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Original Article

Developing a solid-phase method for the enzymatic synthesis of heparan sulfate and chondroitin sulfate backbones

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Despite the recent progress on the solution-phase enzymatic synthesis of heparan sulfate (HS) and chondroitin sulfate (CS), solid-phase enzymatic synthesis has not been fully investigated. Here, we describe the solid-phase enzymatic synthesis of HS and CS backbone oligosaccharides using specialized linkers. We demonstrate the use of immobilized HS linker to synthesize CS, and the use of immobilized CS linker to synthesize HS. The linkers were then digested with chondroitin ABCase and heparin lyases, respectively, to obtain the products. Our findings uncover a potential approach for accelerating the synthesis of structurally homogeneous HS and CS oligosaccharides.

Key words: carbohydrates; chemoenzymatic synthesis; chondroitin sulfate; heparan sulfate; solid phase synthesis..

Chondroitin sulfate (CS) and heparan sulfate (HS) are highly sulfated polysaccharides, widely expressed in the animal kingdom. HS contributes to the regulation of cell differentiation/proliferation (Patel et al. 2014), embryonic development (Fuster and Esko 2005), blood coagulation (Liu and Linhardt 2014) and viral entry (Liu and Thorp 2002). CS is involved in cancer metastasis (Mizumoto et al. 2015), parasitic infections (Fried and Duffy 2015) and neuron growth inhibition after injury (Bradbury et al. 2002; Miyata et al. 2012). HS has the disaccharide-repeating units of glucuronic acid (GlcA) or iduronic acid (IdoA) linked to glucosamine. Both IdoA and glucosamine residues frequently carry sulfo groups. CS on the other hand consists of a disaccharide repeating units of *N*-Acetylgalactosamine (GalNAc) linked to GlcA. Four different types of CS have been identified, including chondroitin sulfate A (CS-A), chondroitin sulfate C (CS-C), chondroitin sulfate D (CS-D) and chondroitin sulfate E (CS-E), which differ by the positions and numbers of sulfo groups in the disaccharide repeating unit. The sulfation patterns in CS and HS dictate the binding affinity to the protein targets to manifest the selectivity in biological functions (Gama et al. 2006). For example, a uniquely distributed sulfated pentasaccharide domain is essential for the anticoagulant activity of HS (Atha et al. 1985). A domain containing 4,6 disulfated *N*-acetyl galactosamine (GalNAc4S6S) residues is proved to be necessary to direct neuronal signaling and inhibits axon growth (Brown et al. 2012; Pulsipher et al. 2014). Recent progress on the synthesis of HS oligosaccharides has also been reported (Ramadan et al. 2022; Zhang et al. 2022). However, the synthesis of structurally homogenous CS and HS oligosaccharides is still challenging through traditional chemical synthesis. Enzymatic and chemoenzymatic synthesis of CS and HS are promising alternatives to prepare structurally defined oligosaccharides because of high efficiency. Arrays of structurally defined HS oligosaccharides and CS oligosaccharides have been synthesized so far by the chemoenzymatic method (Li et al. 2017; Zhang et al. 2017). Furthermore, the method can synthesize gram-scale of structurally complex HS dodecasaccharide with a goal to replace animal sourced heparin, a commonly used anticoagulant drug (Xu et al. 2017).

The enzyme-based methods to synthesize a new class of unnatural chimeric HS-CS oligosaccharides have been reported (Stancanelli et al. 2022). Here we looked for an application of chimeric compounds to develop a solid-phase synthetic method to avoid complex purification procedures that require significant effort, making the process compatible for parallel and automated synthesis. The solid-phase approach has been primarily reported for the chemical synthesis of carbohydrates (Seeberger and Haase 2000). Herein a novel method to obtain pure HS and CS backbone oligosaccharides using a solid-phase enzymatic approach is disclosed.

Results and discussion

The low structural flexibility of HS and CS oligosaccharides in aqueous solution allows us to examine the effectiveness of solid-phase chemoenzymatic synthesis for producing oligosaccharides using those backbones as spacers for the growing compounds. We used two bacterial glycosyltransferases in the current study to synthesize in solution nonsulfated HS and CS oligosaccharides with high efficiency (Sugiura et al. 2016;

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Li et al. 2017). Heparosan synthase 2 from *Pasteurella multocida* (pmHS2) is used to synthesize HS backbone oligosaccharides (Sismey-Ragatz et al. 2007; Liu et al. 2010), whereas KfoC from *E. coli* K4 strain is used to synthesize CS oligosaccharides (Sugiura et al. 2008; Xue et al. 2016). As previously reported (Stancanelli et al. 2022), the synthesis of CS/HS chimeric oligosaccharides initiated from a commercially available acceptor, 4-nitrophenyl-β-D-glucuronide (GlcA-pNP). Here, we prepared two sets of oligosaccharide primers; the first group of linkers (Group A, Fig. 1) contains heparosan in three different sizes, including 7-mer HS linker, 9-mer HS linker and 11-mer HS linker. The synthesis of HS linkers was accomplished using pmHS2 as enzyme. On the other hand, the second group of linkers (Group B, Fig. 1) consists of three different sized CS backbone, 5-mer CS linker, 7-mer CS linker and 9-mer CS-linker. KfoC was the enzyme used for the synthesis of the linkers of CS. These oligosaccharide spacers were then immobilized on agarose support to test the feasibility of solid-phase enzymatic elongation. Immobilized backbones from Group A were incubated with KfoC and UDP-GalNAc followed by another cycle of incubation of KfoC and UDP-[^{13}C] GlcA to form CS hybrid oligosaccharides. The regioselective substrate specificity of heparin lyases was used to selectively degrade the HS linker to release the CS hybrid tetrasaccharide after the synthesis being completed. Given that the supernatant from heparin lyases-treated agarose contained both CS hybrid tetrasaccharide and HS disaccharides, the supernatant was then fractionated to separate CS hybrid oligosaccharide products and HS disaccharides based on the molecular size using a BioGel P-2 column. Isotopically ^{13}C-labeled GlcA was introduced at the non-reducing end of CS tetrasaccharide product, adding a unique molecular mass marker for the product identification. The ESI-MS analysis was performed to confirm the correct molecular weight of newly synthesized CS tetrasaccharide. Three sizes of HS-linkers, including immobilized HS 5-mer, HS-7-mer and HS-11-mer linkers went through the same elongation protocol and subsequent heparin lyases digestion (Fig. 1A). Although the CS-hybrid tetrasaccharide was isolated using HS-9-mer and 11-mer linkers, no product was detected when HS-7-mer served as linker, suggesting that a backbone equal or longer than 9-mer might be required to allow KfoC to perform the elongation reaction. The HS oligosaccharides were synthesized using immobilized CS 5-mer-linker, CS 7-mer-linker and CS 9-mer linker with a similar procedure (Fig. 1B). Both CS 7-mer and CS 9-mer linkers yielded the HS hybrid tetrasaccharide, but not from the use of HS 5-mer linker (Fig. 1B). Both ^{13}C-labeled CS-hybrid tetrasaccharides and HS-hybrid tetrasaccharides were detected by electrospray ionization mass spectrometry (ESI-MS) (Fig. 1C).

We avoided using an even number of oligosaccharides as linkers due to the substrate specificity of KfoC of pmHS2 enzyme. For example, an even number CS oligosaccharide linker has a GalNAc residue at the non-reducing end. The pmHS2 is unable to transfer a GlcA to an oligosaccharide that has a GalNAc at its non-reducing end. An even number HS oligosaccharide has a GlcNAc residue at the non-reducing end. The KfoC is unable to transfer a GlcA to an oligosaccharide that has a GlcNAc at its non-reducing end.

Our next question prior to carrying out the expansive solid-phase synthesis was to ensure the substrate specificities of pmHS2 and KfoC are maintained in the solution format synthesis. To that end, we synthesized compound **1** and compound **2** (Fig. 2). For the synthesis of compound **1**, we started from a HS 9-mer with a sequence of GlcA($\beta1\rightarrow4$)GlcNAc($\alpha1\rightarrow4$)GlcA($\beta1\rightarrow4$)GlcNAc($\alpha1\rightarrow4$) GlcA($\beta1\rightarrow4$)GlcNAc($\alpha1\rightarrow4$)GlcA($\beta1$-4)GlcNAc($\alpha1\rightarrow4$) GlcA-pNA-N_3. After three rounds of elongation reaction using KfoC, we obtained a 12-mer with a structure of **GalNAc($\beta1\rightarrow4$)GlcA($\beta1\rightarrow3$)GalNAc($\beta1\rightarrow4$)**GlcA($\beta1\rightarrow4$) GlcNAc($\alpha1\rightarrow4$)GlcA($\beta1\rightarrow4$)GlcNAc($\alpha1\rightarrow4$)GlcA($\beta1\rightarrow4$) GlcNAc($\alpha1\rightarrow4$)GlcA($\beta1\rightarrow4$)GlcNAc($\alpha1\rightarrow4$)GlcA-pNA-N_3, where, the three additional saccharide residues are shown in bold fold. These three saccharide residues represent the CS structure, containing GalNAc residue. Further, the linkage between GalNAc and GlcA is $\beta1\rightarrow4$ linkage, the linkage between GlcA and GalNAc is $\beta1\rightarrow3$. The structure of compound **1** was confirmed by electrospray ionization mass spectrometry and NMR (Supplemtary Figs S1–S4). The structural analysis (Supplementary Figs S5–S8) of compound **2** confirmed that a HS trisaccharide domain, GlcNAc(a1-4)GlcA(b1-4)GlcNAc(a1-4)-, is added to a CS 7-mer primer. Taken together, our data conclude that KfoC maintains its substrate specificity toward a HS oligosaccharide primer; likewise, pmHS2 maintains its substrate specificity toward a CS oligosaccharide primer.

The synthesis of HS oligosaccharide in the solid-phase format was performed to expand the synthesis of different oligosaccharides. We employed CS-9-mer and HS 7-mer linkers to synthesize CS oligosaccharides and HS oligosaccharides, including two CS hexasaccharide (compound **3**) and CS octasaccharide (compound **4**) and HS tetrasaccharide (compound **5**) and HS hexasaccharide (compound **6**) (Table 1). The ESI-MS spectra and NMR spectra for compounds **3** and **4** are shown in Supplementary Figs S9–S12, and the ESI-MS spectra and NMR spectra for compounds **5** and **6** are shown in Supplementary Figs S13–S16. Because the original synthetic route was diverted to produce different products the isolated amount after BioGel P-2 purification for each compound has been enough only for a partial characterization by NMR. However, the results from ESI-MS confirmed the structures of both CS and HS oligosaccharide products. The ESI-MS spectra and NMR spectra for compounds **3** and **4** are shown in Supplementary Figs S9–S12, and the ESI-MS spectra and NMR spectra for compounds **5** and **6** are shown in Supplementary Figs S13–S16.

Although we demonstrated the feasibility of the synthesis of both CS and HS backbone oligosaccharide using the solid-phase format, this approach encountered certain limitations. First, the solid support used for immobilizing the oligosaccharide substrates prevents the enzymes efficiently reacting with substrates due to the steric hindrance from the support, resulting in low yields (Hsu et al. 2011; Chen et al. 2013). Only a few examples of solid-phase chemoenzymatic synthesis of carbohydrate are reported (Zhang et al. 2020), but none of them offered the ability to carry out the scale-up synthesis. There was an attempt to keep oligosaccharides away from the solid support using extensively long polyethylene-based linkers to reduce the steric hindrance from the support but had very limited success as the linker may fold itself in aqueous solutions (Ruiz et al. 2016). Second, the efficiency for the cleavage of the oligosaccharide from the solid support to retrieve the product is inefficient. In addition, lacking a fast and sensitive method to monitor when the reaction goes to completion is an important drawback of solid phase method.

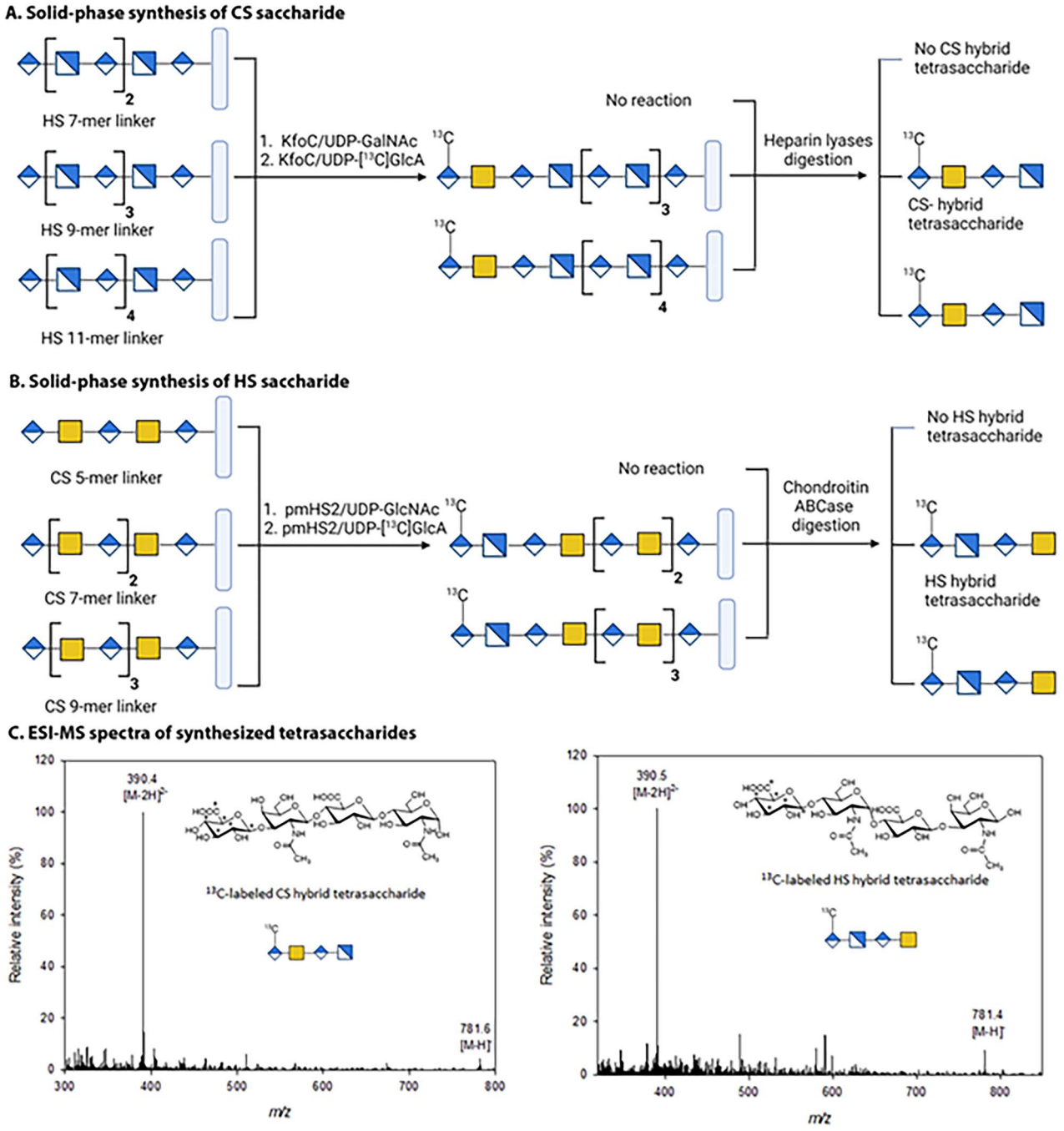

Fig. 1. Schematic synthesis of CS and HS oligosaccharides using the solid-phase synthesis approach. ***Panel A*** shows the synthesis of CS oligosaccharides using different lengths of HS linkers. CS tetrasaccharides were obtained after cleavage with heparin lyases (mixture of heparin lyase I, II and III) only from HS 9-mer and 11-mer linkers. No product obtained when HS 7-mer was used as linker. ***Panel B*** shows the synthesis of HS oligosaccharides using CS 5-mer, 7-mer and 9-mer CS linkers. Both CS 7-mer and 9-mer linkers were capable of releasing HS tetrasaccharides after cleavage with chondroitin lyase (chondroitin ABCase). No product was observed using the CS 5-mer linker. Panel C shows the ESI-MS spectra of two hybrid tetrasaccharides.

High conversion ratio of starting material into product is a critical detail, especially in a multi-step synthetic pathway, to avoid byproducts formation. In our solid phase approach this aspect is additionally emphasized because of the inability to properly purify each compound while bound to the solid support.

To improve the yield for the solid phase chemoenzymatic synthesis, we tried to design a linker that serves as a better primer for the elongation reaction on the beads. Based on our previous research (Stancanelli et al. 2022) the rate-limiting step in the synthesis of chimeric oligosaccharides is the addition of the first amino sugar in the growing backbone when the synthesis of the linker is completed. To address this issue, we used an CS-HS hybrid 8-mer primer with a structure of GlcNAc(α1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3) GalNAc(β1→4) GlcA-pNA-N_3. The product was prepared in solution phase starting from a CS 7-mer (GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)

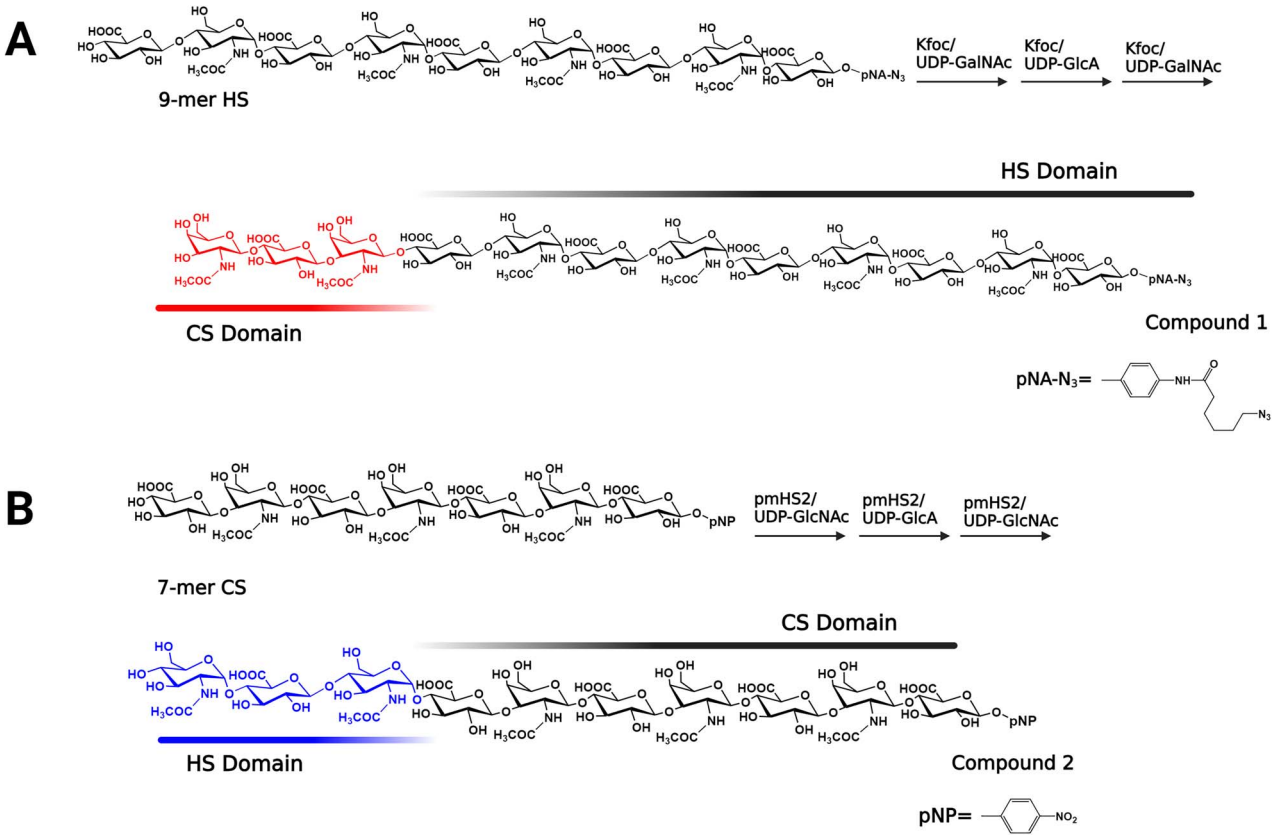

Fig. 2. Synthetic schemes of HS/CS and CS/HS chimeric oligosaccharides using the chemoenzymatic method. **Panel A** shows the synthesis of CS/HS chimeric dodecasaccharides (12-mer, **compound 1**). **Panel B** shows the synthesis of HS/CS chimeric synthesis of HS/CS dodecasaccharide (10-mer, compound 2). In the synthesis of **compound 1**, a pNA-N_3 (para-(6-azido hexanamido)-phenyl) tagged heparosan 9-mer backbone was used as a starting material. In the synthesis of **compound 2**, a pNP (p-nitrophenyl) tagged chondroitin heptasaccharide (7-mer) was used as a starting material. The pNP tag was then converted to a pNA-N3 after 7-mer CS synthesis was completed.

Table 1. Summary of the synthesized oligosaccharides in this study.

	Abbreviated sequences of oligosaccharides	Structural features	Measured MW
Compound **1**	GalNAc(β1→4)GlcA(β1→4)GalNAc(β1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1-4)GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA-pNA-N_3	CS/HS chimeric 12-mer	2524.7 ± 0.4 (2524.2)
Compound **2**	GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4)GlcA-pNP	HS/CS chimeric 10-mer	2035.3 ± 0.4 (2035.7)
Compound **3**	GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→4)GlcNAc	CS 6-mer-H	1155.9 ± 0.1 (1156.0)
Compound **4**	GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4)GlcA(β1→4)GlcNAc	CS 8-mer-H	1535.1 ± 0.5 (1535.3)
Compound **5**	GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GalNAc	HS 4-mer-C	776.0 ± 0.3 (776.7)
Compound **6**	GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GalNAc	HS 6-mer-C	1155.3 ± 0.4 (1156.0)
Compound **7a**	GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GalNAc	HS 7-mer-C	1359.1 ± 0.3 (1359.2)
Compound **7b**	GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GlcNAc(α1→4)GlcA(β1→4)GalNAc-AMAC	HS 7-mer-C	1553.2 ± 0.2 (1553.1)

GalNAc(β1→4)GlcA(β1→3)GalNAc(β1→4) GlcA-pNA-N_3). Comparison of reactions efficiency is reported in in Supplementary Figs S22, leading us to conclude that the extra GlcNAc residue has increased the reactivity for pmHS2 modification. To this end, PmHS2 and UDP-GlcNAc were used to initiate the building of the HS backbone and hence convert the CS 7-mer in the CS-HS hybrid 8-mer. The chimeric CS-HS hybrid 8-mer linker was immobilized on

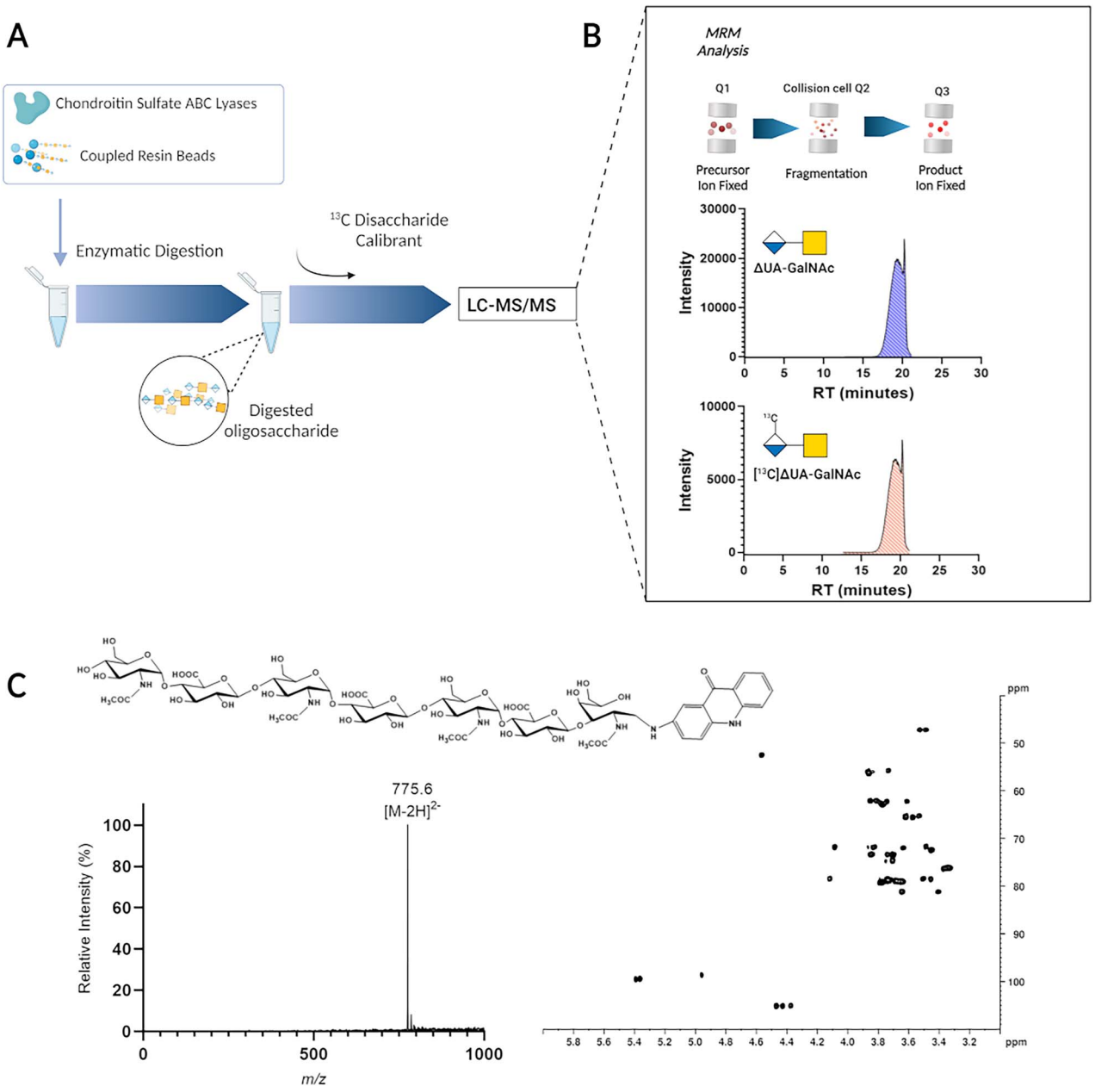

Fig. 3. Quantification protocol via LC-MS of resin-linked oligosaccharides. **Panel A** shows the flowchart to conduct the quantification of oligosaccharide bound to the resin using disaccharide compositional analysis. Two steps are involved. Chondroitin Sulfate ABC Lyase digestion, or step 1. Step 2, disaccharide calibrant is added after the digestion stage. **Panel B** shows MRM (multiple-reaction monitoring) schematic diagram (on top) and LC-MRM chromatograms of ΔUA(β1→3)GalNAc unlabeled and labeled disaccharides used for quantitative analysis. **Panel C** shows ESI-MS and HSQC NMR spectra of compound 7b after cleavage from resin and AMAC functionalization.

agarose support for further elongation. Using this 8-mer, the elongation efficiency was improved, and permitted us to synthesize compound 7a in milligram scales.

We also developed a method to quantify the amount of oligosaccharide bound to the solid support to estimate the binding capacity of the resin. Disaccharide analysis was performed after releasing disaccharide fragments from the solid support by using heparin lyases or chondroitin ABCase. The resulting disaccharides were quantified by a LC-MS/MS method (Antia et al. 2017). In the analysis, ^{13}C-labeled authentic disaccharide standards were employed as internal calibrants (Fig. 3). A small fraction (1 μL) of functionalized resin was digested using chondroitin ABCase. A ^{13}C-labeled disaccharide standard ([^{13}C]ΔUA(β1→3)GalNAc) was added to the digested material and analyzed using the aforementioned LC-MS/MS technique. Comparing the peak areas of the ^{13}C-labeled disaccharide and unlabeled disaccharide permitted us to quantify oligosaccharides attached to the resin (Supplementary Fig. S21).

pmHS2 enzyme has the activity of transferring both GlcA and GlcNAc from UDP-GlcA and UDP-GlcNAc respectively. The solid phase approach gives us the possibility to perform each elongation step without an extensive purification process during the switch of UDP-sugar donors. The presence of unreacted UDP-GlcNAc in the transferring GlcA reaction process may lead to uncontrolled polymerization

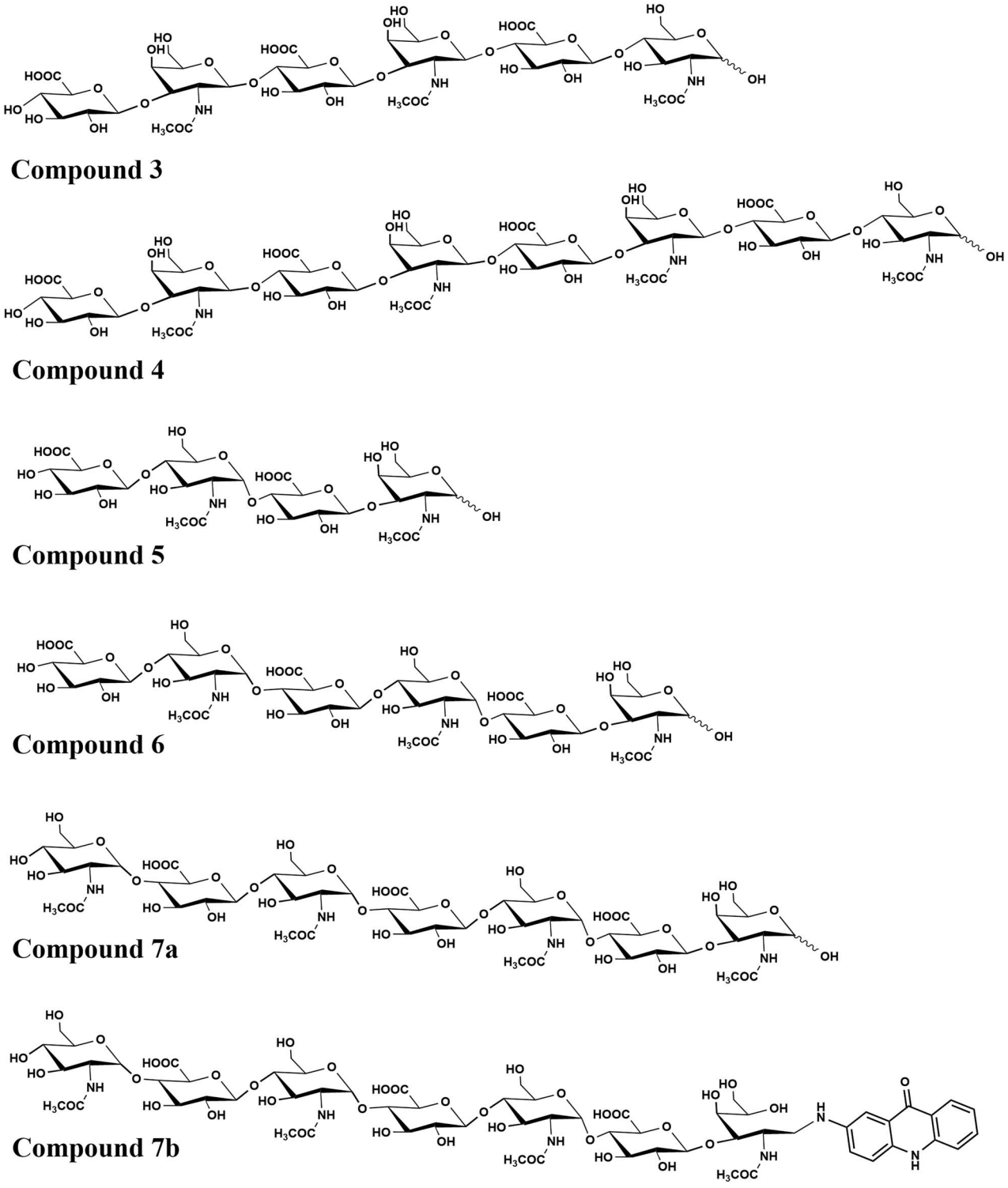

Fig. 4. Structures of two CS oligosaccharides and four HS oligosaccharides synthesized using the solid phase chemoenzymatic approach.

reaction. To removal the unreacted UDP-sugar is essential to control the size of oligosaccharide product during the synthesis. To ensure the completion of each elongation step, we decided to repeat each reaction three times by adding fresh enzyme and the UDP-sugar donor. In addition, a small aliquot of resin was digested and analyzed by LC-MS to confirm the substrate was completely transformed into the product prior to moving into the following elongation step. Five elongation steps were employed to obtain the final heptasaccharide chimeric product (Compound **7a,** Fig. 4 and Table 1) that was released after selective digestion performed by chondroitin ABCase. In accordance with our protocol the digestion was performed three times saving each time the supernatant and digesting the solid support with freshly prepared buffer and enzyme. Combined digested material was then labeled using 2-aminoacridone (2-AMAC). Fluorescence (2-AMAC) tagging of chondroitin and heparan sulfate precursor fragments allowed us to

achieve a better purification using HPLC coupled to a C18 column. Purified material, 1.3 mg, (Compound **7b**, Fig. 4 and Table 1) was then analyzed by NMR spectroscopy and Mass spectrometry for structural characterization and purity confirmation. The ESI-MS spectrum for compound **7a** is shown in Supplementary Fig. S17, and the ESI-MS spectra and NMR spectra for compound **7b** are shown in Supplementary Figs S18–S20.

In summary, we demonstrated the chemoenzymatic synthesis of HS and CS chimeric oligosaccharides using a solid phase approach. Our findings provide a new tool for constructing HS and CS oligosaccharides linkers to keep the substrates away from the solid support to avoid steric hindrance. Our rationale is that both HS and CS linkers have the rigidity in biological buffers (Khan et al. 2010). The length of the linker is tunable through the control of the number of saccharide residues. The synthesis of oligosaccharide linkers can be readily achieved in gram scale, compatible with the need for large scale synthesis. The linkers are degradable by either chondroitin ABCase or heparin lyases, offering an enzyme-based method to cleave the products from the solid support with high efficiency and selectivity. Both recombinant chondroitin ABCase and heparin lyases are available from commercial sources. We demonstrated for the first time the ability of this solid phase chemoenzymatic method to synthesize milligrams of product.

Our next goal is to synthesize a library of structurally diverse CS and HS oligosaccharides, which requires the synthesis of backbone oligosaccharides followed by different sulfation modifications. The solid-phase format is most effectively used to prepare the oligosaccharide backbone with different sizes. After the backbone synthesis is completed, structural diversifications with *N*-sulfotransferase and *O*-sulfotransferases will be performed using a solution-phase format to generate final sulfated products. Therefore, combining both solid-phase and solution phase methods represents an alternative and a more effective approach for the preparation of HS and CS oligosaccharides to provide a library of HS and CS oligosaccharides for ongoing research projects.

Author contributions

Eduardo Stancanelli (Conceptualization [equal], Formal analysis [equal], Investigation [lead], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Wei Liu (Data curation [equal], Formal analysis [equal], Funding acquisition [equal]), Guowei Su (Funding acquisition [equal], Methodology [equal], Writing—review & editing [equal]), Vijayakanth Pagadala (Methodology [equal], Resources [equal]), and Jian Liu (Conceptualization [lead], Funding acquisition [lead], Investigation [equal], Writing—original draft [equal], Writing—review & editing [equal])

Supplementary material

Supplementary material is available at *Glycobiology Journal* online.

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Conflict of interest statement

V.P. and G.S. are employees of Glycan Therapeutics with equity options. J.L. is a founder and chief scientific officer for Glycan Therapeutics Corporation. Other authors declare no conflict of interest.

Supporting information

Experimental procedures and compound structural characterization are present in Supporting Information. The data underlying this article are available in the article and in its online supplementary material.

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