEK293T cells as described under “Experimental Procedures.” The activities of the recombinant sulfotransferases purified from the culture media from the stable cells were 2,000 ~3,000 units/ml (C4ST-1), 3,000 ~6,000 units/ml (C6ST-1), 900~1,600 units/ml (GalNAc4S-6ST), and 300~600 units/ml (UA2ST), respectively. Using these sulfotransferases and sCH-HMDA, we generated various sCS-HMDA species by the sequential addition of sulfate residues at different positions, as shown in Figure 1 (B). Disaccharide compositions and the SD values of the sCS-HMDA derivatives are summarized in Table 2.

Upon incubation with 50 nmol sCH-HMDA, 11.5 µmol PAPS, and 2 kU C4ST-1 at 37°C for 24 h, the product designated as sCSA-HMDA contained up to 95~98% C4S disaccharide units. When a lower amount of C4ST-1 or PAPS was used, the product showed a lower SD (Supplemental Fig. 3). Upon incubation with 10 nmol sCH-HMDA, 2.3 µmol PAPS, and 400 U C6ST-1 at 37°C for 24 h, the product designated as sCSC-HMDA contained up to 92~99% C6S units. When a lower amount of
C6ST-1 or PAPS was used, the product showed a lower SD (Supplemental Fig. 3). The simultaneous reaction of C4ST-1 and C6ST-1, upon incubation with 30 nmol sCH-HMDA, 6.9 µmol PAPS, 720 U C4ST-1, and 520 U C6ST-1 at 37°C for 24 h, yielded a product (sCSAC-HMDA) that contained almost the same amounts of 4S (47.9%) and 6S (50.6%) units. By changing the ratio of C4ST-1 to C6ST-1 in the reaction, the ratio of 4S and 6S units in the product could be altered accordingly (Supplemental Fig. 4). These results indicate the establishment of bioengineering techniques for the generation of CS chains with defined ratios of monosulfated and non-sulfated disaccharide units.

Following this, we reacted sCSA-HMDA with GalNAc4S-6ST to generate sCSE-HMDA, which contained 88.1% diSE (GlcUA-GalNAc(4S6S)) and 10.2% 4S units, with an SD value up to 1.86. GalNAc4S-6ST specifically transferred sulfate to position 6 of GalNAc (4S) residue. Neither sCH-HMDA nor sCSC-HMDA was sulfated with GalNAc4S-6ST (data not shown). UA2ST transferred a small amount of sulfate (approximately 4%) at position 2 of the GlcUA residue of the 6S unit of sCSC-HMDA, and did not transfer any at the residues of the 0S and 4S units of sCH-HMDA and sCSA-HMDA at all (data not shown). In contrast, sCSAC-HMDA was efficiently sulfated with UA2ST. The disaccharide compositions of the product, designated as sCSAD-HMDA, were 41.2% diSD (GlcUA(2S)-GalNAc(6S)) and 4.5% diSB (GlcUA(2S)-GalNAc(4S)) units, with an SD value up to 1.46 (Table 2). We further reacted sCSAD-HMDA with GalNAc4S-6ST. The product, designated as sCSDE-HMDA, contained 20.1% diSE, 39.9% diSD, and 4.9% diSB units (SD = 1.64), exhibiting a highly sulfated hybrid structure composed of three sulfated disaccharide units. This result also indicated that GalNAc4S-6ST catalyzes GlcUA-GalNAc(4S) but not GlcUA(2S)-GalNAc(4S). Next, we reacted sCSE-HMDA with UA2ST. Surprisingly, the product contained 28.8% triS (GlcUA(2S)-GalNAc(4S6S)) unit, which has not been found in natural CS species. This also indicated that UA2ST sulfates diSE and generates the triS unit. As the product contained a substantial amount of the triS unit, we designated it as sTriS-HMDA. By these sequential sulfation reactions, we succeeded in construction of a CS library with defined compositions.

CS-biotin derivatives were prepared from the CS-HMDA species using the Sulfo-NHS-LC-Biotin reagent and were immobilized on streptavidin-coated microplates for ELISA, and on sensor chips for SPR analysis. The chain sizes and disaccharide compositions of the CS-biotin conjugates did not change from their corresponding CS-HMDA origins.

**ELISA**

For the ELISA system, the native and synthetic CS-biotin conjugates were immobilized on streptavidin-coated microplates in a dose-dependent manner (0.001~10 µg/ml). The amounts of anti-CS monoclonal antibodies (MO225, CS56, LY111, and 2H6) bound were measured using the ELISA system as described under “Experimental Procedures” (Figs. 2 and 3). The half-maximal effects (ED50) of the binding activities of the antibodies to the CS derivatives were estimated from the dose response profiles against the concentrations (µg/ml) of the immobilized CS derivatives (Table 3).

MO225 showed high binding affinity to CSC, CSD, and CSE, and lower affinity to CSA, with ED50 values of 0.041, 0.059, 0.08, and 0.53 µg/ml, respectively. CS56 showed highest affinity to CSC, and then to CSD, CSE, and CSA. Their ED50 values were 0.043, 0.08, 0.25, and 0.4 µg/ml, respectively. In contrast, LY111 and 2H6 showed generally lower binding activities to CS than did MO225 and CS56, although they showed higher affinity to CSA than did MO225 and CS56. ED50 values for LY111 were 0.17 (CSA), 0.3 (CSC), 0.7 (CSD), and 0.8 µg/ml (CSE). Those for 2H6 were 0.071 (CSC), 0.11 (CSA), 0.4 (CSE), and 0.5 µg/ml (CSD). None of the antibodies tested bound to CH.

When we performed ELISA using sCS species, MO225 showed high binding affinity to sCSDE, sCSAD, and sCtriS, with ED50 values of 0.0058, 0.012, and 0.031 µg/ml, respectively, indicating their stronger binding affinity than CSD showing the strongest affinity among native CSs. MO225 showed an affinity for sCSAC that was similar to its affinity for CSD, and low binding affinity for cCSE. It did not bind to sCH or sCSA. CS56...
showed similar binding affinities to sCSAD, sCSAC, and sCSDE as to CSC. It did not bind to sCH, sCSA, sCSC, sCSE, and sCtriS. LY111 bound to sCSAC, sCSDE, and sCSAD with higher affinity than to CSA. It did not bind to sCH, sCSC, sCSE, and sCtriS. 2H6 had high binding affinity to sCSAC (ED$_{50}$ = 0.0036 µg/ml), and low affinity to sCSAD (ED$_{50}$ = 0.014 µg/ml) and sCSDE (ED$_{50}$ = 0.023 µg/ml) plates. Again, it did not bind to sCH, sCSA, sCSC, sCSE, and sCtriS.

**SPR assay**

We then analyzed the interaction of the CS-binding cytokines MK and PT with immobilized CS-biotin derivatives. The sensorgrams of various concentrations of MK and PT against the immobilized CS derivatives are shown in Figs. 4 and 5, respectively. The $k_a$, $k_d$, and $K_D$ values of the cytokines for the CS derivatives were calculated using a 1:1 (Langmuir) binding model using the BIAevaluation 4.1 software, and the results are summarized in Table 4.

Both MK and PT bound to all CS derivatives in a dose-dependent manner, but the degree of response was different among them. MK exhibited high responses to CSC, CSD, CSE, sCSC, and sCSAD; these maximum resonance units (Rmax) were 771, 972, 940, 844, 744, 809, respectively. It exhibited moderate responses to CS, sCSA, sCSE, and sCSDE (Rmax: 471, 490, 364, and 343, respectively), and a low response to sCH (Rmax: 189). PT exhibited high responses to sCS, sCSAC, sCSE, and sCSDE (Rmax: 2230, 2620, 3370, and 2820, respectively), moderate responses to CSA, CSD, CSE, and sCSAD (Rmax: 1180, 1410, 1770, and 1950, respectively), low responses to CH, CSC, sCSA, and sCtriS (Rmax: 755, 779, 920, and 980, respectively), and the lowest response to sCH (Rmax: 344).

The $K_D$ values of MK for CSD, CSE, and sCSAD were 78, 80, and 99 nM, respectively. These were 1.3- to 1.7-fold lower than that of CSC, and 5- to 12-fold lower than those of CSA, sCSA, sCSC, sCSAC, and sCSDE. The $K_D$ values of MK for CH, sCSC, sCSE, and sCtriS were over 1,000 nM, and the sCH chip showed the lowest affinity to MK ($K_D$ = 11,200 nM). While the CSC chip had a high association rate constant ($k_a$ = 3.5 × 10$^4$ M$^{-1}$s$^{-1}$), CH, CSA, and the synthetic CS derivatives with the exception of sCSC had lower $k_a$ values (in the order of 10$^2$ M$^{-1}$s$^{-1}$). CSD, sCSAC, and sCSAD had lower dissociation rate constants ($k_d$, in the order of 10$^{-5}$ s$^{-1}$), and CS, CSC, sCH, and sCtriS had higher $k_d$ values (in the order of 10$^{-3}$ s$^{-1}$).

**DISCUSSION**

Recent studies have revealed that CS exhibits various functions via specific binding to physiologically active molecules. Although native CS sources designated as CSA, CSC, CSD, and CSE, are available, their CS disaccharide compositions are not uniform, preventing our understanding of the precise relationship between CS function and structure. In this study, we chemoenzymatically synthesized various CS species with defined compositions, some of which are of uniform structure, and therefore with defined structure. This is the first report of the construction of a CS library with a specified length ($M_r$ ≈ 10k) and defined structures. Using this library, we performed the characterization of individual CS sulfotransferases especially with regard to their substrate specificity, epitope analysis of different anti-CS antibodies, and determination of the CS structure required for binding to MK and PT. These results clearly demonstrate that our CS library is indeed applicable to various studies of CS functions.

For the construction of the CS library, the selection and preparation of enzymes were critical. At present, six CS synthases have been identified in mammals (2,24). However, any one or a mixture of two or more of these enzymes could not elongate CH chain in vitro, and co-expression of at least two of these enzymes in the cell is required for CS polymerization (25). In contrast,
the single enzyme K4CP, a capsular enzyme of bacteria (14) can polymerize the CH chain with a desired length presumably over 500 kDa (Supplemental Fig. 2). In addition, CH prepared using K4CP is totally non-sulfated, whereas chemically desulfated CH species still contain ~10% sulfated disaccharide units.

For the transfer of sulfate groups, we used animal sulfotransferases, which have been well characterized. Regarding their expression, bacterial expression systems have failed to produce sufficiently active enzymes except in a few cases (26-28), and baculoviral infection systems were unsuitable because of chondroitinase activity in the virus-infected medium (29). CS sulfotransferases obtained from mammalian expression systems efficiently modified the CH and CS chains. C4ST-1 and C6ST-1 generated sCSA and sCSC possessing >95% 4S and 6S units, respectively, indicating that 22 disaccharide units out of 23 were uniformly sulfated. In addition, we could generate CS molecules with the desired compositions of 4S and 6S units by changing the reaction conditions (Supplemental Fig. 3), allowing simultaneous reactions with both C4ST-1 and C6ST-1 (Supplemental Fig. 4), and all the chains were sulfated rather equally (Supplemental Fig. 5).

Almost all (~90%) 4S units in sCSA-HMDA were sulfated by GaINAc4S-6ST at the C-6 position of the GaINAc(4S) residue. Though the enzyme was reported to preferentially catalyze GaINAc(4S) residues at the nonreducing end of CSA (6,30), it also catalyzed inside 4S units. Although UA2ST sulfated a GlcUA residue with only 4% of the 6S units in sCSC-HMDA, it transferred ~90% of the 6S units in sCSAC-HMDA, consistent with a previous report (31) showing that UA2ST preferentially sulfates the GlcUA residue of GaINAc(4S)-GlcUA-GaINAc(6S). UA2ST sulfated GlcUA, not IdoUA, residue with 9% of the 4S units in sCSAC-HMDA to form the diSB unit in sCSAD-HMDA with GaINAc4S-6ST. These results suggest that UA2ST requires C4- and/or C6-sulfation in the GaINAc residue adjacent to GlcUA, and that GaINAc4S-6ST does not catalyze GaINAc C6-sulfation after C2 sulfation in the GlcUA residue with UA2ST. The sequential catalytic reaction by GaINAc4S-6ST and UA2ST followed by C4ST and C6ST is strictly defined in vivo.

Epitopes of anti-CS antibodies have been determined by inhibition of interactions with defined oligosaccharides. In the ELISA system, we analyzed the direct interactions of antibodies with CS with defined composition and structure. The most distinct feature was that neither of the CS antibodies tested bound to uniform CS species such as sCH (100% 0S), sCSA (>95% 4S), sCSC (>95% 6S), and sCSE (~88% diSE), except for LY111, which bound to sCSA very weakly, indicating that anti-CS antibodies, in general, require a block of complex CS structures. The interactions with native CS species suggest that these antibodies require minor CS disaccharide residues for binding, in addition to the major CS unit. Another feature is that all the antibodies bound to sCSAC at substantial levels, indicating that the alternate repeating structure of 4S and 6S units is necessary for binding. MO225 bound to sCSDE, sCSAD, and sCtriS, suggesting that it preferentially recognizes a block containing a diSD unit, which confirms previous reports (8). CS56, LY111, and 2H6 bound to sCSAC, sCSAD, and sCSDE, suggesting the preferential requirement of a complex structure composed of various sulfated saccharides. These indicate that these antibodies require combinations of non-uniform mixed sequences in their epitopes, and actually, this is in agreement with the epitope sequences for mAb WF6 (12).

In SPR analysis, native CSD and CSE showed high affinity to MK and PT, consistent with previous reports (33-35), and a comparison between the native and synthetic CS reveals distinct features. MK bound to sCS containing diSD units, but with a lower affinity than to one containing diSE units. Its binding to native CSE may be due to the long chain size (Mr = 100k) and/or the heterogeneous binding motif. MK also bound to sCSA, sCSC, and sCSAC, at certain levels, indicating that it binds to a uniform CS
structure and does not always require a diSD unit. PT binds to sCSC, sCSAC, and sCSAD, similar to MK. As the dissociation constants of these to sCSE were similar, the CSD unit may not be important for stable binding.

The binding motifs of many glycosaminoglycans to various molecules and receptors, such as that of heparan sulfate for heparin-binding growth factors and growth factor receptors, are 8–12 mer oligosaccharides (36). Similarly, CS may contain a binding motif of certain saccharide residues. DS structure, containing IdoUA instead of GlcUA, is important for binding to cytokines, for example heparin cofactor II (37). Chemo-enzymatic synthesis enables the generation of CS chains with specific lengths, distinct compositions, and defined structures. Therefore it will provide a useful tool for studies of physiological functions of CS in development and disease.

REFERENCES


sulfate by uronosyl 2-O-sulfotransferase. J. Biol. Chem. 280, 39115-39123

FOOTNOTES
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Abbreviations
The abbreviations used are: CS, chondroitin sulfate; CH, chondroitin; CSA, chondroitin sulfate A from whale cartilage; CSC, chondroitin sulfate C from shark cartilage; CSD, chondroitin sulfate D from shark fin cartilage; CSE, chondroitin sulfate E from squid skin; DS, dermatan sulfate; GalNAc, N-acetyl-D-galactosamine; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; 0S, GlcUA-GalNAc; 4S, GlcUA-GalNAc(4-sulfate); 6S, GlcUA-GalNAc(6-sulfate); diSD, GlcUA(2-sulfate)-GalNAc(6-sulfate); diSE, GlcUA-GalNAc(4-,6-disulfates); triS, GlcUA(2-sulfate)-GalNAc(4,6-disulfates); K4CP, chondroitin polymerase from E. coli strain K4; C4ST, chondroitin 4-sulfotransferase; C6ST, chondroitin 6-sulfotransferase; GalNAc4S-6ST, GalNAc 4-sulfate 6-sulfotransferase ; UA2ST, uronosyl 2-sulfotransferase ; sCH, synthesized CH with K4CP; sCSA, synthesized CS with C4ST-1; sCSC, synthesized CS with C6ST-1; sCSAC, synthesized CS with C4ST-1 and C6ST-1; sCSE, synthesized CS with GalNAc4S-6ST from sCSA; sCSAD, synthesized CS with UA2ST from sCSC and C6ST-1; sCSDE, synthesized CS with GalNAc4S-6ST from sCSAD; sCtriS, synthesized CS with UA2ST from sCSE; CS-HMDA, hexamethylenediamine-conjugated CS; CS-biotin, biotin-conjugated CS, DTT; dithiothreitol, Mr; average molecular weight, PAPS; adenosine 3′-phosphate 5′-phosphosulphate, SD; degree of sulfation.

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FIGURE LEGENDS

Figure 1. (A) Structures of CS disaccharide units, (B) scheme of enzymatic synthesis of chondroitin polymer and various sulfated CS-HMDA libraries

Figure 2. ELISA profiles of anti-CS antibodies on microplates containing the immobilized native CS-biotin conjugates
The binding of the anti-CS monoclonal antibodies (A: MO225, B: CS56, C: LY111, and D: 2H6) to the immobilized native CS-biotin conjugates (○: CH, □: CSA, Δ: CSC, ▲: CSD, and ■: CSE) in a dose-dependent manner (0.001~10 µg/ml) was determined based on the enzyme reaction of the peroxidase-conjugated secondary antibody with the TMB substrate. Data are the average ± S.D. of three independent experiments.

Figure 3. ELISA profiles of anti-CS antibodies on microplates containing the immobilized synthetic CS-biotin conjugates
The binding of the anti-CS monoclonal antibodies (A: MO225, B: CS56, C: LY111, and D: 2H6) to the immobilized synthetic CS-biotin conjugates (○: sCH, □: sCSA, Δ: sCSC, ●: sCSAC, ▲: sCSAD, ■: sCSE, ◇: sCSDE, and ▼: sCtriS) in a dose-dependent manner (0.001~10 µg/ml) was determined based on the enzyme reaction of the peroxidase-conjugated secondary antibody with the TMB substrate. Data are the average ± S.D. of three independent experiments.

Figure 4. Sensorgrams of MK binding kinetics for native and synthetic CS-biotin conjugates immobilized on SA sensor chips
Various concentrations of MK (…: 50, —: 80, ——: 150, and ——: 250 nM) were injected into the flow cells containing immobilized CS species (A: Blank, B: CH, C: CSA, D: CSC, E: CSD, F: CSE, G: sCH, H: sCSA, I: sCSC, J: sCSAC, K: sCSAD, L: sCSE, M: sCSDE, and N: sCtriS) as described under “Experimental Procedures.” White arrows indicate the beginning of the association phase initiated by the injection of MK solutions, and black arrows indicate the end of the analyte injection and the beginning of the dissociation phase initiated by the running buffer. The values of the vertical axis are expressed in resonance units (RU).

Figure 5. Sensorgrams of PT binding kinetics for native and synthetic CS-biotin conjugates immobilized on SA sensor chips
Various concentrations of PT (…: 50, —: 80, ——: 150, and ——: 250 nM) were injected into the flow cells containing immobilized CS species (A: Blank, B: CH, C: CSA, D: CSC, E: CSD, F: CSE, G: sCH, H: sCSA, I: sCSC, J: sCSAC, K: sCSAD, L: sCSE, M: sCSDE, and N: sCtriS) as described under “Experimental Procedures.” White arrows indicate the beginning of the association phase initiated by the injection of PT solutions, and black arrows indicate the end of the analyte injection and the beginning of the dissociation phase initiated by the running buffer. The values of the vertical axis are expressed in resonance units (RU).
Table 1. Average molecular weights, disaccharide compositions and sulfation degrees of CS-HMDA conjugates

<table>
<thead>
<tr>
<th>Products</th>
<th>Mr</th>
<th>0S</th>
<th>4S</th>
<th>6S</th>
<th>diSE</th>
<th>diSB</th>
<th>diSD</th>
<th>triS</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH-HMDA</td>
<td>6k</td>
<td>90.3%</td>
<td>0.6%</td>
<td>9.1%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.10</td>
</tr>
<tr>
<td>CSA-HMDA</td>
<td>15k</td>
<td>0.5%</td>
<td>77.9%</td>
<td>20.7%</td>
<td>0.4%</td>
<td>–</td>
<td>0.5%</td>
<td>–</td>
<td>1.00</td>
</tr>
<tr>
<td>CSC-HMDA</td>
<td>20k</td>
<td>0.8%</td>
<td>22.4%</td>
<td>66.9%</td>
<td>1.1%</td>
<td>–</td>
<td>8.8%</td>
<td>–</td>
<td>1.09</td>
</tr>
<tr>
<td>CSD-HMDA</td>
<td>20k</td>
<td>1.7%</td>
<td>37.9%</td>
<td>42.8%</td>
<td>1.2%</td>
<td>–</td>
<td>16.5%</td>
<td>–</td>
<td>1.16</td>
</tr>
<tr>
<td>CSE-HMDA</td>
<td>100k</td>
<td>2.6%</td>
<td>26.3%</td>
<td>7.3%</td>
<td>63.8%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.61</td>
</tr>
<tr>
<td>DS-HMDA</td>
<td>20k</td>
<td>0.6%</td>
<td>93.6%</td>
<td>–</td>
<td>–</td>
<td>5.8%</td>
<td>–</td>
<td>–</td>
<td>1.05</td>
</tr>
</tbody>
</table>

^a: Average molecular weight

^b: SD indicates sulfation degree, and means number of sulfate groups as disaccharide unit of CS molecules

^c: – means not detected (= 0%).
Table 2. Disaccharide compositions and sulfation degrees of sCS-HMDA conjugates

<table>
<thead>
<tr>
<th>Products</th>
<th>0S</th>
<th>4S</th>
<th>6S</th>
<th>diSE</th>
<th>diSB</th>
<th>diSD</th>
<th>triS</th>
<th>SD^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCH-HMDA</td>
<td>100% ± 0%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>sCSA-HMDA</td>
<td>3.5% ± 1.5%</td>
<td>96.5% ± 1.5%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>sCSC-HMDA</td>
<td>4.3% ± 3.4%</td>
<td>-</td>
<td>95.7% ± 3.4%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>sCSAC-HMDA</td>
<td>1.6% ± 0.4%</td>
<td>47.9% ± 7.3%</td>
<td>50.6% ± 7.3%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.99 ± 0.15</td>
</tr>
<tr>
<td>sCSAD-HMDA</td>
<td>1.4% ± 0.9%</td>
<td>45.7% ± 4.5%</td>
<td>8.5% ± 3.1%</td>
<td>-</td>
<td>4.5% ± 2.3%</td>
<td>41.2% ± 1.7%</td>
<td>-</td>
<td>1.46 ± 0.16</td>
</tr>
<tr>
<td>sCSE-HMDA</td>
<td>1.7% ± 0.9%</td>
<td>10.2% ± 2.6%</td>
<td>-</td>
<td>88.1% ± 3.8%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.86 ± 0.10</td>
</tr>
<tr>
<td>sCSDE-HMGA</td>
<td>0.9% ± 0.6%</td>
<td>28.4% ± 2.8%</td>
<td>6.0% ± 2.8%</td>
<td>20.1% ± 1.1%</td>
<td>4.9% ± 2.3%</td>
<td>39.9% ± 3.4%</td>
<td>-</td>
<td>1.64 ± 0.16</td>
</tr>
<tr>
<td>CtriS-HMDA</td>
<td>1.5% ± 1.3%</td>
<td>8.2% ± 2.8%</td>
<td>-</td>
<td>61.5% ± 9.9%</td>
<td>-</td>
<td>-</td>
<td>28.8% ± 9.1%</td>
<td>2.18 ± 0.50</td>
</tr>
</tbody>
</table>

^a: SD indicates sulfation degrees, and means number of sulfate groups as disaccharide unit of CS molecules

^b: - means not detected (= 0%).

Data of disaccharide compositions and sulfation degrees are means ± standard deviations of three independent synthetic experiments.
Table 3. ED$_{50}$ values of immobilized CS-biotin species on ELISA system with anti-CS antibodies

<table>
<thead>
<tr>
<th>CS samples</th>
<th>MO225</th>
<th>CS-56</th>
<th>LY111</th>
<th>2H6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED$_{50}$ (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH-biotin</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CSA-biotin</td>
<td>0.526 ± 0.067</td>
<td>0.418 ± 0.090</td>
<td>0.177 ± 0.019</td>
<td>0.111 ± 0.013</td>
</tr>
<tr>
<td>CSC-biotin</td>
<td>0.0423 ± 0.0033</td>
<td>0.0440 ± 0.0072</td>
<td>0.300 ± 0.047</td>
<td>0.0731 ± 0.0076</td>
</tr>
<tr>
<td>CSD-biotin</td>
<td>0.0593 ± 0.0043</td>
<td>0.0834 ± 0.0123</td>
<td>0.691 ± 0.076</td>
<td>0.494 ± 0.054</td>
</tr>
<tr>
<td>CSE-biotin</td>
<td>0.0824 ± 0.0084</td>
<td>0.261 ± 0.026</td>
<td>0.798 ± 0.139</td>
<td>0.403 ± 0.047</td>
</tr>
<tr>
<td>sCH-biotin</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sCSA-biotin</td>
<td>nd</td>
<td>nd</td>
<td>2.09 ± 0.50</td>
<td>nd</td>
</tr>
<tr>
<td>sCSC-biotin</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sCSAC-biotin</td>
<td>0.0604 ± 0.0045</td>
<td>0.0448 ± 0.0048</td>
<td>0.0611 ± 0.0056</td>
<td>0.0359 ± 0.007</td>
</tr>
<tr>
<td>sCSAD-biotin</td>
<td>0.0134 ± 0.0023</td>
<td>0.0412 ± 0.0028</td>
<td>0.134 ± 0.012</td>
<td>0.166 ± 0.014</td>
</tr>
<tr>
<td>sCSE-biotin</td>
<td>0.190 ± 0.017</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>sCSDE-biotin</td>
<td>0.0059 ± 0.0010</td>
<td>0.0442 ± 0.0020</td>
<td>0.0814 ± 0.0111</td>
<td>0.248 ± 0.028</td>
</tr>
<tr>
<td>sCtriS-biotin</td>
<td>0.0329 ± 0.0038</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd: not determined
Table 4. Kinetic parameters of cytokines for the interaction with immobilized CS-biotin species on SPR assay system

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CS samples</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
<th>Rmax (RU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK</td>
<td>CH-biotin</td>
<td>$(7.68 \pm 1.21) \times 10^2$</td>
<td>$(2.80 \pm 0.13) \times 10^{-3}$</td>
<td>3,640</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>CSA-biotin</td>
<td>$(7.29 \pm 1.15) \times 10^2$</td>
<td>$(6.43 \pm 2.21) \times 10^{-4}$</td>
<td>882</td>
<td>471</td>
</tr>
<tr>
<td></td>
<td>CSC-biotin</td>
<td>$(3.50 \pm 0.10) \times 10^4$</td>
<td>$(4.76 \pm 0.20) \times 10^{-3}$</td>
<td>136</td>
<td>771</td>
</tr>
<tr>
<td></td>
<td>CSD-biotin</td>
<td>$(1.06 \pm 0.11) \times 10^3$</td>
<td>$(8.26 \pm 0.44) \times 10^{-5}$</td>
<td>78</td>
<td>972</td>
</tr>
<tr>
<td></td>
<td>CSE-biotin</td>
<td>$(4.72 \pm 0.29) \times 10^3$</td>
<td>$(3.60 \pm 0.34) \times 10^{-4}$</td>
<td>80</td>
<td>940</td>
</tr>
<tr>
<td></td>
<td>sCH-biotin</td>
<td>$(3.40 \pm 0.45) \times 10^2$</td>
<td>$(3.80 \pm 0.12) \times 10^{-3}$</td>
<td>11,200</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>sCSA-biotin</td>
<td>$(1.40 \pm 0.14) \times 10^3$</td>
<td>$(1.38 \pm 0.07) \times 10^{-4}$</td>
<td>986</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>sCSC-biotin</td>
<td>$(1.46 \pm 0.17) \times 10^3$</td>
<td>$(6.70 \pm 0.33) \times 10^{-4}$</td>
<td>459</td>
<td>844</td>
</tr>
<tr>
<td></td>
<td>sCSAC-biotin</td>
<td>$(1.48 \pm 0.12) \times 10^3$</td>
<td>$(7.93 \pm 0.43) \times 10^{-5}$</td>
<td>537</td>
<td>744</td>
</tr>
<tr>
<td></td>
<td>sCSAD-biotin</td>
<td>$(8.05 \pm 0.62) \times 10^2$</td>
<td>$(8.00 \pm 1.07) \times 10^{-5}$</td>
<td>99</td>
<td>809</td>
</tr>
<tr>
<td></td>
<td>sCSE-biotin</td>
<td>$(4.60 \pm 0.35) \times 10^2$</td>
<td>$(6.04 \pm 0.75) \times 10^{-4}$</td>
<td>1,310</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td>sCSDE-biotin</td>
<td>$(5.95 \pm 0.40) \times 10^2$</td>
<td>$(2.99 \pm 0.43) \times 10^{-4}$</td>
<td>503</td>
<td>536</td>
</tr>
<tr>
<td></td>
<td>sCtriS-biotin</td>
<td>$(9.12 \pm 1.90) \times 10^2$</td>
<td>$(5.18 \pm 0.21) \times 10^{-3}$</td>
<td>5,680</td>
<td>343</td>
</tr>
</tbody>
</table>

| PT       | CH-biotin  | $(5.42 \pm 0.09) \times 10^4$ | $(1.19 \pm 0.29) \times 10^{-2}$ | 220      | 755      |
|          | CSA-biotin | $(2.60 \pm 0.05) \times 10^4$ | $(3.57 \pm 0.09) \times 10^{-3}$ | 137      | 1180     |
|          | CSC-biotin | $(3.75 \pm 0.15) \times 10^4$ | $(3.97 \pm 0.24) \times 10^{-3}$ | 106      | 799      |
|          | CSD-biotin | $(3.67 \pm 0.07) \times 10^4$ | $(1.52 \pm 0.06) \times 10^{-3}$ | 42       | 1410     |
|          | CSE-biotin | $(3.03 \pm 0.06) \times 10^4$ | $(1.08 \pm 0.05) \times 10^{-3}$ | 36       | 1770     |
|          | sCH-biotin | $(4.48 \pm 0.10) \times 10^2$ | $(5.34 \pm 0.08) \times 10^{-3}$ | 11,900   | 344      |
|          | sCSA-biotin| $(4.40 \pm 0.46) \times 10^3$ | $(8.24 \pm 1.31) \times 10^{-3}$ | 1,820    | 920      |
|          | sCSC-biotin| $(1.17 \pm 0.09) \times 10^4$ | $(5.03 \pm 0.24) \times 10^{-3}$ | 429      | 2230     |
|          | sCSAC-biotin| $(1.41 \pm 0.74) \times 10^4$ | $(3.81 \pm 0.19) \times 10^{-3}$ | 270      | 2620     |
|          | sCSAD-biotin| $(1.15 \pm 0.06) \times 10^4$ | $(2.97 \pm 0.17) \times 10^{-3}$ | 259      | 1950     |
|          | sCSE-biotin| $(2.56 \pm 0.07) \times 10^4$ | $(2.18 \pm 0.16) \times 10^{-3}$ | 140      | 3370     |
|          | sCSDE-biotin| $(1.45 \pm 0.05) \times 10^4$ | $(2.89 \pm 0.20) \times 10^{-3}$ | 200      | 2820     |
|          | sCtriS-biotin| $(1.74 \pm 0.19) \times 10^3$ | $(4.38 \pm 0.62) \times 10^{-4}$ | 2,520    | 980      |

Rmax: maximum resonance unit at 250 nM of analyte
Fig. 1

(A) 0S (CH) GlcUA-GalNAc

4S (CSA) GlcUA-GalNAc(4S)

6S (CSC) GlcUA-GalNAc(6S)

diSD (CSD) GlcUA(2S)-GalNAc(6S)

diSE (CSE) GlcUA-GalNAc(4S6S)

triS (CtriS) GlcUA(2S)-GalNAc(4S6S)

(B)

CH6-HMDA

K4CP

sCH-HMDA (polymer)

C4ST-1

C6ST-1

sCSA-HMDA

sCSC-HMDA

sCSAC-HMDA

GalNAc4S-6ST

UA2ST

sCSE-HMDA

UA2ST

sTriS-HMDA

UA2ST

sCSAD-HMDA

GalNAc4S-6ST

sCSDE-HMDA
Fig. 2

Concentration of immobilized CS-biotins ($\mu g/ml$)

Absorbance at 450 nm
Fig. 4
Construction of a chondroitin sulfate library with defined structures and analysis of molecular interactions
Nobuo Sugiura, Tatsumasa Shioiri, Mie Chiba, Takashi Sato, Hisashi Narimatsu, Koji Kimata and Hideto Watanabe

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