Carbohydrate Synthesis (1)

DOI: 10.1002/anie.201210132

Automated Solid-Phase Synthesis of Chondroitin Sulfate Glycosaminoglycans**

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Carbohydrates are the most prevalent class of biopolymers on earth. Bound to proteins and lipids, carbohydrates form four structurally and functionally distinct, biologically significant glycoconjugate classes: glycoproteins, glycolipids, glycosylphosphatidylinositol (GPI) anchors, and glycosaminoglycans (GAGs; Figure 1). These structurally diverse macromolecules, which are usually located in the extracellular matrix, are essential for many fundamental cellular processes.[1]

GAGs are acidic, negatively charged polysaccharides that transduce extracellular signals to the interior of the cell.[2] Localization is manifested by connection to a transmembrane core protein, to form a proteoglycan (Figure 1). GAGs are highly variable in size, ranging from 20–200 disaccharide repeating units, backbone composition, and the degree and pattern of sulfation.[2d] Chondroitin sulfate contains *N*-acetyl-β-D-galactosamine and β-D-glucuronic acid and the sulfation and acetylation of particular hydroxy and amino groups varies. The sulfation patterns of GAGs influence the bioactivity of the molecules but limited access to defined GAG structures has impeded mapping structure–activity patterns.[2d]

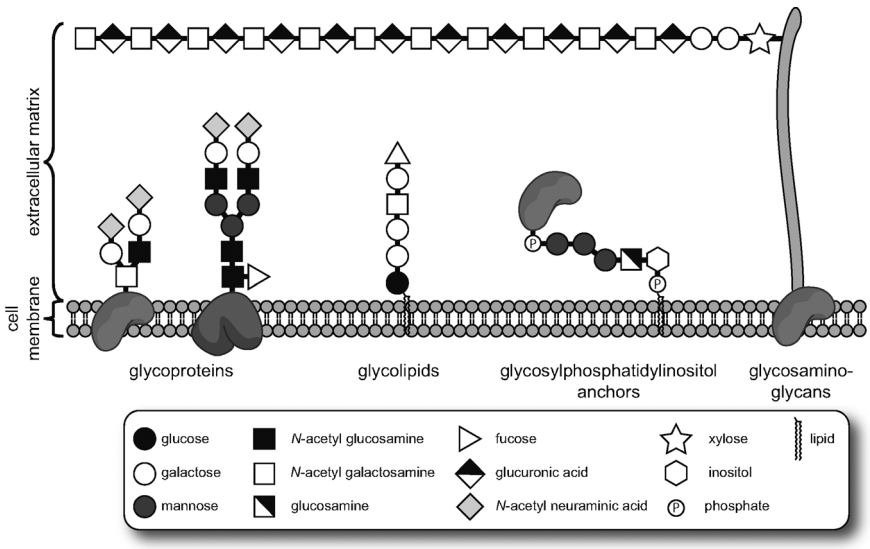

Figure 1. Glycoconjugates of the extracellar matrix. *O*- and *N*-glycans are linked to proteins by the side chains of serine, threonine, or asparagine. Glycolipids are composed of glycans that are attached to lipids and play an essential role in cellular recognition processes. Glycophosphatidylinositols (GPI) anchor proteins via two fatty acids to the cell membrane. Glycosaminoglycans occur as the glycan side chain in proteoglycans.

Tailor-made GAG oligosaccharides can be synthesized chemically[3] or enzymatically,[4] and they have become valuable for analyzing GAG–protein interactions and their biological relevance. Introducing sulfate groups to specific positions of an oligosaccharide chain adds an additional level of complexity on top of the already challenging synthesis of oligosaccharides. Therefore, currently available methods for the assembly of GAG oligosaccharides, including modular approaches, are time-consuming and lack generality as the synthesis of each target molecule poses an individual challenge.

Herein, we describe a novel approach for automated solid-phase synthesis of GAG oligosaccharides that is based in part on established methods for generating the glycan portion of glycoproteins and glycolipids.[5] Key to the success of this procedure was a stable supply of tailor-made differentially protected building blocks, a robust but easily-cleaved linker, to connect the first monosaccharide of the nascent oligosaccharide to the solid support,[6] and the automated synthesizer. A recently-developed automated solid-phase oligosaccharide synthesizer[5b] that allows for fully automated, computer-controlled glycan coupling cycles and the introduction of sulfate groups on solid support was further improved to carry out automated sulfation and modification on solid support.

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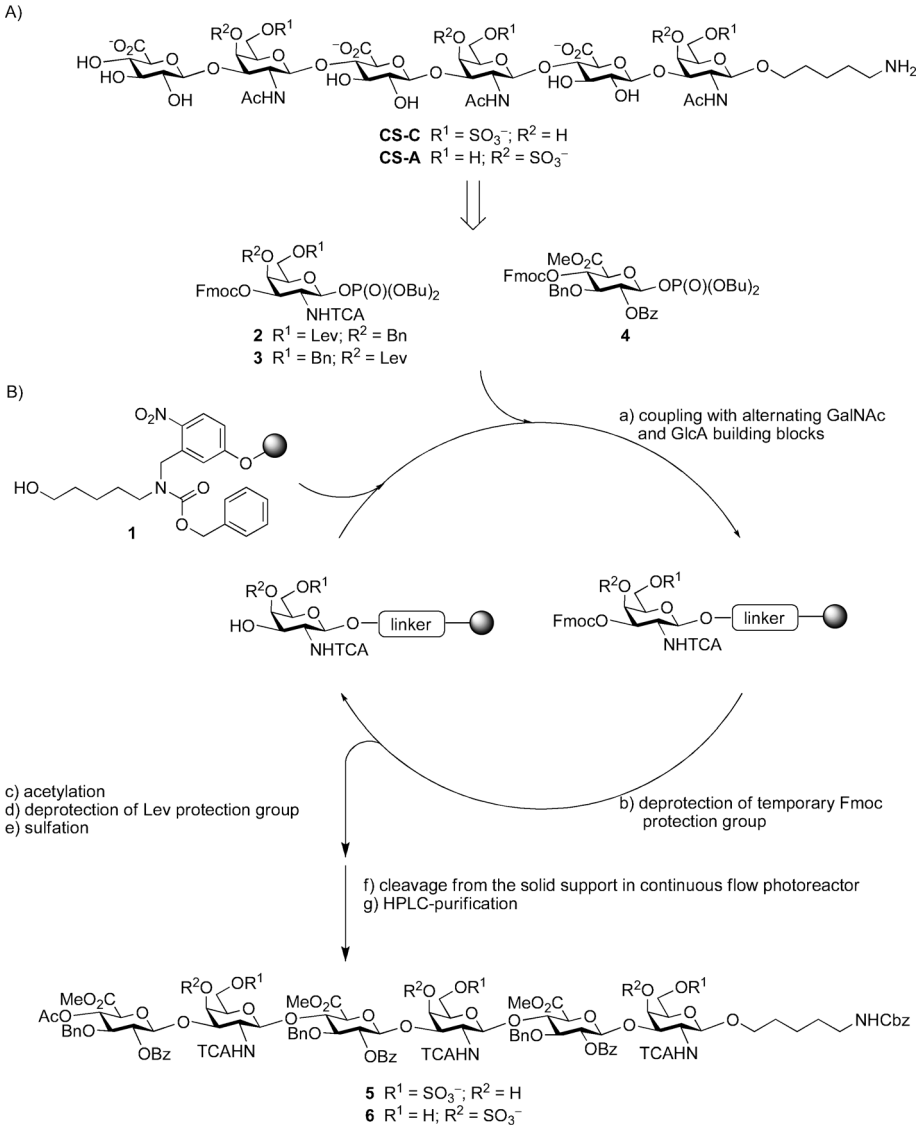
[**] We gratefully thank the Max Planck Society and the European Research Council (ERC Advanced Grant AUTOHEPARIN to P.H.S.) for financial support. The authors would like to thank Dr. D. Kolarich, Dr. M. Hurevich, Dr. M. Schlegel, and O. Calin for helpful discussions, and Dr. Y.-H. Tsai for measuring the NMR spectra. We also would like to thank Dr. V. Mountain for critically editing this manuscript.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201210132.



Scheme 1. A) Retrosynthetic analysis of chondroitin oligosaccharide sequences with different sulfation patterns. B) Automated synthesis of chondroitin hexasaccharides. Reactions and conditions: a) 3×3 equiv building block, TMSOTf, CH_2Cl_2, −15 °C (45 min)→0 °C (15 min); b) 3×20% piperidine in DMF, 25 °C (5 min); c) 3×Ac_2O, pyridine 25 °C (30 min); d) 3×$H_2NNH_2{\cdot}H_2O$, pyridine, AcOH, CH_2Cl_2, 25 °C (60 min); e) 3×SO_3·pyridine, pyridine, DMF, 50 °C (3 h); f) *hν*, CH_2Cl_2, 25 °C (**5**: 13% over 16 steps; **6**: 8% over 16 steps).

Chondroitin sulfate hexasaccharides **CS-C** and **CS-A** (Scheme 1A) were selected as initial target molecules for automated solid-phase GAG synthesis for their biological importance such as in *Plasmodium falciparum* infections[7] and because these highly negatively charged molecules are making their synthesis on a solid phase a significant challenge. Retrosynthetic analysis of the chondroitin sulfate backbone illustrated that hexasaccharides **5** and **6** could be assembled using differentially-protected building blocks derived from two monosaccharides, galactosamine (GalNAc) and glucuronic acid (GlcA; Scheme 1).

A protecting-group strategy was devised using techniques developed for the modular solution phase synthesis of heparin oligosaccharides:[3d] hydroxy groups that were not modified during the reaction were permanently protected by benzyl ethers (Bn); hydroxy groups that would ultimately be sulfated were masked with levulinoyl (Lev) esters that could be efficiently cleaved under mild conditions; and hydroxy groups intended to serve as nucleophiles during glycan chain elongation were protected by fluorenylmethyloxycarbonyl (Fmoc) groups, liberated simply by the mild and selective action of piperidine. Using these parameters, two differentially protected galactosamine phosphate building blocks (**2** and **3**) and a glucuronic acid phosphate building block (**4**) were designed. All building blocks were readily procured in multigram amounts from commercially available galactose and glucose (Scheme 1A; Supporting Information, Figures S2, S3).

Linker selection posed a challenge because of the breadth and variety of chemical reactions that must be withstood during GAG synthesis. The ideal linker can be easily cleaved from solid support at the end of the synthesis, leading directly to a functional group for the chemoselective synthesis of glycan arrays and glycoconjugates.[8] It must simultaneously withstand basic reaction conditions for the removal of Fmoc, Lev, and Bz protecting groups, as well as the strongly acidic conditions for building block coupling reactions. The strongly acidic coupling conditions would cleave the bifunctional linker previously used for the automated synthesis of *N*- and *O*-linked glycans.[5b] Furthermore, the strongly basic conditions necessary to cleave this linker from solid support could favor β-elimination in the glucuronic acid units of the GAG hexasaccharide leading to truncated fragments. Linkers cleaved by metathesis have also been used in automated oligosaccharide synthesis, but require several post-synthetic modification steps in solution to obtain a functional group for attachment to glycan microarrays.[3d]

Consequently, a new orthogonal linker had to be designed. The resilience of a photolabile nitrobenzyl ether-based linker coupled to solid support (**1**, Scheme 1B) was thoroughly investigated by a series of model studies with monosaccharides (data not shown). Light is a powerful and selective reagent that has been used for the cleavage of oligosaccharides and oligopeptides assembled by solid-phase synthesis.[9] However, as light passes through a solution, light intensity decreases with increasing distance from the light

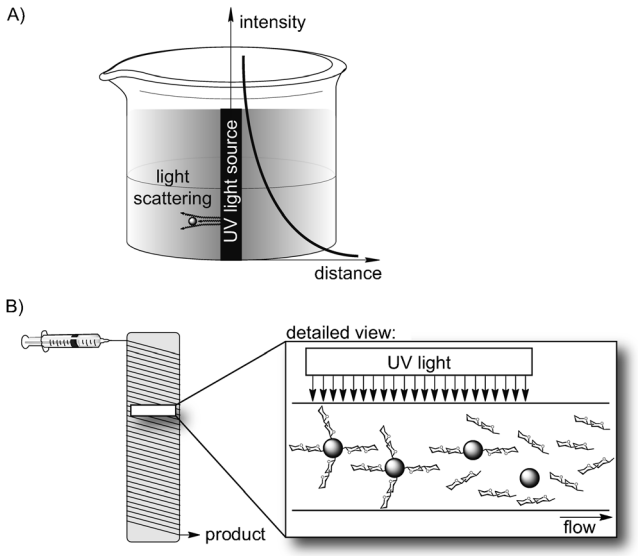


Figure 2. Photocleavage from the resin. A) In suspensions, light intensity decreases with increasing path length. Furthermore, light scattering decreases the efficiency of photoreactions when performed in batch. B) Photocleavage in a continuous-flow reactor: the resin-bound hexasaccharide is transported through a tube located directly adjacent to the light source, thereby ensuring high light intensity throughout the reaction solution and efficient cleavage.

source owing to absorption. Therefore, photocleavage is inefficient in traditional reaction vessels (Figure 2A).[10]

This limitation is likely to be exacerbated when large amounts of resin beads used as solid support are present in the solution, scattering the light. Cleavage from solid support was therefore conducted as a separate step, after automated synthesis, in a continuous-flow reactor, where reactions occur in tubing with small diameters, ensuring efficient irradiation.[11] For this purpose, a new continuous-flow reactor was developed (Figure 2B), constructed of a mercury lamp surrounded by a UV filter (Pyrex, 50% transmittance at 305 nm) and a cooling apparatus. The cleavage reaction occurred in fluorinated ethylene propylene (FEP) tubing that was wrapped around the light source. Resin-bound compounds were transferred from the reaction vessel of the synthesizer into a disposable syringe and pushed through the tubing of the photoreactor with the help of a syringe pump. After cleavage, resin was extracted by filtration, and solvents were removed in vacuo, rendering the crude chondroitin hexasaccharides. To the best of our knowledge, this is the first demonstration of a synthesis system that combines a highly efficient photochemical reaction in continuous flow with solid-phase chemistry.

With a general framework of linker and building blocks in place, different polymeric supports were screened for their influence on the outcome of oligosaccharide assembly. Polystyrene resins performed best and were subsequently used for all studies. Additionally, choices of different leaving groups for the galactosamine and glucuronic acid glycosylating agents were investigated. The phosphate activation resulted in significantly better coupling yields when compared to the corresponding selenoglycosides, thioglycosides, or glycosylimidates (data not shown).

With the synthesis system in place, assembly of chondroitin oligosaccharides **5** and **6** (Scheme 1B) was attempted. Linker-functionalized resin **1**[12] (Scheme 1B) was obtained with a loading[13] of 0.47 mmol g^{-1} and used in both cases. Chondroitin-6-sulfate hexasaccharide **5** was successfully assembled using building blocks **2** and **4** in 16 steps over three days, with a yield of 13% (88% average yield per step). Simply utilizing building blocks **3** and **4** instead generated chondroitin-4-sulfate hexasaccharide **6** with a yield of 8% (86% average yield per step). To assemble the carbohydrate backbones of **5** and **6**, each glycosylation cycle was performed three times, each time using three equivalents of the relevant building block. An equimolar amount of trimethylsilyl trifluoromethanesulfonate (TMSOTf) served as an activator of the glycosylation reaction at −15 °C. Removal of the temporary Fmoc protecting group on the non-reducing building block by the action of piperidine liberated a hydroxy group for further elongation and produced a piperidine–dibenzofulvene adduct that served as a measure of the coupling efficiency of the previous glycosylation reaction by UV absorption quantification. Prior to sulfation, the terminal Fmoc protecting group was replaced by a stable acetyl protecting group to avoid unwanted reactions. All of the levulinoyl esters were then selectively removed with buffered hydrazine. Sulfation of the exposed hydroxy groups required incubation with a sulfur trioxide pyridine complex in pyridine and DMF at 50 °C.

Resin-bound sulfated hexasaccharides were manually transferred from the reaction vessel of the synthesizer to the continuous-flow photoreactor, swollen in dichloromethane, and then cleaved by exposure to UV light. During the cleavage process, the solvent was exchanged for MeOH to ensure the solubility of the negatively charged molecules. After collection, the resin was removed by filtration and the crude products analyzed by reverse-phase HPLC with mass detection (RP-LCMS), then purified by preparative HPLC (Figure 3A). An amino group remains on each hexasaccharide after linker cleavage that is protected by carboxybenzyl (Cbz). This, together with the remaining benzyl ethers, serves as a chromophore enabling UV detection of the target compounds during HPLC purification.

Partially protected chondroitin sulfate hexasaccharides **5** and **6** were fully characterized by nuclear magnetic resonance (NMR) spectroscopy and high resolution mass spectrometry (HR-ESI-MS) (Figure 3B).

For the final deprotection, a hydrogenolysis and saponification approach was envisioned to liberate pure oligosaccharides from any remaining benzyl ether, trichloroacetamides, and benzoyl or acetyl ester protecting groups. This final step also reveals a unique amine on the reducing terminus of the remaining linker that can be used to facilitate selective coupling of the resulting chondroitin hexasaccharides on microarray or nanoparticle surfaces. As characterization and reaction monitoring by TLC of the small quantities of the highly negatively charged molecules is difficult, a straightforward process for the final deprotection has still be to developed to obtain the target structures.

In summary, the combination of an automated carbohydrate synthesizer with a photolabile linker cleaved in a con-

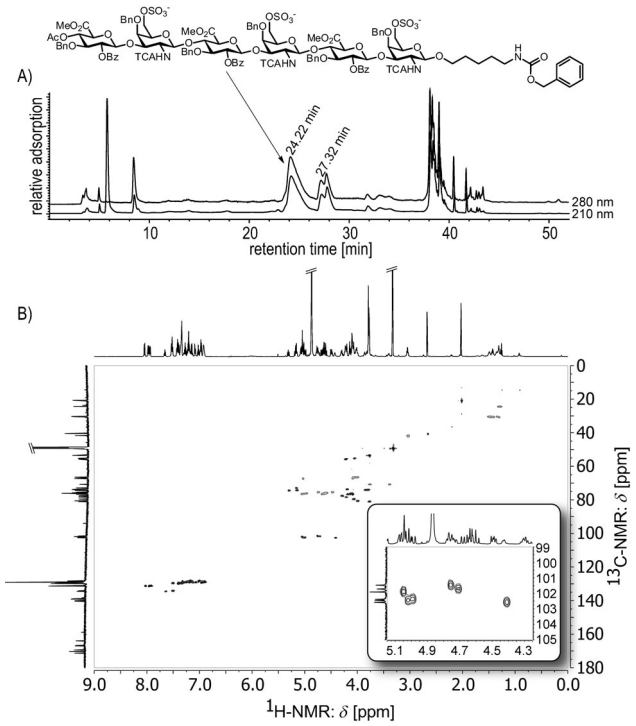


Figure 3. Purification and characterization of chondroitin hexasaccharide **5**. A) Product purification by preparative HPLC. The peak at 24.22 min corresponds to **5**, the peak at 27.32 min to a deletion sequence lacking one GlcA and one GalNAc building block, and the peaks between 38 and 44 min are polystyrene-derived compounds from the Merrifield resin. Conditions: column: C18-Nucleodur (21×250 mm; 5 μm); flow: 10 mL min^{-1}; eluents: 0.01 M NH_4HCO_3 in water/MeCN; gradient: 45% (5 min)→55% (in 40 min)→100% (in 5 min); detection: 210 and 280 nm. B) 600 MHz HSQC NMR spectrum of **5** at 25 °C in CD_3OD.

tinuous-flow photoreactor enabled the synthesis of two chondroitin hexasaccharides as examples for a general method. Using this method with appropriate building blocks will enable access to defined GAG oligosaccharides such as heparin, heparin sulfate, dermatan sulfate, or keratin sulfate in a fraction of the time previously required.

Received: December 19, 2012
Published online: April 15, 2013

Keywords: automated solid-phase synthesis · glycosaminoglycans · photocleavable linker

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