Check for updates

# Recent advances on the one-pot synthesis to assemble size-controlled glycans and glycoconjugates and polysaccharides

Han Xu [a,1], Baoxing Shen [a,1], Meng Qiao [a], Robert J. Linhardt [b,c,**], Xing Zhang [a,*]

[a] *School of Food Science and Pharmaceutical Engineering, Nanjing Normal University, Wenyuan Road 1, Nanjing 210023, China*
[b] *Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy New York, 12180, USA*
[c] *Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy New York, 12180, USA*

## ARTICLE INFO

## ABSTRACT

Glycans and glycoconjugates in nature include macromolecules with important biological activities and widely distributed in all living organisms. These oligosaccharides and polysaccharides play important roles in a variety of normal physiological and pathological processes, such as cell metastasis, signal transduction, intercellular adhesion, inflammation, and immune response. However, the heterogeneity of naturally occurring glycans and glycoconjugates complicates detailed structure-activity relationship studies resulting in an incomplete understanding of their mechanisms of action and hindering further applications. Therefore, the synthesis of homogeneous, or nearly homogeneous, structurally defined glycans is of great significance for the development of carbohydrate-based drugs. One-pot synthesis represents the fastest strategy to assemble oligosaccharides and polysaccharides, although unfortunately, typically relies on random assembly. In this review, we examine the progress that has been made in the controlled one-pot synthesis of homogeneous or nearly homogeneous oligosaccharides and polysaccharides providing a broad spectrum of options to access size-controlled glycan products.

## 1. Introduction

Carbohydrates, the most abundant class of natural products, are polyhydroxyl aldehydes or polyhydroxyl ketones and their poly-condensates and derivatives (Zhang & Ye, 2018). As one of the important class of biological macromolecules, polysaccharides are ubiquitously distributed in nature, present in all living organisms, including animals, plants and microbes (Liu, Willför, & Xu, 2015; Lovegrove et al., 2017; Qin et al., 2019). Moreover, carbohydrates are usually bound to lipids or proteins to form glycoconjugates such as glycolipids, glycoproteins and proteoglycans (Colombo, Pitirollo, & Lay, 2018; Lin, Qiao, Zhang, & Linhardt, 2020). These glycans and glycoconjugates play many critical roles, eliciting a myriad of key biological processes, involving viral and bacterial infection, cell signaling, cell proliferation and differentiation, angiogenesis and metastasis, immune-responses, and neurodegenerative diseases (Boltje, Buskas, & Boons, 2009; Ji, Li et al., 2020; Varki, 1993, 2017). For example, cell-surface glycans directly participate in a wide variety of biological recognition processes including cell adhesion, signaling, development, and the immune response (Dwek, 1996; Haltiwanger & Lowe, 2004; Jefferis, 2009; Macauley, Crocker, & Paulson, 2014; Nimmerjahn & Ravetch, 2008; Varki, 1993). SARS-CoV-2 infection has recently been reported to depend on cellular heparan sulfate and angiotensin-converting enzyme 2 (ACE2), suggesting that exogenous

---

heparin presents potential therapeutic opportunities (Clausen et al., 2020; Kwon et al., 2020).

Polysaccharides generally have two properties associated with their homogeneity. Structural homogeneity refers to the presence of a single repeating unit while molecular weight homogeneity refers to a polydispersity index of 1.0. Most naturally occurring glycans are usually structurally heterogeneous coming from limited sources and with high-cost production. For example, animal-sourced heparin is a widely used polydisperse anticoagulant drug, complicating detailed structure activity relationship studies, thus, hampering its other therapeutic applications (Li & Wang, 2018; Zhang, Lin, Huang, & Linhardt, 2020). In addition, polysaccharides having different molecular weights can possess different physiological and pharmacological functions (Table 1). High molecular weight hyaluronic acid (HMW-HA) has been applied by inhalation to clinically treat inflammation, while low molecular weight hyaluronic acid (LMW-HA) exhibits proinflammatory characteristics (Li, Qiao et al., 2020; Li, Yao et al., 2020). Moreover, impurities are inevitably present due to the incorporation of other similar polysaccharides and bioactive entities such as viruses, prions or growth-modulating factors in animal tissues (Liu & Linhardt, 2014). A crisis resulted from the worldwide distribution of contaminated heparin in 2007 highlighted the importance of developing homogeneous heparin drug instead of a heterogeneous one from an animal source (Guerrini et al., 2010; Szajek et al., 2016). Consequently, efficiently preparing homogeneous, or nearly homogeneous, polysaccharides or structurally well-defined oligosaccharides is at the forefront of the new-generation carbohydrate-based drug development, resulting in reproducible biological properties as well as a safer and more secure supply chain (Fig. 1).
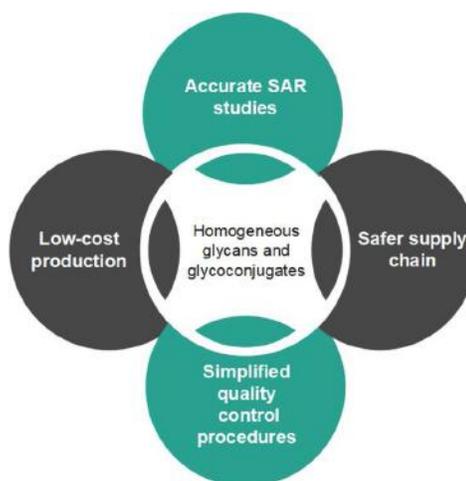


**Fig. 1.** The advantages of size-controlled or homogeneous glycans and glyco-conjugates in the development of carbohydrate-based drugs.

## 2. General approaches for preparing homogeneous glycans

Generally, homogeneous glycans can be synthesized using three strategies: 1. degradation methods; 2. pure chemical synthesis assembly; and 3. enzymatic or chemoenzymatic synthesis assembly. These synthetic approaches hold their own characteristics and scopes of application, respectively, which are be described below (Table 2).

**Table 1**
Representative examples that glycans or glycoconjugates bearing different molecule weights show different properties (HMW: high molecular weight; IMW: intermediate molecular weight; LMW: low molecular weight).

| Polysaccharide/ glycoconjugates | Structure/chain lengths | Activities | Reference |
|---|---|---|---|
| Hyaluronic acid(HA) | HMW-HA | Treats inflammation clinically | (Liu et al., 2008) |
|  | LMW-HA | Exhibits proinflammatory characteristics |  |
| Chitosan | HMW-CHI | High MW chitosan has stronger antifungal activity and affinity for enzymes than low MW chitosan | (Ang, Por, & Yam, 2013; Eikenes, Alfredsen, Christensen, Militz, & Solheim, 2005) |
|  | IMW-CHI |  |  |
|  | LMW-CHI |  |  |
| Pectin | Original pectin | Low solubility, high viscosity, and strong gelatinization | (Yamaguchi et al., 1995) |
|  | LMW-pectin | Low viscosity and high solubility | (Tian, Qiao, Qiu, Deng, & Guo, 2011) |
|  | IMW-pectin | Low viscosity and hypocholesterolemic activities |  |
| Heparin | Unfractionated heparin | Used in surgery and kidney dialysis due to its relatively short half-life and its safety for renal-impaired patients |  |
|  | LMW-heparin | Used in preventing venous thrombosis among high-risk patients for predictable anticoagulant doses, long half-lives, and reduced risks of osteoporosis | (Xu et al., 2011) |
|  | ULMW-heparin | Greatly reduces risks of hit and bleeding |  |
| Oat β-Glucan | H-β-glucan | The viscosity and antioxidant activities decreased obviously with the decreasing of the molecular weight | (Sun et al., 2020) |
|  | I-β-glucan |  |  |
|  | L-β-glucan |  |  |
| Fucoidan | Molecular weights of 3.8, 1.0, and > 8.3 kDa are degraded by $H_2O_2$ | Exhibits superior activity of scavenging hydroxyl radicals and superoxide anions | (Li et al., 2016) |
| Chondroitin Sulfate (CS) | HMW-CS | Treats inflammation clinically | (Ji, Zhang et al., 2020) |
|  | LMW-CS | Exhibits pro-inflammatory characteristics |  |
| N-acetyl chito-oligosaccharides | MW = 1−3 kDa | Exhibit an inhibitory effect against DNA and protein oxidation. | (Ngo, Lee, Kim, & Kim, 2009) |
|  | MW < 1 kDa | NA-COS 1−3 kDa was more effective than NA-COS < 1 kDa in protein oxidation and production of intracellular free radicals in live cells |  |
| Dextran | 9.5 kDa | Inflammatory activity increases with increasing molecular weight | (Kitajima, Takuma, & Morimoto, 2000) |
|  | 54 kDa |  |  |
|  | 520 kDa |  |  |
| Cellulose | HMW | HMW solutions are more viscous than LMW solutions | (Zhang, Zhang, Dai, Tang, & Jin, 2014) |
|  | LMW |  |  |
| Astragalus Polysaccharides | APS-I(MW > 500 kDa) | APS-II had stronger effect on innate and adaptive immunities than APS-I | (Li, Li, Du, & Qin, 2020) |
|  | APS-II(MW = 10 kDa) |  |  |
| Arabinoxylan | WEAX | L-WEAX could hinder starch gelatinization more evidently compared with H-WEAX | (Hou et al., 2020) |
|  | H-WEAX | L-WEAX mainly suppresses the short-term retrogradation of amylose H-WEAX mainly effects the long-term retrogradation |  |

**Table 2**

Comparison of general methods for the synthesis of homogeneous glycans.

| Methods | Strategy | Representative conditions | Comments | References |
|---------|----------|---------------------------|----------|------------|
| Degradation | Chemical degradation | Acid, base, periodate | Provide a relatively narrow molecular weight distribution, difficult to control, complex purification operations | (Trombotto et al., 2008) (Zhang et al., 2017) (Yuan et al., 2015) |
| | Enzymatic degradation | Lyase | | |
| Assembly | Chemical assembly | Chemical glycosylation: using glycosyl halides, thioglycosides or glycosyl imidates as donors | Structurally defined product, tedious synthetic and purification steps, low overall yields | (Petitou & van Boeckel, 2004) |
| | Enzymatic and chemoenzymatic assembly | Glycosyltransferase | Exquisite stereoselectivity and regioselectivity, step-wise reaction, large amounts of enzymes required | (Liu & Linhardt, 2014) (Xu et al., 2017) |
| | One-pot assembly | Glycosyltransferase, free radical polymerization | Simplified operation steps, relatively lower cost, difficult to control | (Qian et al., 2018) |

### 2.1. Degradation strategy

Homogeneous oligosaccharides and polysaccharides, obtained through the degradation of native polysaccharides by chemical or enzymatic reactions (Fig. 2A). By breaking polysaccharides at different sites under acidic, alkaline or oxidative conditions affords oligosaccharides or polysaccharides of certain molecular weights, and is a common approach in the chemical preparation of more homogeneous products (Volpi, Schiller, Stern, & Soltes, 2009; Zhang et al., 2017). For example, in the first step of preparing a series of chitosan oligomer mixtures having a degree of polymerization of 2–12, Domard et al. used acid hydrolysis to partially depolymerize fully *N*-deacetylated chitosan to generate oligomers (Trombotto, Ladaviere, Delolme, & Domard, 2008). However, this degradation method is random and is difficult to control, easily leading to the production of smaller saccharide products, such as monosaccharide or disaccharide (Stern, Asari, & Sugahara, 2006).

Enzymatic degradation can utilize either a lyase or a hydrolase to catalyze the depolymerization of polysaccharides under mild conditions (Stern & Jedrzejas, 2006; Yuan et al., 2015). Although the enzymatic degradation methods can reduce the molecular weight of polysaccharides and provide oligosaccharides having a relatively narrow molecular weight distribution. It is difficult to precisely control the size of oligosaccharides using degradative enzymes, resulting in polydisperse mixtures, and must be followed by complex purification operations to obtain homogeneous polysaccharides or oligosaccharides (Ji, Li et al., 2020; Li, Qiao et al., 2020; Li, Yao et al., 2020).

### 2.2. Assembly strategy

#### 2.2.1. Chemical synthesis

Traditional chemical synthesis is still the major method for preparing structurally defined oligosaccharides in industry (Fig. 2B) (Mende et al.,
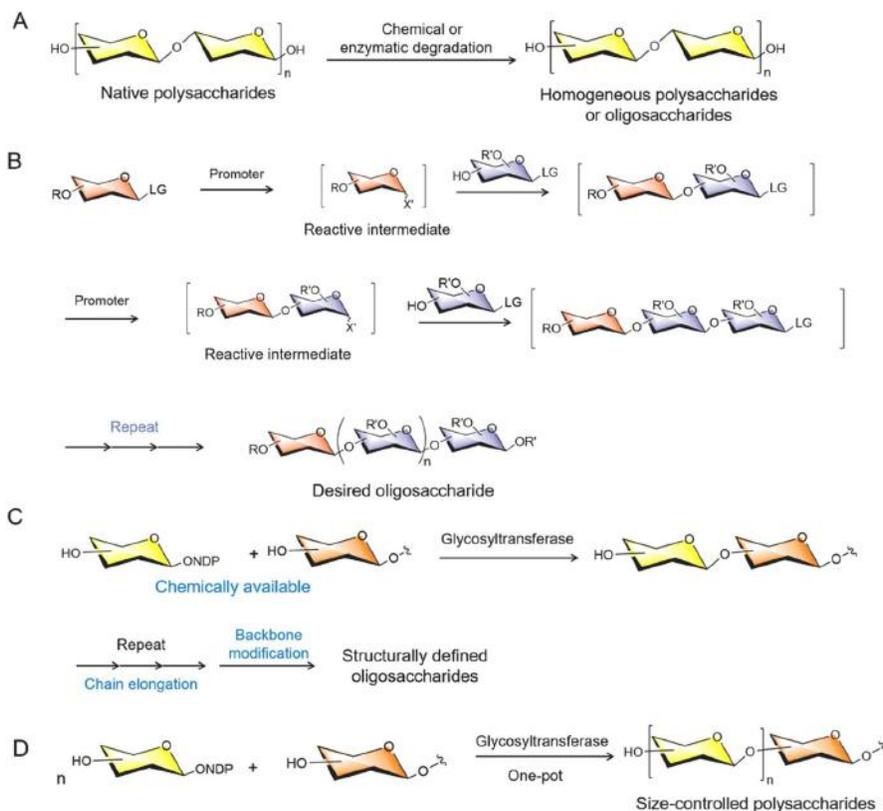


**Fig. 2.** General approaches for preparing homogeneous polysaccharides or structurally defined oligosaccharides. (A) Chemical or enzymatic degradation approach, (B) Pure chemical synthesis, where R and R' are protecting groups, and LG is leaving group, (C) Step-wise enzymatic synthesis assembly, and (D) One-pot polymerization catalyzed by glycosyltransferase.

2016). However, undesired by-products or isomers are prone to produce due to the hardly controlled stereoselectivity and regioselectivity. Furthermore, based on repetitive steps of protection, activation, coupling and deprotection, the chemical synthesis has the drawback of tedious synthetic and purification steps, and the overall yields are generally low, resulting inthe high production cost (Zhang et al., 2020). For example, Arixtra®, a U.S. Food and Drug Administration (FDA)-approved pentasaccharide anticoagulant drug, requires up to 60 chemical steps, and the overall yield is only 0.1 % (Petitou & van Boeckel, 2004). In addition, it is currently difficult to prepare oligosaccharides longer than five sugar units via chemical assembly. Moreover, there are no examples of the chemical synthesis of homogeneous polysaccharides. Therefore, probing more strategies compensating for the shortcomings of purely chemical method is very important for the rapid preparation of oligosaccharides or the preparation of polysaccharides.

### 2.2.2. Enzymatic or chemoenzymatic assembly

Compared with chemical synthesis, enzymatic synthesis has its own advantages including exquisite stereoselectivity and regioselectivity (DeAngelis, Liu, & Linhardt, 2013). Particularly chemoenzymatic synthesis, which integrates the flexibility of chemical methods with the specificity of enzyme-catalyzed reactions, thus, showing better prospects for the synthesis of homogeneous polysaccharides or defined oligosaccharides. In this strategy, the chemical methods are used to prepare natural or unnatural enzymatic substrates, donors or acceptors, and then the glycosyltransferases and the enzymes are responsible for sugar chain elongation and backbone modification, respectively. Nevertheless, enzymatic or chemoenzymatic synthesis are still step-wise and require relatively long reaction steps and large amounts of enzymes. Moreover, the large scale expression of enzymes is challenging and the cost of enzymes is typically high, making it difficult to meet the industrial need (Fig. 2C) (Xu et al., 2017).

### 2.2.3. One-pot assembly

Distinct from the above mentioned approaches, requiring numerous synthetic steps to construct sugar chain, one-pot polymerization undoubtedly represents the fastest way to produce homogeneous polysaccharides or defined oligosaccharides, since multi-step reactions are carried out in a single reaction system (Fig. 2D) (Chen et al., 2013; Yu & Chen, 2016). Moreover, a one-pot strategy, either involving chemical or enzymatic glycosylation, can also be conducted in a single flask where glycosyl donors are added sequentially to the reaction vessel to obtain the required target oligosaccharide (Wen et al., 2018). In this reaction system, the product of the former reaction can be the substrate of the next reaction. Therefore, the target glycan can be prepared without required isolation or purification of intermediates, greatly simplifying operation steps and reducing the cost. Unfortunately, one-pot polymerization is very challenging to control and typically performed in a random way. In this review, we examine the progress that has been made in the controlled one-pot synthesis of homogeneous glycans or oligosaccharides, providing a broad spectrum of options to access these size-controlled biological molecules.

## 3. Techniques for structure characterization/quantification of glycans

Liquid chromatography (LC), gas chromatography (GC), nuclear magnetic resonance (NMR) and mass spectrometry (MS), etc., are common techniques in the structure analysis and quantification of polysaccharides and oligosaccharides. High performance liquid chromatography (HPLC) is widely used in the separation and purification of oligosaccharides, composition analysis, quantitative determination and molecular weight determination (Wang et al., 2018). GC is suitable for the analysis of volatile sugar derivatives, mainly used in the analysis and determination of monosaccharide composition and uronic acid type

(Xie, Gong, & Yu, 2017). However, structure derivatization is necessary and the loss of sample components during this process may occur. MS is utilized for molecular weight determination of glycan (Wei, Tang, Bai, Zaia, & Costello, 2020), and often combined with LC to provide high-resolution, stable and fast continuous analysis platform. Capillary electrophoresis (CE) is an effective technology for separating molecules based on shape, size and charge, with the advantages of high sensitivity, high resolution and high separation efficiency (Ramautar, Somsen, & de Jong, 2017). NMR is widely used in the analysis of the purity and the fine structure of glycans (Kaufmann, Mugge, & Kroh, 2016), but the relatively lower sensitivity limits its certain applications. It's worth noting that the various analytical techniques introduced above can be applied to solve specific analytical problems or coupled to one another to improve the efficiency or accuracy for structure determination.

## 4. Representative synthetic examples of one-pot synthesis

### 4.1. Controlling molar ratio of the donor and acceptor

As sugar nucleotide donors are transferred to glycosyl acceptors by glycosyltransferases or synthases in glycan biosynthetic pathways, controlling the molar ratio of the donor and acceptor may be the promising and simplest way to prepare size-controlled polysaccharides. Fang et al. successfully prepared homogeneous hyaluronic acid (HA) polymers taking HA oligosaccharides as the starting receptors catalyzed by *P. multocida* (PmHAS) in a combined manner, involving the stepwise enzyme-catalyzed oligosaccharide synthesis and one-pot polysaccharide polymerization (Fig. 3A) (Fu et al., 2017). Different lengths of HA oligosaccharide acceptors were crucial in polymerization, and the polymerization initiates faster when HA trisaccharide or tetrasaccharide was used as the acceptor compared to mono- or disaccharide, and thus, displayed a very narrow molecular weight distribution. Moreover, as the chain length of the HA oligosaccharide receptor increases, the polydispersity indices of the formed HA polymer was significantly reduced. In addition, different acceptor/donor molar ratios (UDP-GlcNAc/UDP-GlcA 1:100, 1:500, and 1:1000) could be utilized to prepare various HA polymer in a desired range of chain length. This general synthetic strategy has been further exploited to prepare a library of homogeneous functionalized HA polymers, such as HA-biotin conjugates that can be used as probes for investigation of biological functions.

Using the similar composite strategy, the same group synthesized the homogenous chondroitin polymers with extremely narrow molecular weight distribution taking exogenous trisaccharides as the initial necessary receptors catalyzed by *P. multocida* (PmCS) (Fig. 3B) (Wang et al., 2020). The size of those unsulfated chondroitin polysaccharides (CH) polymers is linearly related to the molar ratios of acceptor/donor in the polymerization. In addition, the polydispersity index of formed chondroitin sulfate polymers is negatively correlated with the length of the corresponding exogenous chondroitin oligosaccharide acceptor, and the synthetic reaction yield can reach 80 %. Most importantly, by incorporating sugar nucleotide derivatives into a synthetic method, the strategy has been further expanded to prepare unnatural zwitterionic and *N*-sulfonated chondroitin polymers, which have potential applications in biological studies.

Recently, Chen et al. established a sequential one-pot multienzyme (OPME) platform for efficient synthesis of homogeneous *Neisseria meningitidis* serogroup W (NmW) capsular polysaccharide (CPS) oligosaccharides in a size-controlled manner using polysaccharide synthase NmSiaDw with a yield of 83–96 % (Li, Yu, Muthana, Freedberg, & Chen, 2020). They have also found that not only the size of the galactoside acceptor substrate but also the ratio of donor and acceptor substrate concentrations can control the size of the oligosaccharides produced.

### 4.2. Utilizing chain-terminators

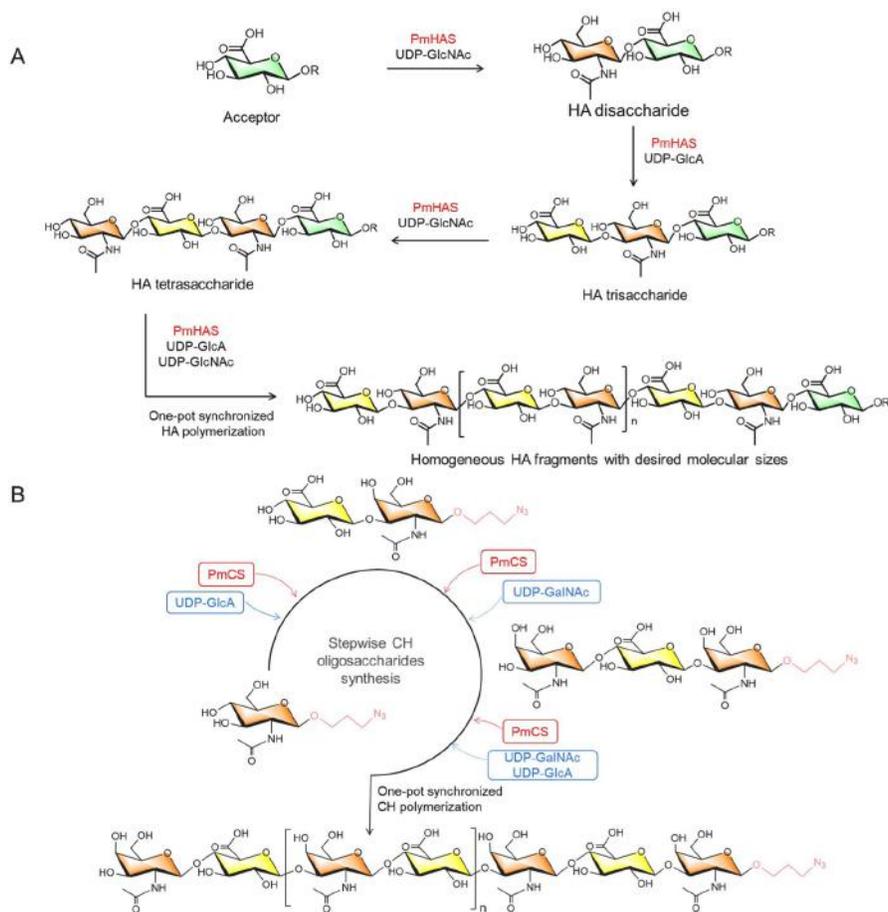In the recent years, substantial efforts have been made in the

**Fig. 3.** Controlling molar ratio of the donor/acceptor to enzymatically prepare homogeneous (A) hyaluronic acid and (B) unsulfated chondroitin polysaccharide.

investigation of glycosyltransferase inhibitors as a means of disrupting the specific biosynthetic pathways of glycans (Komor, Szeja, Komor, Pastuch-Gawolek, & Thiem, 2014). Particularly, a chain-termination strategy, which was originally developed by Sanger et al. as a means of controlling DNA sequence, has been demonstrated a powerful approach to control the chain length of polysaccharides or oligosaccharides. Due to the similarities in bond length and polarization between the C (carbon)−OH (hydroxyl) and C (carbon)-F (fluorine) groups, known as bioisosteres, fluorinated sugars can act as enzymatic

glycosylation inhibitors or chain-terminators (Linclau et al., 2020; Meanwell, 2018). Linhardt et al. reported the chemoenzymatic synthesis of UDP-4FHexNAc as a glycosylation terminator with a satisfactory yield of 45 % and 50 % under the catalysis of the uridine transferase GlmU for the synthesis of heparan sulfate/heparin oligosaccharides with a defined sequence length (Fig. 4). Compared with the natural donors such as UDP-*N*-acetylglucosamine (GlcNAc), UDP-*N*-acetylgalactosamine (GalNAc), and UDP-glucuronic acid (GlcA), the unnatural donors UDP-4FHexNAc lack a C4 hydroxyl group required to form glycosidic
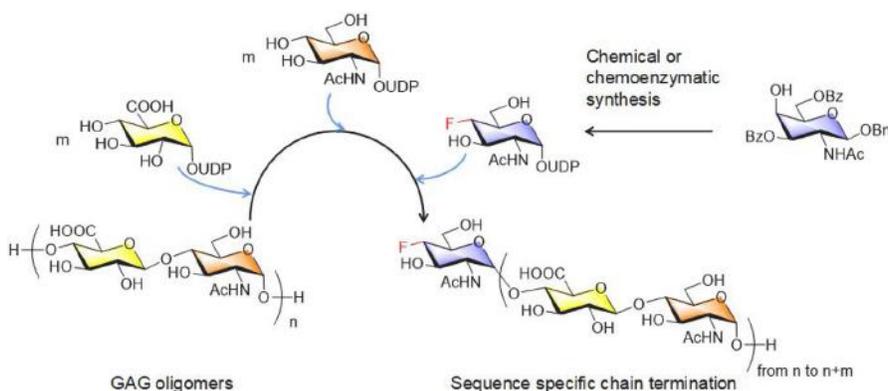


**Fig. 4.** Utilizing UDP-4FHexNAc as chain-terminator to regulate the sugar chain elongation in the glycosyltransferase catalyzed reaction.

bonds. Once this unnatural sugar is incorporated, the sugar chain is no longer extended further, leading to the termination of GAG chain synthesis (Schultz et al., 2017). This unnatural UDP sugar has great potential as a tool for assay development and a reagent for the study of glycoconjugate biosynthesis as well as a glycosylation terminator.

### 4.3. Enzyme engineering technique

Enzyme engineering can significantly improve the properties of enzymes, including broadening their substrate tolerance, increasing the stability, and improving enantioselectivity, thus, making these useful for controlled enzymatic polymerization. As a dual-function polysaccharide synthase, *Pasteurella multocida* heparosan synthase 2 (PmHS2) has both α1→4-*N*-acetylglucosamine transferase (α1→4-GlcNAcT) and β1→4-glucuronyltransferase (β1→4-GlcAT) activities. PmHS2 can efficiently synthesize short oligosaccharides up to hexasaccharides, while the yield of longer oligosaccharides is lower (Wu et al., 2015). Additionally, Δ80PmHS2 with 80 amino acid residues removed from the N-terminus has improved expression levels and stability, however, its reverse glycosylation activity leads to the formation of longer and shorter oligosaccharide byproducts, which increases the polydispersity of oligosaccharide products (Li et al., 2014). Therefore, Chen et al. engineered PmHS2 to generate monofunctional glycosyltransferase mutants, and applied these to the one-pot multi-enzyme method, achieving a high production yield of oligosaccharides and minimizing the generation of byproducts (Fig. 5) (Na et al., 2020). By mutating the key catalytic base residues of domains, D291 N and D569 N mutants of Δ80PmHS2 were generated as single functional β1→4-GlcAT and α1→4GlcNAcT with significantly decreased reverse α1→4-GlcNAcT and reverse β1→4-GlcAT glycosyltransferase activities, respectively. Most importantly, this mutagenesis strategy can be extended to other multifunctional polysaccharide synthases with reverse glycosylation activity to prepare catalysts with higher synthesis efficiency (Na et al., 2020).

Lipopolysaccharides (LPS) are essential outer membrane glycolipids for most gram-negative bacteria (Cress et al., 2014). As one of the components of lipopolysaccharide, O-antigenic polysaccharide (OPS), has a certain resistance to opsonophagocytosis and complement-mediated killing. Moreover, the different lengths of the polysaccharide chain of OPS has a specific purpose, which is very important for its biological functions. Therefore, it is important to understand the molecular mechanism of regulation by polysaccharide chain length (Lerouge & Vanderleyden, 2002). Whitfield *et.al* investigated how to control the expression of the functional protein WbbB, which integrates three key activities (polymerase, terminator and molecular ruler) to regulate the length of sugar molecules (Williams et al., 2017). In the ATP-binding cassette (ABC) transporter-dependent assembly pathway of OPS biosynthesis strategy, the polysaccharides were generated by the polymerase glycosyltransferase modules of WbbB, and then the polymerase activity is opposed using a chain terminator by adding residues that prevent further chain extension once the OPS chains reach the correct length (Greenfield & Whitfield, 2012). The coiled-coil structure between the polymerase and terminator catalytic sites provides the critical determinant of the OPS chain length (King, Berry, Clarke, Morris, & Whitfield, 2014). As a molecular ruler, systematically changing the size of the coiled-coil structure in WbbB can adjust the chain length. Furthermore, the ABC transporter possesses a substrate-specific carbohydrate binding module that recognizes the modified terminal structure offering a quality-control step to ensure the OPS chain lengths fall into a limited size range. In summary, the finding of the regulatory mechanism provides a basis for further understanding of glycan assembly mechanisms and as potential tools for glycoengineering.

In recent years, major efforts have been made towards the preparation of well-defined glycoproteins for both basic research and therapeutic purposes to remove the heterogeneity of naturally occurring glycoproteins (Li & Wang, 2018). For example, Cheng *et.al* formed a series of engineered glycosyltransferase mutants through site-saturated mutagenesis of a key residue in *N*-glycosyltransferase ApNGT (Gln-469), which significantly improves glycosylation efficiency and expands receptor specificity (Song et al., 2017). The most efficient mutant (Q469A) has been proven to efficiently modify proteins with multiple *N*-glycosylation sites, and its relatively extended acceptor selectivity allows fewer alterations toward acceptor proteins to reach uniform glycosylation. As a result, the synthesis of multi-site modified homogeneous sugars by engineered glycosyltransferase mutants provides a promising approach for the potential solution of the macroscopic heterogeneity of glycoproteins.
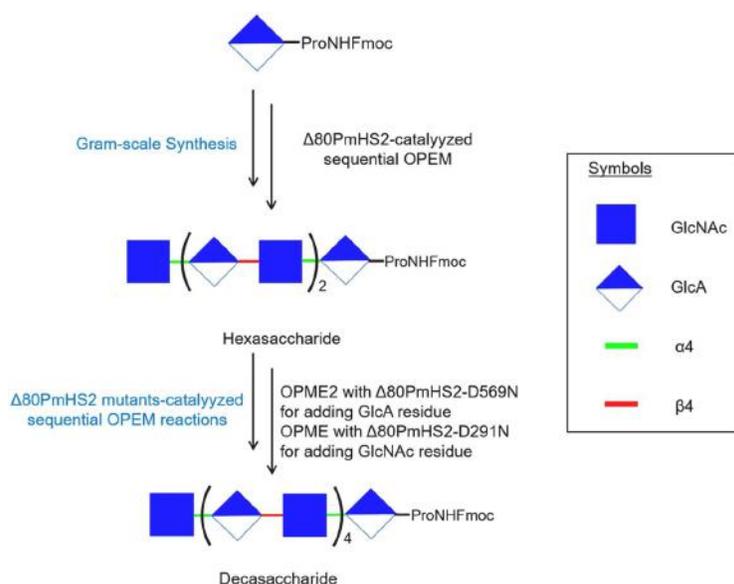


**Fig. 5.** Engineering PmHS2 utilized in the one-pot polymerization to prepare oligosaccharide.

### 4.4. Controlled free radical polymerization

Free radical polymerization is one of the most widely used chemical reactions in the field of polymer synthesis. Although it is still challenging to control the degree of polymerization and polydispersity, significant efforts have been expanded aiming at solving this problem (Meng et al., 2009). Han et al. successfully prepared a series of solid-solid phase change materials namely cellulose acrylate-g-poly (*n*-alkyl acrylate) (CA-g-PAn) (n = 14, 16 and 18) using free radical polymerization (Fig. 6A) (Qian et al., 2018). In this study, the coplymer were synthesized by dissolving different *n*-alkyl acrylates (n = 14, 16 and 18) with CA in dimethylacetamide (DMAc) using acrylic chloride as the coupling reagent. In contrast to most of the strategies that control the formation of sugar chains, this approach focuses on the regulation of non-carbohydrate chain elongation. The resulting special 'homogeneous glycoconjugate' provides good thermal stability and shape stability, thus, has potential applications in thermal energy storage and temperature control, as well as for the fabrication of temperature-sensitive drugs, reagents, fibers and textiles. However, this method has limited applicability in the preparation of other size-controlled polysaccharides. In summary, poor control of free radical polymerization is still a major limitation in accessing homogeneous glycans, and fundamental studies, such as the development of new initiating/catalytic systems as well as an expansion of the range of monomers required in future applications.

Photo-induced reactions are green and sustainable, and these have been widely applied in various aspects owing to their better stereoselectivity and efficiency than traditional methods and these can also be applied in glycosylation (Ghosh, Ghosh, Bardagi, & Konig, 2014). For example, Ye *et.al* developed a glycosyl coupling reaction utilizing the photo-induced method and successfully synthesized tumor-associated KH-1 antigen core nonasaccharide using a one-pot method and was the first successful application of photo-induced glycosylation in the synthesis of complex oligosaccharides (Fig. 6B) (Li, Qiao et al., 2020; Li, Yao et al., 2020). In this work, photo-induced reactions are used to construct all the key glycosidic bonds by employing the light-mediated homolytic cleavage of the C–S bond in thioglycosides with Cu(OTf)$_2$ as an exogenous oxidant, and subsequently using the Umemoto reagent as a stronger oxidant, the fully protected nonasaccharide was obtained in a yield of 90 %. However, this photo-glycosylation applies only to the construction of simple glycosidic bonds, and the application in the assembly of other complex oligosaccharides of biological importance still needs to be further explored (Fig. 7).

### 4.5. Solid-phase synthesis

Fluorous solid-phase extraction (FSPE) is a technique used for sample purification, separation and concentration, in which the sample conjugated to a fluorous tag can bind to the fluorous surface and then be easily released by fluorophilic elution (Zhang, 2003). The advantages of simplicity and wide application make fluorous tagging a good method for the synthesis of high-throughput combinations. Linhardt *et.al* successfully synthesized a series of heparan sulfate oligosaccharides combining the fluorous Boc ([F]Boc) linker and FSPE technique (Cai et al., 2014). Taking the disaccharide with a [F]Boc at the reducing end as the
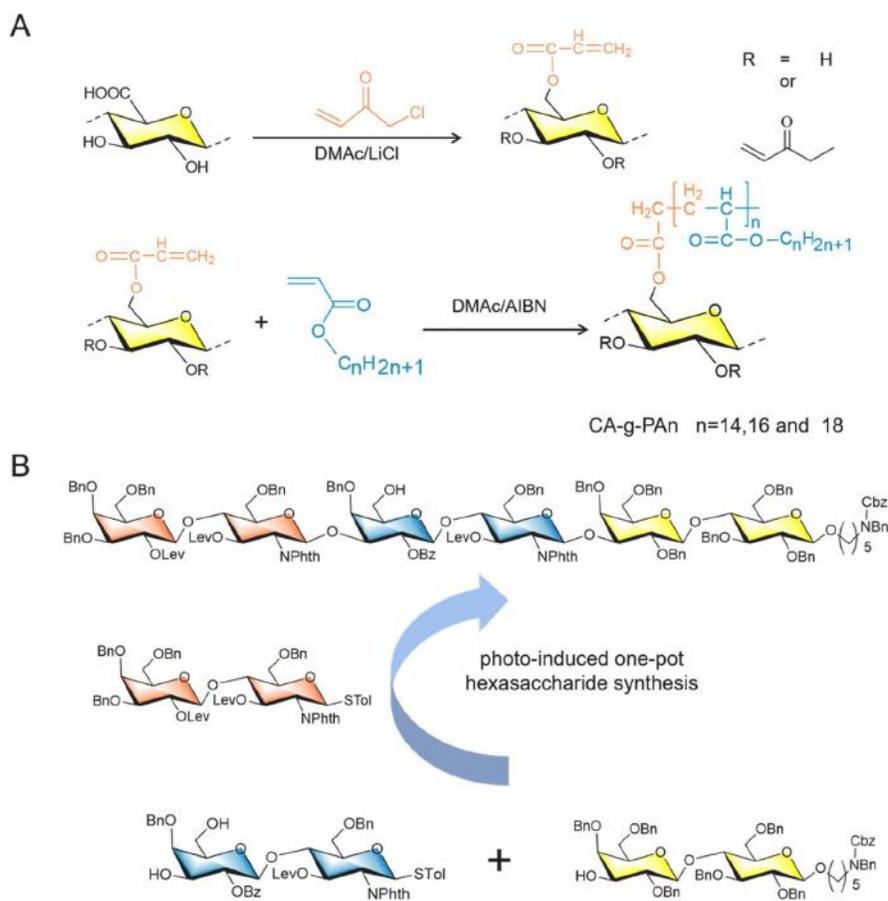


**Fig. 6.** Radical polymerization strategy. (A) Non-carbohydrate chain elongation involving the free radical polymerization of *n*-alkyl acrylates initiated by AIBN. (B) Photo-induced glycosylation in the synthesis of complex oligosaccharides.
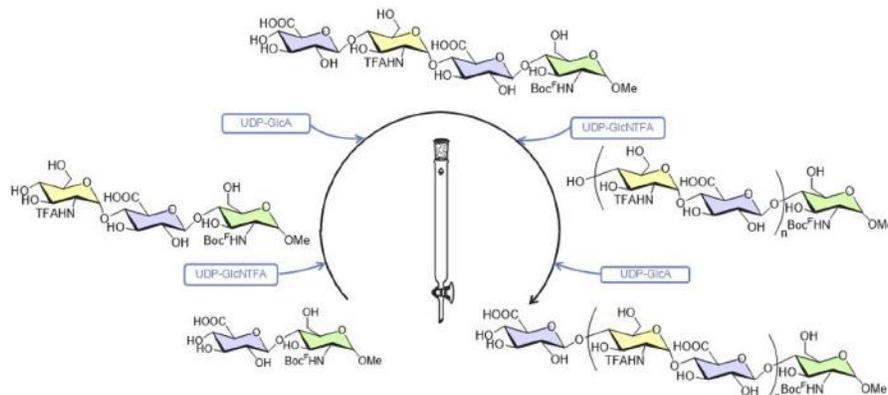
**Fig. 7.** Fluorous solid phase extraction technique utilized in the chemoenzymatic synthesis of heparan sulfate oligosaccharide.

acceptor, the sugar chain was extended with glycosyltransferases KifA and PmHS2 and modified by 6-OSTs followed by removing unwanted deletion sequences from the glycosylation mixture by FSPE to prepared oligosaccharides in one-pot method. It is noteworthy that the [F]Boc linker does not interfere with enzymatic recognition for both elongation and specific sulfation. Although different from the common one-pot polymerization that multi-step reactions are carried out in a single run, this one-pot strategy also avoids tedious isolation and purification of intermediates.

## 5. Conclusion and perspectives

In conclusion, in this review, we provide an overview of the synthetic strategies to obtain size-controllable or homogeneous glycans using a one-pot method. Although traditional chemical methods are still the major ways of preparing homogeneous oligosaccharides, problems such as lengthy steps, complicated purification operations and limited product size still exist. The one-pot method greatly simplifies the operation procedure and reduces the cost of preparing homogeneous polysaccharides. While this method holds promise, many challenges remain in this field and it is far from kilogram scale synthesis. Approaches such as controlling the molar ratio of donor-acceptor, using terminator, enzyme mutation, free radical polymerization, and solid phase synthesis, provide a variety of options for preparing homogeneous glycans, that can also increase the overall yield, reduce the generation of by-products, and facilitate further industrial applications in the future.

## Acknowledgements

## References

Ang, L. F., Por, L. Y., & Yam, M. F. (2013). Study on different molecular weights of chitosan as an immobilization matrix for a glucose biosensor. *PloS One, 8*(8).

Boltje, T. J., Buskas, T., & Boons, G. J. (2009). Opportunities and challenges in synthetic oligosaccharide and glycoconjugate research. *Nature Chemistry, 1*(8), 611–622.

Cai, C., Dickinson, D. M., Li, L., Masuko, S., Suflita, M., Schultz, V., et al. (2014). Fluorous-assisted chemoenzymatic synthesis of heparan sulfate oligosaccharides. *Organic Letters, 16*(8), 2240–2243.

Chen, Y., Li, Y., Yu, H., Sugiarto, G., Thon, V., Hwang, J., et al. (2013). Tailored design and synthesis of heparan sulfate oligosaccharide analogues using sequential one-pot multienzyme systems. *Angewandte Chemie-International Edition, 52*(45), 11852–11856.

Clausen, T. M., Sandoval, D. R., Spliid, C. B., Pihl, J., Perrett, H. R., Painter, C. D., et al. (2020). SARS-CoV-2 infection depends on cellular heparan sulfate and ACE2. *Cell*.

Colombo, C., Pitirollo, O., & Lay, L. (2018). Recent advances in the synthesis of glycoconjugates for vaccine development. *Molecules, 23*(7).

Cress, B. F., Englaender, J. A., He, W., Kasper, D., Linhardt, R. J., & Koffas, M. A. G. (2014). Masquerading microbial pathogens: Capsular polysaccharides mimic host-tissue molecules. *FEMS Microbiology Reviews, 38*(4), 660–697.

DeAngelis, P. L., Liu, J., & Linhardt, R. J. (2013). Chemoenzymatic synthesis of glycosaminoglycans: Re-creating, re-modeling and re-designing nature's longest or most complex carbohydrate chains. *Glycobiology, 23*(7), 764–777.

Dwek, R. A. (1996). Glycobiology: Toward understanding the function of sugars. *Chemical Reviews, 96*(2), 683–720.

Eikenes, M., Alfredsen, G., Christensen, B. E., Militz, H., & Solheim, H. (2005). Comparison of chitosans with different molecular weights as possible wood preservatives. *Journal of Wood Science, 51*(4), 387–394.

Fu, X., Shang, W. J., Wang, S. S., Liu, Y. P., Qu, J. Y., Chen, X., et al. (2017). A general strategy for the synthesis of homogeneous hyaluronan conjugates and their biological applications. *Chemical Communications, 53*(25), 3555–3558.

Ghosh, I., Ghosh, T., Bardagi, J. I., & Konig, B. (2014). Reduction of aryl halides by consecutive visible light-induced electron transfer processes. *Science, 346*(6210), 725–728.

Greenfield, L. K., & Whitfield, C. (2012). Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways. *Carbohydrate Research, 356*, 12–24.

Guerrini, M., Shriver, Z., Naggi, A., Casu, B., Linhardt, R. J., Torri, G., et al. (2010). Oversulfated chondroitin sulfate is the major contaminant in suspect heparin lots collected in February/March of 2008. *Nature Biotechnology, 28*, 207–211.

Haltiwanger, R. S., & Lowe, J. B. (2004). Role of glycosylation in development. *Annual Review of Biochemistry, 73*, 491–537.

Hou, C. D., Zhao, X. H., Tian, M. Q., Zhou, Y. L., Yang, R. Q., Gu, Z. X., et al. (2020). Impact of water extractable arabinoxylan with different molecular weight on the gelatinization and retrogradation behavior of wheat starch. *Food Chemistry, 318*.

Jefferis, R. (2009). Glycosylation as a strategy to improve antibody-based therapeutics. *Nature Reviews Drug Discovery, 8*(3), 226–234.

Ji, Y., Li, B. Z., Qiao, M., Li, J. M., Xu, H., Zhang, L. H., et al. (2020). Advances on the in vivo and in vitro glycosylations of flavonoids. *Applied Microbiology and Biotechnology, 104*(15), 6587–6600.

Ji, Y., Zhang, S., Qiao, M., Jiao, R., Li, J., Song, P., et al. (2020). Synthesis of structurally defined chondroitin sulfate: Paving the way to the structure-activity relationship studies. *Carbohydrate Polymers, 248*.

Kaufmann, M., Mugge, C., & Kroh, L. W. (2016). Carbohydrate isomer systems examination by dynamic NMR-Spectroscopy. *Deutsche Lebensmittel-Rundschau : Zeitschrift Fur Lebensmittelkunde Und Lebensmittelrecht, 112*(3), 94–98.

King, J. D., Berry, S., Clarke, B. R., Morris, R. J., & Whitfield, C. (2014). Lipopolysaccharide O antigen size distribution is determined by a chain extension complex of variable stoichiometry in Escherichia coli O9a. *Proceedings of the National Academy of Sciences of the United States of America, 111*(17), 6407–6412.

Kitajima, S., Takuma, S., & Morimoto, M. (2000). Histological analysis of murine colitis induced by dextran sulfate sodium of different molecular weights. *Experimental Animals, 49*(1), 9–15.

Komor, K., Szeja, W., Komor, R., Pastuch-Gawolek, G., & Thiem, J. (2014). Synthesis of fucosylated uridine conjugates as potential glycosyltransferase inhibitors. *Acta Poloniae Pharmaceutica, 71*(6), 1083–1089.

Kwon, P. S., Oh, H., Kwon, S. J., Jin, W., Zhang, F., Fraser, K., et al. (2020). Sulfated polysaccharides effectively inhibit SARS-CoV-2 in vitro. *Cell Discovery, 6*, 50.

Lerouge, I., & Vanderleyden, J. (2002). O-antigen structural variation: Mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiology Reviews, 26* (1), 17–47.

Li, C., & Wang, L. X. (2018). Chemoenzymatic methods for the synthesis of glycoproteins. *Chemical Reviews, 118*(17), 8359–8413.

Li, S. J., Xiong, Q. P., Lai, X. P., Li, X., Wan, M., Zhang, J. N., et al. (2016). Molecular modification of polysaccharides and resulting bioactivities. *Comprehensive Reviews in Food Science and Food Safety, 15*(2), 237–250.

Li, Y. H., Yu, H., Thon, V., Chen, Y., Muthana, M. M., Qu, J. Y., et al. (2014). Donor substrate promiscuity of the N-acetylglucosaminyltransferase activities of Pasteurella multocida heparosan synthase 2 (PmHS2) and Escherichia coli K5 KfiA. *Applied Microbiology and Biotechnology, 98*(3), 1127–1134.

Li, K., Li, S. Y., Du, Y. G., & Qin, X. M. (2020). Screening and structure study of active components of Astragalus polysaccharide for injection based on different molecular weights. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 1152*.

Li, J., Qiao, M., Ji, Y., Lin, L., Zhang, X., & Linhardt, R. J. (2020). Chemical, enzymatic and biological synthesis of hyaluronic acids. *International Journal of Biological Macromolecules, 152*, 199–206.

Li, B.-H., Yao, W., Yang, H., Wu, C., Xiong, D.-C., Yin, Y., et al. (2020). Total synthesis of tumor-associated KH-1 antigen core nonasaccharide via photo-induced glycosylation. *Organic Chemistry Frontiers, 7*(10), 1255–1259.

Li, R. Y., Yu, H., Muthana, S. M., Freedberg, D. I., & Chen, X. (2020). Size-Controlled chemoenzymatic synthesis of homogeneous oligosaccharides of Neisseria meningitidis W capsular polysaccharide. *ACS Catalysis, 10*(4), 2791–2798.

Lin, L., Qiao, M., Zhang, X., & Linhardt, R. J. (2020). Site-selective reactions for the synthesis of glycoconjugates in polysaccharide vaccine development. *Carbohydrate Polymers, 230*, Article 115643.

Linclau, B., Arda, A., Reichardt, N.-C., Sollogoub, M., Unione, L., Vincent, S. P., et al. (2020). Fluorinated carbohydrates as chemical probes for molecular recognition studies. Current status and perspectives. *Chemical Society Reviews, 49*(12), 3863–3888.

Liu, J., & Linhardt, R. J. (2014). Chemoenzymatic synthesis of heparan sulfate and heparin. *Natural Product Reports, 31*(12), 1676–1685.

Liu, J., Willför, S., & Xu, C. (2015). A review of bioactive plant polysaccharides: Biological activities, functionalization, and biomedical applications. *Bioactive Carbohydrates and Dietary Fibre, 5*(1), 31–61.

Liu, Y.-Y., Lee, C.-H., Dedaj, R., Zhao, H., Mrabat, H., Sheidlin, A., et al. (2008). High-molecular-weight hyaluronan - a possible new treatment for sepsis-induced lung injury: A preclinical study in mechanically ventilated rats. *Critical Care, 12*(4).

Lovegrove, A., Edwards, C. H., De Noni, I., Patel, H., El, S. N., Grassby, T., et al. (2017). Role of polysaccharides in food, digestion, and health. *Critical Reviews in Food Science and Nutrition, 57*(2), 237–253.

Macauley, M. S., Crocker, P. R., & Paulson, J. C. (2014). Siglec-mediated regulation of immune cell function in disease. *Nature Reviews Immunology, 14*(10), 653–666.

Meanwell, N. A. (2018). Fluorine and fluorinated motifs in the design and application of bioisosteres for drug design. *Journal of Medicinal Chemistry, 61*(14), 5822–5880.

Mende, M., Bednarek, C., Wawryszyn, M., Sauter, P., Biskup, M. B., Schepers, U., et al. (2016). Chemical synthesis of glycosaminoglycans. *Chemical Reviews, 116*(14), 8193–8255.

Meng, T., Gao, X., Zhang, J., Yuan, J. Y., Zhang, Y. Z., & He, J. S. (2009). Graft copolymers prepared by atom transfer radical polymerization (ATRP) from cellulose. *Polymer, 50*(2), 447–454.

Na, L., Yu, H., McArthur, J. B., Ghosh, T., Asbell, T., & Chen, X. (2020). Engineer P. multocida heparosan synthase 2 (PmHS2) for size-controlled synthesis of longer heparosan oligosaccharides. *ACS Catalysis, 10*(11), 6113–6118.

Ngo, D. N., Lee, S. H., Kim, M. M., & Kim, S. K. (2009). Production of chitin oligosaccharides with different molecular weights and their antioxidant effect in RAW 264.7 cells. *Journal of Functional Foods, 1*(2), 188–198.

Nimmerjahn, F., & Ravetch, J. V. (2008). Fc gamma receptors as regulators of immune responses. *Nature Reviews Immunology, 8*(1), 34–47.

Petitou, M., & van Boeckel, C. A. A. (2004). A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? *Angewandte Chemie-International Edition, 43*(24), 3118–3133.

Qian, Y., Han, N., Bo, Y., Tan, L., Zhang, L., & Zhang, X. (2018). Homogeneous synthesis of cellulose acrylate-g-poly (n-alkyl acrylate) solid-solid phase change materials via free radical polymerization. *Carbohydrate Polymers, 193*, 129–136.

Qin, J., Wang, H. Y., Zhuang, D., Meng, F. C., Zhang, X., Huang, H. (2019). Structural characterization and immunoregulatory activity of two polysaccharides from the rhizomes of Atractylodes lancea (Thunb.) DC. *International Journal of Biological Macromolecules, 136*, 341–351.

Ramautar, R., Somsen, G. W., & de Jong, G. J. (2017). CE-MS for metabolomics: Developments and applications in the period 2014-2016. *Electrophoresis, 38*(1), 190–202.

Schultz, V. L., Zhang, X., Linkens, K., Rimel, J., Green, D. E., DeAngelis, P. L., et al. (2017). Chemoenzymatic synthesis of 4-Fluoro-N-acetylhexosamine uridine diphosphate donors: Chain terminators in glycosaminoglycan synthesis. *The Journal of Organic Chemistry, 82*(4), 2243–2248.

Song, Q. T., Wu, Z. G., Fan, F. Y., Song, W. R., Zhang, P. R., Wang, L., et al. (2017). Production of homogeneous glycoprotein with multisite modifications by an engineered N-glycosyltransferase mutant. *The Journal of Biological Chemistry, 292* (21), 8856–8863.

Stern, R., & Jedrzejas, M. J. (2006). Hyaluronidases: Their genomics, structures, and mechanisms of action. *Chemical Reviews, 106*(3), 818–839.

Stern, R., Asari, A. A., & Sugahara, K. N. (2006). Hyaluronan fragments: An information-rich system. *European Journal of Cell Biology, 85*(8), 699–715.

Sun, T., Li, J. R., Qin, Y. Y., Xie, J., Xue, B., Li, X. H., et al. (2020). Rheological and functional properties of oat beta-glucan with different molecular weight. *Journal of Molecular Structure, 1209*.

Szajek, A. Y., Chess, E., Johansen, K., Gratzl, G., Gray, E., Keire, D., et al. (2016). The US regulatory and pharmacopeia response to the global heparin contamination crisis. *Nature Biotechnology, 34*(6), 625–630.

Tian, Y. X., Qiao, S. T., Qiu, N. X., Deng, H., & Guo, Y. R. (2011). Characterization of apple pectin fractionated by ultrafiltration. *Food Science, 30*(24), 197–201.

Trombotto, S., Ladaviere, C., Delolme, F., & Domard, A. (2008). Chemical preparation and structural characterization of a homogeneous series of chitin/chitosan oligomers. *Biomacromolecules, 9*(7), 1731–1738.

Varki, A. (1993). Biological roles of oligosaccharides: All of the theories are correct. *Glycobiology, 3*(2), 97–130.

Varki, A. (2017). Biological roles of glycans. *Glycobiology, 27*(1), 3–49.

Volpi, N., Schiller, J., Stern, R., & Soltes, L. (2009). Role, metabolism, chemical modifications and applications of hyaluronan. *Current Medicinal Chemistry, 16*(14), 1718–1745.

Wang, Y. Q., Li, S., Xu, X., Tan, Y. J., Liu, X. W., & Fang, J. Q. (2020). Chemoenzymatic synthesis of homogeneous chondroitin polymers and its derivatives. *Carbohydrate Polymers, 232*.

Wang, Z. J., Zhang, T. J., Xie, S. S., Liu, X. Y., Li, H. M., Linhardt, R. J., et al. (2018). Sequencing the oligosaccharide pool in the low molecular weight heparin dalteparin with offline HPLC and ESI-MS/MS. *Carbohydrate Polymers, 183*, 81–90.

Wei, J., Tang, Y., Bai, Y., Zaia, J., Costello, C. E., et al. (2020). Toward automatic and comprehensive glycan characterization by online PGC-LC-EED MS/MS. *Analytical Chemistry, 92*(1), 782–791.

Wen, L. Q., Edmunds, G., Gibbons, C., Zhang, J. B., Gadi, M. R., Zhu, H. L., et al. (2018). Toward automated enzymatic synthesis of oligosaccharides. *Chemical Reviews, 118* (17), 8151–8187.

Williams, D. M., Ovchinnikova, O. G., Koizumi, A., Mainprize, I. L., Kimber, M. S., Lowary, T. L., et al. (2017). Single polysaccharide assembly protein that integrates polymerization, termination, and chain-length quality control. *Proceedings of the National Academy of Sciences of the United States of America, 114*(7), E1215–E1223.

Wu, B., Wei, N., Thon, V., Wei, M., Yu, Z., Xu, Y., et al. (2015). Facile chemoenzymatic synthesis of biotinylated heparosan hexasaccharide. *Organic & Biomolecular Chemistry, 13*(18), 5098–5101.

Xie, W.-Q., Gong, Y.-X., & Yu, K.-X. (2017). Determination of total sugar content in lignocellulosic hydrolysates by using a reaction headspace gas chromatographic technique. *Cellulose, 24*(11), 4591–4597.

Xu, Y., Chandarajoti, K., Zhang, X., Pagadala, V., Dou, W., Hoppensteadt, D. M., et al. (2017). Synthetic oligosaccharides can replace animal-sourced low-molecular weight heparins. *Science Translational Medicine, 9*(406).

Xu, Y. M., Masuko, S., Takieddin, M., Xu, H. M., Liu, R. P., Jing, J., et al. (2011). Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins. *Science, 334*(6055), 498–501.

Yamaguchi, F., Uchida, S., Watabe, S., Kojima, H., Shimizu, N., & Hatanaka, C. (1995). Relationship between molecular weights of pectin and hypocholesterolemic effects in rats. *Bioscience, Biotechnology, and Biochemistry, 59*(11), 2130–2131.

Yu, H., & Chen, X. (2016). One-pot multienzyme (OPME) systems for chemoenzymatic synthesis of carbohydrates. *Organic & Biomolecular Chemistry, 14*(10), 2809–2818.

Yuan, P. H., Lv, M. X., Jin, P., Wang, M., Du, G. C., Chen, J., et al. (2015). Enzymatic production of specifically distributed hyaluronan oligosaccharides. *Carbohydrate Polymers, 129*, 194–200.

Zhang, W. (2003). Fluorous technologies for solution-phase high-throughput organic synthesis. *Tetrahedron, 59*(25), 4475–4489.

Zhang, G. L., & Ye, X. S. (2018). Synthetic Glycans and Glycomimetics: A promising alternative to natural polysaccharides. *Chemistry, 24*(26), 6696–6704.

Zhang, X., Lin, L., Huang, H., & Linhardt, R. J. (2020). Chemoenzymatic synthesis of glycosaminoglycans. *Accounts of Chemical Research, 53*(2), 335–346.

Zhang, X., Xu, Y. M., Hsieh, P. H., Liu, J., Lin, L., Schmidt, E. P., et al. (2017). Chemoenzymatic synthesis of unmodified heparin oligosaccharides: Cleavage of p-nitrophenyl glucuronide by alkaline and Smith degradation. *Organic & Biomolecular Chemistry, 15*(5), 1222–1227.

Zhang, X., Zhang, L., Dai, Y., Tang, W., & Jin, R. (2014). Rheological property of solutions of high molecular weight bacterial cellulose in LiCI/N, N-dimethylacetamide. *New Chemical Materials, 42*(10), 140–142.