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Review

In vitro synthesis of artificial polysaccharides by glycosidases and glycosynthases

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Abstract—Artificial polysaccharides produced by in vitro enzymatic synthesis are new biomaterials with defined structures that either mimic natural polysaccharides or have unnatural structures and functionalities. This review summarizes recent developments in the in vitro polysaccharide synthesis by *endo*-glycosidases, grouped in two major strategies: (a) native retaining *endo*-glycosidases under kinetically controlled conditions (transglycosylation with activated glycosyl donors), and (b) glycosynthases, engineered glycosidases devoid of hydrolase activity but with high transglycosylation activity. Polysaccharides are obtained by enzymatic polymerization of simple glycosyl donors by repetitive condensation. This approach not only provides a powerful methodology to produce polysaccharides with defined structures and morphologies as novel biomaterials, but is also a valuable tool to analyze the mechanisms of polymerization and packing to acquire high-order molecular assemblies.

Keywords: Glycosidases; Glycosynthases; Polysaccharides; Enzymatic synthesis; Biomaterials

Contents

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1. Introduction

Oligosaccharides and polysaccharides are key biomolecules in essentially all living organisms. They have multiple functions including serving as structural components of cell walls, energy storage, cell recognition, regulation of signaling, cell differentiation, cell proliferation, and immune responses.[1–4] The biosynthesis of naturally occurring oligo- and polysaccharides is a complex process that involves formation of glycosidic bonds between their constituent monosaccharide units and side chain modifications to produce specific functional group derivatizations. Glycosyltransferases, transglycosidases, and phosphorylases are the enzymes responsible for glycosyl bond formation, whereas accessory enzymes such as estearases, epimerases, and sulfotransferases modify the carbohydrate structures to produce the functional biomolecules.[4–7]

Polysaccharides are highly diverse in structure and biological functions. They are essential for many fields of research, for example, for biochemical studies in glycobiology, as potential drugs directed to enzymes or receptors involved in their function and metabolism, and as advanced materials due to their biocompatibility, structure-forming capacity, and environmentally benign properties.[8–13] The development of efficient synthetic methodologies for their preparation has therefore been in high demand. Methods for both chemical and enzymatic syntheses have experienced notable advances in the last decade with the aim of producing either polysaccharides resembling the natural products or novel polysaccharide mimetics for biomedical applications and for biomaterials development.

Chemical synthesis has evolved greatly because improved glycosyl donors and advanced synthetic methodologies have been developed (recent reviews[14–18]). However, perfect control of the regio- and stereochemistry of glycosylation is still a difficult problem, and the synthesis of complex oligo- and polysaccharides is often limited to the milligram scale and difficult to scale-up. Enzymatic synthesis is an alternative that overcomes these limitations because of their high catalytic activity, lack of undesirable side-reactions, mild reaction conditions, and high regio- and stereoselectivity.[19–22]

In nature, glycosyltransferases (GT) are responsible for glycoside bond formation. They catalyze the transfer of a monosaccharide from a sugar nucleotide donor (in Leloir GTs) to an acceptor, acting processively in homopolysaccharide biosynthesis or in combination with other GTs to produce heteropolysaccharides. Although the use of glycosyltransferases is still limited as a general tool for in vitro carbohydrate synthesis, a number of recombinant enzymes are available and different methodologies are being developed for large-scale synthesis. Good examples are the synthesis of hyaluran using the hyaluran synthase from *Streptococcus equisimilis* via an enzymatic process with coupled regeneration of the sugar nucleotide[23] or the use of engineered *Bacillus subtilis* cells expressing the hyaluran synthase enzyme and producing hyaluronic acid in the 1 MDa range.[24]

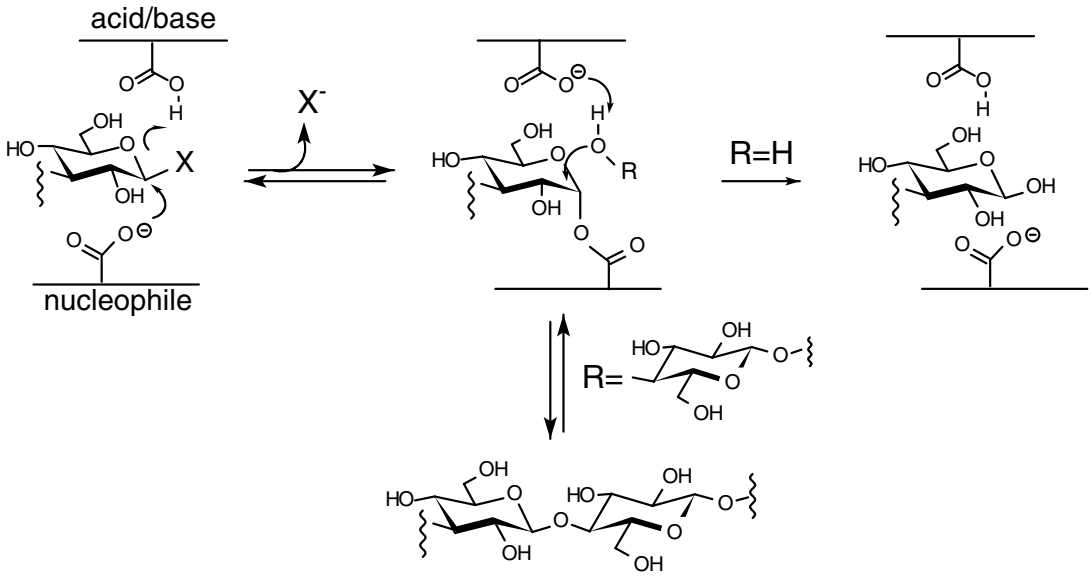
Glycosidases (GH) are degrading enzymes that catalyze the hydrolysis of glycosidic bonds, but their normal hydrolytic reaction can be reversed under appropriate conditions.[25] Therefore, glycosidases have been extensively studied as biocatalysts for oligo- and polysaccharide synthesis. They are stable enzymes, easy to produce, and a large number of enzymes from different organisms and with different specificities are available. In addition, the glycosyl donors required are cheap compounds and easy to obtain in a multigram scale.

Retaining glycosidases follow a double-displacement reaction mechanism via the formation and hydrolysis of a glycosyl-enzyme intermediate.[26–29] The canonical mechanism involves two steps with general acid–base catalysis: in the first step (*glycosylation*) the amino acid residue acting as a general acid protonates the glycosidic oxygen, while the deprotonated carboxylate functioning as a nucleophile attacks the anomeric center with concomitant C–O breaking of the scissile glycosidic bond leading to a covalent glycosyl-enzyme intermediate. The second *deglycosylation* step involves the attack by a molecule of water assisted by the conjugate base of the general acid residue, which renders the free sugar with overall retention of configuration, and the enzyme returns to its initial protonation state (Scheme 1). Under conditions that favor reversal of their normal hydrolytic reaction, retaining glycosidases have been extensively used as catalysts in oligosaccharide synthesis. This may be achieved either by displacing the equilibrium toward glycosidic bond formation (*thermodynamically controlled condensation*) or by using activated glycosyl donors (*kinetically controlled transglycosylation*).

This review focuses on recent developments on the enzymatic synthesis of polysaccharides by retaining *endo*-glycosidases. This refers to enzymatic polymerization of simple glycosyl donors by repetitive condensation (transglycosylation) to produce synthetic polysaccharides resembling either the natural products or mimetics with unnatural structures or functionalities. In vitro enzymatic synthesis not only provides a powerful methodology to produce polysaccharide derivatives with defined structures, but is also a valuable tool to analyze the mechanisms of polymerization and packing to acquire high-order molecular assemblies.

Two general strategies are currently under development:

(a) The use of wild-type glycosidases under kinetically controlled conditions (transglycosylation). The competing hydrolase activity is reduced by modifying the reaction conditions, and the insolubility of



Scheme 1. The mechanism of retaining glycosidases. Activated donors (X = F, $PhNO_2$, etc.) and sugar donors (X = glycosyl) are able to transglycosylate a sugar acceptor under kinetically controlled conditions.

the formed polysaccharide products often displaces the reaction toward transglycosylation. The alternative application of thermodynamically controlled conditions (reversal of hydrolysis or condensation) has low efficiency and it is limited to the preparation of simple disaccharides or alkyl glycosides,[30,31] and such conditions have not been explored for enzymatic polymerization.

(b) The use of glycosynthases, engineered glycosidases devoid of hydrolase activity but with high transglycosylation activity. This is an emerging technology that has just started to be explored for the production of artificial polysaccharides by self-transglycosylation of activated glycosyl donors. Because the active site has been engineered to abolish hydrolase activity, the products formed are stable and, in principle, higher molecular weight oligomers and higher product yields can be obtained.

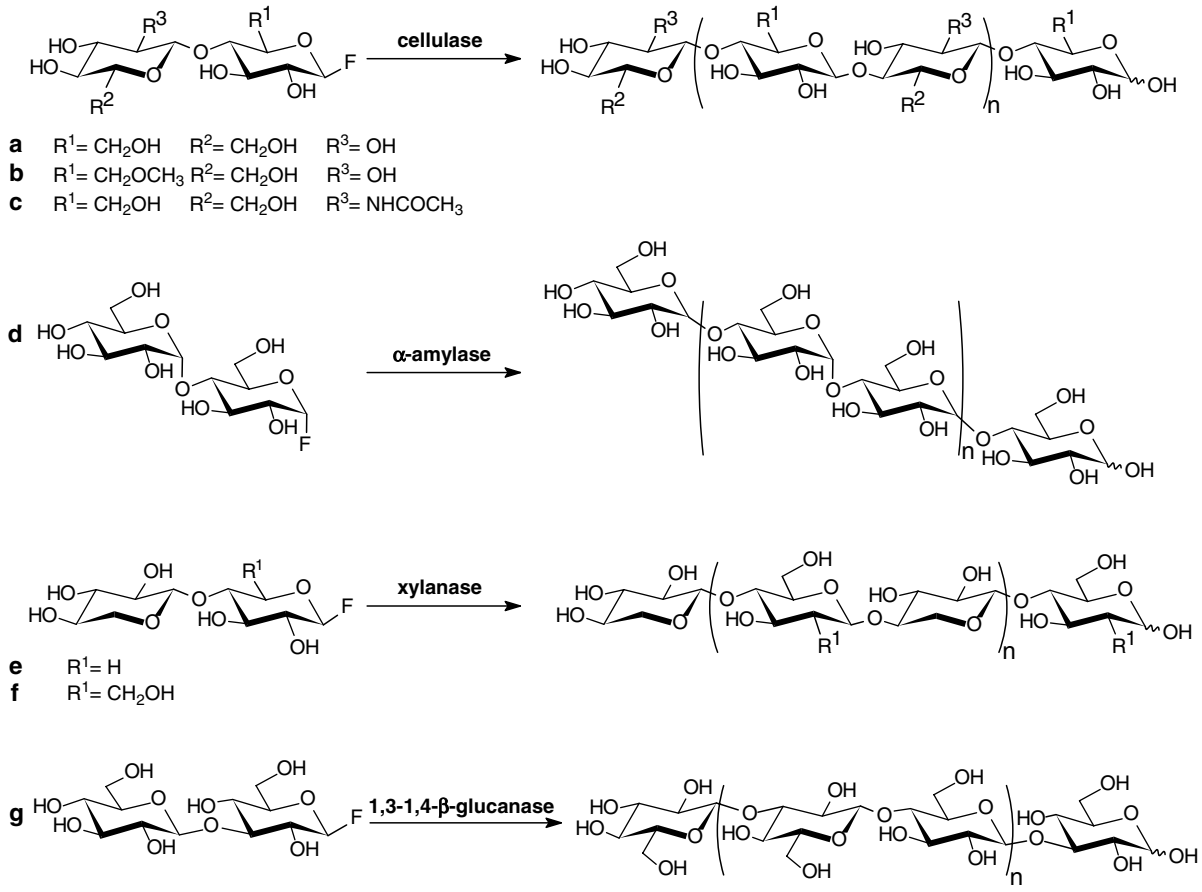
2. Enzymatic polymerization by *endo*-glycosidases. Transglycosylation under kinetic control

Transglycosylation by wild-type enzymes, or mutants near the active site, which are designed or selected to enhance transglycosylation, was introduced in the early 1990s for polysaccharide synthesis. These glycosidase-catalyzed polymerizations can be grouped by the type of glycosyl donor used for polymerization and the enzyme family according to its mechanism. That is, enzymes having a catalytic nucleophile and enzymes acting by substrate-assisted catalysis, because donor activation differs in either case.

2.1. Polymerization by *endo*-glycosidases with activated substrates

In the case of enzymes that act on activated substrates, the catalytic machinery of the enzyme remains intact and efficient transglycosylation reactions are achieved by the use of the activated donors and modification of the reaction conditions, that is, by the use of organic co-solvents, thus combining kinetic and thermodynamic control. Depending on the glycosidase family, two general approaches have been developed.

2.1.1. Retaining glycosidases with an active site catalytic nucleophile. This group comprises most of the retaining glycosidase families and are characterized by the presence of both essential catalytic residues: a general acid/base and the catalytic nucleophile. The enzymes operate by a double-displacement mechanism with formation of a covalent intermediate with the enzyme nucleophile. Common activated glycosyl donors for polymerization are glycosyl fluorides (nitrophenyl glycosides are also used for transglycosylation reactions with *exo*-glycosidases, but are not used for *endo*-glycosidase-catalyzed polymerizations). Classical examples of this approach in the late 1990s used di- or trisaccharide glycosyl fluoride donors as substrates for self-condensation by cellulases, xylanases, amylases, and β-glucanases. These approaches are being currently refined to produce modified polysaccharides by using modified donors in enzyme-catalyzed polymerizations. A summary of the main artificial polysaccharides obtained by this class of enzymes is as follows (Scheme 2).



Scheme 2. Artificial polysaccharides produced by retaining *endo*-glycosidases whose mechanism involves the participation of an enzyme nucleophile. Typically, activated glycosyl donors for self-condensation are glycosyl fluorides.

Artificial cellulose produced by cellulase-catalyzed polycondensation of β-cellobiosyl fluoride in acetonitrile/acetate buffer was the first in vitro polysaccharide synthesized by enzymatic polymerization[32–34] (Scheme 2a). Depending on the cellulase origin and reaction conditions, an artificial cellulose with a degree of polymerization of about 22 glucose units was reached in 54% yield. The high-order molecular assembly was also dependent on the enzyme source and purity. When a crude cellulase was employed, the cellulose II allomorph, a stable crystalline form with anti-parallel-chain packing, was obtained. Interestingly, metastable crystalline cellulose I with parallel-chain packing was obtained with a partially purified cellulase preparation from *Trichoderma viride*. However, with purified EGII from the same organism, a highly crystalline cellulose II was again isolated.[35,36] Because other enzymes and protein components were present in the different extracts, these results indicate that the relative intermolecular direction of growing glucan chains is controlled in the propagating process of enzymatic polymerization.[37]

More recently, a cellulase from *T. viride* was engineered to remove the cellulose-binding domain and the efficiency of cellulose synthesis was improved. Deletion of the cellulose-binding domain did not interfere in the polymerization ability but prevented hydrolysis of cellulose due to the difficult access of the enzyme to the new crystalline material. The turn-over was as high as with the full length enzyme and the crystalline product was stable in the polymerization solution.[38]

An unnatural cellulose derivative has also been prepared by enzymatic polymerization of 6-*O*-methyl-β-cellobiosyl fluoride as substrate of cellulase, giving rise to an alternating 6-O-methylated cellulose derivative[39] (Scheme 2b). Similarly, thiocello-oligosaccharides were synthesized using 4-thio-β-cellobiosyl fluoride as donor in acetonitrile/buffer system by the *Humicola insolens* cellulase Cel7B.[40] This enzyme has also been used for the preparation of a bifunctionalized fluorogenic cellotetraoside as substrate for the screening of cellulases.[41]

A hybrid cellulose–chitin polysaccharide was produced with a cellulase from *T. viride* as well as with a chitinase from *Bacillus* sp. (see later). The first enzyme accepted β-D-Glc*p*NAc-(1→4)-β-D-Glc*p* fluoride as donor and acceptor to produce an insoluble polysaccharide formed mainly of eight glycosidic units in 63% yield[42] (Scheme 2c). Compared to artificial cellulose or artificial chitin, the polymerization degree is lower and does not

present any crystalline structure. The physical and bioactive properties of this molecule and its resemblance to cellulose as a structural polysaccharide, or chitin, as bioactive polysaccharide, are still unknown.

Maltooligosaccharides were prepared by polymerization of α-maltosyl fluoride using an α-amylase in various organic–water mixture of solvents[43,44] (Scheme 2d). Without organic co-solvent, oligosaccharides up to maltopentaose were observed at the early stages of the polymerization, but they were eventually converted to glucose and maltose. The formation of odd-numbered maltooligosaccharides from a disaccharide donor could be due to enzymatic hydrolysis of products during the reaction.

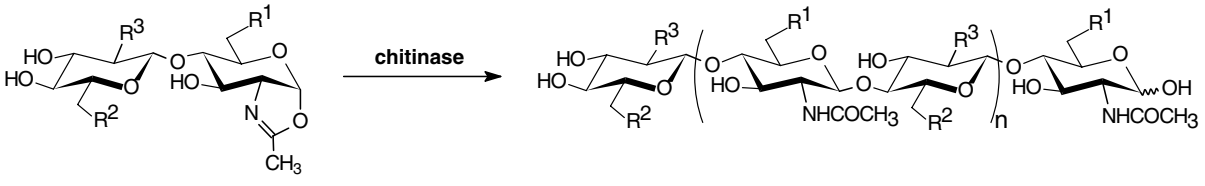
Artificial xylan was also produced by enzymatic polymerization of β-xylobiosyl fluoride in acetonitrile/acetate buffer with an enzyme preparation containing xylanase activity.[45] This synthetic xylan (Scheme 2e) with all β-(1→4) glycosidic bonds differs from the natural xylan (principal component of hemicellulose in plant cell walls) in that the natural polymer also contains other monosaccharides (e.g., methyl-glucuronic acid, L-arabinose) as minor units in the side chain.

A hybrid cellulose–xylan polysaccharide was obtained with a xylanase using a disaccharide donor composed of two different monosaccharyl units (Scheme 2f). Condensation of β-D-Xyl*p*-β-(1→4)-β-D-Glc*p*-fluoride gave rise to a hybrid polymer with a degree of polymerization up to 12 saccharide units with all β-(1→4) glycosidic linkages.[46]

Mixed linked β-(1→3)-(1→4) glucans have also been produced by enzymatic transglycosylation catalyzed by a *Bacillus* β-(1→3)-(1→4)-glucanase. β-Glycosyl fluorides are good glycosyl donors but not *p*-nitrophenyl β-glycosides.[47] Self-condensation of β-laminaribiosyl fluoride or β-glucosyl-β-(1→4)-laminaribiosyl fluoride gave a mixture of oligomers, the reactions proceeding up to the formation of an insoluble polymeric material (Scheme 2g). Gluco-oligomers containing different combinations of β-(1→3) and β-(1→4) glycosidic linkages have been produced in this way.[47–49]

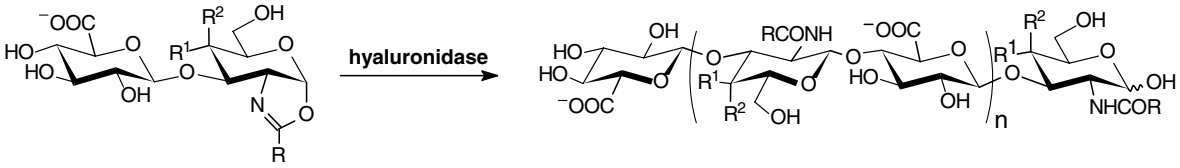
2.1.2. Retaining glycosidases lacking the active site nucleophile (substrate-assisted catalysis). Family 18 (chitinases) and family 56 (hyaluronidases) operate by substrate-assisted catalysis for the hydrolysis of their natural polysaccharide substrates. The 2-acetamido group of the monosaccharide unit in subsite −1 acts as the nucleophile to generate an oxazolinium intermediate. Activated donors developed for enzyme-catalyzed self-condensation are stable 1,2-oxazoline derivatives that mimic the intermediate in the normal hydrolase reaction by these enzymes. Ring-opening polyaddition of these donors can proceed consecutively and, although enzymatic or chemical hydrolysis of the oxazoline derivative take place as side reactions, polymeric products were obtained in yields between 20% and 75% with degrees of polymerization from 6 to 88 depending on the enzyme and the reaction conditions. With this methodology, homopolysaccharides such as chitin, chitosan, and derivatives (Scheme 3), and heteropolysaccharides such as hyaluronic and chondroitin derivatives have been successfully produced (Scheme 4).

2.1.2.1. Chitin and chitosan derivatives by family 18 chitinases. The first enzymatic synthesis of chitin used chitobiose oxazoline as the glycosyl donor for polycondensation and employed chitinase from *Bacillus* sp. (Scheme 3a). By choosing the appropriate pH value (i.e., 10.6), the hydrolytic activity of the enzyme was significantly suppressed and self-condensation of the N,N^{II}-diacetyl-chitobiose oxazoline derivative led to artificial chitin oligomers.[37,50,51] The synthetic chitin had the repeating structure of β-(1→4)-linked GlcNAc without any of the glucosamine units normally found in naturally occurring chitin. The molecular weight was determined to be >4000, corresponding mainly to 16 disaccharide units.



a R^1= OH R^2= OH R^3= $NHCOCH_3$
b R^1= OH R^2= OH R^3= NH_2
c R^1= OH R^2= OH R^3= OH
d R^1= OH R^2= OH R^3= $NHSO_3Na$
e R^1= F R^2= OH R^3= $NHCOCH_3$
f R^1= OH R^2= F R^3= $NHCOCH_3$
g R^1= F R^2= F R^3= $NHCOCH_3$

Scheme 3. Artificial chitin-type polysaccharide produced by family 18 chitinases. Activated glycosyl donors are oxazoline derivatives.



a	R^1= OH	R^2= H	R= CH_3
b	R^1= OH	R^2= H	R= CH_2CH_2
c	R^1= OH	R^2= H	R= $CH_2CH_2CH_3$
d	R^1= OH	R^2= H	R= $CH{=}CH_2$
e	R^1= H	R^2= OH	R= CH_3
f	R^1= H	R^2= OH	R= CH_2CH_2
g	R^1= H	R^2= OH	R= $CH{=}CH_2$
h	R^1= H	R^2= OSO_3Na	R= CH_3

Scheme 4. Artificial hyaluran and chondroitin-type polysaccharides produced by family 56 hyaluronidases using oxazoline derivatives as activated glycosyl donors.

More recently, several chitinases were assayed with different *N*-acetyl-chitobiose oxazoline derivatives in which C-2^{II}, C-3 or C-6 was functionalized. Not only could the *N*-acetyl group at C-2^{II} be recognized as a substrate in the cleft-like catalytic domain of the enzymes, but also hydroxyl, amino or even anionic groups such as sulfonamide. The C-6 positions of *N*-acetyl-chitobiose oxazoline were also varied by replacement with carboxylate and fluorine groups. The yields of the polymerization reaction and molecular weights of the products depend on the stability of the oxazoline derivative, the chitinase origin and the reaction conditions. In all cases, the polymerization reaction proceeds with total control of regioselectivity and stereochemistry leading to different chitin hybrids with well-defined structure (see below). 3-O-Methylated chitin oligomers were also attempted using the disaccharides 3^{I}-*O*-methyl, 3^{II}-*O*-methyl and 3^{I},3^{II}-di-*O*-methyl-diacetylchitobiose oxazolines.[52] The most efficient enzymatic reaction by *Bacillus* sp. chitinase was on the 3-*O*-methyl donor derivative but despite this, only small oligomers were obtained.

A hybrid chitin–chitosan polysaccharide was obtained with the commercial chitinases from *Bacillus* sp, *Streptomyces griseus* and *Serratia marcescens*. The self-condensation of an *N*-acetyl-chitobiose oxazoline derivative with an amino group at the C-2 position on its non-reducing end yielded an alternating structure of β-(1→4)-linked *N*-acetylglucosamine and glucosamine[53] (Scheme 3b). Compared to natural chitin and chitosan polysaccharides, the different polymers were soluble in aqueous media and mean molecular weight values ranged from octa- to dodecasaccharide products. The chitinases from *Bacillus* sp. and *Serratia marcescens* were the most effective for the production of these hybrids with yields as high as 75%.

A cellulose–chitin hybrid was obtained using a chitinase from *Bacillus* sp. Self-condensation of β-Glc-(1→4)-GlcNAc oxazoline at pH 11.0 reached a maximal yield of 46% and the molecular weight of the product corresponded mainly to the dodecasaccharide (Scheme 3c). The polysaccharide is similar to that obtained with cellulase (see above), but with a glucose instead of a GlcNAc at the non-reducing end.[54]

A hybrid of natural chitin and unnatural N-sulfonated chitosan was also obtained using a N^{II}-sulfonyl-N^{I}-acetylchitobiose oxazoline donor (Scheme 3d). Chitinases from *Bacillus* sp., *S. marcescens*, *S. griseus*, and *A. hydrophila* were assayed and the first three catalyzed the polymerization reaction producing water-soluble polysaccharides. Their molecular weight distributions and yields depended on the enzyme used and the reaction conditions. Using *Bacillus* sp. at pH 10.5, the maximum yield was around 50% with a mean molecular weight of 1900, which corresponds mainly to octa and decasaccharides. In contrast, chitinase from *S. marcescens* at pH 8.0 yielded the highest yield of 62% with a molecular weight of 4000, corresponding to 18–20 glycosidic units.[55]

Fluorinated chitin derivatives were also synthesized with the chitinase from *Bacillus* sp. The monomer 6-deoxy-6-fluoro-*N*-acetylglucosamine oxazoline was not polymerized,[56] but the disaccharides 6^{II}-deoxy-6^{II}-fluoro, 6-deoxy-6-fluoro, and 6,6^{II}-dideoxy-6,6^{II}-difluoro-diacetylchitobiose oxazolines were self-condensed via specific β-(1→4) linkages and rendered alternating 6-fluorinated and fully 6-fluorinated chitin derivatives (Scheme 3e–g). The molecular weight of the products changed slightly depending on reaction conditions and corresponded mainly to hexa and octasaccharides, and maximum yields were of 50–60% for the alternating fluorinated chitins, and 23% for the fully fluorinated analog when the reaction was carried out at pH 8–9 and 30–40 °C. The introduction of the fluorine groups did not interfere in higher order assembly and at least the alternating fluorinated chitin presented the same crystalline structure and anti-parallel molecular chain alignment as natural chitin and synthetic chitin.[57]

The chitinase from *Bacillus* sp. is also able to use carboxylate-functionalized chitobiose oxazolines such as N,N^{II}-diacetyl-6^{II}-*O*-carboxymethylchitobiose and D-glucuronyl-β-(1→4)-*N*-acetyl-D-glucosamine derivatives as glycosyl donors but not the N,N^{II}-diacetyl-6-*O*-carboxymethylchitobiose oxazoline. The single step condensation with different glycosyl acceptors maintained the same stereo and regioselectivity and yielded chitooligosaccharides with a carboxylate at O-6^{II} or a glucuronic unit at the non-reducing terminus, respectively.[58] Although donor self-condensation was not studied, these results open up the possibility of obtaining carboxyl-functionalized chitopolysaccharides as carbohydrate-based materials.

2.1.2.2. Hyaluran and chondroitin derivatives by family 56 hyaluronidases. Hyaluronidases from family 56 are *endo*-glycosidases that hydrolyze hyaluronic acid in β-(1→4)-glycosidic linkages between GlcNAc and GlcA residues by substrate-assisted catalysis via an oxazolinium intermediate.[59] Hyaluronidases can also hydrolyze condroitin, condroitin sulfate, and dermatan sulfate at β-(1→4)-*N*-acetyl-D-galactosaminyl linkages.[60] Different glycosaminoglycans have been recently obtained by hyaluronidase-catalyzed ring-opening polyaddition of the corresponding oxazoline donor. The enzymes accept a broad range of oxazoline derivatives with total control of regioselectivity and stereochemistry of polymerization to afford either hyaluran, chondroitin or dermatan derivatives[61] (Scheme 4). Like chitinases, the pH optimum for condensation reactions is shifted relative to the pH optimum of the hydrolase reaction and product hydrolysis is minimized at more basic pH values.[60]

The first mimetic of natural hyaluran was obtained by polyaddition of β-GlcA-(1→3)-GlcNAc oxazoline using bovine testicular hyaluronidase (BTH) and ovine testicular hyaluronidase (OTH).[62] The donor self-condensed via β-(1→4) linkages to produce polysaccharides in aqueous buffer at pH 7.5 at 30 °C (Scheme 4a), but polymerization did not take place upon addition of organic co-solvents, due to the instability of the enzyme in the media.[63] With OTH, the reaction contained a mixture of oligomers with a mean molecular weight over 5500, which corresponds to 28–30 glycosyl units, in 78% yield. In contrast, BTH gave a distribution with mean molecular weight of 7800 and 53% yield. However, when the precipitated polymers were isolated from the soluble fraction, a chitin polysaccharide with molecular weight of 13,300 (66 saccharyl units) was obtained with the OTH enzyme in 53% yield, whereas the polysaccharide obtained with the BTH enzyme had a molecular weight of 17,700 (88 saccharyl units) in 34% yield.

Modified hyaluronan derivatives with different *N*-acyl groups instead of the *N*-acetyl substitution found in natural glycosaminoglycans were also synthesized.[63] *N*-acyl-hyalobiuronate oxazolines with propionyl, butyryl, *sec*-butyryl, pentanoyl, benzoyl, and acryloyl groups were assayed with OTH and BTH. Best results were obtained for the *N*-propionyl, *N*-butyryl, and *N*-acryloyl hyaluronic acid derivatives (Scheme 4b–d). The OTH enzyme gave polymeric products with molecular weights of 8000–9700, corresponding to 38 and 46 saccharyl units, in 40–50% yield. Interestingly, the vinyl group of the *N*-acryloyl hyaluronic acid can lead to functionalized polysaccharides and carbohydrate-based polymers. In addition, copolymerization reactions with combinations of donors gave the corresponding hetero-hyaluronan derivatives in similar yields.[61]

Synthetic chondroitin has been produced by enzymatic polymerization of *N*-acetyl-chondrosine (β-GlcA-(1→3)-GalNAc) oxazoline by hyaluronidases[64] (Scheme 4e). The highest yield was achieved using OTH at pH 7.5 and 30 °C, which provided a precipitated polymer of mean molecular weight of 2100 in 50% yield. However, higher oligomers with molecular weights of about 4600 were obtained with shorter reaction times at pH between 7.5 and 8.5 in yields of 6–20%. This molecular weight corresponds to 22–24 saccharyl units, which is similar to that of naturally occurring chondroitin.[65] In any case, yields and molecular weights of these polymers are lower compared to those obtained for hyaluronic acid because donor hydrolysis is more extensive in this case.

Modified chondroitin derivatives have also been produced using *N*-acyl-chondrosine oxazolines with different *N*-acyl groups.[64] *N*-Acryloyl and *N*-propionyl chondroitin derivatives were achieved in 46% and 19% yields with molecular weights of 2700 (12–14 saccharide units) and 3400 (16–18 units), respectively, after long reaction times (Scheme 4f and g). In contrast, very low yields were obtained with the corresponding *N*-butyryl and *N*-*sec*-butyl oxazolines, and the *N*-benzoyl oxazoline was not recognized by the enzyme.

Chondroitin 4-sulfate (Ch4S), bearing sulfate groups at C-4 in all *N*-acetyl-galactosamine units, which mimics the natural Ch4S or chondroitin sulfate A (ChS-A), was also synthesized using hyaluronidases.[66] The OTH enzyme polymerized the 4-sulfated *N*-acetyl-chondroisine oxazoline derivative and produced the corresponding chondroitin 4-sulfate with a mean molecular weight of 11,700 in 75% yield at pH 7.5 and 30 °C (Scheme 4h). The size of the product is similar to that of naturally occurring Ch4S, but depending on reaction conditions such as enzyme amount, donor concentration, pH, and temperature, polymers with molecular weight from 9000 to 18,400 were obtained. The highest molecular weight value corresponds to 72–74 saccharyl units and the product was obtained in 68% yield when 10% of the wild-type enzyme and 0.2 M donor are reacted at pH 7.5 and 30 °C. The sulfation degree could be controlled by copolymerization of the oxazoline monomer of *N*-acetyl-chondrosine and the C-4 sulfated derivative.[61]

Mimetics of other natural chondroitin sulfates with different sulfation patterns (i.e., Ch6S or ChS-C, Ch2,6S or ChS-D, and Ch4,6S or ChS-E) could not be obtained by hyaluronidase-catalyzed polymerization of the corresponding chondroisine oxazoline derivatives because they were not recognized as acceptors. In these instances, only hydrolysis of the oxazoline took place.[66]

A hybrid hyaluronic–chondroitin polysaccharide has been produced by copolymerization of *N*-acetyl-hyalobiuronate and the *N*-acetyl-chondrosine oxazolines with the OTH enzyme. The copolymer composition was controllable depending on the monomer ratio, and oligomers with molecular weights up to 6000 were isolated.[61]

2.1.3. Summary: polymerization by *endo*-glycosidases with activated substrates. In summary, the use of wild-type glycosidases with activated donors as glycosyl fluoride or oxazoline derivatives leads to polysaccharides by donor self-condensation in moderate yields in the range of 50–75%. However, the degree of polymerization of the product depends greatly on reaction conditions and type of glycosyl donor. For enzymes utilizing a glycosyl fluoride donor, the degrees of polymerization of the produced polysaccharides do not exceed 10–30 monosaccharide units. For hyaluronidases with oxazoline donors containing charged substituents, the degrees of polymerization are significantly higher, in the range of 25–80 monosaccharide units. Apart from engineered cells expressing glycosyltransferases as hyaluran synthases,[24] no other enzymatic approach or synthetic methodology can achieve glycosaminoglycan-type polysaccharides in these high yields and diversity of chain length. In addition to the balance between hydrolytic and transglycosylation activities of each particular enzyme, the product solubility greatly influences the size of the polymer. Although organic co-solvents are often used, they are present in low amounts so as to preserve enzyme stability and, therefore these solvents do not significantly improve product solubility.

2.2. Polymerization by retaining glycosidases with unmodified carbohydrate substrates

Kinetically controlled transglycosylation by wild-type retaining glycosidases has also been studied and developed with unmodified sugar donors. That is, donor substrates in which the leaving group in the transfer reaction to an acceptor is a carbohydrate moiety instead of the fluoride, nitrophenyl or oxazoline leaving groups commonly used in the activated donors described in Section 2.1. Based on the enzyme mechanism, this approach is a normal transglycosylation reaction of a glycosyl moiety from an oligosaccharide donor to an acceptor. In general, these donors are less activated and hydrolysis predominates, requiring engineering of the reaction conditions (enzyme immobilization, organic solvents, etc.) and/or enzyme engineering (site-specific mutagenesis and directed evolution approaches) to make transglycosylation practical. Although these approaches have been extensively studied for the preparation of simple oligosaccharides,[67–70] there are few examples of application to polysaccharide synthesis. In contrast to these enzymes are natural transglycosylases, enzymes with the same mechanism as retaining glycosidases and classified in the same enzyme families, but whose predominant activity is transglycosylation. A number of them are involved in the biosynthesis of natural polysaccharides and therefore in vitro enzymatic syntheses have been successfully developed.

2.2.1. Retaining glycosidases. Because substrate hydrolysis is the predominant reaction in aqueous solution, modification of the reaction conditions to favor transglycosylation to an acceptor (the same molecule for self-transglycosylation leading to oligomers and polymers) is required to make synthesis practical. Relevant examples are the production of food-grade oligosaccharides,[71–73] poly-*N*-acetyl-lactosamines[74,75] or the β-(1→3)-glucooligosaccharides with marine β-(1→3)-D-glucanase.[76] Operational conditions and enzyme engineering are current approaches to improve transglycosylation activity, and a number of examples have been recently reported. However, it is out of the scope of this review because low molecular weight oligosaccharides are produced, and no polysaccharides have yet been obtained by these approaches.

2.2.2. Transglycosylases. Among transglycosylases, several families catalyze the consecutive transfer of simple saccharide donors, thus leading to oligo- and polysaccharides. The most explored are glucansucrases and fructosyltransferases, which use sucrose as donor substrate. Glucansucrases transfer the glucosyl moiety of sucrose to afford different α-glucans. Depending on the new linkages, amylose, dextran, mutan, alternan, and reuteran are synthesized. By contrast, fructosyltransferases transfer the fructosyl moiety resulting in the synthesis of fructooligosaccharides and/or fructan polymers such as levan or inulin. These enzymes differ markedly in product specificities, hydrolysis/transglycosylation activity ratios, and oligomers to polymers ratio (recent reviews[77,78]). Current efforts for enzyme development focus on side-directed mutagenesis, directed evolution, and chimeric enzymes construction to modulate the different activities and improve product properties and yields.

Recent examples include an insoluble amylose-like polymer produced by the amylosucrase from *Neisseria polysaccharea*, which is a remarkable transglucosidase from family GH13. By directed evolution, amylosucrase variants were obtained having the highest polymerase

activity found up to date,[79] with 60% increased activity, or producing longer polymers than the wild-type enzyme.[80] Likewise, higher molecular weight glucans and fructans were synthesized by bacterial glucansucrases and fructosyltransferases. These homo-polysaccharides can reach up to 5×10^7 Da, and their molecular mass and linkage type distributions can be altered.[79,81–83] For example, because the variations in glucosidic linkage specificity observed in different glucansucrases from family 70 appear to be based on relatively small differences in amino acid sequences in their sugar-binding acceptor subsites, mutants of reuteransucrase *Lactobacillus reuteri* 121 resulted in the synthesis of an α-glucan containing only a very small percentage of α-(1→4) linkages and furthermore an increased percentage of α-(1→6) linkages. This was a rational transformation of a reuteransucrase into dextransucrase.[83]

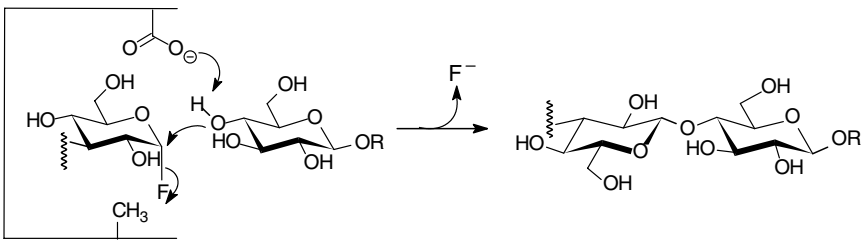
3. Enzymatic polymerization by *endo*-glycosynthases: engineered glycosidases for efficient transglycosylation

A recent approach based on the redesign of the enzyme mechanism of glycosidases has been developed for efficient synthetic applications. The glycosynthase concept was introduced in 1998 on both *exo*-glycosidase[84] and *endo*-glycosidases.[85] These enzymes have their catalytic nucleophile substituted by a non-nucleophilic residue and are able to catalyze the transglycosylation of glycosyl fluoride donors with opposite anomeric configuration compared to the normal substrate of the wild-type enzyme. Mutation of the catalytic nucleophile disables the enzyme as a hydrolase because no glycosyl-enzyme intermediate can be formed. However, an activated glycosyl donor with an anomeric configuration opposed to that of the donor substrate in the wild-type reaction (i.e., an α-glycosyl fluoride for a β-glycosidase) mimics the glycosyl-enzyme intermediate and is therefore able to react with an acceptor (Scheme 5). The cavity created in the active site by mutation of the carboxylate residue acting as nucleophile in the wild-type enzyme by a smaller residue allows binding of the glycosyl fluoride with the opposite anomeric configuration. As with the wild-type enzyme, transglycosylation is kinetically favored but the transglycosylation reaction is now irreversible because of the lack of the catalytic nucleophile; the product is no longer hydrolyzed and accumulates to give high transglycosylation yields. This approach represents an important improvement compared to kinetically controlled transglycosylation by wild-type enzymes. This methodology has been extended to other *exo*- and *endo*-glycosidases, covering a broad repertoire of glycosyl units and glycosidic linkages in enzymatic synthesis of oligosaccharides and glycoconjugates (Table 1).[86–100]

Compared to *exo*-glycosynthases, *endo*-glycosynthases have an extended active site, which is able to accept complex oligosaccharides. When the glycosyl fluoride serves as the donor and acceptor, self-condensation proceeds consecutively and high molecular weight polymers can, in principle, be formed. Enzymatic polymerization has been reported for four out of the seven currently developed *endo*-glycosynthases: E197 mutants of cellulase Cel7B from *Humicola insolens*, E231 mutants of β-(1→3)-glucanase from barley, E134 mutants of β-(1→3)-(1→4)-glucanase from *Bacillus licheniformis*, and glycine mutants of several xylanases (Scheme 6). These enzymes are able to synthesize β-glycans in high yields, and with the potential of producing oligomers with higher degree of polymerization than wild-type glycosidases under kinetically controlled transglycosylation.

3.1. β-(1→4)-Glucans (synthetic cellulose-type polysaccharides)

The E197A mutant of cellulase Cel7B from *Humicola insolens* was shown to condense α-cellobiosyl and α-lactosyl fluoride donors to different acceptors via β-(1→4) linkages.[86] Interestingly, this enzyme presents an active site capable of binding diverse donors functionalized at C-6^{II} with amino, bromo or azido groups or even with glycosyl substitution, which makes this glycosynthase and its more active E197S homologue[86,103] a versatile tool for a broad variety of synthetic applications. This glycosynthase has been used to synthesize cellodextrins,[104] functionalized cellooligosaccharides,[105] xyloglycans oligomers and xylo-glucooligosaccharides,[106,107] and glycosylated flavonoids.[108]



Scheme 5. Mechanism of glycosynthases. Mutation of the catalytic nucleophile by a small non-nucleophilic residue renders a hydrolytically inactive enzyme that is able to accept an activated glycosyl fluoride with the 'wrong' anomeric configuration and catalyze transglycosylation to an acceptor.

Table 1. Glycosynthases derived from *exo*- and *endo*-glycosidases to date

Parental glycosidase	Family	Mutated residue	Reaction C[a]/P[b]	Linkage formed	Reference
exo-Glycosidases					
Agrobacterium sp. β-glucosidase	GH 1	E358	C	β-(1→4) β-(1→3)[c]	84
Sulfolobus solfataricus β-glycosidase	GH 1	E387	C	β-(1→3/4/6)	87
Thermosphaera aggregans β-glycosidase	GH 1	E386	C	β-(1→3/4/6)	91
Pyrococcus furiosus β-glycosidase	GH 1	E372	C	β-(1→3)	91
Thermus thermophilus β-glycosidase	GH 1	E338	C	β-(1→3) (β-(1→6))	95
Streptomyces sp. β-glucosidase	GH 1	E383	C	β-(1→3) (β-(1→4))[c]	96
Oryza sativa β-glucosidase	GH 1	E414	C/P	β-(1→4)	102
Cellulomonas fimi β-mannosidase	GH 2	E519	C	β-(1→3) β-(1→4)[d]	88
Escherichia coli β-galactosidase	GH 2	E537	C	β-(1→6)	89
Termotogoma maritima β-glucuronidase	GH 2	E476	C	β-(1→4)	97
Schizosaccharomyces pombe α-glucosidase	GH 31	D481	C	α-(1→6/4)	90
Geobacillus stearothermophilus β-xylosidase	GH 32	E335	C	β-(1→4)	101
endo-Glycosidases					
Rhodococcus sp. glycoceramidase	GH 5	E351	C	β-(1→1)	100
Humicola insolens cellulase	GH 7	E197	C/P	β-(1→4)	86
Clostridium stercorarium β-(1→4)-xylanase	GH 10	E293	C/P	β-(1→4)	98
Bacillus halodurans β-(1→4)-xylanase	GH 10	E301	C/P	β-(1→4)	98
Cellulomonas fimi Cex β-(1→4)-xylanase	GH 10	E233	C/P	β-(1→4)	98
Thermotoga maritima β-(1→4)-xylanase	GH 10	E259	C/P	β-(1→4)	98
Cellulomonas fimi CFX β-(1→4)-xylanase	GH 10	E235	C	β-(1→4)	99
Geobacillus stearothermophilus β-(1→4)-xylanase	GH 10	E265	C/P	β-(1→4)	101
Bacillus licheniformis β-(1→3)-(1→4)-glucanase	GH 16	E134	C/P	β-(1→4)	85
Pyrococcus furiosus β-(1→3/4)-glucanase	GH 16	E170	C	β-(1→4) β-(1→3)[d]	94
Hordeum vulgare β-(1→3)-glucanase	GH 17	E231	C/P	β-(1→3)	92
Cellvibrio japonicus β-mannanase	GH 26	E320	C	β-(1→4)	93

[a] Donor and acceptor condensation, and eventually, product elongation leading to short oligomers.
[b] Polymerization by donor self-condensation to produce artificial polysaccharides.
[c] Depending on the acceptor, a mixture of both linkages is obtained.
[d] Regioselectivity changes depending on the acceptor.

The E197A cellulase can also produce β-(1→4)-glucans.[86] Polymerization of α-cellobiosyl fluoride gave an insoluble β-(1→4)-glucan that is consistent with low molecular mass cellulose II (Scheme 6a). This enzyme was also able to polymerize cellobiosyl fluoride donors modified at C-6^{II} with amino, bromo or thioglycosyl groups to generate functionalized polysaccharides. These chemical functionalities open up possibilities to later modify and graft the polysaccharides.

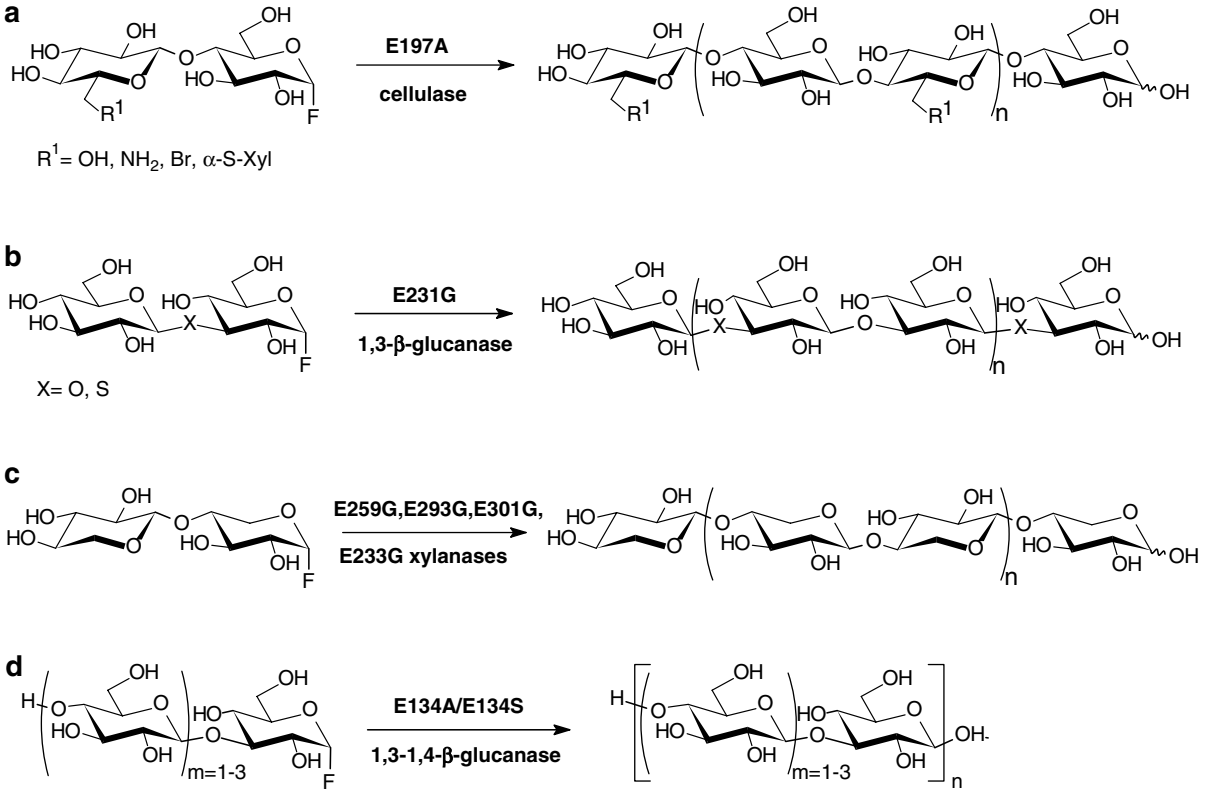
3.2. β-(1→3)-Glucans (synthetic laminarin-type polysaccharides)

Glycosynthase mutants of β-(1→3)-glucanase from barley are able to synthesize homooligomers of β-(1→3)-linked glycosyl units.[92] Self-condensation of α-laminaribiosyl fluoride by the E231G mutant yielded an insoluble β-(1→3)-glucan (Scheme 6b). The polysaccharide has a degree of polymerization of about 30 and it is structurally similar to recrystallized curdlan.

This mutant was also capable of self-condensing 3-thio-α-laminaribiosyl fluoride and producing oligosaccharides with alternating S- and O-linkages. In this case, the rate of formation and the degree of polymerization were lower than those of the homologous homopolysaccharide, but the novel heteropolysaccharide was more resistant to hydrolysis by β-(1→3)-glucanase.[92] In addition, because the 3-azido-3-deoxy-laminaribiosyl fluoride was also accepted as a donor for the condensation reaction,[109] functionalized β-(1→3)-glucans could also be achieved. This glycosynthase activity offers the possibility of synthesizing tailor-made β-(1→3)-glucans that can present valuable therapeutic applications as antitumor agents and immunomodulators.[1]

3.3. β-(1→4)-Xylans

Recently, different endoxylanases from family 10 were converted to glycosynthases. The glycine mutants of the xylanases from *Thermotoga maritima* (E259G), *Clostridium stercorarium* (E293G), *Bacillus halodurans* (E301G), and *Cellulomonas fimi Cex* (E233G) afforded polymeric β-(1→4)-xylan from α-xylobiosyl fluoride donor[98] (Scheme 6c). The yields varied between 29% and 69% depending on the xylanase origin and the average degree of polymerization was estimated to be 22. In the same way, the xylanase mutant E265G from *Geobacillus stearothermophilus* (XT6) was recently reported to yield xylan polymers from six to over 30 xylose units.[101] On the other hand, the E235G *endo*-(1→4)-xylanase



Scheme 6. Glycosynthase-catalyzed polymerizations by self-condensation of activated glycosyl donors.

mutant from *Cellulomonas fimi* (CFX) was able to synthesize xylooligomers with a lower degree of polymerization.[99] However, in this case, the enzymatic oligomerization did not proceed by self-condensation of the α-xylobiosyl fluoride as it did not act as an acceptor, but polymerization took place through elongation of the first condensation product between the donor and *p*-nitrophenyl xylobioside acceptor. After several additions of donor over time, oligomers from tetra to dodecasaccharides were formed in yields varying from 30% to 1%, respectively.

3.4. β-(1→3)-(1→4)-Glucans (artificial (1→3)-(1→4) mixed-linked β-glucans)

The E134A mutant of β-(1→3)-(1→4)-glucanase from *Bacillus licheniformis* can afford β-(1→3)-(1→4)-hetero-ligo- and polysaccharides via formation of β-(1→4) linkages.[85] The α-laminaribiosyl fluoride donor was condensed to different gluco-, cello- and laminariobiosides acceptors in high yields. The glycosynthase mechanism was carefully analyzed with this enzyme showing a shifted pH profile as compared to the wild-type enzyme, consistent with general base catalysis. The series of donors $(\beta\text{-Glc-}(1\rightarrow4))_n\beta\text{-Glc}(1\rightarrow3)\text{-}\alpha\text{-GlcF}$ $(n = 0\text{–}2)$ showed increased reactivity with size of the donor,[110] and several β-(1→3)-(1→4) mixed-linked oligosaccharides with defined sequence have been obtained.[110–112] Remarkably, due to the differential specificity with cellulases, one-pot reactions by sequential addition of two glycosynthases, the E197A Cel7B cellulase and the E134A β-(1→3)-(1→4)-glucanase, produced defined oligosaccharides in yields as high as 80% in purified products.[112]

The ability for donor self-condensation was exploited for the in vitro synthesis of the first heteropolysaccharides with alternating β-(1→3) and β-(1→4) linkages (Scheme 6d). Self-condensation of α-laminariobiosyl fluoride yielded artificial β-(1→3)-(1→4)-glucans with new properties compared to the glycans present in nature due to their regular structure with repeating →4)-β-Glc-(1→3)-β-Glc units.[113] The occurrence of β-(1→3)-(1→4)-glucans is limited to grasses and certain lichens. They are linear polysaccharides with a polymerization degree that can be as high as 1200 and with a heterogenous proportion of (1→3) and (1→4) linkages, in which the amount of β-(1→3) bonds does not exceed 25–30%. Light microscopy and TEM revealed that the in vitro product was composed of lower molecular mass polysaccharides forming spherulites of platelet crystals with a crystalline structure similar to the cellulose I crystals.[113]

Recently, polymerization applications of this glycosynthase were expanded using the tri- and tetrasaccharyl fluoride donors (Planas and co., unpublished results). The yields of these polymerization reactions are high

(80%) and the corresponding β-(1→3)-(1→4)-glucans with different repeating units, →4)-β-Glc-(1→4)-β-Glc-(1→3)-β-Glc and →4)-β-Glc-(1→4)-β-Glc-(1→4)-β-Glc-(1→3)-β-Glc, were obtained. While the polymer from laminaribiosyl fluoride self-condensation presented 50% of β-(1→3) linkages, the tetrasaccharide donor provided polymers with 25% β-(1→3) linkages, similar to natural lichenans and cereal β-glucan, but with a homogeneous repetitive structure.

The polymerization degree clearly depends on the enzyme turn-over. When the more active E134S glycosynthase was used, the molecular weight profile was shifted to higher molecular weight values, and polymers with M_n corresponding to 108 glucose units and a polymerization degree of 27 were reached. The microstructure of these polymers is composed of spherulites for the polymers coming from di- and tetrasaccharide donors, but not for those coming from the trisaccharyl fluoride donor. Therefore, this glycosynthase can afford mimetics of the natural β-(1→3)-(1→4)-glucans with well-defined structures and the desired β-(1→3) and β-(1→4) linkage ratio.

The other reported *endo*-glycosynthases that a priori can afford polysaccharides are those derived from (1→3/4)-glucanase and mannanase. The (1→3/4)-glucanase from *Pyrococcus furiosus* (GH16 family)[94] was the first thermophilic enzyme transformed into a glycosynthase. The E170A mutant was able to condense α-laminaribiosyl fluoride to different acceptors, but because the condensation reactions had no absolute regiospecificity and the yields did not exceed 30%, polymerization reactions were not explored. As opposed to the mannanase from *Cellulomonas fimi* for which nucleophile mutants did not have glycosynthase activity, the E320G mutant of mannanase from *Cellvibrio japonius* (family 26)[93] transferred α-mannobiosyl fluoride to glycoside acceptors via β-(1→4)-linkage. This enzyme offers a new synthetic strategy for β-manno-oligosaccharides, which are complicated to prepare by conventional chemical synthesis. Unfortunately, the α-mannobiosyl fluoride is not accepted in the acceptor subsites and enzymatic polymerization cannot take place.

Apart from *endo*-glycosynthases, the glycosynthase derived from rice β-glucosidase is the first reported *exo*-glycosynthase that can produce long chain cellooligosaccharides.[102] In contrast to the donor self-condensation ability of *endo*-glycosynthases, this enzyme exploits its long acceptor binding subsite and elongates condensation products to insoluble polymers with a degree of polymerization from 3 to at least 11 in yields up to 80%.

4. Conclusions

In conclusion, transglycosylation reactions catalyzed by retaining *endo*-glycosidases have been adapted for efficient polysaccharides synthesis. Through the use of either wild-type enzymes or glycosynthase mutants, it is possible to access different mimetics of natural polysaccharides and artificial polysaccharides with well-defined structures and morphologies. Compared to enzymatic polymerization by native glycosidases with activated donor as glycosyl fluorides or oxazoline derivatives, the main advantage of the glycosynthase strategy is the lack of product hydrolysis, resulting in higher polymer yields and degrees of polymerization. Further directed evolution of these enzymes as well as discovery of new glycosidases with different specificities will expand the scope of polysaccharide synthesis.

These methodologies offer a range of possibilities for the synthesis of biomaterials. New perspectives such as the ability of using modified donors to introduce functionalizable groups into polysaccharide structures, controlling the degree of polymerization, and the preparation of novel composite materials by enzymatic polymerization in the presence of other polymers are just emerging, and a number of applications in advanced biomaterials are awaiting to come in the near future.

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