

Glycosaminoglycans (GAGs) are a class of naturally occurring polysaccharides that play vital roles in cellular functions. GAGs (eg, hyaluronic acid, heparosan, chondroitin, chondroitin sulfate, heparan sulfate and heparin) have also been utilised in biopharmaceutical and nutraceutical industries. Animal-sourced GAGs can contain process impurities and contaminants, which may result in adverse effects. To overcome this issue, efforts have focused on the industrial production of animal-component free GAGs. This review focuses on the production of tailored biosynthetic GAGs that have specific and novel functions, including biosynthetic heparins, potential anti-viral and drug delivery applications.

Introduction

GAGs are a family of linear polysaccharides containing repeating disaccharide units of uronic acid and *N*-acetyl glucosamine (GlcNAc) or *N*-acetyl galactosamine (GalNAc). Non-sulfated GAGs (eg, hyaluronic acid, heparosan and chondroitin) are biosynthesised in certain prokaryotes. These non-sulfated GAGs are bacterial capsular polysaccharides (CPS) and contribute to the pathogenicity and survival of these bacteria in their host. These non-sulfated GAGs are also produced in animal cells and, in some cases, are the precursor backbones for sulfated GAGs (eg, heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate). For example, heparosan is the non-sulfated linear polysaccharide composed of the repeating disaccharide unit $[\rightarrow 4) \beta\text{-D-glucuronic acid (GlcA) (1\rightarrow 4) \alpha\text{-D-(GlcNAc) (1\rightarrow 3),}]_n$. The heparosan chain is sequentially modified by tissue-specific

sulfotransferases and C5-epimerase to form sulfated heparan sulfate and highly sulfated heparin. Chondroitin, $[\rightarrow 4)\beta\text{-D-GlcA (1\rightarrow 3)\alpha\text{-D-GalNAc (1\rightarrow 3),}]_n$, is similarly transformed to chondroitin sulfate or dermatan sulfate. The sulfated domains within these sulfated GAGs serve as the binding sites for specific biological macromolecules, including blood proteins and viral capsular proteins. For instance, heparin is produced chiefly in the mast cells and comprised of highly sulfated pentasaccharide domains that bind to antithrombin III, preventing coagulation. Unfractionated heparins and low molecular weight heparins have been widely used as an anticoagulant drug.

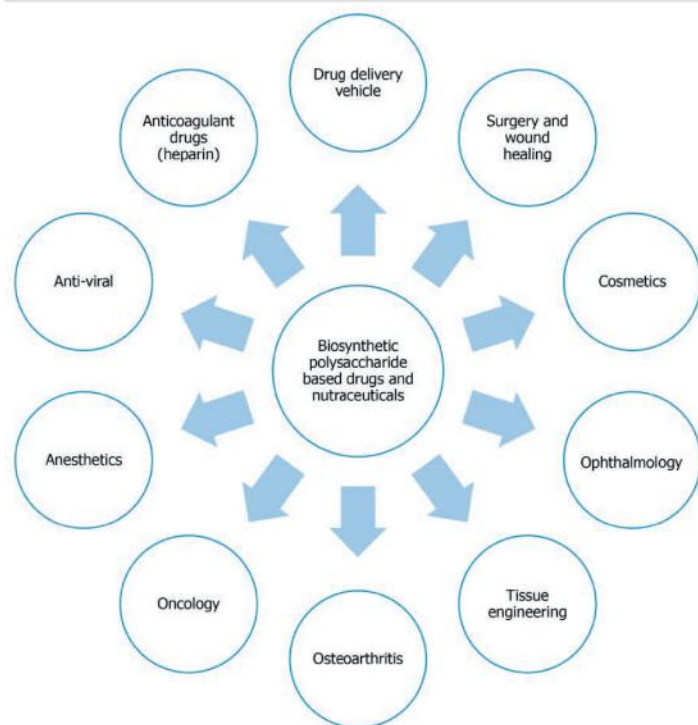
GAGs are present on the cell surface, in the extracellular matrix and form an integral component of the connective tissue systems. Both non-sulfated and sulfated GAGs play vital regulatory roles in biological and cellular functions, including

homeostasis, cell growth, development, cardiovascular disease, cancer, infection and wound repair. GAGs have also been widely utilised in the biopharmaceutical and nutraceutical industries:

- Hyaluronic acid and its derivatives are used in a wide range of pharmaceuticals, such as Euflexxa, Healon, Orthovisc, Restylane, Synvisc and Sefrafilm. In 2019, the global market for hyaluronic acid was valued at \$9.1 billion.
- Both hyaluronan and heparosan are being explored as potential drug delivery vehicles¹
- Heparin is a widely used anticoagulant drug with a global market value of \$6.49 billion in 2018
- Chondroitin sulfate-type A is a popular dietary supplement and in 2019, the global market for chondroitin sulfate was valued at \$1.14 billion. »

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Figure 1



Biosynthetic polysaccharide-based drugs and nutraceuticals

The conventional industrial production of hyaluronic acid, chondroitin sulfate and heparin relies on animal tissues: hyaluronic acid is extracted from rooster combs and bovine/porcine eyes; chondroitin sulfate is extracted from animal cartilage; and heparin is extracted from porcine/bovine intestine and lung tissues. Animal-sourced products may contain process impurities and contaminants, including transmissible infective agents (prions, viruses, bacteria residues), other polysaccharides, (macro)-molecules, proteins, lipids and nucleic acids. For example, in 2008 heparin contamination/adulteration with over-sulfated chondroitin sulfate led to adverse effects, including death, in patients. The incident demonstrated a need to develop a well-characterised biomanufacturing process and sensitive analytical tools. Additionally, the supply chain of animal-sourced GAGs is unstable and may be impacted by adverse events.

This review article focuses on advances in the production of biosynthetic polysaccharide-based drugs and

nutraceuticals from non-animal sources and their potential applications (Figure 1).

Biosynthetic polysaccharide-based drugs and nutraceuticals from non-animal sources

The biomanufacturing of polysaccharide-based drugs and nutraceuticals has been possible due to advances in strain characterisation methods, bioprocess optimisation tools, sensitive analytical techniques and cell engineering strategies. Strain characterisation tools have empowered the identification of natural microbial producers of simple polysaccharides. Examples of strain characterisation tools include high-coverage, whole-genome sequencing, rapid annotation using a subsystem technology server with Glimmer3 as a gene caller, metabolic reconstruction and comparative genomics analysis.² Certain simple prokaryotes have the necessary genes to biosynthesise non-sulfated GAGs. For example, *E. coli* K4 is a natural producer of chondroitin,

Streptococcus equi is a natural producer of hyaluronic acid and *Pasteurella multocida* type D strain, *E. coli* K5 and *E. coli* Nissle 1917 are natural producers of heparosan. Analytical methods critical for biomanufacturing of GAGs^{3,4} include nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS), which can define GAG structure and disaccharide composition. The molecular weight (MW) and polydispersity, critical to quality (CTQs) attributes, rely on gel permeation chromatography. A relatively mono-dispersed MW distribution is a desirable CTQ attribute for downstream processing and applications.

Microbial production of non-sulfated GAGs (hyaluronic acid, heparosan and chondroitin) has been established. One example is the use of high cell fed-batch density fermentation of *E. coli* K5 in a chemically defined media for heparosan production.⁵ Non-sulfated GAGs can be further modified using chemicals and enzymes to form highly sulfated GAGs (eg, chondroitin sulfate and heparin). For example, *S. equi* has been utilised for the production of hyaluronic acid.⁶ The MW of GAG needs to be modified based on its application. For hyaluronic acid, very highly controlled fermentation parameters and downstream processing results in hyaluronic acid with very high MW. An example of fermentation-derived chondroitin sulfate is Mythocondro[®]-Gnosis.⁷ *E. coli* O5:K4:H4 strain U1-41 (ATCC 23502) has been adapted to high cell density fermentation for the production of non-sulfated chondroitin. Post-fermentation, the chondroitin is purified, processed and chemically modified using regioselective sulfation to produce chondroitin sulfate. An example of fermentation-derived heparin is biosynthetic heparin.⁸ *E. coli* K5 (Serovar O10:K5:H4; ATCC #23506) and *E. coli* Nissle 1917 (Serovar O6:K5:H1) have been adapted to high cell density fermentation for the production of heparosan. The heparosan recovered from fermentation, once purified, represents an important nutraceutical that has the potential to be used as an inert drug delivery vehicle. In addition, purified heparosan can be sequentially chemoenzymatically modified to produce biosynthetic heparin. The heparin biosynthetic enzymes are of mammalian-origin and have been

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expressed and purified using high cell density fermentation technology. The possibility of using well characterised and controlled chemoenzymatic modification reactions could generate tailored biosynthetic GAGs that have specific and novel functions.⁴⁸⁻⁵⁰ For example, biosynthetic heparin and biosynthetic TriS-heparin (an intermediate in the biosynthetic heparin synthesis pathway) show antiviral activity.⁴¹⁰ Researchers used a competitive surface plasmon resonance (SPR) to evaluate *in vitro* interactions of sulfated polysaccharides, including biosynthetic heparin and biosynthetic non-anticoagulant TriS-heparin, with the SARS-CoV-2 Spike (S) glycoprotein.

An alternative approach to generating biosynthetic GAGs includes the synthesis of defined oligosaccharides, which may be further chemoenzymatically modified.³¹¹ Low-molecular weight heparin (LMWH) was synthesised using a similar approach.³ The synthesis of LMWH (dodecasaccharides, 12-mer) was initiated with a *p*-nitrophenyl

glucuronide and uridine diphosphate sugar donor and the chain was modified using heparin biosynthetic enzymes expressed in microbial and insect cell lines. The researchers evaluated the 12-mers for >98 percent purity, chemical structure, safety and efficacy. Chemical composition can be determined using heparin fragment analysis or chain mapping with heparin lyases. The enzymatic cleavage of the 12-mer with heparin lyases further confirmed the structural assignments. The safety and efficacy was evaluated using both *in vitro* and *in vivo* experiments. The researchers have compared 12-mers with unfractionated heparin (UFH) and US Food and Drug Administration (FDA)-approved LMWH drugs, such as enoxaparin and fondaparinux. These 12-mers can be chemoenzymatically synthesised in gram scale, can be neutralised, like heparin, using protamine and showed no toxic effects. These data are promising and together suggest the possibility of synthesising homogenous oligosaccharides that can replace animal-

sourced polysaccharide-based drugs and nutraceuticals. Additional non-clinical and clinical studies will be essential to demonstrate the safety and efficacy of the products.

Conclusion

Advances in synthetic biology and metabolic engineering tools have led to research focusing on strain improvements and the creation of in-cell microbial factories that can synthesise tailored polysaccharides with defined functionalities.¹² For example, synthetic biology tools have been used to study the heparosan pathway in *E. coli* to increase the heparosan yield.^{13,14} The metabolic pathway for GAG synthesis is interspersed with other cellular pathways. For example, glucose is used as the precursor molecule for GAG building blocks as well as cell metabolism. Fine-tuning the metabolic pathway to maintain cell health and growth and increase the desired GAG production is desirable.^{15,16} Synthetic biology tools have been instrumental in the study and metabolic engineering of microbial and mammalian cells. Examples of synthetic biology tools include: *in silico* strategies, optimisation of genetic promoters, programmable genetic controls, modular vector systems, complementation systems and CRISPR-mediated metabolic engineering. 

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