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COMMUNICATION

Using Automated Glycan Assembly (AGA) for the Practical Synthesis of Heparan Sulfate Oligosaccharide Precursors

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Herein we report synthesis of complex heparan sulfate oligosaccharide precursors by automated glycan assembly using disaccharide donor building blocks. Rapid access to a hexasaccharide was achieved through iterative solid phase glycosylations on a photolabile resin using the Glyconeer™, an automated oligosaccharide synthesiser, followed by photochemical cleavage and glycan purification using simple flash column chromatography.

Heparan sulfate (HS) is a strongly anionic linear polysaccharide belonging to the glycosaminoglycan (GAG) family of macromolecules. HS chains are synthesized in the Golgi apparatus[1] and comprise of a repeating *N*-acetyl glucosamine (GlcN)- D-glucuronic acid (GlcA) disaccharide unit. Modifications in the synthesis of HS chains include *N*-deacetylation/*N*-sulfation of GlcN residues; epimerization of GlcA to L-iduronic acid (IdoA); sulfation at the 2-*O* position of IdoA and sulfation at the 6-*O* and 3-*O* positions of GlcN.[2] Owing to the high content of charged groups in HS, many proteins bind to HS through electrostatic interactions.[3] HS chains can also be covalently attached to a core protein forming glycoproteins, known as Heparan Sulfate Proteoglycans (HSPGs). The functional role of the many forms of HS in different biological processes has been extensively studied[4] particularly in growth factor mediated signalling,[5] chemotaxis,[6, 7] and as a protein component of the extracellular matrix (ECM),[8] where HSPGs are essential for maintaining the integrity of the ECM and basement membrane (BM) and modulating cell mobility.[3]

In vivo experiments[9, 10] have also highlighted the interaction of HS and heparin (a HS analogue with higher degree of sulfation) with amyloid-β peptide (Aβ) plaques whose aggregation and deposition in brain parenchyma[11] is a histopathological hallmark of Alzheimer's disease (AD). These aggregates are formed of Aβ peptides 40-42 amino acids long which are products of sequential cleavage of the transmembrane protein amyloid β precursor protein (APP) by β-secretase (β-site APP cleaving enzyme 1: BACE1) and γ-secretase.[12] Although HS has been observed to co-deposit in Aβ plaques, several studies have reported that HS oligosaccharides **1** of varying lengths,[13, 14] can also modulate the activity of BACE1, whereby HS chains bind at or near the active site of BACE1 and prevent its access to the protein APP (Figure 1).[15] However, despite success in establishing the interactions between HS and Aβ peptides and BACE1, the heterogeneity of HS glycans purified from natural sources[16] has limited their utility for determining detailed structure-activity relationships. Thus the synthesis of pure and homogeneous HS oligosaccharides is crucial for facilitating further biological studies. To date, the synthesis of HS oligosaccharides has been executed via chemoenzymatic,[17-20] solution-phase[21-25] and solid-phase approaches,[26] relying on many manual operations. But recently, automated glycan assembly (AGA)[27, 28] has emerged as a powerful tool for the facile synthesis of biologically important carbohydrate libraries;[29] including sulfated oligosaccharides and their protected precursors.[30-32] Herein we report an iterative automated solid-phase synthesis of HS oligosaccharide precursors using the Glyconeer™,[33] an automated oligosaccharide synthesizer, which can provide rapid access to oligosaccharides via AGA.

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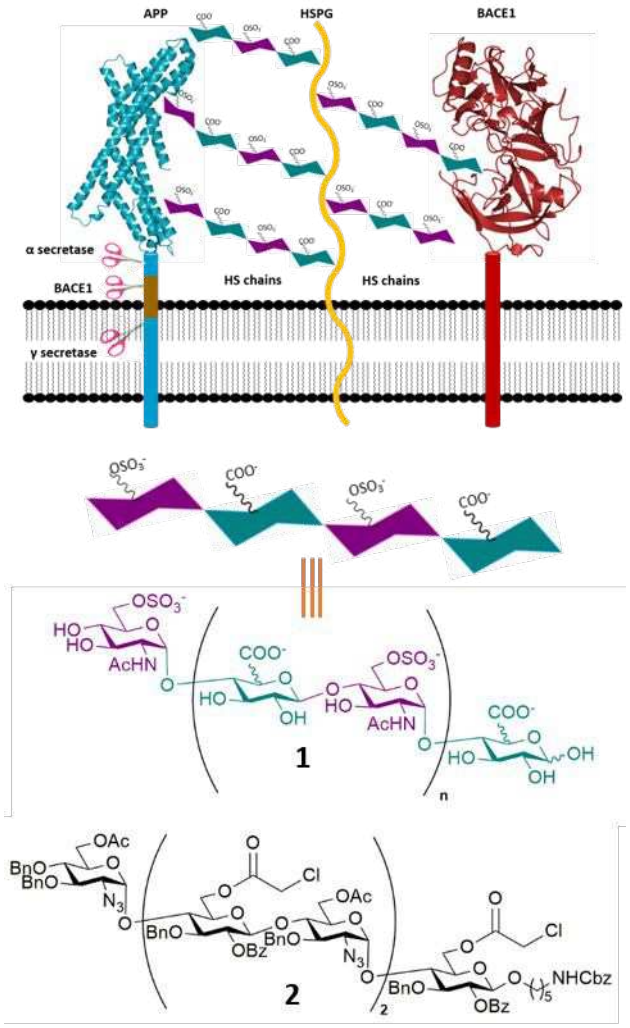

Figure 1: Cartoon illustration of the inhibition of BACE1 by HS chains. HS is depicted in simplified form **1** which may vary greatly in length (n can reach 125), and the oligosaccharide **2**, targeted as proof of principle which is a precursor to a HS hexasaccharide.

As a proof of principle and because BACE1 inhibition was previously reported with a similar length oligosaccharide,[13] we targeted the synthesis of a HS hexasaccharide precursor **2** with an orthogonal protecting group pattern. Specifically, the presence of the chloroacetate group at the *O*-6 position in the glucose moiety enables introduction of glucuronic acid through suitable deprotection and oxidation, whilst the azido group in the GlcN sugar can also be processed into the *N*-acetamido group post glycosylation (Figure 1).[14] We opted to utilise the disaccharide trichloroacetimidate (TCA) building block **3** as a donor in the Glyconeer™ to access the target hexasaccharide (Figure 2), firstly because the challenging α-glycosidic linkage required between GlcN and GlcA in the final product was already in place[14] and secondly, we anticipated that the benzoyl ester group at *O*-2 of GlcA would direct glycosylation following TCA activation in a stereoselective manner, ensuring only the formation of β-glycosidic linkages during AGA. The *O*-4 position of the GlcN is also masked with a temporary orthogonal Fmoc protecting group which is suitable for deprotection on resin, prior to the anticipated elongation of the oligosaccharide through repetitive glycosylation using the disaccharide building block. For chain termination and to aid downstream purification, we designed building block **4** containing a semi-permanent benzyl group at *O*-4 in place of Fmoc for the final glycosylation.

The disaccharide **3** was first synthesized in solution by the glycosylation between glucopyranoside donor (SI**1**) and the acceptor (SI**2**) in 71% yield following a reported method (See supporting information, Scheme S1).[14] The *p*-methoxyphenyl group of the product was hydrolysed by ceric ammonium nitrate, followed by TCA installation.[34] This TCA donor proved stable enough to be used as a building block on the Glyconeer™ and was chosen as its glycosylation had previously been optimised in solution.[14] Additionally the donor provided a facile method for recycling excess donor hydrolysed during automated synthesis, through reinstallation of the TCA group directly from the hemiacetal **5**, thus increasing the efficiency of the methodology. Notably, the entire synthesizer is kept under argon atmosphere to ensure complete exclusion of water; thereby minimizing any hydrolysis of the activated building blocks prior to delivery into the reaction vessel. Merrifield resin functionalised with a photolabile nitrobenzyl ether-based linker **7** (loading 0.40 mmol g^{-1}) was chosen as a solid support for the assembly of the oligosaccharide, due to its stability in both acidic and basic solutions.[35]

The synthesis of the oligosaccharide briefly includes iterative glycosylation of the resin and the building blocks followed by Fmoc deprotection and acid wash cycles in the Glyconeer™. The desired length oligosaccharide was then cleaved from the resin photochemically prior to purification (Figure 2). Glycosylation of the resin with the disaccharide building block **3** commenced with a preprogrammed glycosylation-deprotection module (see supporting information Table S1) using trimethylsilyl trifluoromethanesulfonate (TMSOTf) as the activating agent, followed by 5% piperidine in DMF as the Fmoc deprotecting solution. Optimisation of the first glycosylation module (attachment to the solid support) revealed that using 10 equiv. of donor building block **3** in the first cycle afforded higher conversions (75%) than two-cycles using 5 equiv. of donor (68%), or two-cycles using 3.5 equiv. of donor (43%). To maximise attachment of the sugar onto the resin we therefore opted to use 10 equiv. of donor **3** in the first cycle followed by a further 5 equiv. of donor in a second cycle. Resin loading was calculated following glycosylation, by cleaving a small portion of the resin and performing UV-based DBU-Fmoc assay[36] (which measures the amount of dibenzofulvene adduct released after Fmoc deprotection in solution). A washing step with Lewis acid (TMSOTf in dry CH_2Cl_2 at -30 °C for 1 min) was also introduced before each glycosylation module to ensure removal of any residual base from the previous deprotection step.

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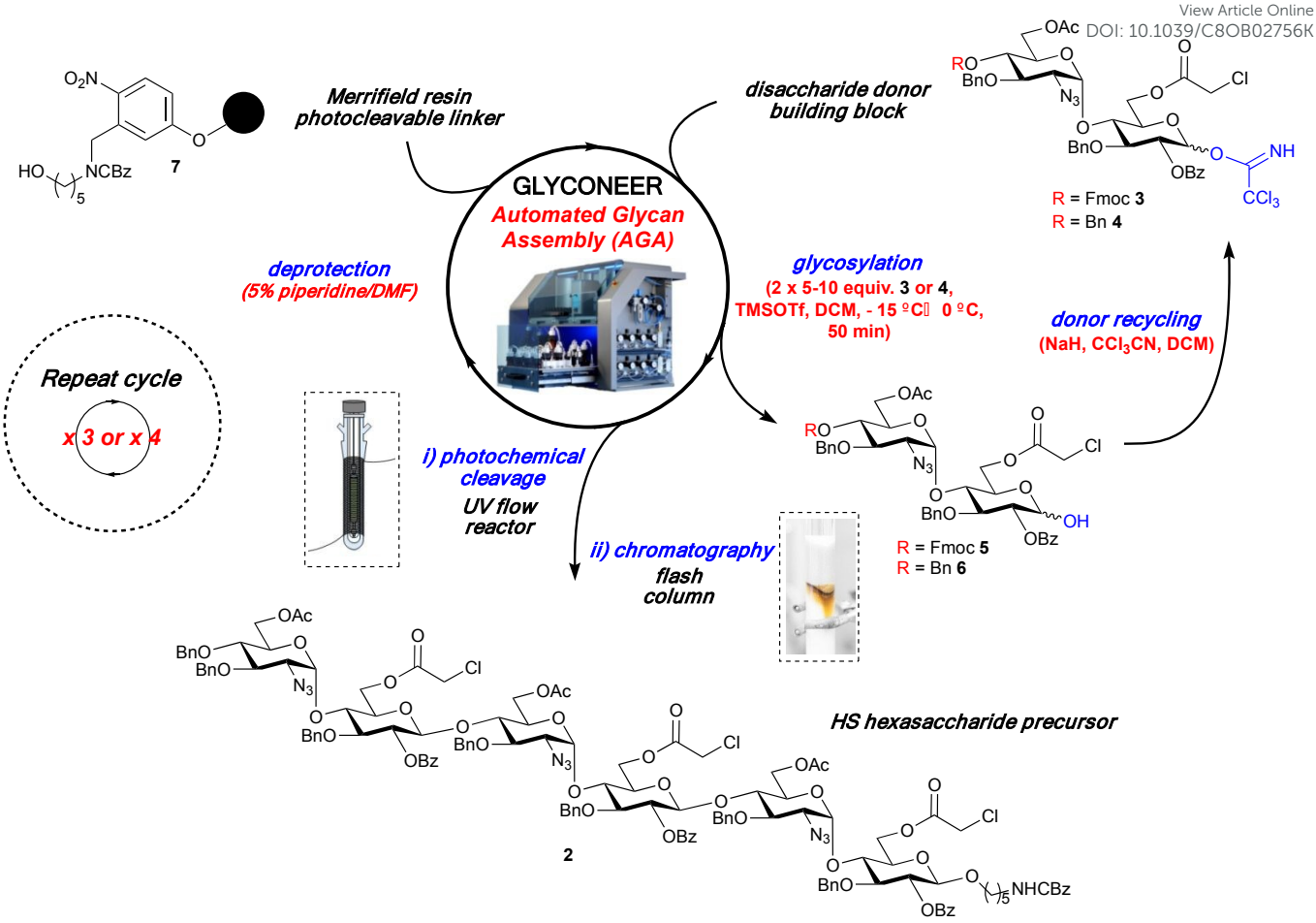

Figure 2: Schematic illustration of automated glycan assembly of hexasaccharide **2**, highlighting the individual steps of glycosylation and deprotection which are performed in a repetitive cycle on the Glyconeer, followed by photochemical resin cleavage in a UV flow reactor, flash column chromatography purification, and TCA donor recycling.

Once the glycosylation conditions for the TCA disaccharide donors were standardised, the AGA of hexasaccharide **2** was achieved *via* iterative glycosylations using a further glycosylation-deprotection module (see Supporting Information, Module 2, Section 3). After completion of each synthetic cycle, total removal of Fmoc group from the resin-bound glycan was monitored by measuring the UV signal through an in-line UV detector. The oligosaccharide was cleaved from the photolabile resin using an in-house photoreactor consisting of a mercury lamp of 400 W fitted with FEP tubing and connection to a cooling tap.[37] Specifically the resin containing the glycan was suspended in CH_2Cl_2 and subsequently passed through a sample injector to the tubing surrounding the mercury lamp before filtration and concentration of the filtrate *in vacuo*. The crude protected glycan could then be analysed rapidly through MALDI-TOF MS. Although some target hexasaccharide was observed by MALDI-TOF MS after three iterative glycosylations, the major product was the tetrasaccharide, a deletion sequence, which are common in solid phase synthesis and often result from an incomplete or unsuccessful coupling reaction during the programmed cycle. Therefore, in order to maximize the yield of hexasaccharide, four consecutive glycosylations were performed to account for the anticipated inefficiencies in the solid-phase glycosylation, and convert any tetrasaccharide deletion sequence into the hexasaccharide. Following crude product analysis, on this occasion, the hexasaccharide **2** was formed as the major product, with only a small amount of tetrasaccharide by-product (Figure S3 and S4). Although improved capping procedures have recently been reported to minimise such deletion sequence formation,[38] we opted not to use capping to ensure the minimum reaction time per cycle. Here, the use of a disaccharide building block was especially advantageous as deletion sequences were more disparate in size and polarity (tetra vs hexasaccharide) than would have been observed using monosaccharide building blocks. This facilitated the purification of the final hexasaccharide from any deletion sequences in 30% overall yield on a 15 mg scale, using only readily accessible silica gel column chromatography. Importantly the use of disaccharide building block strategy obviated the requirement for a highly optimised, costly and low yielding preparative HPLC purification, as has commonly been utilised following AGA.

A comparison to the synthesis of a similar hexasaccharide performed in solution using identical donor building blocks,[14] reveals that the automated synthesis reported here compares

favourably in terms of overall yield, but is far more time and resource efficient, taking less than a quarter of the time and requiring only one purification step as opposed to at least four in solution. Also, the excess hydrolysed donor **5** formed throughout the synthesis was automatically collected in the fraction collector, and could be subsequently recycled back into the TCA donor in solution. At the 0.02 mmol scale (50 mg resin, 0.40 mmol g^{-1} loading) employed here, 1.02 g of TCA donor was required for the complete hexasaccharide synthesis, but 657 mg of hemicacetal **5** was recovered which was then subjected to TCA reinstallation, with an overall donor recycling efficiency of ~60%.

Finally, 1D and 2D NMR (Figure 3) corroborated the presence of 3 α- and 3 β- anomeric protons as expected from the final product. In total, desired HS hexasaccharide precursor **2** was synthesized in 6 hours from the stable, scalable disaccharide donors, highlighting the potential of the Glyconeer™ to deliver rapid access to HS precursors, which can then be differentially sulfated and subjected to global deprotection as and when required.

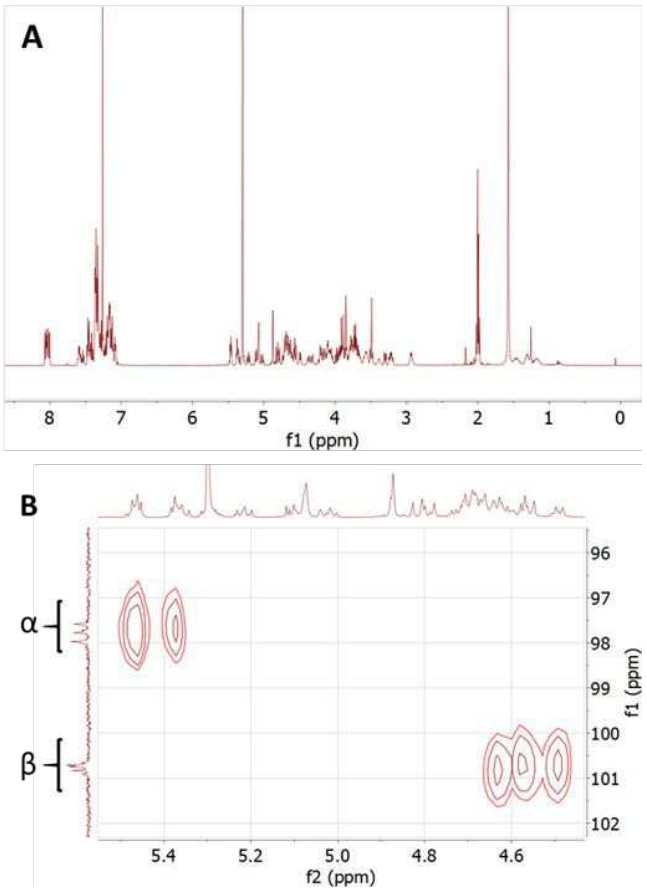

Figure 3: A) 1H NMR of pure hexasaccharide **2** and **B)** 1H-13C HSQC expansion of the anomeric region, highlighting the presence of three α-anomeric linkages, and three β-anomeric linkages.

Conclusions

In conclusion, the automated synthesis of an orthogonally protected HS hexasaccharide precursor was achieved efficiently using the Glyconeer™, providing scope to further explore the therapeutic role of HS in Alzheimer's disease in greater molecular detail in the future. The cleavage of the oligosaccharide from the solid support was demonstrated using an in-house photoreactor, enabling practical analysis of the crude oligosaccharide in solution. The use of flash chromatography enabled a convenient practical purification of the target glycan without the need for preparative HPLC, removing deletion sequences in the process. Although by-products such as deletion sequences formed in AGA can be reduced using capping procedures, the requirement for four iterative glycosylations to achieve three glycosidic linkages in this example highlights that further optimisation of glycosylation procedures in automated synthesis is still essential and represents a key area for exploration by synthetic carbohydrate chemists. [19, 33]

Conflicts of interest

"There are no conflicts to declare".

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