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REVIEW

Bioengineered production of glycosaminoglycans and their analogues

Weihua Jin[1,2] · Fuming Zhang[2] · Robert J. Linhardt[2,3]

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Abstract
Glycosaminoglycans (GAGs) are a class of linear polysaccharides, consisting of alternating disaccharide sequences of uronic acid and hexosamines (or galactose) with and without sulfation. They can interact with various proteins, such as growth factors, receptors and cell adhesion molecules, endowing these with various biological and pharmacological activities. Such activities make GAGs useful in health care products and medicines. Currently, all GAGs, with the exception of hyaluronan, are produced by extraction from animal tissues. However, limited availability, poor control of animal tissues, impurities, viruses, prions, endotoxins, contamination and other problems have increased the interest in new approaches for GAG production. These new approaches include GAGs production by chemical synthesis, chemoenzymatic synthesis and metabolic engineering. One chemically synthesized heparin pentasaccharide, fondaparinux sodium, is in clinical use. Mostly, hyaluronan today is prepared by microbial fermentation, largely replacing hyaluronan from rooster comb. The recent gram scale chemoenzymatic synthesis of a heparin dodecasaccharide suggests its potential to replace currently used animal-sourced low molecular weight heparin (LMWH). Despite these considerable successes, such high-tech approaches still cannot meet worldwide demands for GAGs. This review gives a brief introduction on the manufacturing of unfractionated and low molecular weight heparins, the chemical synthesis and chemoenzymatic synthesis of GAGs and focuses on the progress in the bioengineered preparation of GAGs, particularly heparin.

Keywords Glycosaminoglycans · Metabolic engineering · Chemoenzymatic synthesis · Heparin

Introduction

Glycosaminoglycans (GAGs) are a class of linear polysaccharides, consisting of alternating disaccharides units, as shown in Fig. 1. GAGs are usually divided into four major types: heparin and heparan sulfate (HS), chondroitin sulfate (CS) and dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (hyaluronic acid, HA). With the exception of keratan sulfate, the disaccharide units of GAGs include anuronic acid [glucuronic acid (GlcA) or iduronic acid (IdoA)] and ahexosamine (glucosamine or galactosamine). In keratan sulfate, the hexosamine residues are replaced by galactose. GAGs have a variety of "fine structures" (i.e., fully characterized chemical structures) because of their variable molecular weight and sulfation patterns, which are attributed to variation in its biosynthetic pathway, associated with translation and location of biosynthetic enzymes such as glycosyltransferases, sulfotransferases and epimerases. The differences in the expression levels of these enzymes in different cells and tissues result in the structure heterogeneity of GAGs and their roles in various physiological and pathological processes [1].

With the exception of hyaluronic acid, GAGs are normally covalently linked to different core proteins to form the proteoglycans (PGs) that make up much of the extracellular matrix (ECM) [2]. GAGs, either on their own or as a component of PGs, interact with a large number of different proteins, including growth factors, receptors and chemokines

✉ Fuming Zhang
zhangf2@rpi.edu

✉ Robert J. Linhardt
linhar@rpi.edu

[1] College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, China

[2] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

[3] Department of Biological Science, Departments of Chemistry and Chemical Biology and Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

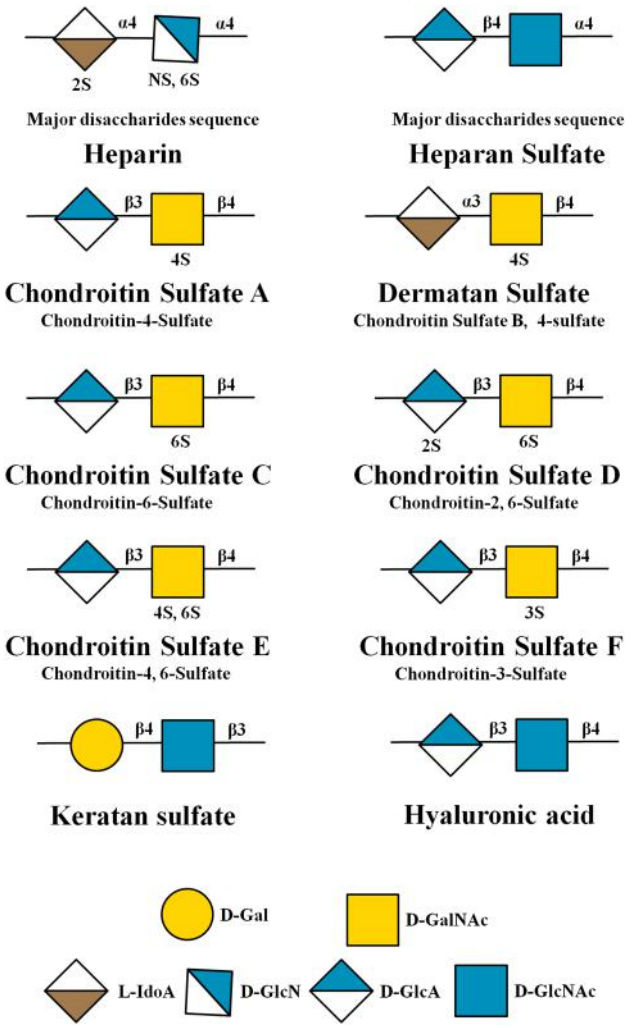

Fig. 1 Chemical structures of glycosaminoglycans. α3, α4, β3 and β4 represent the glyosidic linkage to the "C3" or "C4" carbons of the adjacent saccharide residue. The 2S, NS, 3S, 4S and 6S represent the sulfation position of the corresponding residues

[3–7]. These interactions play vital roles in the various physiological processes such as regulation, cell migration, growth and differentiation, and pathological processes such as metastasis, neurodegeneration and inflammation [8–13].

Owing to the various biological activities of GAGs, methods used in the manufacture of GAGs are particularly important. Traditionally, most GAGs are produced by extraction from animal tissues. The animal tissue-based extractions have many disadvantages such as poor control of animal tissues, impurities, endotoxin and potential viral and prion infection. These problems have driven research in new approaches for GAGs production including chemical synthesis, chemoenzymatic synthesis and metabolic engineering. This review gives a brief introduction of the manufacturing of unfractionated and low molecular weight heparins, the chemical synthesis and chemoenzymatic synthesis of GAGs and focuses on progress in the bioengineered preparation of GAGs, particularly heparin.

Pharmaceutical heparin or unfractionated heparin and low molecular weight heparins (LMWHs)

Heparin is widely used as an anticoagulant drug. Heparin is biosynthesized in the endoplasmic reticulum (ER) and Golgi of mast cells, which can be found at high levels in the liver, intestines and lungs [14]. Pharmaceutical heparin is primarily extracted from porcine intestines. However, other sources, such as bovine intestine and bovine lung, are currently being studied as potential substitutes [14, 15]. Commercial heparin is prepared through a five-step procedure (Fig. 2): (1) preparation of tissues; (2) extraction; (3) recovery of raw heparin; (4) purification; and (5) purified heparin recovery [16–18]. Only the heparin purification and recovery steps 4 and 5 are performed under current good manufacturing practice (cGMP) to obtain heparin as an active pharmaceutical ingredient (API) [18, 19].

Low molecular weight heparins (LMWHs) are manufactured by the controlled chemical or enzymatic depolymerization of heparin-active pharmaceutical ingredient (API) (Fig. 2) [15, 16]. There are a number of methods used for the preparation of LMWHs. Controlled and selective oxidation, using hydrogen peroxide or nitrous acid is often used to obtain LMWH that is then purified by ultrafiltration, fractional precipitation or anion exchange chromatography [15, 16, 20]. LMWH can also be obtained by enzymatic degradation using a heparinase through the β-eliminative cleavage or a hydrolysis reaction [15, 16, 21].

There was a heparin crisis resulting in the deaths of over 100 patients reportedly involving the adulteration of heparin with oversulfated chondroitin sulfate adversely impacting the supply of heparin in 2008 [19, 22]. The crisis raised a question about the quality control of heparin from animal tissues. The animal tissue supply chain at slaughterhouses lacks CGMP oversight [16]. Thus, heparin can be obtained from multiple animal species and different tissues (porcine intestines, bovine lung, ovine intestines and bovine intestines) and blended [23–26]. Moreover, animal source material can be infected with viral or prion diseases, including ones causing porcine epidemics, bovine spongiform encephalopathy and scrapies [27–29]. Finally, there have been spot shortages of commercial animal-derived heparin, emphasizing a need to develop alternative sources through synthesis or metabolic engineering.

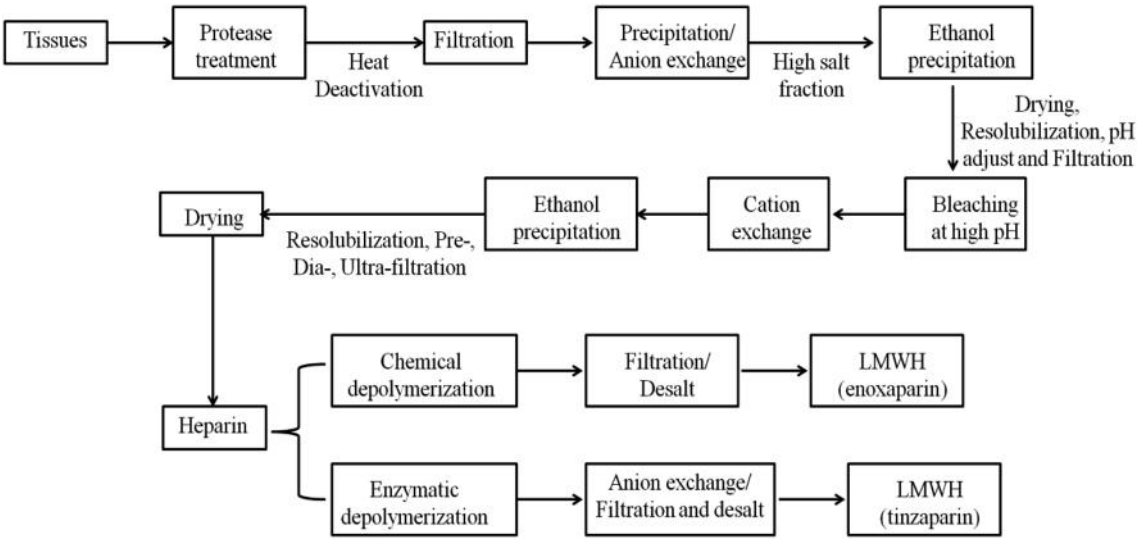

Fig. 2 The preparation of commercial heparin and low molecular weight heparins (LMWHs). Other methods (not shown) include oxidative methods such as treatment with hydrogen peroxide, nitrous acid, or radiative energy

Chemical synthesis of GAGs and their analogues

There are a number of studies and reviews on the chemical synthesis of GAGs and their analogues (i.e., linear polyanions with GAG-like properties) [30–33]. In the chemical synthesis of GAGs, three general considerations include building block design, elongation strategy and elongation chemistry [30]. With respect to building block design for GAGs synthesis, there are several challenges: the introduction of protection groups; control of the carboxylate functionality of the uronic acid moiety; control of the amino functionality of the hexosamine; and control of the sulfation pattern. There are two major strategies for GAGs chain elongation, stepwise and convergent synthesis. The general methods used in chemical synthesis of GAGs, using heparin as an example, are summarized in Fig. 3. Solid-phase GAG synthesis poses additional challenges including the optimization of solid supports, linker, attachment, and the chemistry of the glycosylation reaction and the cleavage of product from the resin [30, 34]. The most commonly used elongation method involves Schmidt glycosylation using trichloroacetimidates. In addition, glycosyl bromides and thioglycoside donors have also been used in glycosylation reactions. Some challenges still remain in the chemical synthesis of GAGs, such as low product yields, too many steps, particularly for selective protection and deprotection, and high costs. Moreover, it remains difficult to synthesize large oligosaccharides and polysaccharides.

Chemoenzymatic synthesis of GAGs and their analogues

There are many excellent studies and reviews on the chemoenzymatic synthesis of GAGs and their analogues [35–44]. The key elements required for the chemoenzymatic synthesis of GAGs are enzymes, co-factors, sugar substrates and sulfo donors [44, 45]. Most of the enzymes for biosynthesis of heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate have been well characterized [44, 45]. The synthetic scheme for chemoenzymatic synthesis of low molecular weight heparin is shown in Fig. 4. Chemoenzymatic synthesis involves two major steps: the first is to build the backbone using different sugar substrates and enzymes and the second is to modify the backbone using epimerases and sulfotransferases. Even though the chemoenzymatic synthesis of heparin can reach the gram scale in research laboratories, due to the improvement of enzyme efficiency, expression levels and improvements in the synthesis of enzyme co-factors, questions still remain concerning the cost and scalability of such processes.

Metabolic engineering for GAGs and their analogues

Metabolic engineering is the process of optimizing genetic and regulatory process that results in the production of a desired product accompanied by the suppression of competing pathways, through the transfer of product-specific

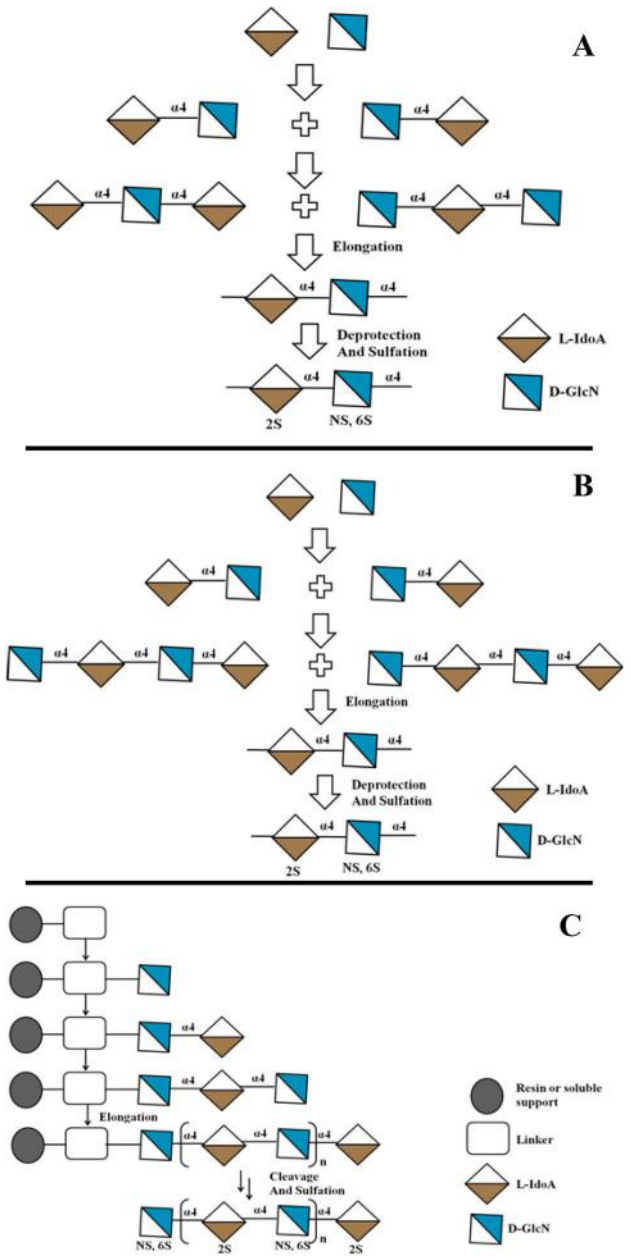

Fig. 3 **a** Strategy for the stepwise synthesis of heparin; **b** strategy for the convergent synthesis of heparin; and **c** strategy for the solid-phase synthesis of heparin

enzymes or complete metabolic pathways from the intractable host organism into a more easily manipulated and readily available engineered microorganism [15, 46–50].

The potential shortage and safety concerns about commercial GAGs have pushed a new direction from traditional animal-sourced methods of GAG production to biomanufacturing. Synthetic biology techniques are becoming increasingly important tools for pathway optimization and metabolic engineering [15]. Some GAGs including chondroitin and hyaluronic acid have been prepared using metabolic engineering [45, 51–57]. Currently, commercial scale production of hyaluronan relies on bacterial expression systems in *Streptococci* and endotoxin-free microorganisms such as *Bacilli*.

In general, there are three issues regarding the biomanufacturing of GAGs, productivity, efficiency and cost. Even though much work has been carried out on the biomanufacturing of non-sulfated heparin-precursor, heparosan and the chondroitin backbone of chondroitin sulfate by fermentation, modifications involving epimerization and specific sulfations are still required to produce the desired final GAGs products [15, 58].

Heparin is biosynthesized as a proteoglycan in the Golgi of eukaryotic mast cells [8, 59]. Heparin might 1 day be biosynthesized in eukaryotic systems, such as insect cells, yeast and Chinese hamster ovary (CHO) cells, having a Golgi [15, 18, 60]. However, the engineering of bacterial expression systems for GAG production is much more complex.

For the biomanufacturing of other GAGs and their analogues, there are currently two systems under evaluation, eukaryotic systems (including mammalian cells) and prokaryotic cells. Eukaryotic systems and mammalian cells can be engineered to produce the heparan sulfate, and 1 day might be capable of producing heparin [18, 61, 62]. Yeast, the simplest eukaryote, is capable of generating some of the essential glycosylation patterns found in mammals; unfortunately, yeast does not produce heparan sulfate [18]. However, the use of yeast for the expression of sulfotransferases, including *N*-sulfotransferases (NST), 3-*O*-sulfotransferases (3-*O*ST), 2-*O*-sulfotransferases (2-*O*ST) and 6-*O*-sulfotransferases (6-*O*ST), and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in yeast had been reported [63]. CHO cells are mammalian cell lines that are capable of producing heparan sulfate. However, CHO cells only express 2/4 NST, 1/3 6-*O*ST but none of 3-*O*ST-1, and do not have granules [64–66]. Genetic engineering of CHO cells can result in the production of heparan sulfate and heparin capable of antithrombin binding, heparin cofactor binding, and herpes simplex virus entry [67, 68]. Unfortunately, the production levels of engineering CHO cells are still relatively low in comparison with the high levels in mammalian mast cells [65]. While further increases in yield and activity could be improved by changes in the fermentation condition, feeding strategies, media composition and through genetic engineering, it is doubtful that a eukaryotic expression system could ever produce the 100 metric ton quantities needed to fill the worldwide market [69, 70].

With respect to prokaryotic expression systems, several microorganisms have been reported for the biosynthesis of GAGs and their analogues. The capsular polysaccharide (CPS) of *Escherichia coli* K4 consists of a repeating disaccharide unit [→4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→] branched with β-linked fructose at C3 of GlcA [71, 72].

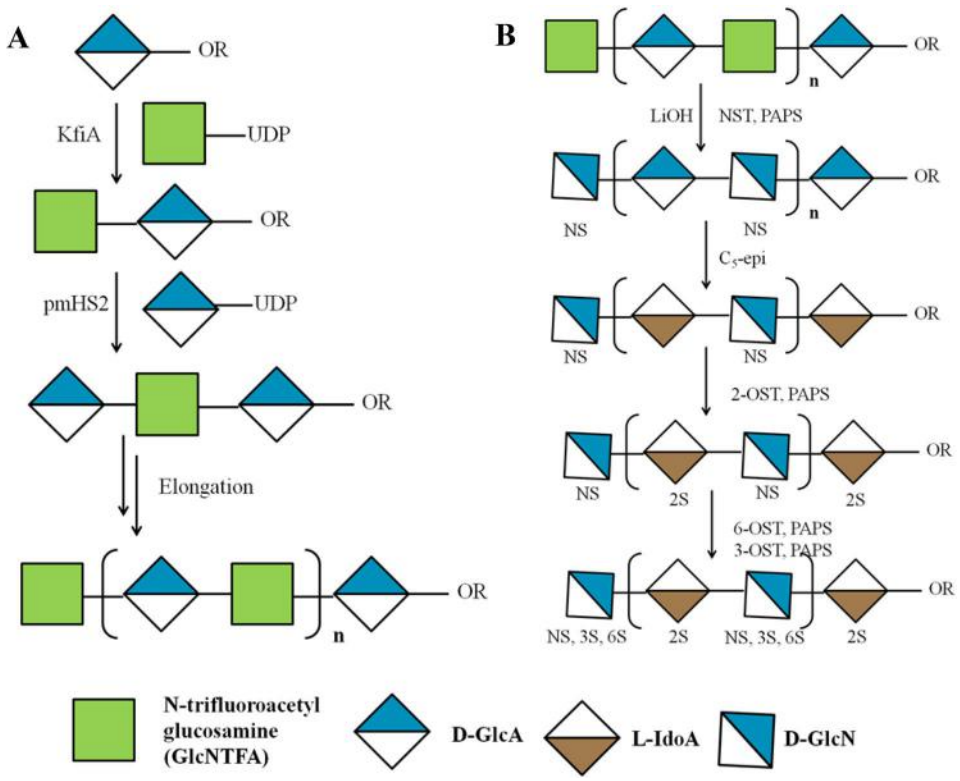

Fig. 4 Chemoenzymatic synthesis of low molecular weight heparin. **a** Sugar backbone synthesis relies on two bacterial glycosyltransferases KfiA and pmHS2 and can transfer glucosamine and glucoronic acid residues to an acceptor substrate, respectively. **b** Modification of backbone relies on four sulfotransferases (NST, 2-OST, 3-OST and 6-OST) and one epimerase (C_5-epi). PAPS co-factor corresponds to 3′-phosphoadenosine-5′-phosphosulfate and OR represents the protection group at the reducing end

After th e*kfoE* gene was knocked out, non-fructosylated chondroitin was obtained, which is the polysaccharide backbone of chondroitin sulfate [56]. Chondroitin production can reach maximum levels of 2.4 g/L in a modified rich defined medium using oxygen-stat fed batch bioreactor after re-arranging the gene sequence in the order of *kfoC*, *kfoA* and *kfoF* in a pETM6_PCAF construct and expressing in the non-pathogenic *E. coli* BL21 Star (DE3) strain [15, 56]. Similarly, heparosan, the backbone of heparin and heparan sulfate is the CPS of *E. coli* K5, which act as the principal protection of these enteric bacteria against intrinsic host defense within the cell surface [15, 56]. Many microorganisms, such as *E. coli* K5, K-12, BL21 and *Pasteurella multocida* are capable of producing heparosan. In the case of *E. coli* K5, the chain size of heparosan produced is considerably larger than that of heparan sulfate or heparin. Studies show that the region 2 genes of the K5 gene cluster control heparosan biosynthesis [73]. By optimizing heparosan production through genetic engineering, fermentation and metabolic engineering by the expression of a lyase gene and the overexpression of two glycosyltransferases genes (*kfiA* and *kfiC*) heparosan yields can be increased [74]. For example, *E. coli* K5 produces 15 g/L heparosan in a defined medium using exponential fed-batch glucose supplement with oxygen enrichment [75]. A recombinant bacterial strain *E. coli* K-12 that contains the cloned genes *kfiABCD* can express heparosan of different molecular weights [15]. Non-pathogenic *E. coli* BL21 can be engineered to produce both chondroitin and heparosan although the yields are lower than obtained from *E. coli* K4 and K5. A competitive relationship between *E. coli* K-12 cell growth and heparosan production has been observed in a mineral culture medium under fed-batch cultivation [76]. This competitive relationship was also found in the biosynthesis of hyaluronic acid [77]. *Bacillus subtilis* and *B. magaterium* can also be prepared to express heparosan, chondroitin and hyaluronic acid. It was reported that heparosan synthase encoding genes from *E. coli* K5 (*kfiA* and *kfiC*), the chondroitin pathway genes (*kfoA* and *kfoC*) and the *Streptococcus hasA* gene were cloned and inserted into *Bacillus* to express the corresponding GAG analogues [78–80]. For example, *Streptococcus hasA* gene had been expressed in *B. subtilis* A1645 with a mineral salts medium, resulting in the production of hyaluronic acid [78].

Conclusion and future prospective

Many GAGs, particularly heparin, chondroitin sulfate and hyaluronan acid, are commercial products widely used in pharmaceutical, nutraceutical and cosmetics industries. Currently, most of these GAGs are produced by the traditional method by extracting from animal tissues in metric ton quantities. This method of GAG preparation can be influenced by many factors, such as a shortage of animals, environmental factors and virus, prion and endotoxin impurities. Therefore, new methods for the manufacturing of GAGs are urgently

needed. Chemical synthesis represents a good method to prepare low molecular weight GAG oligosaccharides and their analogues, for example fondaparinux sodium [81, 82]. However, chemical synthesis is unsuitable for larger GAG oligosaccharides and polysaccharides. Chemoenzymatic synthesis has advantages over chemical synthesis as it does require the selective protection and de-protection of intermediates making synthesis both more controlled and eco-friendly. Challenges still remain in obtaining high expression levels of highly efficient enzymes at low cost as well as sugar substrates, donors and cofactors. Improved yields, large-scale synthesis and low costs are the most important bottlenecks in chemoenzymatic synthesis. With new developments of synthetic biology and genetic engineering, metabolic engineering holds considerable promise in the biomanufacturing of GAGs and analogues. Engineering eukaryotic cells is currently achievable but cannot produce sufficient amounts to meet current market demands and will be very costly [69]. The metabolic engineering of prokaryotic organisms should offer a cost-effective large-scale method for the production of GAGs. Unfortunately, there are many issues that still must be solved for the production of complex GAGs, such as heparan sulfate and heparin. In the near term, we expect to see the first production of metabolically engineered chondroitin sulfate. Such a major breakthrough should further accelerate research in this area.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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