Biochimica et Biophysica Acta 1860 (2016) 844–855

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Impact of donor binding on polymerization catalyzed by KfoC by regulating the affinity of enzyme for acceptor

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ARTICLE INFO

Article history:
Received 1 September 2015
Received in revised form 16 January 2016
Accepted 19 January 2016
Available online 21 January 2016

Keywords:
Carbohydrate biosynthesis
Glycosaminoglycan
Chondroitin polymerase
Substrate specificity
Catalytic mechanism

ABSTRACT

Background: Currently marketed chondroitin sulfate isolated from animal sources and structurally quite heterogeneous. Synthesis of structurally defined chondroitin sulfate is highly desired. The capsular polysaccharide from *Escherichia coli* strain K4 is similar to chondroitin, and its biosynthesis requires a chondroitin polymerase (KfoC). The essential step toward de novo enzymatic synthesis of chondroitin sulfate, synthesis of chondroitin, could be achieved by employing this enzyme.
Methods: Structurally defined acceptors and donor-sugars were prepared by chemoenzymatic approaches. In addition, surface plasmon resonance was employed to determine the binding affinities of individual substrates and donor–acceptor pairs for KfoC.
Results: KfoC has broad donor substrate specificity and acceptor promiscuity, making it an attractive tool enzyme for use in structurally-defined chimeric glycosaminoglycan oligosaccharide synthesis *in vitro*. In addition, the binding of donor substrate molecules regulated the affinity of KfoC for acceptors, then influenced the glycosyl transferase reaction catalyzed by this chondroitin polymerase.
Conclusion and general significance: These results assist in the development of enzymatic synthesis approaches toward chimeric glycosaminoglycan oligosaccharides and designing future strategies for directed evolution of KfoC in order to create mutants toward user-defined goals.

1. Introduction

Chondroitin sulfate proteoglycans (CSPGs), members of the proteoglycan family, play vital functional roles in numerous biological processes and have been implicated in many diseases, including Alzheimer's disease, cancer, and osteoarthritis (OA) [1–6]. Chondroitin sulfate (CS) is covalently linked as a side chain to the core protein of CSPGs through a tetrasaccharide [7]. This unbranched polysaccharide consists of disaccharide repeating units of glucuronic acid (GlcUA) and *N*-acetylgalactosamine (GalNAc) with β 1–3 and β 1–4 linkages, each capable of carrying sulfo groups.

CS has been used therapeutically as an important drug for treating OA [4,8]. The CS currently marketed is isolated from animal sources, such as bovine or shark cartilage [9]. Therefore, most commercial CS polysaccharides and oligosaccharides are structurally quite heterogeneous with respect to size and sulfation, which causes potential safety issues and limits further development as a drug [10]. Therefore, it is highly desirable to develop effective synthetic strategies to produce non-animal sourced, homogeneous CS. Chemoenzymatic synthesis is an efficient solution to combat such problems and has leapt forward in recent years. For example, a series of synthetic structurally defined heparin oligosaccharides were prepared by cost-effective chemoenzymatic methods with high efficiency [11,12]. Full understanding of the substrate specificities and catalytic modes of the microbial-derived and animal-derived enzymes involved in the heparin synthesis was essential in the development of this method [13–16].

Fortunately, some bacteria, such as *Escherichia coli* strain K4, *Pasteurella multocida* type F and *Avibacterium paragallinarum* genotype I, possess CS backbone (chondroitin) that is identical to human and animal CS or its derivatives [17–21]. However, the amount of polysaccharide produced by these strains is not sufficient to allow their use as an industrial platform for the production of chondroitin [22]. A series of microbial-derived chondroitin polymerizing enzymes have been identified and studied *in vitro* [22–26], making it possible to produce homogeneous CS oligosaccharides through a similar strategy to that used for the *in vitro* synthesis of heparin. To expand this approach, understanding of

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http://dx.doi.org/10.1016/j.bbagen.2016.01.018
0304-4165/

the control mechanism for the microbial biosynthesis of CS and uncovering the substrate tolerance of CS biosynthesis enzymes are necessary.

E. coli K4 capsular polysaccharide consists of a GalNAc and GlcUA repeating disaccharide to which a fructose is β-linked at position C-3 of the GlcUA residues [19]. Previous studies have indicated that the biosynthesis of this polysaccharide requires a chondroitin polymerase (KfoC) [23]. This enzyme has two glycosyl transferase activities, *N*-acetylgalactosaminyltransferase (GalNAc-T) and D-glucuronyltransferase (GlcUA-T). KfoC alternatively incorporates GalNAc and GlcUA residues, from uridine 5′-diphosphate-*N*-acetylgalactosamine (UDP-GalNAc) and UDP-glucuronic acid (UDP-GlcUA), to the nonreducing ends of the oligo- or polysaccharides. However, understanding of the substrate specificity of this enzyme is still incomplete, which is primarily due to the unavailability of pure substrates. Here, a series of structurally defined glycosaminoglycan (GAG) oligosaccharides, such as the backbone oligosaccharides of heparan sulfate (HS), CS and hyaluronic acid (HA), and their *N*-modified derivatives, were prepared and used to systematically investigate the acceptor substrate tolerance of KfoC. Moreover, a number of UDP-sugar analogs were used to test the donor substrate specificity. Last, substrate–enzyme interactions, especially the effects of the initial substance binding on the affinity of secondary substance for enzyme–substrate complex were investigated. These experiments resulted in the interesting new finding that the binding of donor molecules regulates the affinity of KfoC for acceptor molecules. These results could help in the development of enzymatic synthesis approaches toward chimeric GAG oligosaccharides. It is also envisaged that the studies on substrate–enzyme interactions will assist in designing future strategies for directed evolution of KfoC in order to create mutants with broader substrate specificities and enhanced catalytic activities.

2. Materials and methods

2.1. Polymerase activity of recombinant KfoC

KfoC (GenBank accession number AB079602) was recombinantly expressed in *E. coli* BL21 (DE3) cells as a soluble *N*-His_6-tagged fusion protein and purified by appropriate affinity chromatography, as described in the supplemental information. GalNAc and GlcUA transferase activity assays were carried out on purified KfoC using defined acceptors. To determine the GalNAc transferase activity, enzyme (1 μg) was

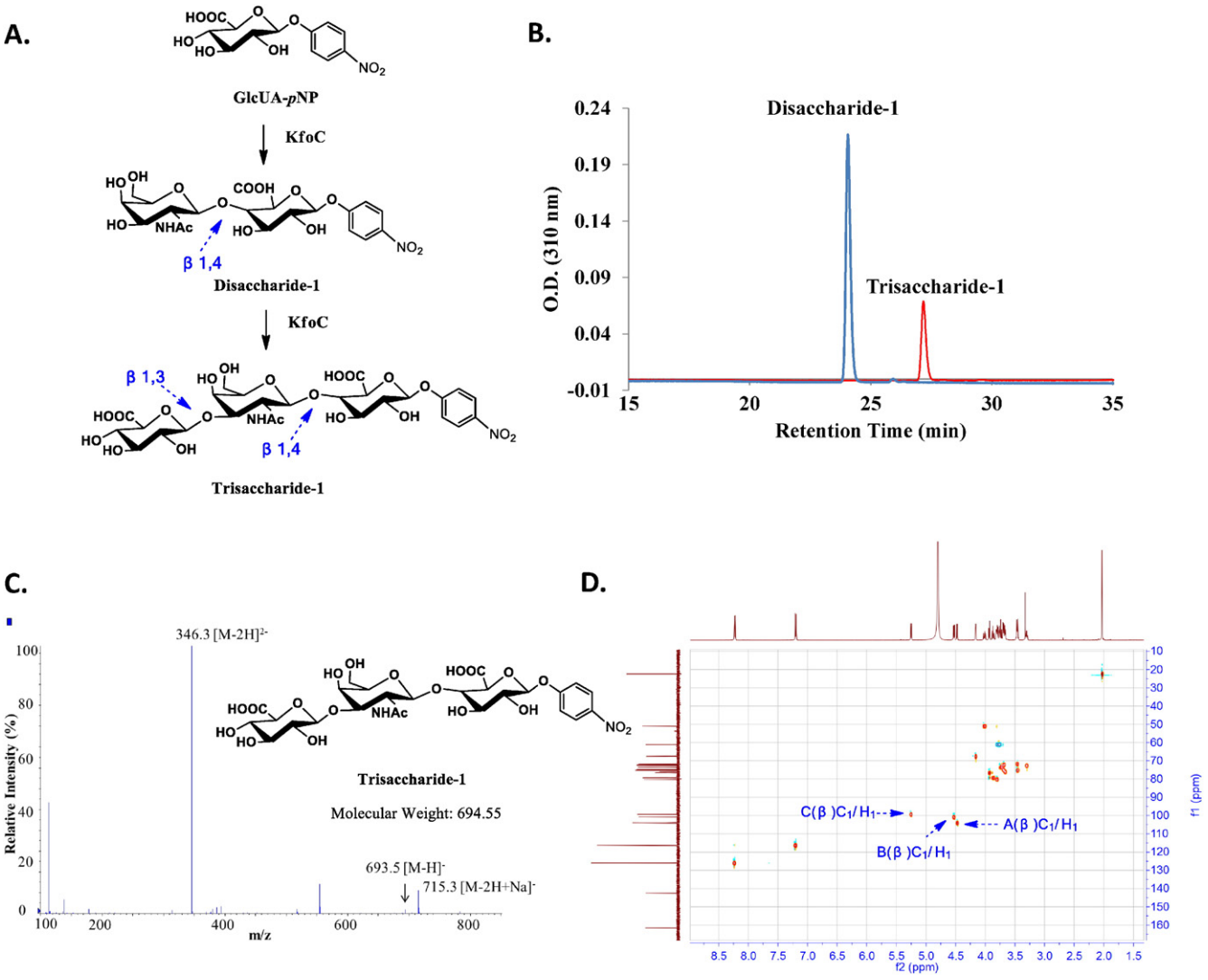

Fig. 1. Determination of the bifunctional polymerase activity of KfoC. KfoC activity was determined using GlcUA-*p*NP as an initiating acceptor. UDP-GalNAc and UDP-GlcUA were used as monosaccharide donors to determine the *N*-acetylgalactosaminyltransferase and glucuronysyltransferase activities, respectively. (A) Targeted structures and schematic synthesis of trisaccharide-1. (B) PAMN-HPLC analysis of the *N*-acetylgalactosaminyltransferase product (disaccharide-1) and glucuronysyltransferase product (trisaccharide-1). (C) ESI-MS analysis of trisaccharide-1. (D) 1D and 2D correlation spectra of trisaccharide-1. Anomeric protons resonate at δ5.25 (d, $J = 7.6$ Hz, 1 H), 4.53 (d, $J = 8.5$ Hz, 1 H), and 4.48 (d, $J = 7.9$ Hz, 1 H). All the coupling constants of anomeric protons (~8 Hz) indicate β-linkages.

incubated in 1 ml buffer (pH 7.0) containing 50 mM Tris–HCl, 0.04 mM 1-O-(*para*-nitrophenyl)-glucuronide (GlcUA-*p*NP) (Sigma Chemical Co., St. Louis, MO, USA), 20 mM $MnCl_2$, and 0.06 mM UDP-GalNAc for 4 h at 37 °C. The reaction was stopped by boiling for 2 min, followed by analysis using polyamine-based anion exchange (PAMN)-HPLC to demonstrate the formation of the GalNAc-containing disaccharide. For PAMN-HPLC, the column (Shimogyo-ku, Kyoto, Japan) was eluted with a linear gradient of KH_2PO_4 from 0 to 0.4 M over 40 min at a flow rate of 0.5 ml/min. The *para*-nitrophenyl (*p*NP) group can be detected at 310 nm.

After determination of the GalNAc transferase activity of KfoC, 50 μmol of GlcUA-*p*NP, 55 μmol of UDP-GalNAc and 2 mg of KfoC were incubated in a 100 ml reaction system at 37 °C for 12 h. Upon the complete consumption of UDP-GalNAc assayed by PAMN-HPLC, 2 mg of KfoC and 80 μmol of UDP-GlcUA were added into the reaction mixture and allowed to incubate overnight at 37 °C. Then, the product was also analyzed using PAMN-HPLC, followed by purification using a Bio-Gel P-2 column (0.75 × 200 cm) from Bio-Rad (Richmond, CA, USA), which was equilibrated with 0.1 M ammonium bicarbonate, at a flow rate of 4 ml/h [16]. The fractions containing product were located by O.D. 310 nm detection, followed by electrospray ionization mass spectrometry (ESI-MS) analysis.

2.2. Preparation of UDP-sugars

UDP-*N*-trifluoroacetylated glucosamine (UDP-GlcNTFA), UDP-glucosamine (UDP-$GlcNH_2$) and UDP-acetylmannosamine (UDP-ManNAc) were prepared in this study. UDP-GlcNTFA was synthesized by *N*-acetylhexosamine 1-kinase (NahK) and glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU), starting from glucosamine (Sigma Chemical Co., St. Louis, MO), following the protocol described previously [27–29]. The NTFA group of UDP-GlcNTFA was subsequently removed under mildly basic conditions to produce the corresponding UDP-glucosamine (UDP-$GlcNH_2$) [27]. UDP-ManNAc was synthesized by NahK and UDP-sugar pyrophosphorylase, starting from ManNAc [30].

2.3. Preparation of oligosaccharide acceptors

A total of five structurally defined oligosaccharides were prepared in this study. The preparation of trisaccharide-HP and its *N*-modification derivatives (trisaccharide-NTFA, trisaccharide-NH_2, and trisaccharide-NS) followed essentially the same procedures described in our previous work [11,16,31,32]. Briefly, the synthesis was initiated from a commercially available monosaccharide with a *p*NP group (GlcUA-*p*NP). The

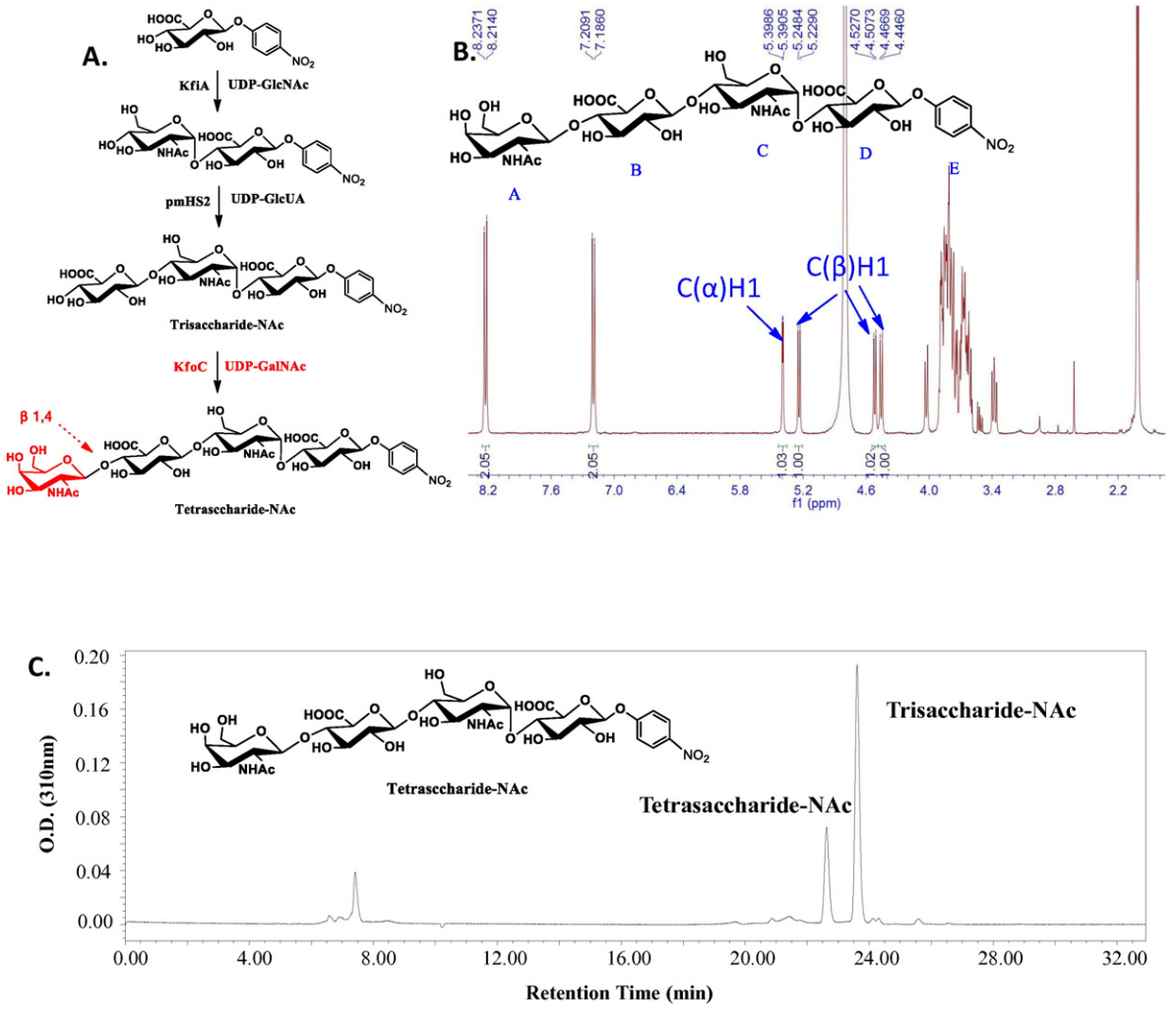

Fig. 2. Structural analysis of the chimeric oligosaccharide synthesized using heparan sulfate backbone trisaccharide as acceptor. (A) Targeted structures and schematic synthesis of tetrasaccharide-NAc. GlcUA-*p*NP was used as the initial acceptor and it was elongated by KfiA and pmHS2 (heparosan synthase 2 from *P. multocida*), by the addition of GlcNAc and GlcUA residues, respectively. Then, the trisaccharide-NAc generated was elongated by KfoC by adding a GalNAc residue at the C4 position of GlcUA via a β-linkage. (B) 1 H spectra of tetrasaccharide-NAc. The anomeric protons resonate at δ5.29 (d, $J = 3.3$ Hz, 1 H), 5.14 (d, $J = 7.7$ Hz, 1 H), 4.42 (d, $J = 7.9$ Hz, 1 H), and 4.36 (d, $J = 8.3$ Hz, 1 H). Coupling constants of 3.3 Hz (an α-linkage) and ~8 Hz demonstrated that the glycosidic bond between the GalNAc and GlcUA residues remains the β-(1,4) linkage. (C) PAMN-HPLC analysis of generation of tetrasaccharide-NAc from trisaccharide-NAc.

elongation was completed with UDP-monosaccharide donors (UDP-GlcUA, UDP-GlcNAc, or UDP-GlcNTFA) and bacterial glycosyltransferases *N*-acetyl-D-glucosaminyltransferase (KfiA) and *P. multocida* heparosan synthase 2 (PmHS2) in buffer containing 50 mM Tris–HCl (pH 7.0) and 20 mM $MnCl_2$. Then, trisaccharide-NTFA was dried and re-suspended in a solution containing CH_3OH, H_2O, and $(C_2H_5)3$ N (*v*/v/*v*, 2:2:1) at 37 °C for 6 h. The samples were dried and redissolved in H_2O, resulting in de-*N*-trifluoroacetylated trisaccharide-NH_2. Trisaccharide-NH_2 was incubated with *N*-sulfotransferase (180 μg/ml) and 0.75 mM 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to yield trisaccharide-NS. The product was confirmed by ESI-MS analysis. A high ratio of PAPS to oligosaccharide was employed to deliver the desired product with high purity and yield during the *N*-sulfation step.

Another acceptor, tetrasaccharide-HA, was the completely degraded product of HA incubated with bovine testis hyaluronidase from Sigma [33]. Then, the degraded products were separated and purified by fractionation using Bio-Gel P-10 (Bio-Rad), which was eluted with a buffer containing 25 mM Tris (pH 7.2) and 1 M NaCl at a flow rate of 6 ml/h. The eluent was monitored and confirmed by ESI-MS.

2.4. ESI-MS and NMR analysis

MS analyses were performed on a Thermo LCQ-Deca. The samples were dissolved in 50% methanol. Experiments were performed in negative ionization mode with a spray voltage of 5 kV and a capillary temperature of 275 °C.

The purified chondroitin trisaccharide (trisaccharide-1) and chimeric tetrasaccharide (tetrasaccharide-NAc) were both characterized by nuclear magnetic resonance (NMR) using protocols described previously [34,35]. Samples were dissolved in 0.5 ml D_2O (99.8%, J&K Scientific Ltd., Beijing, China). Trisaccharide-1 was analyzed on an Agilent 600 MHz instrument (Agilent Technologies, CA), and tetrasaccharide-NAc was investigated on a Bruker 400 MHz spectrometer (Bruker BioSpin, Billerica, MA). All 1D and 2D spectra were obtained at 22 °C, and the spectra were processed using MestReNova software (Mestrelab Research, Escondido, CA, USA).

2.5. Determination of the preference for acceptors

A typical elongation reaction was conducted in a total volume of 1 ml (0.04 mM trisaccharide acceptor, 0.04 mM UDP-GalNAc, 50 mM Tris–

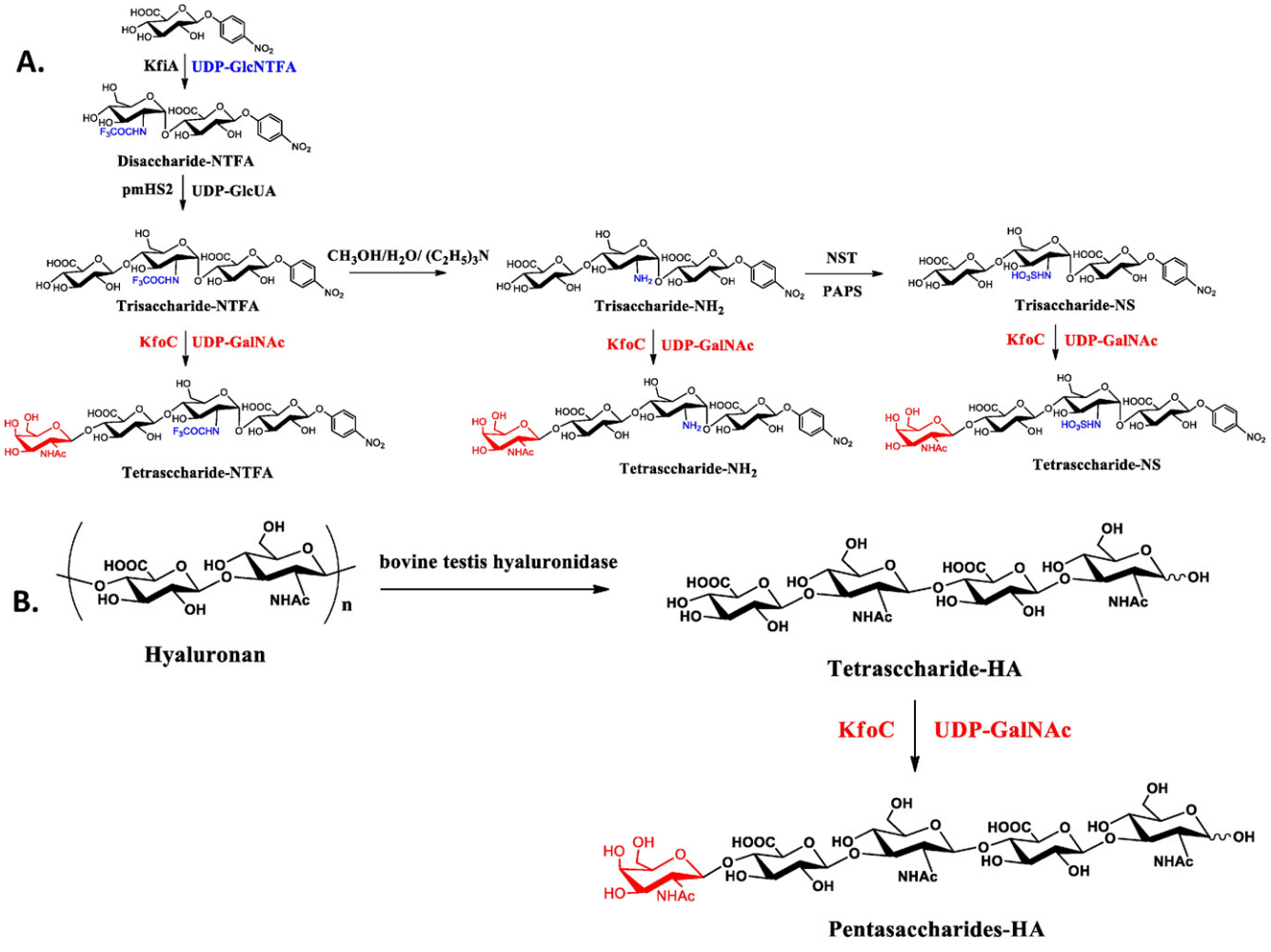

Fig. 3. Preparation of structurally defined oligosaccharides. Four oligosaccharides with various structures were prepared and used as acceptors to determine the *N*-acetylgalactosaminyltransferase activity of KfoC. (A) Targeted structures and schematic synthesis of *N*-modified derivatives of HS backbone tetrasaccharide. The enzymatic synthesis steps are presented in Experimental procedures. Abbreviations: NST, *N*-sulfotransferase; pmHS2, heparosan synthase 2 from *P. multocida*; KfiA, *N*-acetylglucosaminyl transferase from *E. coli* strain K5; KfoC, chondroitin polymerase from *E. coli* strain K4; NTFA, *N*-trifluoroacetyl; GlcNTFA, *N*-trifluoroacetylated glucosamine; GlcUA, glucuronic acid; GlcNS, *N*-sulfoglucosamine; GlcNAc, *N*-acetylated glucosamine; PAPS, 3′-phosphoadenosine-5′-phosphosulfate. The synthesized oligosaccharides were incubated with KfoC to test the acceptor tolerance of the *N*-acetylgalactosaminyltransferase activity. (B) Process of the preparation of hyaluronan tetrasaccharide. Hyaluronan polysaccharide was degraded by bovine testis hyaluronidase. The complete degradation product, tetrasaccharide-HA, was purified by P2 column, followed by incubation with KfoC to test the acceptor tolerance of the *N*-acetylgalactosaminyltransferase activity of KfoC.

HCl, 20 mM Mn^{2+}, pH 7.0) containing 1 μg of purified KfoC for 5 min or 4 h at 37 °C, followed by the subsequent heating of the mixture at 100 °C for 5 min and separation by centrifugation. The reaction mixture was monitored by (PAMN)-HPLC.

Then, the weight-average molecular weight ($\overline{M}$w) and saccharide size dispersity of the polymerization products resulting from the treatment of these two trisaccharides (HP-trisaccharide and CS-trisaccharide) with KfoC and both donors (UDP-GalNAc and UDP-GlcUA) were identified by size exclusion chromatography-multi-angle laser light scattering (MALLS-SEC), following the protocol described previously [36]. Briefly, 0.02 mmole acceptor was incubated with high concentrations of UDP-GalNAc (1.4 mmol) and UDP-GlcUA (1.4 mmol) in a total volume of 15 ml (50 mM Tris–HCl, 20 mM Mn^{2+}, pH 7.0) for 16 h at 37 °C. 1 mg enzyme was also added in the reaction mixtures per 4 h for four times. Then the polymerization products were filtered and analyzed by MALLS (Wyatt, CA, USA) and size exclusion chromatography (SEC) that was equipped with Shodex OHpak SB-803 HQ column (Showa Denko K. K, Japan) at a flow rate of 0.5 ml/min.

2.6. Preparation of surface plasmon resonance chip with immobilized KfoC

To determine the binding kinetic parameters of each substrate and the effects of donor molecule binding on the affinity for acceptor molecules, KfoC was tethered to a CM5 sensor chip (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The surfaces of chips were activated with *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) for 30 min, then KfoC (80 μg/ml) was immobilized in immobilization buffer (10 mM sodium acetate) with pH values in the range 4.0–6.0. Results demonstrated that the His-tagged KfoC was tethered successfully and the optimal pH value of the enzyme solution for the resonance unit (RU) response was 4.5. Then the dissociative carboxyl groups of the CM5 chip were inactivated by ethanolamine-HCl. PBS (20 mM, pH 7.4) and NaCl (1 M) solutions were used as running and regeneration buffer, respectively.

2.7. Surface plasmon resonance analysis

Surface plasmon resonance (SPR) measurements were performed using a Biacore 3000 system at 25 °C. First, the association rate (k_a), dissociation rate (k_d), and the equilibrium dissociation constant (K_D) of each donor and acceptor substrate were measured. For example, GlcUA-*p*NP at various concentrations (0, 20, 40, 80 and 160 μM) flowed across the surface of the CM5 chip with immobilized KfoC at a flow rate of 30 μl/min for 10 min, resulting in multiple sensorgrams of GlcUA-*p*NP binding to KfoC. Subsequently, UDP-GalNAc, UDP-GlcNAc, UDP-GlcNTFA, and GlcUA-GalNAc-GlcUA-*p*NP were respectively analyzed with the same protocol. Second, donor and acceptor solution were injected one after the other so that KfoC captured the substrates in a particular order. For example, 15 μl UDP-GalNAc (200 μM) flowed across the sensor chip, then 15 μl GlcUA-*p*NP (200 μM) was injected across the chip now with immobilized enzyme–UDP-GalNAc complex. The difference between the resonance before and after GlcUA-*p*NP injection was calculated to estimate the regulatory role of UDP-GalNAc binding on the affinity of KfoC for the acceptor GlcUA-*p*NP. Similarly,

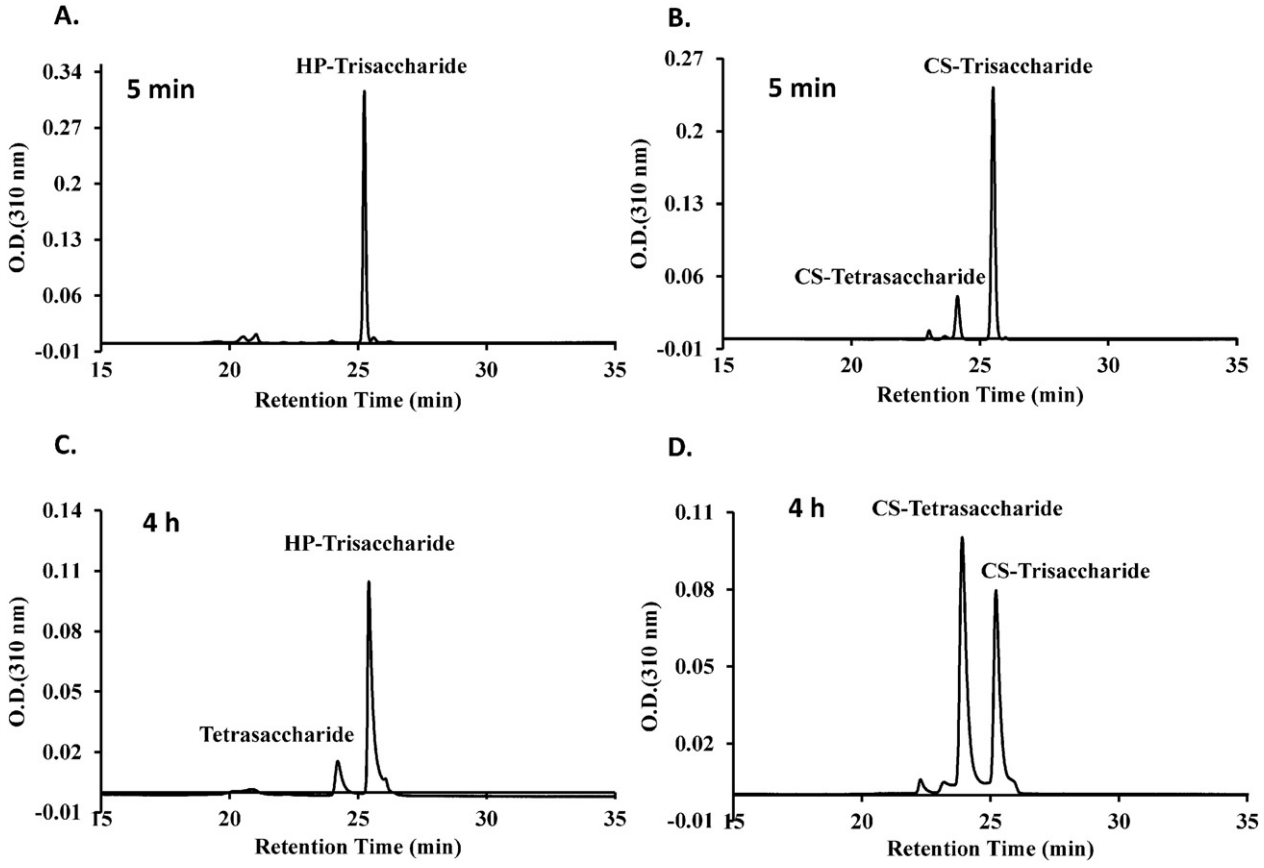

Fig. 4. PAMN-HPLC analysis of the elongation products resulting from the treatment of two trisaccharides (HP-trisaccharide and CS-trisaccharide) with KfoC. A typical elongation reaction was conducted in a total volume of 1 ml (0.04 mM trisaccharide acceptor, 0.04 mM UDP-GalNAc, 50 mM Tris–HCl, 20 mM Mn^{2+}, pH 7.0) containing 1 μg of purified KfoC for 5 min or 4 h at 37 °C, followed by the subsequent heating of the mixture at 100 °C for 5 min and separation by centrifugation. The reaction mixture was monitored by (PAMN)-HPLC. (A) PAMN-HPLC analysis of generation of tetrasacchride from HP-trisaccharide treated for 5 min. (B) PAMN-HPLC analysis of generation of tetrasacchride from CS-trisaccharide treated for 5 min. (C) PAMN-HPLC analysis of generation of tetrasacchride from HP-trisaccharide treated for 4 h. (D) PAMN-HPLC analysis of generation of tetrasacchride from CS-trisaccharide treated for 4 h.

ΔRU for another six pairs, including UDP-GlcNAc and GlcUA-*p*NP, UDP-GalNAc and trisaccharide-1, UDP-GlcNAc and trisaccharide-1, trisaccharide-1 and UDP-GalNAc, trisaccharide-1 and UDP-GlcNAc, and UDP-GlcNTFA and trisaccharide-1, were respectively analyzed using the same protocol. Another two groups (UDP-GlcUA/GlcUA-*p*NP pair and UDP-GalNAc/GalNAc-*p*NP pair) were employed as non-functional controls. The data presented represent the average of three independent determinations ± standard deviation.

Additional methods are presented in the supplemental information.

3. Results and discussion

3.1. Determination of polymerase activity

KfoC is a bifunctional enzyme with both *N*-acetylgalactosaminyltransferase and glucuronysyltransferase activities in *E. coli* strain K4 [23,26,37,38]. The potential application of this enzyme in synthesizing structurally defined CS backbone prompted us to investigate its acceptor and UDP-sugar donor substrate specificities. The full-length KfoC gene was cloned from *E. coli* K4 genomic DNA and expressed as an *N*-terminal His_6 fusion protein, as described in the supplemental information.

The activities of KfoC were determined using GlcUA-*p*NP as an initiating acceptor. Meanwhile, UDP-GalNAc and UDP-GlcUA were used as monosaccharide donors to determine the *N*-acetylgalactosaminyltransferase and glucuronysyltransferase activities, respectively (Fig. 1A). The products (disaccharide-1 and trisaccharide-1) were resolved stepwise using PAMN-HPLC (Fig. 1B). GlcUA-*p*NP and UDP-GalNAc were incubated with KfoC at 37 °C for 8 h. An obvious peak that eluted from the HPLC column at 24 min was observed, while GlcUA-*p*NP was eluted at 26 min, suggesting that a disaccharide was generated by the *N*-acetyl galactosaminyltransferase (GalNAc-T) action of KfoC. Then, UDP-GlcUA and additional KfoC were added into the reaction products containing disaccharide-1, and incubated at 37 °C for 8 h. A novel peak was observed eluting at 27 min, suggesting that a trisaccharide was produced by the glucuronysyltransferase action of KfoC. This trisaccharide was purified and confirmed by ESI-MS and NMR. The molecular mass of trisaccharide-1 was determined to be 694.4 ± 0.2 Da, which is almost identical to the calculated molecular mass of the anticipated trisaccharide (694.2 Da) (Fig. 1C). Synthesized trisaccharide-1 and its intermediates were also analyzed by 1D ^{1}H-NMR, ^{13}C-NMR and 2D-correlation spectroscopy (Fig. 1D). All the results indicated that the bonds between the A–B and B–C rings were β-linkages. As expected, the residues of oligosaccharides synthesized by KfoC are linked by the same glycosidic bonds as in natural chondroitin.

The requirement for the size of the acceptor for KfoC was investigated in a previous publication by incubating the enzyme with various partially digested products of CS and chondroitin, resulting in the conclusion that the acceptor should at least be longer than a tetrasaccharide [23]. However, our data clearly showed that a monosaccharide with a *p*NP group could serve as the acceptor for KfoC. It appears that the *p*NP tag played some role in encouraging the tolerance of the size specificity of the acceptor for KfoC. Similarly, Williams *et al.* reported that a fluorescein tag facilitated the GlcUA group to be extended by *P. multocida* HA synthase (PmHAS), a very similar enzyme to KfoC, used as a synthetic acceptor [24]. Therefore, the activity determination

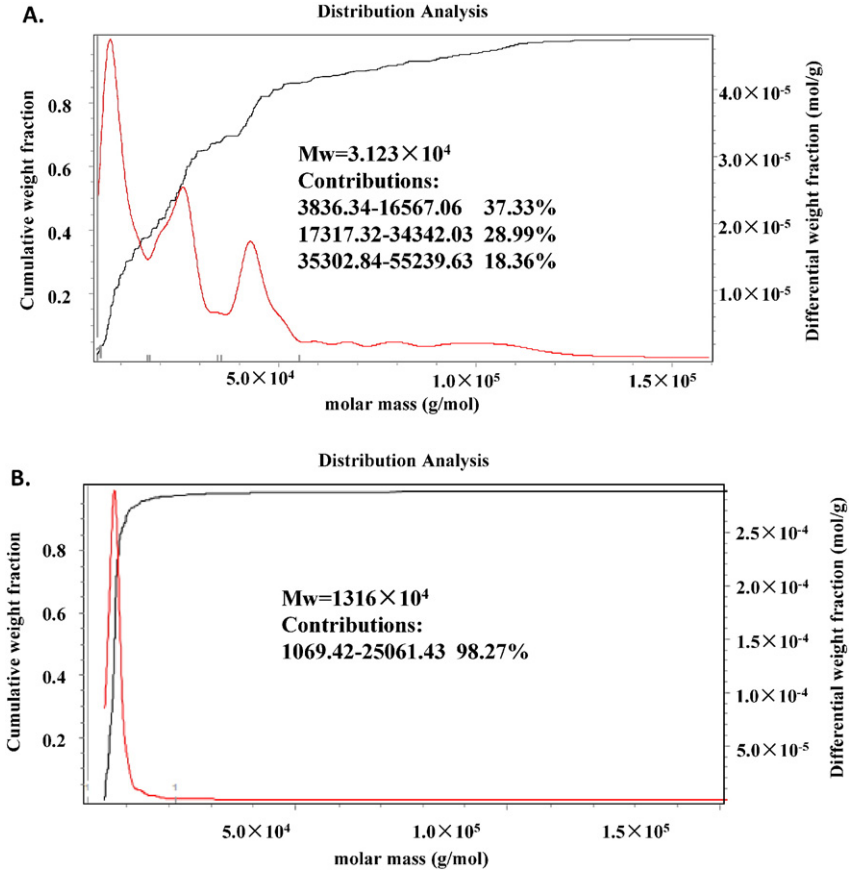

Fig. 5. MALLS-SEC analysis of the polymerization products resulting from the treatment of two trisaccharides (HP-trisaccharide and CS-trisaccharide) with KfoC. The weight-average molecular weight ($\overline{M}$w) and saccharide size dispersity of the polymerization products resulting from the treatment of these two trisaccharides (HP-trisaccharide and CS-trisaccharide) with KfoC and both donors (UDP-GalNAc and UDP-GlcUA) were identified by size exclusion chromatography-multi-angle laser light scattering (MALLS-SEC). (A) MALLS-SEC analysis of the polymerization products resulting from the treatment of HP-trisaccharide with KfoC. (B) MALLS-SEC analysis of the polymerization products resulting from the treatment of CS-trisaccharide with KfoC.

Table 1
Surface plasmon resonance binding studies with immobilized KfoC and individual substrates.

	Analyte	K_D (M)[I]	k_d (1/s)[II]	k_a (1/Ms)[III]
Acceptor	GlcUA-*p*NP	2.3×10^{-6}[V]	1×10^{-5}[V]	4.4[V]
	Trisaccharide-1[IV]	$<5.3 \times 10^{-12}$[VI]	$<1 \times 10^{-5}$[VI]	1.9×10^{6}[VI]
Donor	UDP-GalNAc	1.2×10^{-11}[VII]	1.1×10^{-5}[VII]	9.5×10^{5}[VII]
	UDP-GlcNAc	2.1×10^{-6}[VII]	1×10^{-5}[VII]	4.7[VII]
	UDP-GlcNTFA	4×10^{-8}[IX]	1×10^{-5}[IX]	2.5×10^{2}[IX]

I. The equilibrium dissociation constant.
II. The association rate.
III. The dissociation rate.
IV. GlcUA-(β1,3)-GalNAc-(β1,4)-GlcUA-*p*NP was prepared as described in experimental procedures and shown in Fig. 1A. The MW was determined by ESI-MS.
V. The data from surface plasmon resonance (SPR) analysis shown in supplemental Fig. S 7A.
VI. The data from SPR analysis shown in supplemental Fig. S 7B.
VII. The data from SPR analysis shown in supplemental Fig. S 8A.
VII. The data from SPR analysis shown in supplemental Fig. S 8B.
IX. The data from SPR analysis shown in supplemental Fig. S 8C.

and oligosaccharide synthesis in the present work were initiated from a commercially available monosaccharide with a *p*NP group, GlcUA-*p*NP, and the reaction progress could be easily monitored by exploiting the UV absorbance of the *p*NP tag at 310 nm.

The effects of divalent metal ions and pH on the GalNAc-T activity of KfoC were investigated using GlcUA-*p*NP as the acceptor, as described in supplemental information (supplemental Fig. S1). These results demonstrated that KfoC exhibit its optimal activity in the presence of Mn^{2+} ions and the reaction buffer was close to pH 7.0.

3.2. Donor specificities of the N-acetylgalactosaminyltransferase activity of KfoC

The donor specificities of KfoC were investigated by incubating the enzyme with various UDP-sugars in the presence of the acceptor trisaccharide-1 (GlcUA-(β1,3)-GalNAc-(β1,4)-GlcUA-*p*NP), and the products were analyzed by PAMN-HPLC (supplemental Fig. S2). It appears that KfoC is capable of transferring GlcNAc and ManNAc residues to trisaccharide-1, while we could not detect elongation products when

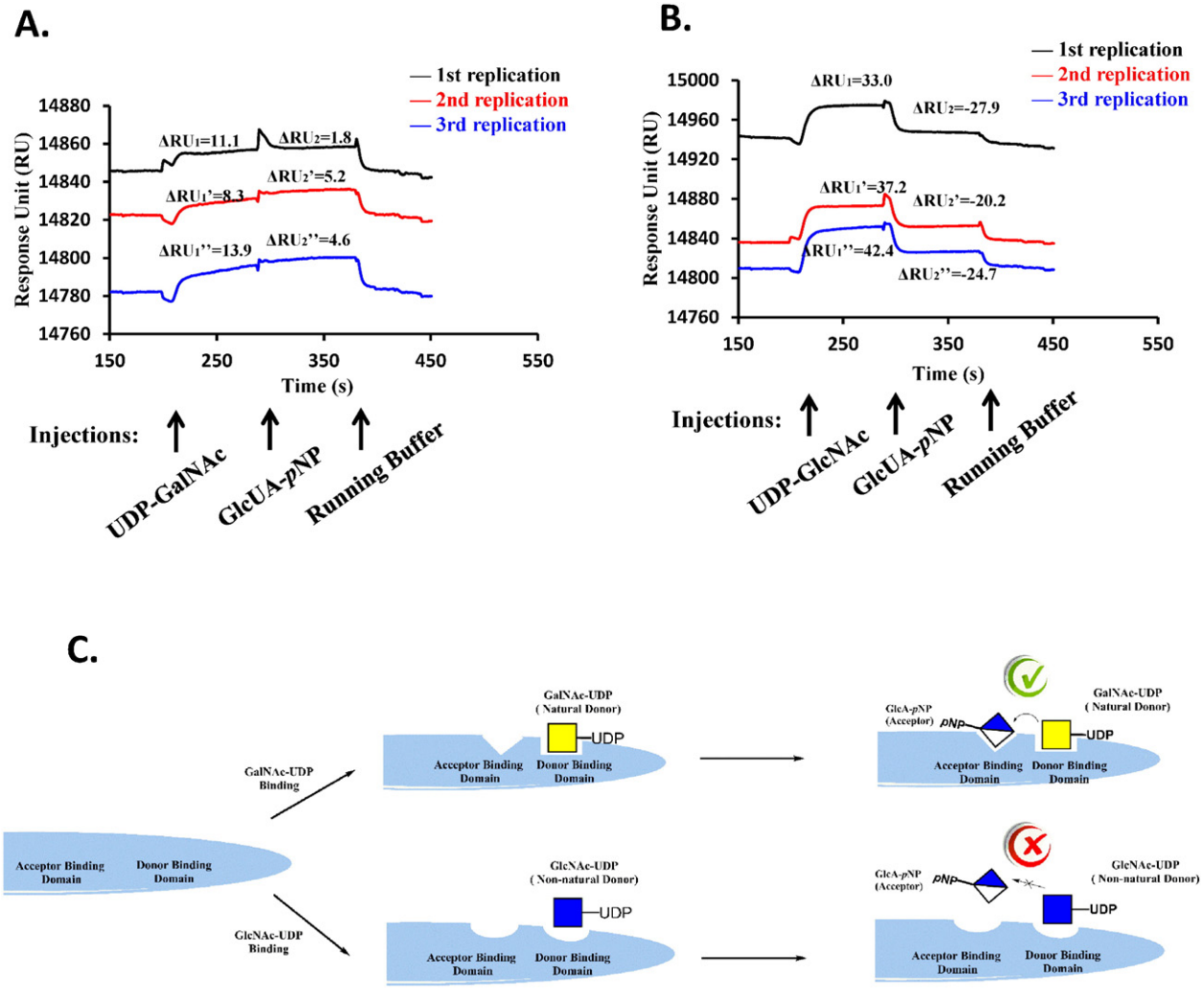

Fig. 6. The hypothetical mechanism for donor binding influencing on the affinity of enzyme for acceptor and the glycosyl transferase reaction. Surface plasmon resonance (SPR) has been used extensively in the field of substrate–enzyme interactions [44]. Here, it was employed to analyse the effects of the initial substrate binding on the affinity in subsequent substrate binding to the enzyme-substrate complex. To guarantee that KfoC captured the substrates in a particular order, the first substance and the secondary one were injected sequentially across the surface of a CM5 chip containing immobilized KfoC. The difference between the resonance units before and after the secondary substrate injection was calculated. (A) Sensorgrams for the UDP-GalNAc/GlcUA-*p*NP pair. The difference between the resonance units before and after GlcUA-*p*NP injection increased when UDP-GalNAc was injected initially. (B) Sensorgrams for the UDP-GlcNAc/GlcUA-*p*NP pair. (C) Within the glycosyl transferase reaction catalyzed by KfoC using GlcUA-*p*NP as acceptor, the tertiary structure of the enzyme bound to UDP-GalNAc clearly elevates catalytic efficiency by upregulating the affinity of GlcUA-*p*NP for the enzyme-donor complex, compared with the holo structure changed upon the binding of UDP-GlcNAc. It implied the reason why UDP-GalNAc could react with *p*NP-monosaccharide while UDP-GlcNAc could not.

Table 2
Impact of first substance binding on the affinity of enzyme for secondary substance.

Analyte				
First substance	Secondary substance	$\Delta RU_1^{II\ XII}$	$\Delta RU_2^{III\ XII}$	Impact [IV]
UDP-GalNAc	GlcUA-*p*NP	11.1 ± 2.8^{V}	3.9 ± 1.8^{V}	Up-regulated
UDP-GlcNAc	GlcUA-*p*NP	37.5 ± 4.7^{VI}	-24.3 ± 3.9^{VI}	Down-regulated
UDP-GalNAc	Trisaccharide-1[I]	11.5 ± 3.2^{VII}	72.3 ± 2.5^{VII}	Up-regulated
UDP-GlcNAc	Trisaccharide-1[I]	39.6 ± 8.1^{VII}	54.5 ± 3.9^{VII}	Up-regulated
UDP-GlcNTFA	Trisaccharide-1[I]	204.5 ± 64.0^{IX}	-106.1 ± 57.8^{IX}	Down-regulated
Trisaccharide-1[I]	UDP-GalNAc	58.4 ± 9.1^{X}	-60.9 ± 20.6^{X}	Down-regulated
Trisaccharide-1[I]	UDP-GlcNAc	51.9 ± 4.3^{XI}	-4.0 ± 3.3^{XI}	Down-regulated

I. GlcUA-(β1,3)-GalNAc-(β1,4)-GlcUA-*p*NP was prepared as described in experimental procedures and shown in Fig. 1A. The MW was determined by ESI-MS.
II. The difference between the resonance units (RU) before and after the first substance injection.
III. The difference between RU before and after the secondary substance injection.
IV. The impact of first substance binding on the affinity of enzyme for secondary substance.
V. The data from SPR analysis shown in Fig. 5A.
VI. The data from SPR analysis shown in Fig. 5B.
VII. The data from SPR analysis shown in supplemental Fig. 5C.
VII. The data from SPR analysis shown in supplemental Fig. 5D.
IX. The data from SPR analysis shown in supplemental Fig. 5E.
X. The data from SPR analysis shown in supplemental Fig. 5F.
XI. The data from SPR analysis shown in supplemental Fig. 5G.
XII. The data represented the average of these three independent determinations ± standard deviation.

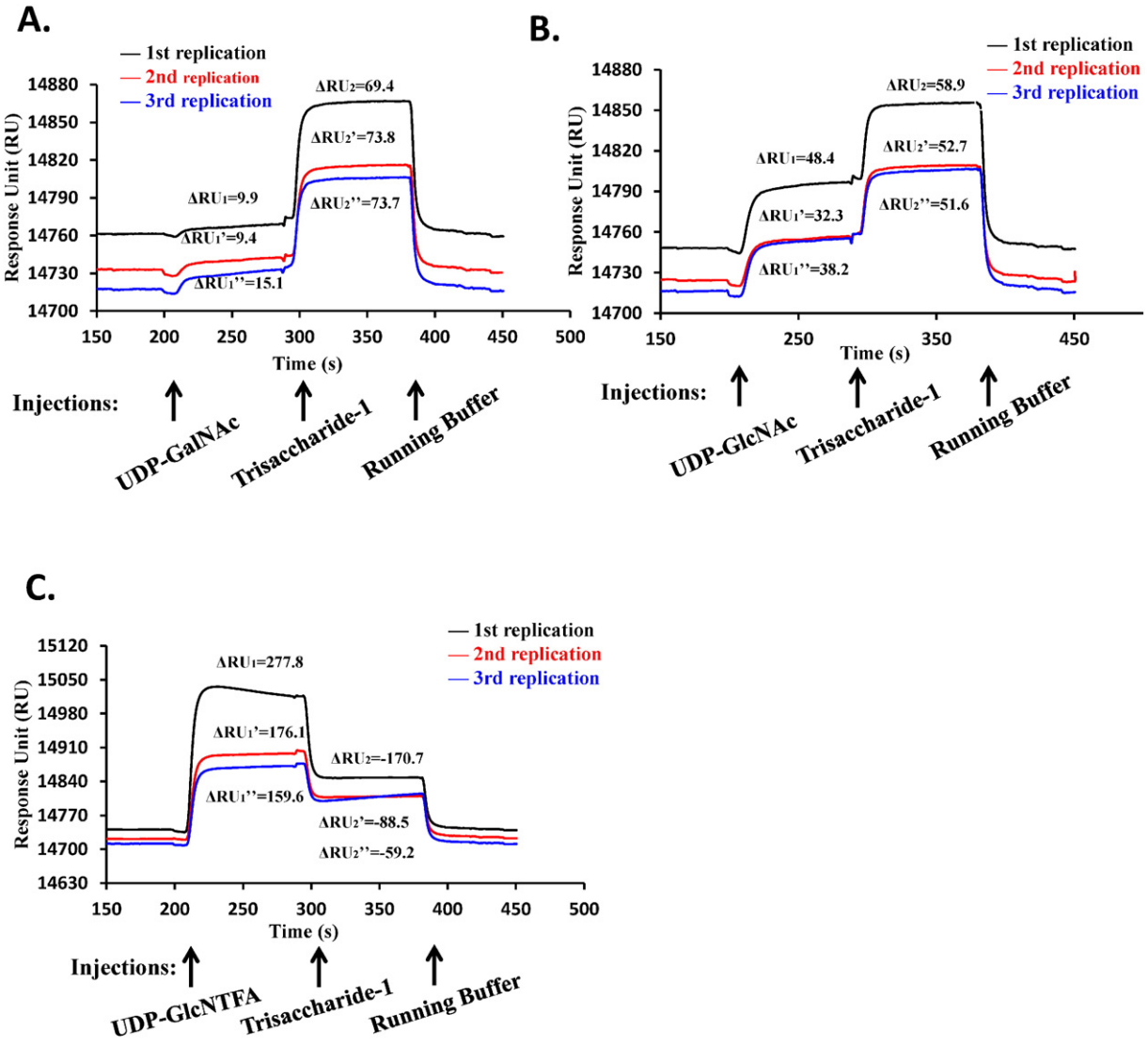

Fig. 7. Sensorgrams for the acceptor–donor pairs. (A) UDP-GalNAc/trisaccharide-1 pair, (B) UDP-GlcNAc/trisaccharide-1 pair, (C) UDP-GlcNTFA/trisaccharide-1 pair.

UDP-GlcNTFA or UDP-$GlcNH_2$ were used as donors. These results suggest that KfoC shows broad tolerance toward the orientation of the hydroxyl group (the difference between GalNAc and GlcNAc is at the C4 position), as well as the *N*-acetyl group (the difference between GalNAc and ManNAc is at the C2 position). Unexpectedly, we failed to transfer a *N*-trifluoroacetylated glucosamine (GlcNTFA) residue from UPD-GlcNTFA to trisaccharide-1, though this unnatural analog has a similar chemical conformation to UDP-GlcNAc. We further studied the donor specificity when monosaccharide with a *p*NP group (GlcUA-*p*NP) was used as the acceptor. Only the natural substrate UDP-GalNAc could serve as a donor for KfoC with this short acceptor, demonstrating that the size of the acceptor also affects the donor tolerance.

3.3. Acceptor promiscuity of the N-acetylgalactosaminyltransferase activity of KfoC

In 2002, Koji Kimata's group determined the specificity of recombinant K4 chondroitin polymerase for acceptor substrates [23]. They found chondroitin or chondroitin sulfate polymers and oligosaccharides longer than a tetrasaccharide apparently serve as better acceptors than hyaluronic acid polymers and hexasaccharide. Dermatan sulfate and heparin polysaccharides were not good acceptors for KfoC. Recently, an advanced chemoenzymatic method was recently developed for the efficient synthesis of glycosaminoglycans (GAG) oligosaccharides with high purity [11,39,40]. These developments have allowed us to prepare structurally defined oligosaccharide substrates to probe this issue in greater detail. In total, five oligosaccharides with various structures were prepared and used as acceptors to determine the *N-acetylgalactosaminyltransferase* GalNAc-transferase activity. First, trisaccharide-NAc with the structure GlcUA-(β1,4)-GlcNAc-(α1,4)-GlcUA-*p*NP was incubated with the enzyme in the presence of UDP-GalNAc (Fig. 2A). After the reaction, an obvious novel oligosaccharide, tetrasaccharide-NAc, was detected via PAMN-HPLC (Fig. 2C), suggesting that KfoC could transfer the monosaccharide residue to the HS backbone oligosaccharide. As shown in Fig. 2B, there are one α-linkage and three β-linkages within elongation product resulting from the treatment of heparosan-like trisaccharide acceptor with KfoC and UDP-GalNAc. And the α-linkage and two β-linkages are from acceptor. Thus the new glycosidic bond between the GalNAc and GlcUA residues remains the β-linkage. As expected, NMR data for tetrasaccharide-NAc demonstrated that the glycosidic bond between the GalNAc and GlcUA residues remained a β-(1,4) linkage (Fig. 2B). Similarly, we showed that *N*-modified derivatives of trisaccharide-NAc (trisaccharide-NTFA, trisaccharide-NH_2 and trisaccharide-NS) and tetrasaccharide-HA (the

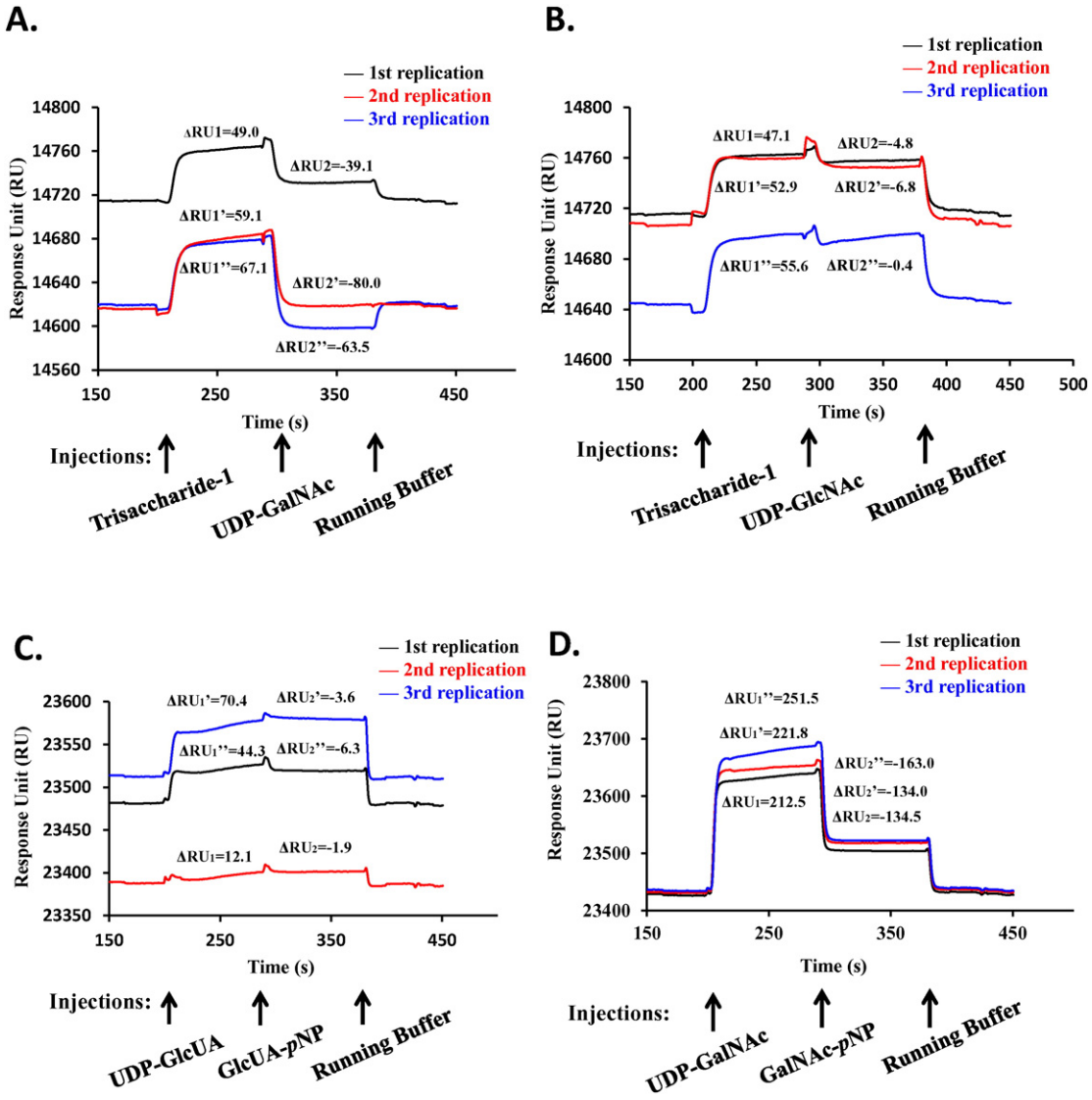

Fig. 8. Sensorgrams for the acceptor–donor pairs. (A) trisaccharide-1/UDP-GalNAc pair, (B) trisaccharide-1/UDP-GlcNAc pair, (C) UDP-GlcUA/GlcUA-*p*NP pair, (D) UDP-GalNAc/GalNAc-*p*NP pair.

enzymatic degradation product of HA) could also be substrates for KfoC (Fig. 3; supplemental Figs. S3–5). Therefore, it appears that the backbone oligosaccharides of HS, CS and HA and their *N*-modified-derivatives were tolerable acceptors in the β-(1,4) GalNAc-T activity of KfoC. In addition, our data clearly showed that a commercially available monosaccharide with a *p*NP group, GlcUA-pNP, could serve as the acceptor for KfoC. It appears that the *p*NP tag played some role in encouraging the tolerance of the size specificity of the acceptor for KfoC. The catalytic functions and broad substrate specificities of KfoC make it an attractive enzyme for use in structurally defined chimeric GAG oligosaccharide synthesis *in vitro*.

As mentioned in DeAngelis *et al.*'s previous review [10], a variety of chimeric GAGs have been reported [32,36,41]. Our conclusion about the substrate promiscuities of KfoC will allow efficient chemoenzymatic synthesis of chimeric GAGs composed of chondroitin, hyaluronan (HA) and/or heparan sulfate (HS). Despite the applications of chimeric GAGs have not been reported, structurally defined chimeric GAG oligosaccharide or their derivatives could be candidate inhibitors of mammalian glucuronidases that cleave HS, CS and HA. These enzymes, such as heparanase and hyaluronidase, have been linked to numerous human diseases, such as cancer, diabetes, and Alzheimer disease [42,43]. In addition, homologous chimeric GAG oligosaccharide also could be used as model substrates in the determination of catalytic mechanism of glucuronidase and polysaccharide lyases towards GAGs *in vitro*.

Then, the elongation products resulting from the treatment of two trisaccharides (HP-trisaccharide and CS-trisaccharide) with KfoC and UDP-GalNAc were identified by PAMN-HPLC, thereby providing an opportunity to determine whether this enzyme exhibited a preference for acceptor for polymerization. The reactions were incubated for 5 min and 4 h at 37 °C, followed by a subsequent period of heating at 100 °C for 5 min. The yield of desired tetrasaccharide when CS-trisaccharide was used as acceptor was higher than that when HP-trisaccharide was used as acceptor (Fig. 4ABCD), clearly suggesting that the natural acceptor was preferred for KfoC for polymerization.

Lastly, the weight-average molecular weight ($\overline{M}$w) and saccharide size dispersity of the polymerization products resulting from the treatment of these two trisaccharides (HP-trisaccharide and CS-trisaccharide) with KfoC and both donors (UDP-GalNAc and UDP-GlcUA) were identified by size exclusion chromatography-multi-angle laser light scattering (MALLS-SEC). Our interesting results demonstrated that reaction with CS-trisaccharide as acceptor was successful to produce nearly monodisperse polysaccharides at the range of 1069.42 to 25,061.43 (accounts for 98.27%) (Fig. 5A). When a preferred acceptor was used for polymerization, we hypothesize that all the chains are parallel elongated and the extended products displayed quasi-mono disperse size distributions. In contrast, for reactions with non-preferred acceptor (HP-trisaccharide), the new chain is formed in a slow process, and some first such chains to be initiated become preferable acceptor, yielding polysaccharide products with a wide size distribution (Fig. 5B).

3.4. Kinetic analysis of KfoC toward individual substrates

A better understanding of the interaction of KfoC with substrates and their derivatives could help in the development of enzymatic synthesis approaches for GAG oligosaccharides. SPR has been used extensively in the field of substrate–enzyme interactions [44]. According to the equation A + B = AB, k_a was measured from the forward reaction, and k_d was measured from the reverse reaction. K_D (k_d/k_a) is the quantitative measurement of affinity for binding of a ligand to an enzyme; the lower the K_D value, the higher the affinity of the substrate for the enzyme.

The kinetics of individual nucleotide donor sugars and acceptors binding to KfoC were measured by SPR (Table 1, supplemental Figs. S7–8). Three nucleotide donor sugars and two acceptors exhibited quite varied association rates with KfoC, though their dissociation rates were similar. UDP-GalNAc showed a lower K_D value ($K_D = 1.21 \times 10^{-11}$ M) than UDP-GlcNAc ($K_D = 2.1 \times 10^{-6}$ M) and UDP-GlcNTFA ($K_D = 4 \times 10^{-8}$ M), suggesting that the natural monosaccharide donor has higher affinity for the enzyme than the non-natural sugars. UDP-GlcNTFA exhibited an increased affinity for KfoC compared with UDP-GlcNAc. In agreement with a previous hypothesis [45], it appears that hydrophobic interactions between the enzyme and the fluorine atoms within UDP-GlcNTFA were conducive to the affiliation of KfoC for donors. Unexpectedly, our previous study of donor specificities of KfoC clearly indicated that UDP-GlcNAc was a good donor while on use of trisaccharide-1 as the acceptor, a GlcNTFA residue could not be transferred from UDP-GlcNTFA, a molecule with higher affinity for the enzyme compared with UDP-GlcNAc, to trisaccharide-1. These results suggested that strong affinity between the donor and the enzyme is a necessary but not a sufficient condition for glycosyl transfer reactions. Therefore, solely improving the affinity of the nucleotide donor sugar for the enzyme by protein engineering would not be an efficacious strategy for extending the substrate specificity. The trisaccharide-1 and GlcUA-*p*NP bound to KfoC with association constants of $1.9 \times 10^6\ M^{-1}\ s^{-1}$ and $4.4\ M^{-1}\ s^{-1}$, respectively. As DeAngelis hypothesized, the length of acceptors would somewhat influence the polymerase reaction and elevated monosaccharide transfer efficiency could occur as the saccharide chain becomes longer [24,45]. Liu showed that the catalytic efficiency of *E. coli* K5 KfiA increased as the acceptor became longer [13]. According to our data, it appears that the enzyme–acceptor association rate influenced by the length of the acceptor was the crucial factor determining monosaccharide transfer efficiency in polymerization.

3.5. Examination of the impact of donor binding on the affinity of enzyme for acceptors

KfoC has two glycosyl transferase activities, GalNAc-T and GlcUA-T. Therefore, two types of chemicals, nucleotide donor sugars and oligosaccharide acceptors, are substrates of KfoC. The effects of the initial substrate binding on the affinity in subsequent substrate binding to the enzyme–substrate complex have not been fully elucidated. Another aim of the present study was therefore to examine the impact of donor binding on the affinity of the enzyme for acceptors, and to delineate the regulating role on the polymerization reaction. The Biacore system is effective for the determination of kinetic constants and interaction of acceptor–donor pairs [46].

As described above, KfoC failed to utilize UDP-GlcNAc as donor to build a new glycosidic bond when GlcUA-*p*NP was used as the acceptor, while UDP-GalNAc was a highly-efficient donor to KfoC when GlcUA-*p*NP and trisaccharide-1 were used as acceptors. Thus we have an ideal model system to study whether or not a glycosyl transfer reaction will occur. Here, to guarantee that KfoC captured the substrates in a particular order, the donor and GlcUA-*p*NP were injected sequentially across the CM5 chip immobilized KfoC. The difference between RU values before and after GlcUA-*p*NP injection was calculated and is shown in Fig. 6 and Table 2. The ΔRU value for GlcUA-*p*NP ($\Delta RU = 3.9 \pm 1.8$) increased when UDP-GalNAc was injected initially (Fig. 6A). In contrast, the affinity of GlcUA-*p*NP for KfoC decreased when UDP-GlcNAc was injected initially (Fig. 6B). These data suggest that UDP-GalNAc, the natural donor for KfoC, might promote combination between GlcUA-*p*NP and the enzyme, even though the acceptor has low affinity for the enzyme in the absence of UDP-GalNAc (Fig. 6A). On the contrary, non-natural donors could not upregulate short acceptor binding to the enzyme, resulting in no glycosyl transfer reaction (Figs. 7 and 8).

The biological functions of enzymes are frequently associated with the formation of complexes with their ligands [47,48]. It is the same for glycosyltransferases, such as KfoC. Within this model system, in the glycosyl transferase reaction catalyzed by KfoC using GlcUA-*p*NP as acceptor, the tertiary structure of the enzyme bound to UDP-

GalNAc clearly elevates catalytic efficiency by upregulating the affinity of GlcUA-*p*NP for the enzyme–donor complex, compared with the holo structure changed upon the binding of UDP-GlcNAc (Fig. 6C). It implied the reason why UDP-GalNAc could react with *p*NP-monosaccharide while UDP-GlcNAc could not. Meanwhile, the ΔRU values of trisaccharide-1 in the presence of UDP-GalNAc were also higher ($\Delta RU = 72.3 \pm 2.5$) than in the presence of UDP-GlcNAc ($\Delta RU = 54.5 \pm 3.9$). Interestingly, the affinities for UDP-sugars were downregulated if KfoC was initially incubated with trisaccharide-1. For these two non-functional donor or acceptor pairs, the ΔRU values for acceptor were both negative ($\Delta RU = -3.9 \pm 2.4$ and -143.8 ± 19.1) when donors were injected initially. Notably, when KfoC failed to transfer one donor to the non-reducing end of acceptor, if this non-reactive pair of donor and acceptor were injected sequentially across the chip surface, the difference between the resonance units before and after the acceptor injection was always negative. These interesting findings might support the hypothesis proposed by DeAngelis that UDP-sugars bind to the glycotransferase first during the polymerization [45], although direct proof is still required.

4. Conclusions

KfoC has a broad substrate specificity. Backbone oligosaccharides of GAG and their *N*-modified-derivatives are tolerable acceptors in the β-(1,4) GalNAc transferase activity of KfoC. Monosaccharide with a *para*-nitrophenyl group could serve as the acceptor from KfoC when UDP-GalNAc was donor. These results support the notion that KfoC is an attractive enzyme for use in structurally-defined chimeric glycosaminoglycan oligosaccharide synthesis in vitro. DeAngelis hypothesized that the length of acceptors would somewhat influence the polymerase reaction by regulating the monosaccharide transfer efficiency [24,46]. Indeed, the affinity for the enzyme was not sufficient to estimate whether a given UDP-sugar would function as a donor or not within a glycosyl transferase reaction. Our findings also demonstrate that the enzyme–acceptor association rate, influenced by the length of the acceptor, is the crucial factor determining monosaccharide transfer efficiency in polymerization. Meanwhile, our data clearly show the impact of the binding of donor molecules on the affinity of KfoC for acceptor molecules, thus regulating the efficiency of biosynthesis of polysaccharides. Further investigation will reveal whether this impact is universal among reactions catalyzed by glycosyltransferases, and assist in designing strategies for directed evolution of glycosyltransferases toward user-defined goals.

Conflict of interest

None declared.

Author contributions

JS, PL and FW designed and coordinated the work. JX carried out the experiments. JX and LJ conducted 1D and 2D NMR analysis. JX and XZ conducted SPR analysis. JS, JX wrote the manuscript. All of the authors have read and approved the final manuscript.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (Project no. 31300673), the Specialized Research Fund for the Doctoral Program of Higher Education (Project no. 20130131120046), and China–Australia Centre for Health Sciences Research Foundation (Project no. 2015GJ01) as well as The Fundamental Research Funds (Project no. 2014YQ002) of Shandong University. We gratefully thank Dr. Cao Hongzhi and Dr. Liu Chunhui for discussion and suggestions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2016.01.018.

References

[1] E. Pantazaka, E. Papadimitriou, Chondroitin sulfate-cell membrane effectors as regulators of growth factor-mediated vascular and cancer cell migration, Biochim. Biophys. Acta Gen. Subj. 1840 (2014) 2643–2650, http://dx.doi.org/10.1016/j.bbagen.2014.01.009.
[2] D.A. DeWitt, P.L. Richey, D. Praprotnik, J. Silver, G. Perry, Chondroitin sulfate proteoglycans are a common component of neuronal inclusions and astrocytic reaction in neurodegenerative diseases, Brain Res. 656 (1994) 205–209, http://dx.doi.org/10.1016/0006-8993(94)91386-2.
[3] S. Haylock-Jacobs, M.B. Keough, L. Lau, V.W. Yong, Chondroitin sulphate proteoglycans: extracellular matrix proteins that regulate immunity of the central nervous system, Autoimmun. Rev. 10 (2011) 766–772, http://dx.doi.org/10.1016/j.autrev.2011.05.019.
[4] D. Uebelhart, Clinical review of chondroitin sulfate in osteoarthritis, Osteoarthr. Cartil. 16 (Supplement 3) (2008) S19–S21, http://dx.doi.org/10.1016/j.joca.2008.06.006.
[5] D.J. Silver, J. Silver, Contributions of chondroitin sulfate proteoglycans to neurodevelopment, injury, and cancer, Curr. Opin. Neurobiol. 27 (2014) 171–178, http://dx.doi.org/10.1016/j.conb.2014.03.016.
[6] V.H. Pomin, Sulfated glycans in inflammation, Eur. J. Med. Chem. 92 (2015) 353–369, http://dx.doi.org/10.1016/j.ejmech.2015.01.002.
[7] S. Mizumoto, S. Yamada, K. Sugahara, Molecular interactions between chondroitin-dermatan sulfate and growth factors/receptors/matrix proteins, Curr. Opin. Struct. Biol. 34 (2015) 35–42, http://dx.doi.org/10.1016/j.sbi.2015.06.004.
[8] T.E. McAlindon, J.B. Driban, G.H. Lo, Osteoarthritis year 2011 in review: clinical, Osteoarthr. Cartil. 20 (2012) 197–200, http://dx.doi.org/10.1016/j.joca.2011.12.015.
[9] U. Bhaskar, E. Sterner, A.M. Hickey, A. Onishi, F. Zhang, J.S. Dordick, et al., Engineering of routes to heparin and related polysaccharides, Appl. Microbiol. Biotechnol. 93 (2012) 1–16, http://dx.doi.org/10.1007/s00253-011-3641-4.
[10] P.L. DeAngelis, J. Liu, R.J. Linhardt, Chemoenzymatic synthesis of glycosaminoglycans: re-creating, re-modeling and re-designing nature's longest or most complex carbohydrate chains, Glycobiology 23 (2013) 764–777.
[11] Y. Xu, S. Masuko, M. Takieddin, H. Xu, R. Liu, J. Jing, et al., Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins, Science 334 (2011) 498–501, http://dx.doi.org/10.1126/science.1207478.
[12] Y. Xu, C. Cai, K. Chandarajoti, P.-H. Hsieh, L. Li, T.Q. Pham, et al., Homogeneous low-molecular-weight heparins with reversible anticoagulant activity, Nat. Chem. Biol. 10 (2014) 248–250, http://dx.doi.org/10.1038/nchembio.1459.
[13] M. Chen, A. Bridges, J. Liu, Determination of the substrate specificities of N-acetyl-d-glucosaminyltransferase†, Biochemistry 45 (2006) 12358–12365, http://dx.doi.org/10.1021/bi060844g.
[14] J. Sheng, R. Liu, Y. Xu, J. Liu, The dominating role of N-deacetylase/N-sulfotransferase 1 in forming domain structures in heparan sulfate, J. Biol. Chem. 286 (2011) 19768–19776, http://dx.doi.org/10.1074/jbc.M111.224311.
[15] J. Liu, A.F. Moon, J. Sheng, L.C. Pedersen, Understanding the substrate specificity of the heparan sulfate sulfotransferases by an integrated biosynthetic and crystallographic approach, Curr. Opin. Struct. Biol. 22 (2012) 550–557, http://dx.doi.org/10.1016/j.sbi.2012.07.004.
[16] J. Sheng, Y. Xu, S.B. Dulaney, X. Huang, J. Liu, Uncovering biphasic catalytic mode of C5-epimerase in heparan sulfate biosynthesis, J. Biol. Chem. 287 (2012) 20996–21002, http://dx.doi.org/10.1074/jbc.M112.359885.
[17] N. Guirguis, R. Schneerson, A. Bax, W. Egan, J.B. Robbins, J. Shiloach, et al., *Escherichia coli* K51 and K93 capsular polysaccharides are crossreactive with the group A capsular polysaccharide of Neisseria meningitidis. Immunochemical, biological, and epidemiological studies, J. Exp. Med. 162 (1985) 1837–1851.
[18] R.B. Rimler, K.R. Rhoades, Serogroup F, a new capsule serogroup of *Pasteurella multocida*, J. Clin. Microbiol. 25 (1987) 615–618.
[19] M.-L. Rodriguez, B. Jann, K. Jann, Structure and serological characteristics of the capsular K4 antigen of *Escherichia coli* O5:K4:H4, a fructose-containing polysaccharide with a chondroitin backbone, Eur. J. Biochem. 177 (1988) 117–124, http://dx.doi.org/10.1111/j.1432-1033.1988.tb14351.x-i2.
[20] J.-R. Wu, P.-Y. Chen, J.-H. Shien, C.-L. Shyu, H.K. Shieh, F. Chang, et al., Analysis of the biosynthesis genes and chemical components of the capsule of *Avibacterium paragallinarum*, Vet. Microbiol. 145 (2010) 90–99, http://dx.doi.org/10.1016/j.vetmic.2010.03.002.
[21] M.E. Richmond, S. DeLuca, J.E. Silbert, Biosynthesis of chondroitin sulfate. Assembly of chondroitin on microsomal primers, Biochemistry (Mosc) 12 (1973) 3904–3910.
[22] A. Zanfardino, O.F. Restaino, E. Notomista, D. Cimini, C. Schiraldi, M.D. Rosa, et al., Isolation of an *Escherichia coli* K4 kfoC mutant over-producing capsular chondroitin, Microb. Cell Factories 9 (2010) 34, http://dx.doi.org/10.1186/1475-2859-9-34.
[23] T. Ninomiya, N. Sugiura, A. Tawada, K. Sugimoto, H. Watanabe, K. Kimata, Molecular cloning and characterization of chondroitin polymerase from *Escherichia coli* strain

K4, J. Biol. Chem. 277 (2002) 21567–21575, http://dx.doi.org/10.1074/jbc.M201719200.
[24] K.J. Williams, K.M. Halkes, J.P. Kamerling, P.L. DeAngelis, Critical elements of oligosaccharide acceptor substrates for the *Pasteurella multocida* hyaluronan synthase, J. Biol. Chem. 281 (2006) 5391–5397, http://dx.doi.org/10.1074/jbc.M510439200.
[25] M. Sobhany, Y. Kakuta, N. Sugiura, K. Kimata, M. Negishi, The chondroitin polymerase K4CP and the molecular mechanism of selective bindings of donor substrates to two active sites, J. Biol. Chem. 283 (2008) 32328–32333, http://dx.doi.org/10.1074/jbc.M804332200.
[26] T. Osawa, N. Sugiura, H. Shimada, R. Hirooka, A. Tsuji, T. Shirakawa, et al., Crystal structure of chondroitin polymerase from *Escherichia coli* K4, Biochem. Biophys. Res. Commun. 378 (2009) 10–14, http://dx.doi.org/10.1016/j.bbrc.2008.08.121.
[27] Y. Chen, V. Thon, Y. Li, H. Yu, L. Ding, K. Lau, et al., One-pot three-enzyme synthesis of UDP-GlcNAc derivatives, Chem. Commun. 47 (2011) 10815–10817, http://dx.doi.org/10.1039/C1CC14034E.
[28] G. Zhao, W. Guan, L. Cai, P.G. Wang, Enzymatic route to preparative-scale synthesis of UDP–GlcNAc/GalNAc, their analogues and GDP–fucose, Nat. Protoc. 5 (2010) 636–646, http://dx.doi.org/10.1038/nprot.2010.3.
[29] S. Masuko, S. Bera, D.E. Green, M. Weïwer, J. Liu, P.L. DeAngelis, et al., Chemoenzymatic synthesis of uridine diphosphate-GlcNAc and uridine diphosphate-GalNAc analogs for the preparation of unnatural glycosaminoglycans, J. Organomet. Chem. 77 (2012) 1449–1456, http://dx.doi.org/10.1021/jo202322k.
[30] M.M. Muthana, J. Qu, Y. Li, L. Zhang, H. Yu, L. Ding, et al., Efficient one-pot multienzyme synthesis of UDP-sugars using a promiscuous UDP-sugar pyrophosphorylase from Bifidobacterium longum (BLUSP), Chem. Commun. 48 (2012) 2728–2730, http://dx.doi.org/10.1039/C2CC17577K.
[31] C. Liu, J. Sheng, J.M. Krahn, L. Perera, Y. Xu, P.-H. Hsieh, et al., Molecular mechanism of substrate specificity for heparan sulfate 2-O-sulfotransferase, J. Biol. Chem. 289 (2014) 13407–13418, http://dx.doi.org/10.1074/jbc.M113.530535.
[32] B.S. Tracy, F.Y. Avci, R.J. Linhardt, P.L. DeAngelis, Acceptor specificity of the *Pasteurella hyaluronan* and chondroitin synthases and production of chimeric glycosaminoglycans, J. Biol. Chem. 282 (2007) 337–344, http://dx.doi.org/10.1074/jbc.M607569200.
[33] H. Saitoh, K. Takagaki, M. Majima, T. Nakamura, A. Matsuki, M. Kasai, et al., Enzymic reconstruction of glycosaminoglycan oligosaccharide chains using the transglycosylation reaction of bovine testicular hyaluronidase, J. Biol. Chem. 270 (1995) 3741–3747.
[34] W. He, L. Fu, G. Li, J. Andrew Jones, R.J. Linhardt, M. Koffas, Production of chondroitin in metabolically engineered *E. coli*, Metab. Eng. 27 (2015) 92–100, http://dx.doi.org/10.1016/j.ymben.2014.11.003.
[35] Z. Wang, Z. Zhang, S.A. McCallum, R.J. Linhardt, Nuclear magnetic resonance quantification for monitoring heparosan K5 capsular polysaccharide production, Anal. Biochem. 398 (2010) 275–277, http://dx.doi.org/10.1016/j.ab.2009.12.005.
[36] W. Jing, P.L. DeAngelis, Synchronized chemoenzymatic synthesis of monodisperse hyaluronan polymers, J. Biol. Chem. 279 (2004) 42345–42349, http://dx.doi.org/10.1074/jbc.M402744200.
[37] P.L. DeAngelis, L.C. Oatman, D.F. Gay, Rapid chemoenzymatic synthesis of monodisperse hyaluronan oligosaccharides with immobilized enzyme reactors, J. Biol. Chem. 278 (2003) 35199–35203, http://dx.doi.org/10.1074/jbc.M306431200.
[38] W. Jing, P.L. DeAngelis, Analysis of the two active sites of the hyaluronan synthase and the chondroitin synthase of *Pasteurella multocida*, Glycobiology 13 (2003) 661–671, http://dx.doi.org/10.1093/glycob/cwg085.
[39] J. Liu, R.J. Linhardt, Chemoenzymatic synthesis of heparan sulfate and heparin, Nat. Prod. Rep. 31 (2014) 1676–1685, http://dx.doi.org/10.1039/C4NP00076E.
[40] Y. Chen, Y. Li, H. Yu, G. Sugiarto, V. Thon, J. Hwang, et al., Tailored design and synthesis of heparan sulfate oligosaccharide analogues using sequential one-pot multienzyme systems, Angew. Chem. Int. Ed. 52 (2013) 11852–11856, http://dx.doi.org/10.1002/anie.201305667.
[41] M. Endo, I. Kakizaki, Synthesis of neoproteoglycans using the transglycosylation reaction as a reverse reaction of endo-glycosidases, Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 88 (2012) 327–344, http://dx.doi.org/10.2183/pjab.88.327.
[42] N.S. El-Safory, A.E. Fazary, C.-K. Lee, Hyaluronidases, a group of glycosidases: current and future perspectives, Carbohydr. Polym. 81 (2010) 165–181, http://dx.doi.org/10.1016/j.carbpol.2010.02.047.
[43] I. Vlodavsky, M. Elkin, G. Abboud-Jarrous, F. Levi-Adam, L. Fuks, I. Shafat, et al., Heparanase: one molecule with multiple functions in cancer progression, Connect. Tissue Res. 49 (2008) 207–210, http://dx.doi.org/10.1080/03008200802143281.
[44] R.L. Rich, D.G. Myszka, Higher-throughput, label-free, real-time molecular interaction analysis, Anal. Biochem. 361 (2007) 1–6, http://dx.doi.org/10.1016/j.ab.2006.10.040.
[45] N.J. Otto, D.E. Green, S. Masuko, A. Mayer, M.E. Tanner, R.J. Linhardt, et al., Structure/function analysis of *Pasteurella multocida* heparosan synthases, J. Biol. Chem. 287 (2012) 7203–7212, http://dx.doi.org/10.1074/jbc.M111.311704.
[46] D. Solís, N.V. Bovin, A.P. Davis, J. Jiménez-Barbero, A. Romero, R. Roy, et al., A guide into glycosciences: how chemistry, biochemistry and biology cooperate to crack the sugar code, Biochim. Biophys. Acta Gen. Subj. 1850 (2015) 186–235, http://dx.doi.org/10.1016/j.bbagen.2014.03.016.
[47] J. Holm, S.W. Bruun, S.I. Hansen, The complex interplay between ligand binding and conformational structure of the folate binding protein (folate receptor): biological perspectives, Biochim. Biophys. Acta Protein Proteomics 1854 (2015) 1249–1259, http://dx.doi.org/10.1016/j.bbapap.2015.06.009.
[48] D. Seeliger, B.L. de Groot, Conformational transitions upon ligand binding: holo-structure prediction from apo conformations, PLoS Comput. Biol. 6 (2010), e1000634http://dx.doi.org/10.1371/journal.pcbi.1000634.