Synthesis of a chondroitin sulfate disaccharide library and a GAG-binding protein interaction analysis

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ABSTRACT:

Chondroitin sulfate (CS), which belongs to the glycosaminoglycan (GAG) superfamily, is a linear sulfated polysaccharide involved in various biological processes. CS structure is very heterogeneous and contains various sulfation patterns owing to the multiple and random enzymatic modifications that occur during its biosynthesis. The resultant microdomain structure in the CS chain interacts with specific biomolecules to regulate biological functions. Therefore, an analysis of the structure-activity relationship of CS at the molecular level is necessary to clarify their biofunctions. In this study, we designed the common intermediate possessing an orthogonally removable protective group and systematically synthesized all 16 types of CS disaccharide structure generated by sulfation. In addition, we demonstrated the on-time analysis of

the binding properties of GAG-binding proteins using “Sugar Chip” immobilized CS disaccharide structures by surface plasmon resonance (SPR) imaging, indicating that our chip technology is effective for the evaluation of binding properties.

KEYWORDS:

synthesis; chondroitin sulfate; library; Sugar Chip; glycosaminoglycan

MANUSCRIPT TEXT:

Chondroitin sulfate (CS) is a linear, sulfated polysaccharide belonging to the glycosaminoglycan (GAG) superfamily, like heparin (HP) and heparan sulfate (HS). CS is widely found on the surface and in the extracellular matrix of cells including those in nerve and cartilage tissue, playing significant roles in many biological processes such as neurite formation, cell differentiation, proliferation, viral infections, etc.¹⁻⁴ CS structure includes a repeating $\beta(1,3)$ -linked disaccharide unit composed of glucuronic acid and *N*-acetylgalactosamine. CS structure is very heterogeneous and contains various sulfation patterns due to the multiple and random enzymatic modifications that occurs during its biosynthesis.⁵ Consequently, there are many CS microdomain structures, which are thought to regulate the activity of numerous biomolecules, such as growth factors, cytokines, viral proteins, and coagulation factors, through binding interactions.^{6,7} The CS microdomain structures and the structure-function relationships at the molecular level, however, are very difficult to determine due to the naturally occurring structural diversity. Therefore, structurally defined CS sequences are necessary for the precise investigation of the mode of CS action on target molecules.

Although the synthesis of CS oligosaccharides has been studied extensively,⁸⁻¹⁸ the design and synthesis of the building blocks for generating a CS library is still challenging. In the present study, to exhaustively investigate the binding properties of proteins or viruses at the molecular level, we demonstrated the systematic synthesis of all 16 kinds of CS partial disaccharide structures possessing a diverse sulfation set. They were prepared on “Sugar Chips” by immobilizing them onto gold-coated chips, and their binding properties against basic fibroblast growth factor (bFGF) were evaluated by a surface plasmon resonance (SPR) analysis.

For the systematic synthesis of all 16 kinds of CS disaccharide structures, a common trisaccharide intermediate **1** was designed, which contains a diverse set of protective groups for generating various sulfation patterns. Regioselective sulfation was achieved by the selective removal of benzylidene, benzoyl (Bz), and/or 4-acetaminobenzyl (AAB) groups, and subsequent sulfation. The glucose (Glc) moiety at the reductive terminal acts as a hydrophilic spacer when the sugar component is immobilized on an SPR sensor chip. The trisaccharide **1** can be prepared from each monosaccharide building block derived from glucose and galactosamine, respectively.

The preparation of monosaccharide building blocks was carried out as shown in Scheme 1. The *N*-acetylgalactosamine (GalNAc) moiety was derived from

galactosamine hydrochloride in 3 steps in the usual protective manner. The *N*-trichloroethoxycarbonyl (Troc) group was introduced for β -selective glycosylation with glucose moiety. The glucuronic acid (GlcA) moiety was prepared from diisopropylidene-glucose **5**. The *p*-nitrobenzyl (PNB) group¹⁹ was introduced at 3 positions. The obtained **7** was transformed to allyl glycoside **8** by acidic treatment with allyl alcohol followed by benzylidenation. After the introduction of a Bz group, reductive cleavage of the benzylidene group was carried out under conditions with BF₃ etherate and Me₂NH•BH₃ to afford 6-OH compound **10**. The obtained **10** was then converted to methyl glucuronate **11** by TEMPO oxidation and subsequent methyl esterification. Removal of the allyl group of compound **11** afforded GlcA moiety **12**.

With the monosaccharide building blocks obtained, the synthesis of key trisaccharide intermediate **1** was carried out as shown in Scheme 2. The GlcA moiety was converted to glycosyl imidate, which was subjected to glycosylation with GalNAc moiety **5** to afford disaccharide **14** in a high yield. After the removal of the allyl group of disaccharide **14**, the resulting 1-OH group was imidated for the next glycosylation. Trisaccharide **18** was obtained by the glycosylation of Glc moiety **17**^{20,21} with disaccharide donor **16**. Reduction of the nitro group on PNB and the Troc group followed by acetylation gave the desired key trisaccharide intermediate **1**.

Selective cleavage of a benzylidene group of trisaccharide **1** is the key step in the synthesis of a CS disaccharide library. Although many useful reaction conditions have been found in the case of monosaccharides,²² conditions for the selective cleavage of a benzylidene group on a galactosamine residue are limited. Therefore, we examined various conditions leading to three compounds: 4,6-diol **19**, 4-OH **20**, and 6-OH **21**. The results of the selective cleavage of the benzylidene group are shown in Table 1. Diol **19** was easily obtained by standard acidic hydrolysis with trifluoroacetic acid (TFA) (Run 1). Reductive cleavage was then investigated (Runs 2–12). When triethylsilane (Et₃SiH) as a reductant²³ and trifluoromethanesulfonic acid (TfOH) as a promoter were used, 4-OH **20** was obtained (Run 5); when triethylsilane (Et₃SiH) as a reductant and dichlorophenylborane (PhBCl₂) as a promoter were used, 6-OH **21** was obtained at a high yield with excellent selectivity (Run 12).

Since the reactions at the branching point in the synthetic pathways were successfully achieved, syntheses of 16 kinds of CS disaccharide structures were then carried out (Scheme 3). Protective groups such as the *p*-acetaminobenzyl (AAB) and ester groups were orthogonally removed under appropriate conditions, i.e., the AAB

group was removed by DDQ oxidation and the ester group was removed by basic hydrolysis, regardless of the presence of sulfate groups. Yields for the deprotection of the AAB group were around 50% and nearly 50% of starting materials were recovered. Basic hydrolysis of the benzoyl group and methyl ester resulted in high yields. Resulting hydroxy groups were then sulfated by the $\text{SO}_3\cdot\text{pyridine}$ complex. Sulfate groups tolerated the deprotection conditions of AAB and/or ester groups. All of sulfated compounds were subjected to gel-filtration in Sephadex LH-20 columns with MeOH before hydrogenolysis. Hydrogenolysis of sulfated compounds was carried out in a 100-ml stainless steel autoclave under the standard conditions using Pd/C. The trisaccharides obtained were roughly purified by ODS columns and Sephadex G15, and subjected to conjugation with linker molecule **38** according to a method previously reported.^{20,21} The resulting ligand conjugates **39-54** were purified by ODS columns.

After synthesizing the CS disaccharide library, the binding interactions of CS ligand conjugates were analyzed using an SPR imaging sensor. Ligand conjugates were immobilized onto gold chips according to a previous method.^{20,21} Basic fibroblast growth factor (bFGF) was chosen as a CS-binding protein; it strongly binds to the CS-E structure.²⁴ As shown in Figure 2, the binding pattern varied depending on CS structure. Of CS disaccharides possessing mono- or disulfate groups like CS-A (GlcA-GalNAc4S), CS-C (GlcA-GalNAc6S), CS-D (GlcA2S-GalNAc6S), and/or CS-E (GlcA-GalNAc4S6S), which are the major forms occurring in nature, the CS-E and CS-D structures bound tightly to bFGF. CS disaccharides with minor disulfate patterns such as GlcA2S3S-GalNAc (CS-R), GlcA2S-GalNAc4S (CS-B), GlcA3S-GalNAc4S (CS-K), and GlcA3S-GalNAc6S (CS-L), also bound to bFGF with significant binding intensity. In addition, oversulfated disaccharides such as trisulfate and tetrasulfate, which are rarely found in nature, also bound tightly to bFGF. Although the effect of electrostatic interactions between oversulfated disaccharides and proteins should also be considered, the effect of structural interactions is important as evidenced by the variation in binding intensities observed for trisulfated disaccharides. The K_D values calculated using a 1:1 Langmuir binding model are summarized in Table 2 and K_D values on the nanomolar scale were observed in all cases. The SPR signal intensity depends on the protein binding level; however, no model has been proposed to estimate such a parameter. Detailed investigations are ongoing, but these results indicate that the structure-function relationship of CS with respect to GAG-binding proteins at the molecular level can be analyzed on-time by using our CS Sugar Chip.

In conclusion, we designed a trisaccharide intermediate for a CS disaccharide library that possesses orthogonally cleavable protective groups and is capable of generating diverse sulfation patterns. The trisaccharide intermediate was efficiently synthesized using appropriate monosaccharide moieties. The selective cleavage of benzylidene groups on the GalNAc moiety was successfully achieved by choosing appropriate deprotection conditions. Ester and AAB groups were orthogonally deprotected irrespective of the presence or absence of sulfate groups. All 16 kinds of CS disaccharide structures were systematically synthesized from the trisaccharide intermediate and converted to CS ligand conjugates to fabricate the Sugar Chip. The on-time Sugar Chip binding analysis using synthesized CS disaccharides enabled the analysis of the binding property of GAG-binding proteins at the molecular level. Our Sugar Chip is a powerful tool for the analysis of the binding property with GAG-binding proteins, as well as for the high-throughput screening of protein and cell samples.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at ????????

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Legends to Schemes and Figures

Fig. 1. Diversity of CS disaccharide structures and trisaccharide intermediate 1

Scheme 1. Synthesis of monosaccharide building blocks. (a) TrocCl, NaHCO₃ in H₂O; (b) Dowex (H⁺ form) in AllylOH; (c) PhCH(OMe)₂, TsOH•H₂O in CH₃CN, 60% (3 steps); (d) PNBBBr, Ag₂O in Et₂O; (e) Dowex (H⁺ form) in AllylOH; (f) PhCH(OMe)₂, TsOH•H₂O in CH₃CN, 68% (3 steps); (g) BzCl, Pyr. in CH₂Cl₂, 0 °C → rt, 87%; (h) BF₃•Et₂O, Me₂NH•BH₃ in CH₂Cl₂, -18 °C, 84%; (i) TEMPO, NaClO, KBr, NaHCO₃, H₂O in CH₃CN; (j) TMSCHN₂ in MeOH, 96% (2 steps); (k) Ir[(cod)(PMePh₂)₂]PF₆, H₂ in THF; (l) I₂, H₂O in THF, 97%

Scheme 2. Synthesis of trisaccharide intermediate **1**. (a) CCl₃CN, Cs₂CO₃ in CH₂Cl₂; (b) **5**, TMSOTf in CH₂Cl₂, -20 → 4 °C, 80% (2 steps); (c) Ir[(cod)(PMePh₂)₂]PF₆, H₂ in THF; I₂, H₂O in THF, 78% (2 steps); (d) CCl₃CN, Cs₂CO₃ in CH₂Cl₂; (e) TMSOTf in CH₂Cl₂, -20 → 4 °C, 73% (2 steps); (f) Zn-Cu in AcOH; Ac₂O in Pyr., 80% (2 steps)

Table 1. Selective cleavage of the benzylidene group.

Run	Additives (eq)	Solvent	Temp.	Time (h)	Yield (%)		
					19	20	21
1	10% TFA, H ₂ O	CH ₂ Cl ₂	0 °C→rt	5	95	*	*
2 ^a	Et ₃ SiH (3.0), TFA (2.0), MS4A	CH ₂ Cl ₂	0 °C	2	*	*	*
3 ^a	Et ₃ SiH (3.0), BF ₃ •Et ₂ O (2.0), MS4A	CH ₂ Cl ₂	0 °C	2	*	*	*
4 ^a	Et ₃ SiH (3.0), TfOH (3.4), MS4A	CH ₂ Cl ₂	-78 °C	3	*	55	*
5	Et ₃ SiH (6.0), TfOH (6.8), MS4A	CH ₂ Cl ₂	-78 °C	3	*	88	*
6 ^a	BH ₃ •THF (5.0), Yb(OTf) ₃ (3.0)	CH ₂ Cl ₂	rt	2	20	*	*
7 ^a	BH ₃ •THF (5.0), Cu(OTf) ₂ (3.0)	CH ₂ Cl ₂	rt	2	50	*	*
8 ^a	BH ₃ •THF (5.0), La(OTf) ₃ (3.0)	CH ₂ Cl ₂	rt	2	*	*	*
9 ^a	BH ₃ •THF (3.0), CoCl ₂ (3.0)	THF	0 °C→rt	2	*	*	*
10 ^a	BH ₃ •HNMe ₂ (5.0), BF ₃ •Et ₂ O (5.0), MS4A	CH ₂ Cl ₂	0 °C	2	*	*	*
11 ^a	BH ₃ •HNMe ₂ (6.0), PhBCl ₂ (5.0), MS4A	CH ₂ Cl ₂	-78 °C	2	*	*	*
12	Et ₃ SiH (3.0), PhBCl ₂ (2.0), MS4A	CH ₂ Cl ₂	-78 °C	0.2	*	*	97

^aStarting material was recovered. * trace

Scheme 3. Synthesis of 16 CS disaccharide ligand conjugates. (a) LiOH aq., H_2O_2 in THF then NaOH aq. in MeOH; (b) NaOH aq. in MeOH/THF; (c) DDQ, H_2O in CH_2Cl_2 ; (d) TFA, H_2O in CH_2Cl_2 ; (e) $\text{SO}_3\cdot\text{Pyr.}$ in Pyr., 50 °C; (f) $\text{Pd}(\text{OH})_2/\text{C}$, H_2 (7.5 atm) in MeOH/ H_2O /AcOH; Yield: 67% for **22** (from **1**, 2 steps); (g) Pd/C , H_2 (7.5 atm) in MeOH/ H_2O /AcOH; Yields: 47% for **23** (from **1**, 3 steps), 38% for **24** (from **1**, 4 steps), 24% for **25** (from **1**, 4 steps), 19% for **26** (from **1**, 5 steps), 71% for **27** (from **19**, 3 steps), 59% for **28** (from **19**, 3 steps), 42% for **29** (from **19**, 4 steps), 19% for **30** (from **20**, 3 steps), 81% for **31** (from **20**, 3 steps), 25% for **32** (from **20**, 4 steps), 42% for **34** (from **21**, 3 steps), 73% for **35** (from **21**, 3 steps), 24% for **36** (from **21**, 4 steps); (h) **38** then NaBH_3CN in DMAc/ H_2O /AcOH, 37 °C; Yields: 53% for **39**, 69% for **40**, 51% for **41**, 63% for **42**, 18% for **43**, 54% for **44**, 21% for **45**, 63% for **46**, 46% for **47**, 60% for **48**, 36% for **49**, 30% for **50**, 90% for **51**, 6% for **52** (from **20**, 5 steps), 7% for **53** (from **21**, 5 steps), 26% for **54**.

Fig. 2. Binding intensity with bFGF. Bar graph profiles of various protein concentrations. The error bars indicate SDs. Glc indicates Glc α 1-4Glc ligand conjugate, used as a negative control.

Table 2. Kinetic parameters for the interaction of bFGF with CS disaccharide structures

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
39	ND	ND	ND
40	ND	ND	ND
41	1.2×10^5	4.2×10^{-3}	36
42	1.3×10^5	8.2×10^{-3}	62
43	8.6×10^4	2.2×10^{-3}	25
44	9.1×10^4	1.5×10^{-3}	16
45	5.0×10^4	2.1×10^{-3}	41
46	4.1×10^4	1.7×10^{-3}	41
47	9.9×10^4	2.3×10^{-5}	23
48	3.4×10^4	5.0×10^{-3}	147
49	4.1×10^5	3.9×10^{-5}	95
50	3.8×10^4	8.2×10^{-4}	21
51	4.2×10^4	2.5×10^{-3}	60
52	3.9×10^4	2.1×10^{-3}	52
53	3.8×10^4	3.8×10^{-3}	99
54	4.4×10^4	2.2×10^{-4}	4.9

ND means not determined due to feeble interaction.

Graphical Abstract

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