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# Functionalized celluloses with regular substitution pattern by glycosynthase-catalyzed polymerization

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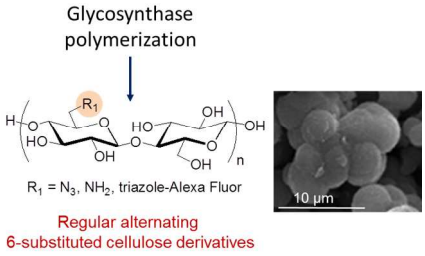
KEYWORDS: Cellulose • glycosynthase • functionalization • sequence control • regular pattern • *Humicola insolens* cellulase

## ABSTRACT

Control of the monomer sequence in polymers is extraordinarily difficult by chemical synthesis, though Nature routinely exerts such control, including in the biosynthesis of polysaccharides. This inability has prevented us from being able to match the exquisite structure-activity control exhibited in biosynthesis of bioactive natural polysaccharides. We here address a powerful approach, whereby enzyme-catalyzed polymerization of properly modified building blocks is introduced as a simple route affording polysaccharides with controlled sequence and functionalization pattern. Targeting cellulose as a versatile scaffold for novel biomaterials, we describe the preparation of a perfectly alternating polysaccharide with repeat unit 6'-azido-6'-deoxycellobiose by a glycosynthase-catalyzed polymerization using the *H. insolens* cellulase Cel7B E197A mutant, and its further functionalization to give novel modified cellulose derivatives with a regular substitution pattern.


Figure for the abstract



## 1. INTRODUCTION

Synthetic aminopolysaccharide mimetics of natural chitosans have attracted recent interest in materials science to modify and improve the physicochemical and biological properties of chitosan-based biomaterials. Chitosan, a linear heteropolysaccharide of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and glucosamine ($GlcNH_2$) units, is obtained by chemical or enzymatic partial de-*N*-acetylation of chitin, with variable degrees of acetylation and random distribution of cationic glucosaminyl units along the polysaccharide chain.[1] The cationic nature of chitosans makes them versatile materials with a broad range of applications.[2–6] They are biocompatible and possess mucoadhesive, bacteriostatic, and hemostatic properties. The positively charged chitosans are able to bind and encapsulate anionic compounds such as proteins and DNA, and interact with negatively charged compounds found in mucus and on cell surfaces, which improves the cell adhesion and proliferation properties of chitosan-based scaffolds. However, applications in drug and gene delivery systems suffer from low transfection efficiency.[7]

With the aim of mimicking and improving the biomedical properties of chitosans, different procedures to modify neutral polysaccharides have been reported.[8] Modifications of cellulose, laminarin, curdlan, and other natural polysaccharides are performed by chemical procedures, *i.e.* by functionalizing the C-6 positions with amino-substituted side chains or azido groups than can be reduced to amines or reacted by "Click" chemistry. The properties of these functionalized polysaccharides will depend on their molecular weight and degree of substitution. However, they are polydisperse with random distribution of the functional groups, requiring new methodologies to produce defined and reproducible polymeric materials.

Sequence control is one of the great remaining problems in polysaccharide synthesis. Nature creates polysaccharides often with a very high degree of control over sequence leading to

highly specific biological activity, for example the active sequences of glycosaminoglycans heparin[9] and chondroitin sulfate[10] that are vital to their interactions with proteins, governing their ability to prevent thrombosis. Laborious, multi-step, low-yielding chemical syntheses have been necessary to produce even oligosaccharides of controlled sequence.[11]

Enzymatic synthesis opens up access to regular and homogeneous polysaccharides. Glycosyltransferases are the natural enzymes catalyzing glycoside bond formation in the biosynthesis of oligo- and polysaccharides, but have limited applicability for preparative *in vitro* synthesis due to the requirement of expensive glycosyl donors (*i.e.* sugar nucleotides) and limited stability of these enzymes. Glycosidases, which catalyze the hydrolysis of oligo and polysaccharides, have been demonstrated to be effective catalysts for carbohydrate synthesis under medium engineered conditions and using activated glycosyl donors (kinetically controlled transglycosylation). In this way, glycosidase-catalyzed polymerizations of simple glycosyl donors are regio- and stereospecific for the newly-formed glycosidic bonds. Kobayashi pioneered the application of cellulase-catalyzed polymerization of activated disaccharides, preparing synthetic cellulose for the first time by polymerization of β-cellobiosyl fluoride in acetonitrile/acetate buffer[12] and 6-O-methylated celluloses by polymerization of the corresponding donors.[13] Few other polysaccharides (*e.g.* amylose, xylan, mixed-linked β-glucans, alternating cellulose-chitin, and cellulose-xylan hybrids) have been prepared by kinetically-controlled transglycosylation using retaining glycosidases,[14,15] with moderate yields due to the intrinsic hydrolase activity of the enzymes. More recently, glycosynthases have proved their efficiency for the enzymatic synthesis of oligosaccharides and glycoconjugates. Glycosynthases are mutated retaining glycoside hydrolases in which the catalytic nucleophile has been replaced by a smaller and inert residue.[16,17] They are hydrolytically inactive but efficiently catalyze glycoside bond formation when using glycosyl fluoride donors that have the opposite

anomeric configuration to the normal substrate of the parental wild-type enzyme.[18–21] The concept has been extended to glycosidases acting by substrate assisted catalysis (*i.e.* hexosaminidases) using oxazoline donors with engineered hydrolytically inactive mutant enzymes.[19] The glycosynthase technology was expanded to the synthesis of polysaccharides by promoting the donor self-condensation reaction.[14] We reported the first non-natural polysaccharide, an alternating mixed-linked β-1,3 and β-1,4 glucan by polymerization of α-laminaribiosyl fluoride with a glycosynthase variant of a *Bacillus* 1,3-1,4-β-glucanase.[22] Other polysaccharides such as cellulose,[23] laminarin,[24] xylans,[25] 1,3-1,4-β-glucans,[26] and branched xyloglucans[27,28] have also been successfully achieved. Interestingly, Fort et al.[23] explored the ability of the glycosynthase derived from the cellulase Cel7B from *Humicola insolens* (*Hi*Cel7B E197A) to accept different functionalized cellobiosyl donors. The enzyme was able to accept α-cellobiosyl fluorides that had functionalities at C-6' position such as bromine, amino and thioglucosyl groups. This particular glycosynthase offers a broad range of synthetic applications since it has also been employed for the synthesis of xyloglucan oligosaccharides.[27]

We envision the glycosynthase technology as a potential tool for the efficient synthesis of functionalized polysaccharides: simple glycosyl donors prepared with the specific modifications can be accepted and polymerized by the glycosynthase enzyme to produce modified polysaccharides. This glycosynthase approach could provide a new generation of polysaccharides with controlled functionalization patterns and polymerization degrees different from those obtained by chemical modification routes. We here report the preparation of a perfectly alternating polysaccharide with repeat 6'-azido-6'-deoxycellobiose by a glycosynthase catalyzed polymerization of a 6'-azido-cellobiosyl donor using the cellulase *Hi*Cel7B E197A mutant, and its further functionalization to give modified cellulose derivatives with a regular substitution pattern.

## 2. EXPERIMENTAL SECTION

**2.1. Structural characterization.** $^{1}H$ and $^{13}C$ NMR, COSY, TOCSY, and HSQC spectra were recorded on a Varian Gemini 400 MHz spectrometer operated at 298 K. For $^{1}H$ NMR, the TMS peak (0 ppm) was used as reference. When using MeOD as solvent, the central peak of the MeOD multiplet (3.31 ppm) was used as a reference. For $^{13}C$ NMR, the central peak of the $CDCl_3$ triplet (77.16 ppm) was used as a reference. When using MeOD as solvent, the central peak of the MeOD multiplet (49.00 ppm) was used as a reference. Synthetic cellulose and functionalized cellulose $^{13}C$ NMR spectra were obtained on Varian INOVA and Varian UNITY 400 MHz spectrometers with a minimum of 5,000 scans in $CDCl_3$. Chemical shifts are reported relative to the solvent peaks.

A Thermo Electron Nicolet 8700 FTIR was used to perform infrared spectroscopy analyses of the samples as pressed KBr pellets. All samples were dried in a vacuum oven prior to analysis to avoid moisture. *In situ* FTIR spectra were obtained using a Mettler Toledo ReactIR 45M with a SiComp fiber optic ATR probe.

HPSEC analyses to determine molecular mass profiles were performed on an Agilent 1100 HPLC system equipped with a refractive index detector using a PSS Gram column (8.0 x 300 mm, 100Å, 10 μm) and a PSS Gram pre-column (9.0 x 50 mm, 100 Å, 10 μm) maintained at 50ºC, and DMSO with lithium bromide (5 g/L) as eluent at a flow rate of 0.5 mL/min. The calibration curve was obtained with dextrans as standards (American Polymer Standards Corporation DXT1 – DXT55 kDa). Freeze dried polymers and standards were dissolved in DMSO and filtered. From the chromatograms, $M_p$ (molecular mass of the peak maximum), $M_w$ (weight average molecular mass), $M_n$ (number average molecular mass), DP (degree of polymerization), and PDI (polydispersity index) were calculated.[29]

MALDI-TOF spectra were obtained in a Bruker Daltonics spectrometer with 2,5-dihydroxybenzoic acid as matrix. Reflectron, positive ion mode, 19 kV acceleration voltage, and 20 kV reflector voltage were used.

For SEM experiments, the dried product was fixed on a graphite tape, coated with gold/palladium by ion-sputtering, and observed at an accelerating voltage of 10 kV and working distance of 16 mm using a JEOL JSM-5310 microscope.

Fluorescent polysaccharides were observed with a Stereoscopic microscope Nikon Eclipse TE 2000-U using a Piston GFP filter, excitation 450-490 nm, emission: 500-530 nm, and images were acquired with a cooled CCD camera.

**2.2. Synthesis of donor and acceptor substrates.** α-Cellobiosyl fluoride (CelαF) (**1**) and 6'-azido-α-cellobiosyl fluoride (6'-$N_3$-CelαF) (**2**) were prepared as reported in Fort *et al.*[1] 4-Nitrophenyl-β-D-glucopyranoside (GlcpNP) (**3**) was from Sigma.

4-Nitrophenyl 6-azido-6-deoxy-β-D-glucopyranoside (6-$N_3$-GlcpNP) (**4**) was prepared by tosylation of the 6-OH group of **3**, acetylation with $Ac_2O$/py/DMAP, substitution of the tosyl group by azide, and finally de-*O*-acetylation by reaction with sodium methoxide in methanol, as follows:

4-Nitrophenyl-β-D-glucopyranoside (**3**) (2.59 g, 8.59 mmol) was dissolved in dry pyridine (26 mL) and the solution was cooled in an ice bath. A cold solution of tosyl chloride (4.91 g, 25.78 mmol) in dry pyridine (26 mL) was added and the reaction mixture was stirred for 22 hours under nitrogen atmosphere at room temperature. Acetic anhydride (52 mL) and N,N-dimethylaminopyridine (DMAP) (25 mg) were directly added to the solution. The mixture was stirred for 24 hours at room temperature. Finally, methanol (100 mL) was added to quench the reaction and, after evaporation until dryness, the product was redissolved with ethyl acetate,

washed with water, the organic phase dried, and the solvent evaporated under reduced pressure to yield 9.17 g of 4-nitrophenyl 6-*O*-tosyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (compound **I).** Compound **I** (9.17 g, 15.77 mmol) was dissolved in dry DMF (183 mL). Sodium azide (2.23 g, 34.37 mmol) was added and the solution stirred at 80 ºC for 14 hours. A saturated aqueous solution of sodium bicarbonate and ethyl acetate was added, and the organic phase was washed with water, dried, and the solvent evaporated. The product was purified by flash chromatography (toluene-ethyl acetate 20:1). 4-Nitrophenyl 6-azido-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (compound **II**) (1.94 g, 4.31 mmol) was obtained in 50% overall yield from **3**. $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 170.2, 169.6, 169.3 (CO), 126.0, 116.8 (C-Ar), 98.1 (C-1), 74.0 (C-5), 72.4, 71.0, 69.3 (C-2, C-3, C-4), 51.3 (C-6), 20.7 ($CH_3$). $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, 2H, $J_{Ar}$ = 9.1 Hz, arom), 7.10 (d, 2H, $J_{Ar}$ = 9.1 Hz, arom), 5.33 – 5.30 (m, 2H, H2, H3), 5.24 (d, 1H, $J_{1\text{-}2}$ = 7.9 Hz, H1), 5.09 (m, 1H, H4), 3.85 (m, 1H, H5), 3.44 (dd, 1H, $J_{5,6b}$ = 7.4 Hz, $J_{6a,6b}$ = 13.4 Hz, H-6$^b$), 3.35 (dd, 1H, $J_{5,6a}$ = 2.7 Hz, $J_{6a,6b}$ = 13.4 H-6$^a$), 2.07-2.05 (m, 9H, $CH_3$). ESI-MS (m/z): 470.1514 $[M+NH_4]^+$. Compound **II** (200 mg, 0.44 mmol) was dissolved in methanol (7 mL) and sodium methoxide (1 mM in methanol). The mixture was stirred for 6 hours at room temperature. Then, the mixture was neutralized with Amberlite IR 120 resin (H+), filtrated and freeze-dried. 4-Nitrophenyl 6-azido-6-deoxy-β-D-glucopyranoside (**4**) was obtained in 98% yield.

**2.3. Cel7B E197A protein.** The *Humicola insolens* Cel7B E197A enzyme was kindly provided by Novozymes (Denmark). The protein was >95% homogeneous as judged by SDS-PAGE. Concentration was determined by UV spectrophotometry ($\varepsilon_{280}$= 6.68 x $10^5$ $M^{-1}\cdot cm^{-1}$).

**2.4. Kinetics of donor-acceptor condensations catalyzed by the *Hi*Cel7B E197A glycosynthase.** Reactions were done in 96-well microplates. Donor and acceptor substrates in 100 mM phosphate pH 7.8 were pre-incubated at 35ºC for 5 min, then the reactions were initiated by addition of the *Hi*Cel7B E197A enzyme, and kept at 35 °C (final reaction volume of 0.3 mL). Aliquots were withdrawn at regular time intervals, diluted 1:10 with formic acid 2% (v/v) to stop the enzymatic reaction, and analyzed by HPLC (Agilent HPLC equipment, NovaPak C18 (4 μm, 3.9 × 150 mm) column (Waters), flow rate 1 mL/min, 8.5% MeOH in water when using cellobiosyl fluoride donor (**1**) and 12% MeOH in water for 6'-azido-cellobiosyl fluoride donor (**2**), UV detector at 300 nm). Initial rates ($v_o$) were obtained from the linear progress curves of product formation (normalized area *vs.* time) at initial reaction times (< 10% conversion) and expressed as $v_o$/[E].

For the determination of specific activities, reactions were done at 1 mM donor (**1** or **2**), 7 mM acceptor (**3** or **4**), and 0.83 μM enzyme. For determination of kinetic parameters, reactions were done at constant acceptor (**3**) concentration of 20 mM, varying donor (**1** or **2**) concentrations (0.025 to 2 mM), and 0.1 or 0.5 μM enzyme for reactions with donor **1** or **2**, respectively. Kinetic parameters $k_{cat}$, $K_M$, and $k_{cat}/K_M$ were calculated by nonlinear fitting of $v_0$ *vs.* donor concentration data to the Michaelis Menten model or substrate inhibition model.

**2.5. Glycosynthase-catalyzed polymerizations.** Reaction mixtures (0.5 mL) consisting of 30 - 50 mg donor substrate (**1** or **2**), phosphate buffer (100 mM, pH = 7.8) and *Hi*Cel7B E197A enzyme (4.5 - 56 μM, concentrations as indicated) were incubated at 40ºC and 250 rpm. A precipitate was formed during the reaction and no donor was observed by TLC (ACN/$H_2O$ 8:2) after 24 hours. The precipitated product was isolated by centrifugation at 13,000 rpm for 3 minutes, and the precipitate was thoroughly washed with cold water. Finally, the product was

freeze-dried to yield water-insoluble polymers as white powders. Supernatants were also lyophilized to recover soluble oligomers. The products were analyzed by HPSEC to determine the polymer parameters ($M_w$, $M_n$, $M_p$, DP, PDI).

*Synthetic cellulose* (**5**).- Compound **1** (30 mg, 0.087 mmol) was dissolved in phosphate buffer (0.1M, pH 7.8, 0.5 mL) and incubated with *Hi*Cel7B E197A enzyme (56 μM) at 40 °C. After 24 h, the isolated water-insoluble polymer was freeze-dried obtaining a white powder (29 mg, 92% yield).

*Alternating 6-azido-6-deoxycellulose* (**6**).- Compound **2** (30 mg, 0.081 mmol) was dissolved in phosphate buffer (0.1M, pH 7.8, 0.5 mL) and incubated with *Hi*Cel7B E197A enzyme (56 μM) at 40 °C. After 24 h the isolated water-insoluble polymer was freeze-dried obtaining a white powder (26 mg, 82% yield). FTIR: 3431 $cm^{-1}$ (OH), 2112 $cm^{-1}$ ($N_3$).

*Acetylated alternating 6-azido-6-deoxycellulose* (**7**).- Polymer **6** (26 mg) was mixed with pyridine (118 μL, 20 equiv), acetic anhydride (138 μL, 20 equiv) and DMAP (1 mg). The reaction solution was stirred for 24 h at 80 ºC. The product was isolated by pouring the reaction mixture into 2 mL deionized water, followed by filtration. The precipitate was dried overnight in a vacuum oven at 50 °C. DS = 2.1 determined by $^1$H NMR spectroscopy. FTIR: 3439 $cm^{-1}$ (OH), 2109 $cm^{-1}$ ($N_3$), 1757 $cm^{-1}$ (C=O). 1H NMR (DMSO, 400 MHz) δ 5.11-5.06 (m, 2H, H-3, H-3'), 4.73 (d, 1H, H-1'), 4.54 (d, 1H, H-1), 4.56-4.52 (m, 2H, H-2, H-2'), 4.24 (d, 1H, J6a,6b = 11.1 Hz, H-6a), 4.05-3.99 (m, 1H, H-6b), 3.83-3.74 (m, 2H, H-5, H-5'), 3.74-3.70 (m, 1H, H-4'), 3.65-3.60 (m, 1H, H-4), 3.54 (d, 1H, J6'a,6'b = 12 Hz, H-6'a), 3.49-3.39 (m, 1H, H-6'b), 2.07-1.86 (m, 15H, CH3).

13C NMR (DMSO, 100 MHz) δ 170.59 – 169.25 (C=O), 99.62 (C-1'), 99.27 (C-1), 76.84 (C-4), 75.73 (C-4'), 73.29 (C-5), 72.64 (C-3, C-3'), 72.17 (C-5'), 71.96 (C-2, C-2'), 62.61 (C-6), 50.37

(C-6'), 20.95 – 20.53 (CH3). HSQC, COSY, and TOCSY experiments are given in *Supporting Information*.

**2.6. Functionalization of polysaccharides 6 and 7.**

*Conjugated alternating 6-azido-6-deoxycellulose with Alexa Fluor® 488 alkyne* (**8**).- A solution of Alexa Fluor® 488 alkyne (Life Technologies) (2.0 mg, 2.56 μmol) in water (1 mL) and freshly prepared solution of sodium L-ascorbate (1M, 20 mL, Sigma-Aldrich) were added to a solution of polymer **6** (1.0 mg, 2.6 μmol/anhydrous cellobiose unit) in DMSO (1 mL). Then, 7.5% aqueous cupric sulfate (24 μL, 7.21 μmol) was added to the reaction mixture, which was stirred overnight at 24ºC. The unreacted Alexa Fluor reagent was removed by dialysis (Pur-A-Lyzer$^{TM}$ Mega 1000 Dialysis Kit, Sigma-Aldrich) against water. The functionalized polymer **8** (1.0 mg, 1.07 μmol) was obtained after lyophilization in 41 % yield. FTIR: 3431 $cm^{-1}$ (OH, $NH_2$), 1763 $cm^{-1}$ (C=O).

*Acetylated alternating 6-aminocellulose* (**9**).- Polymer **7** (20 mg) was dissolved in 2 mL of THF, followed by the dropwise addition of 13 μL of deionized water. Then, $Ph_3P$ (2 eq. per acetylated glucosyl unit) was added, and the solution allowed to react at room temperature for 12h in a sealed flask. The solution was transferred to a 3500 MWCO dialysis tubing that was placed in a beaker containing 600 mL ethanol. As the dialysis proceeded, a precipitate was slowly formed. After one day of dialysis, the contents inside the tubing were removed, and the precipitate was isolated by filtration. The precipitate (polymer **9**) was dried in a vacuum oven at 50 °C. FTIR: 3431 $cm^{-1}$ (OH, $NH_2$), 1763 $cm^{-1}$ (C=O). $^{13}C$ NMR (TDF, 100 MHz) δ: 171.62 – 170.35 (C=O), 100.26, 100.25 (C-1, C-1'), 64.15 (C-6), 43.25 (C-6'), 21.74 – 20.37 ($CH_3$).

## 3. RESULTS AND DISCUSSION

### 3.1. Kinetics of *Hi*Cel7B E197A glycosynthase with azido substrates.

To evaluate the ability of the *Hi*Cel7B E197A glycosynthase to accept azido-substituted substrates, different donor-acceptor pairs were kinetically analyzed (Figure 1). With CelαF (**1**) as donor substrate, specific activities with acceptors GlcpNP (**3**) and $6N_3$-GlcpNP (**4**) were essentially the same, indicating that the azido group can be accommodated in subsite +1 of the enzyme's binding cleft. With $6'N_3$-CelαF (**2**) donor and GlcpNP (**3**) acceptor substrates, activity was just 2-fold lower than the reference **1**+**3** reaction, indicating that subsite -2 also tolerates an azido substitution. Kinetics with different donor concentrations at saturating GlcpNP acceptor concentration are plotted in Figure 2, and kinetic parameters given in Table 1. CelαF (**1**) follows saturation kinetics with $k_{cat}$ of 17 $min^{-1}$, whereas $6'N_3$-CelαF (**2**) has the same $k_{cat}$ but shows substrate inhibition at high donor concentration. Therefore, subsites -2 and +1 accept the 6-azido substitution, a requirement to evaluate donor self-condensation to yield alternating 6-azido polymers.

### 3.2. Glycosynthase-catalyzed polymerizations.

Glycosynthase-catalyzed polymerizations were performed at high donor concentration, pH 7.8 and 40 ºC (Figure 3, Table 2). Polymeric products precipitated as a white powder after 24 h reactions. TLC analysis showed that the polymerizations proceeded quantitatively and neither fluoride donors nor their hydrolysis products were detected. The water-insoluble polymers were isolated in high yield (> 90%) after centrifugation, water washes, and lyophilization, and then structurally characterized.

Analysis of the degree of polymerization and molecular mass distribution of the insoluble polysaccharides by HPSEC showed that cellulose (**5**) (Table 2) presents a monomodal profile

with a $M_w$ value of 4.3 kDa which corresponds to $(4Glc\beta4Glc\beta)_n$, n = 13, and the alternating 6-azido-6-deoxycellulose (**6**) has a $M_w$ value of 5.8 kDa ($(4[6N_3Glc]\beta4Glc\beta)_n$, n = 17) (Figure 4). Similar results were obtained after acetylation of the 6-azido polysaccharide with $Ac_2O$ and pyridine (polymer **7**). MALDI-TOF MS shows a series of peaks up to 5.5 kDa corresponding to the low molecular mass oligomers, peaks separated by Δm/z of 324 and 349 for polymer **5** and **6**, respectively, which correspond to the theoretical mass of the repeating (Glcβ4Glc) unit for polymer **5** and the ($6N_3Glc\beta4Glc$) unit for polymer **6**, and Δm/z of 560 for the acetylated polymer **7** Figure S1, *Supporting Information*). Analysis of the acetylated polymer **7** by $^1H$ NMR confirmed the presence of the azide after acetylation with a degree of acetylation (DA) of 2.2 (maximum theoretical DA = 2.56) (Figure 5A). $^{13}C$ NMR (Figure 5B) showed the formation of the new β-1,4 glycosidic linkage ($\delta_C$ 76 ppm) and disappearance of the signal at $\delta_C$ 69 ppm assignable to the unsubstituted C4 of the non-reducing end of the cellobiosyl donor. The signal at $\delta_C$ 62 ppm corresponds to the acetylated C6 positions while the signal at $\delta_C$ 51 ppm was assigned to the azidated C-6' positions consistent with the presence of azide groups on alternating glucosyl units. 2D NMR experiments (COSY, TOCSY, and HSQC) allowed the assignment of the two different spin systems for 6-azido-glucosyl and glucosyl units (Figure S2, *Supporting Information*). The FTIR spectrum of the alternating 6-azido-6-deoxycellulose (**6**) (Figure 6A) shows an intense stretching band of the azido group at 2112 $cm^{-1}$, and the absorption band of the sugar OH groups at 3431 $cm^{-1}$, whereas the acetylated polymer **7** (Figure 6B) retains the azide band at 2112 $cm^{-1}$, a new band at 1753 $cm^{-1}$ for the C=O of the acetyl groups, but still contains a small absorption band at 3431 $cm^{-1}$ due to free hydroxyl groups resulting from the incomplete acetylation reaction.

Polysaccharide morphology was analyzed by SEM revealing that the 6-azido-6-deoxycellulose (**6**) formed porous spherulites like the non-modified cellulose (**5**), although they

seem to be larger, with 5-7 μm versus 2-4 μm average diameter, respectively (Figure 7). These artificial polysaccharides notably differ from natural cellulose such as MCC (microcrystalline cellulose), whose particles of 10-50 μm are composed of aggregate bundles of multi-sized cellulose microfibrils.[30] The spherulite morphology with variable porosity seems to be common for artificial polysaccharides produced by *in vitro* enzymatic syntheses, as previously seen in the first artificial cellulose obtained by transglycosylation with *T. viridiae* cellulase[31] or alternating β-1,3/1,4 glucan obtained by a glycosynthase-catalyzed polymerization.[22,26]

Substituted celluloses at C6 have been achieved by chemical modification of MCC, where $PPh_3$/NBS bromination of cellulose is completely selective at C6,[32] but further $S_N2$ displacement of the bromide is limited due to the poor solubility of the 6-bromo-6-deoxycellulose. Edgar and coworkers[33] showed that esterification of brominated cellulose broadens the potential for further modifications. In this way, 6-azido-6-deoxy-2,3-di-*O*-acyl-cellulose was obtained from MCC resulting in a polysaccharide of DP 50 and DS (azide) of 0.92.[34] The enzymatic protocol here reported provides access to novel structures, where azide substitutions are regularly distributed at C6 of alternating glucosyl units on a cellulose chain with free hydroxyl groups, with DP of about 40 and DS (azide) of 0.5. It is notable that nature employs this very architectural motif of amine substitution on alternating monosaccharides in all of the active glycosaminoglycans. By extension, the same strategy with homologous azido-cellooligosaccharide donors (*i.e.* 6''-azido-cellotriosyl and 6'''-azido-cellotetraosyl fluoride donors) should afford other regularly functionalized celluloses with different substitution patterns.

### 3.3. Functionalization of polysaccharides 6 and 7

The azido cellulose **6** and the acetylated derivative **7** are reactive intermediates for further modifications (Figure 3). The accessibility and reactivity of the 6-azido groups was first tested

by "click" chemistry.[35] The alternating 6-azido-6-deoxycellulose (**6**) was subjected to a copper-catalyzed azide/alkyne cycloaddition (Huisgen reaction)[36] with the alkyne-functionalized Alexa Fluor dye. After isolation of the water soluble product (**8**) by dialysis and lyophilization, FTIR spectroscopy (Figure 6C) showed the appearance of a new absorption band at 1621 $cm^{-1}$ corresponding to the carbonyl group of the Alexa Fluor amide function, and the disappearance of the stretching band of the azide groups at 2112 $cm^{-1}$, although some residual band was apparent due to incomplete reaction. The dye-labeled polymer **8** was observed as a green fluorescent solid by fluorescence microscopy. Next, reduction of the azido groups to amines was studied. Staudinger reduction has been recently applied to acylated 6-azido-6-deoxycelluloses with DS (azido) of 0.9-0.95 to selectively reduce the azido groups to amines in the presence of easily reducible ester groups.[34] The same protocol was here applied to reduce the acetylated alternating 6-azido polymer **7** with the aim of obtaining a novel 6-amino-6-deoxy cellulose derivative with a regular pattern of one amino group every two glucosyl units in the polysaccharide chain. The reaction was performed by reacting the acetylated polymer **7** dissolved in THF with $PPh_3$ to form a phosphazide which evolves to an iminophosphorane. It is then hydrolyzed by water to produce the polymer **9** and triphenylphosphine oxide. Unfortunately, this new polysaccharide has poor solubility in water and common organic solvents, as did the reported acylated 6-aminocelluloses obtained from MCC.[34] Due to the solubility issues, the reaction was performed in deuterated THF and the $^{13}C$ NMR spectrum of the crude reaction mixture was directly recorded (Figure 5C). The spectrum showed the formation of the amine ($\delta_C$ 43 ppm assigned to C6'-$NH_2$). FTIR analysis confirms the presence of the free amine (broad N−H stretching band at 3390 $cm^{-1}$ and the N−H bend at 1570 $cm^{-1}$) but shows a residual signal of the azide at 2112 $cm^{-1}$, while the carbonyl signal of the acetyl groups remains at 1753 $cm^{-1}$ (Figure 6D).

## 4. CONCLUSIONS

As opposed to most chemically modified celluloses where the substitution pattern is intrinsically random, the glycosynthase-catalyzed polymerization of properly modified glycosyl donors gives access to novel functionalized celluloses with defined and regular substitution patterns. We have shown that a 6'-azido-cellobiosyl donor is readily polymerized to give alternating 6-azido-6-deoxycellulose with a regular pattern of one azido group every two glucosyl units, which can be further reacted to install different functional groups or substituents. The strategy can be extended to other functionalized donors, *i.e.* tri- or tetrasaccharide donors, to afford other regularly functionalized glycopolymers with different substitution patterns. Likewise, other functional groups may be introduced in the glycosyl donor (i.e. –COOH, -$CONH_2$, -SH) provided that the donor is accepted as a substrate by the glycosynthase, which otherwise can be engineered for the desired specificity by protein engineering and directed evolution approaches. This strategy provides access to novel polysaccharide structures with promising applications.

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Notes

The authors declare no competing financial interest

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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## REFERENCES


(1) Varki A., Cummings R.D., Esko J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M. E. *Essentials of Glycobiology*; Second.; Cold Spring Harbor Press: Cold Spring Harbor (NY), 2009.

(2) Dash, M.; Chiellini, F.; Ottenbrite, R. M.; Chiellini, E. *Prog. Polym. Sci.* **2011**, *36*, 981–1014.

(3) Kumar, M. N. V. R.; Muzzarelli, R. a a; Muzzarelli, C.; Sashiwa, H.; Domb, a J. *Chem. Rev.* **2004**, *104*, 6017–6084.

(4) Patel, M. P.; Patel, R. R.; Patel, J. K. *J Pharm Pharm. Sci* **2010**, *13*, 536–557.

(5) Duceppe, N.; Tabrizian, M. *Expert. Opin. Drug Deliv.* **2010**, *7*, 1191–1207.

(6) Shukla, S. K.; Mishra, A. K.; Arotiba, O. a; Mamba, B. B. *Int. J. Biol. Macromol.* **2013**, *59*, 46–58.

(7) Jayakumar, R.; Chennazhi, K. P.; Muzzarelli, R. a. a.; Tamura, H.; Nair, S. V.; Selvamurugan, N. *Carbohydr. Polym.* **2010**, *79*, 1–8.

(8) Cumpstey, I. *ISRN Org. Chem.* **2013**, *2013*, 1–27.

(9) Desai, U. R.; Petitou, M.; Bjo, I.; Olson, S. T. *J. Biol. Chem.* **1998**, *273*, 7478–7487.

(10) Gama, C. I.; Tully, S. E.; Sotogaku, N.; Clark, P. M.; Rawat, M.; Vaidehi, N.; Goddard, W. a; Nishi, A.; Hsieh-Wilson, L. C. *Nat. Chem. Biol.* **2006**, *2*, 467–473.

(11) Rawat, M.; Gama, C. I.; Matson, J. B.; Hsieh-wilson, L. C. *J. Am. Chem. Soc.* **2008**, *130*, 2959–2961.

(12) Kobayashi, S.; Kashiwa, K.; Kawasaki, T.; Shoda, S. *J. Am. Chem. Soc.* **1991**, *113*, 3079–3084.

(13) Okamoto, E.; Kiyosada, T.; Shoda, S.-I.; Kobayashi, S. *Cellulose* **1997**, *4*, 161–172.

(14) Faijes, M.; Planas, A. *Carbohydr. Res.* **2007**, *342*, 1581–1594.

(15) Kadokawa, J.; Kobayashi, S. *Curr. Opin. Chem. Biol.* **2010**, *14*, 145–153.

(16) Mackenzie, L. F.; Wang, Q.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **1998**, *120*, 5583–5584.

(17) Malet, C.; Planas, A. *FEBS Lett.* **1998**, *440*, 208–212.

(18) Shaikh, F. A.; Withers, S. G. *Biochem. Cell Biol.* **2008**, *86*, 169–177.

(19) Wang, L.-X.; Huang, W. *Curr. Opin. Chem. Biol.* **2009**, *13*, 592–600.

(20) Cobucci-Ponzano, B.; Moracci, M. *Nat. Prod. Rep.* **2012**, *29*, 697–709.

(21) Armstrong, Z.; Withers, S. G. *Biopolymers* **2013**, *99*, 666–674.

(22) Faijes, M.; Imai, T.; Bulone, V.; Planas, A. *Biochem. J* **2004**, *380*, 635–641.

(23) Fort, S.; Boyer, V.; Greffe, L.; Davies, G. J.; Moroz, O.; Christiansen, L.; Schülein, M.; Cottaz, S.; Driguez, H. *J. Am. Chem. Soc.* **2000**, *122*, 5429–5437.

(24) Hrmova, M.; Imai, T.; Rutten, S. J.; Fairweather, J. K.; Pelosi, L.; Bulone, V.; Driguez, H.; Fincher, G. B. *J. Biol. Chem.* **2002**, *277*, 30102–30111.

(25) Sugimura, M.; Nishimoto, M.; Kitaoka, M. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1210–1217.

(26) Pérez, X.; Faijes, M.; Planas, A. *Biomacromolecules* **2011**, *12*, 494–501.

(27) Gullfot, F.; Ibatullin, F. M.; Sundqvist, G.; Davies, G. J.; Brumer, H. *Biomacromolecules* **2009**, *10*, 1782–1788.

(28) Spadiut, O.; Ibatullin, F. M.; Peart, J.; Gullfot, F.; Martinez-Fleites, C.; Ruda, M.; Xu, C.; Sundqvist, G.; Davies, G. J.; Brumer, H. *J. Am. Chem. Soc.* **2011**, *133*, 10892–10900.

(29) Carraher, C. E. J. *Seymour/Carraher's Polymer Chemistry*; 2007.

(30) Moon, R. J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. *Chem. Soc. Rev.* **2011**, *40*, 3941–3994.

(31) Kobayashi, S.; Hobson, L. J.; Sakamoto, J.; Kimura, S.; Sugiyama, J.; Imai, T.; Itoh, T. *Biomacromolecules* **2000**, *1*, 168–173.

(32) Furuhata, K.; Koganei, K.; Chang, H.; Aoki, N. *Carbohydr. Res.* **1992**, *230*, 165–177.

(33) Fox, S. C.; Edgar, K. J. *Cellulose* **2011**, *18*, 1305–1314.

(34) Fox, S. C.; Edgar, K. J. *Biomacromolecules* **2012**, *13*, 992–1001.

(35) Liebert, T.; Hänsch, C.; Heinze, T. *Macromol. Rapid Commun.* **2006**, *27*, 208–213.

(36) Meldal, M.; Tornøe, C. W. *Chem. Rev.* **2008**, *108*, 2952–3015.

## TABLES

**Table 1.** Kinetic parameters of *Hi*Cel7B E197A glycosynthase for donors CelαF (**1**) and 6'-$N_3$-CelαF (**2**) at saturating (20 mM) GlcpNP (**3**) acceptor in 100 mM phosphate buffer pH 7.8, 35ºC.

| Donor | $k_{cat}$ ($min^{-1}$) | $K_M$ (mM) | $K_I$ (mM) | $k_{cat}/K_M$ ($mM^{-1}min^{-1}$) |
|---|---|---|---|---|
| **1** | 17.1 ± 0.9 | 0.10 ± 0.03 | -- | 173 |
| **2** | 16.4 ± 2.4 | 0.15 ± 0.04 | 1.5 ± 0.5 | 113 |

**Table 2.** Enzymatic polymerizations by the *Hi*Cel7B E197A glycosynthase. Polysaccharide parameters determined by HPSEC.

| P | donor | [donor] (mM) | yield[a] (%) | $M_w$[b] (kDa) | n (DP)[c] | $M_n$[d] (kDa) | PDI[e] | $M_p$[f] (kDa) |
|---|---|---|---|---|---|---|---|---|
| **5** | **1** | 174 | 92 | 4.3 | 13 (26) | 2.9 | 1.5 | 3.7 |
| **6** | **2** | 162 | 90 | 5.8 | 17 (34) | 4.9 | 1.2 | 4.7 |
| **7**[g] | **2** | 162 | 88 | 9.0 | 16 (32) | 7 | 1.2 | 8 |

Conditions: [enzyme]= 56 µM, 100 mM phosphate buffer, pH 7.8, 40ºC. [a] Average yield from 4 repetitions; [b] $M_w$, weight average molecular mass; [c] n, number of condensations, (DP) degree of polymerization for $M_w$. [d] $M_n$, number average molecular mass. [e] PDI, polydispersity index; [f] $M_p$, molecular mass of the peak maximum in the chromatograms. [g] Glycosynthase reaction followed by acetylation, parameters of the acetylated polymer.

## FIGURE LEGENDS

**Figure 1.** Donor-acceptor condensations catalyzed by the *Hi*Cel7B E197A glycosynthase. [1] Specific activities (s.a.) at 1 mM donor (**1** or **2**), 7 mM acceptor (**3** or **4**), 0.83 μM enzyme in 100 mM phosphate buffer pH 7.8, 35ºC.

**Figure 2.** Kinetics of *Hi*Cel7B E197A glycosynthase for donors CelαF (**1**) and 6'-$N_3$-CelαF (**2**) at saturating (20 mM) GlcpNP (**3**) acceptor in 100 mM phosphate buffer pH 7.8, 35ºC.

**Figure 3.** Glycosynthase-catalyzed polymerizations of cellobiosyl donors **1** and **2**, and further derivatization of the alternating 6-azido-6-deoxycellulose polymers.

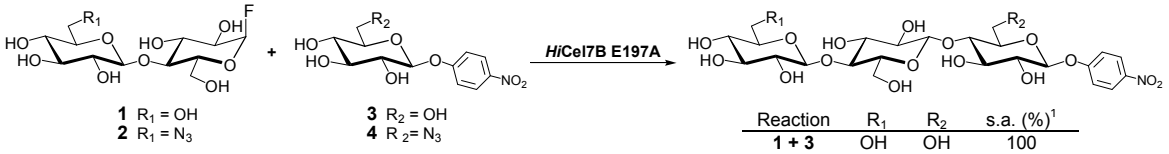
**Figure 4.** Size exclusion chromatography (HPSEC) of artificial polysaccharides cellulose (**5**) and 6-azido 6-deoxycellulose (**6**).

**Figure 5.** NMR spectra of acetylated alternating 6-azido-6-deoxycellulose (**7**) and acetylated alternating 6-amino-6-deoxycellulose (**9**). (A) $^{1}$H NMR ($CDCl_3$) of **7**. (B) $^{13}$C NMR ($CDCl_3$) of **7**. (C) $^{13}$C NMR (TDF) of **9**. Monodimensional and 2D spectra (COSY, TOCSY, and HSQC) of **7** in DMSO are given in Figure S2, *Supporting Information*, and assignments of proton and carbon chemical shifts are detailed in the *Experimental Section*.

**Figure 6.** FTIR spectra of alternating 6-azido 6-deoxycellulose and their derivatives. A) 6-Azido 6-deoxycellulose (**6**). B) Acetylated 6-azido 6-deoxycellulose (**7**). C) Conjugated 6-azido 6-deoxycellulose with Alexa Fluor (**8**). D) Acetylated 6-amino 6-deoxycellulose (**9**).

**Figure 7.** SEM of the polysaccharides **5** (left) and **6** (right).

FIGURE 1




| Reaction | $R_1$ | $R_2$ | s.a. (%)[1] |
|---|---|---|---|
| **1 + 3** | OH | OH | 100 |
| **1 + 4** | OH | $N_3$ | 102 |
| **2 + 3** | $N_3$ | OH | 48 |

FIGURE 2

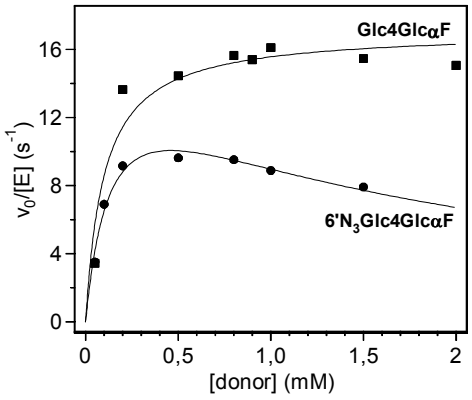



FIGURE 3

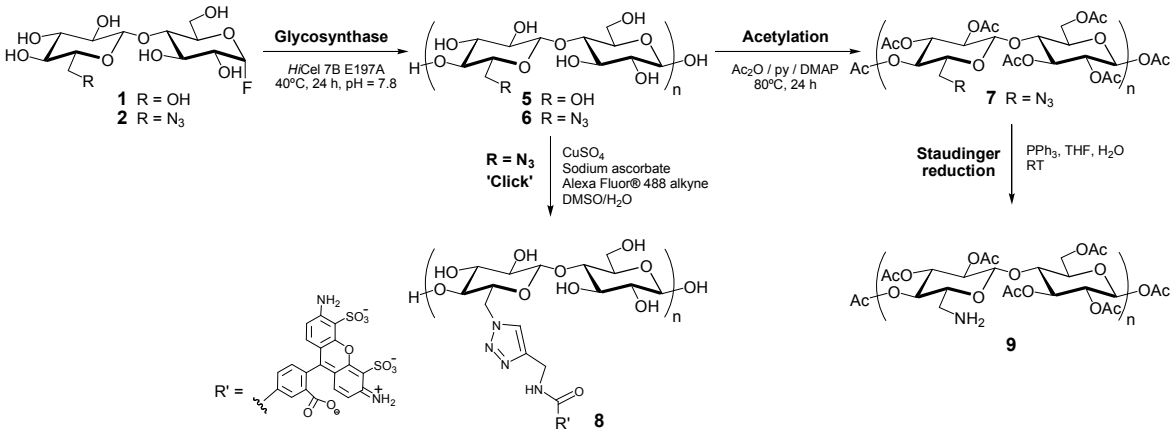


FIGURE 4

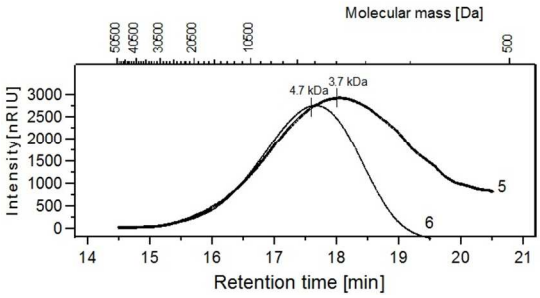



FIGURE 5

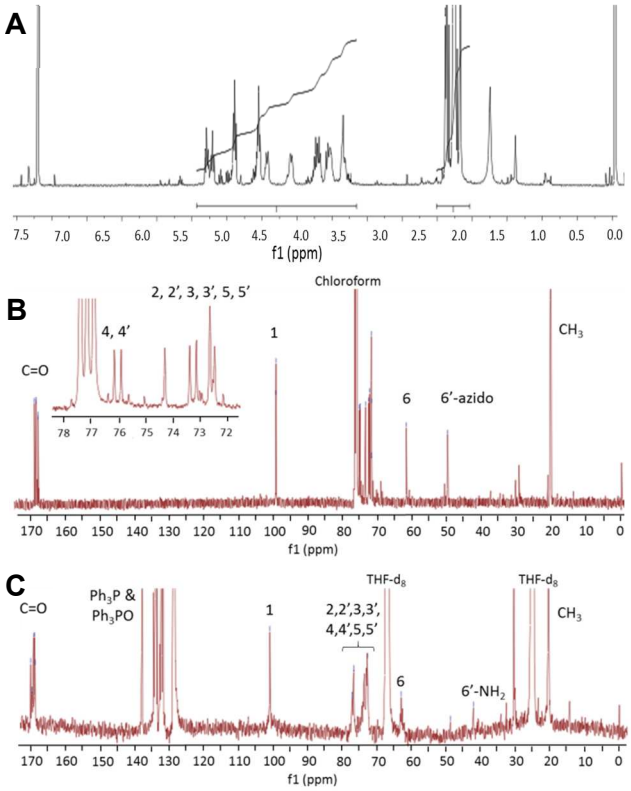


FIGURE 6

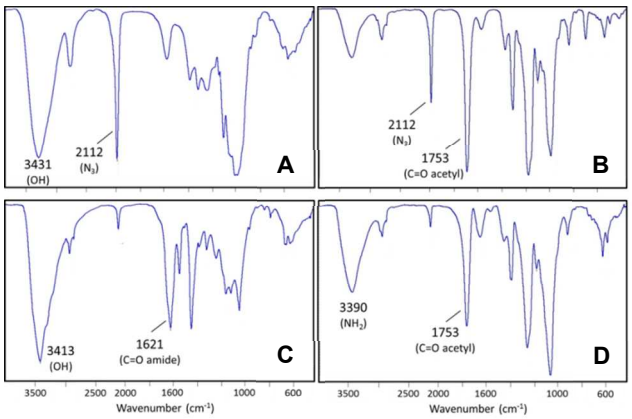
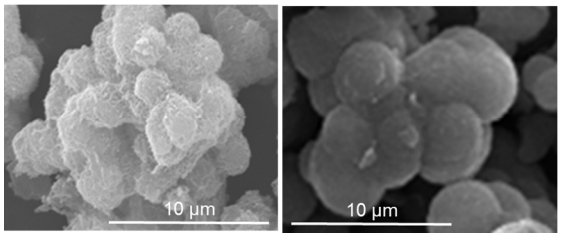



FIGURE 7



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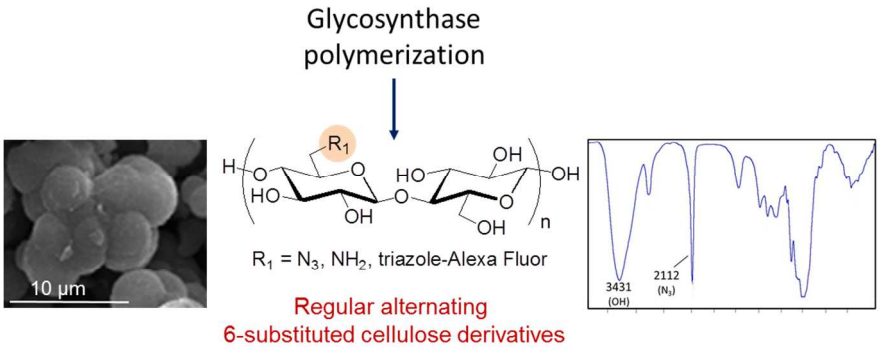
Glycosynthase
polymerization
10 μm
R1
H
O
HO
OH
HO
OH
OH
OH
n
R1 = N3, NH2, triazole-Alexa Fluor
Regular alternating
6-substituted cellulose derivatives
3431
(OH)
2112
(N3)