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The road to animal-free glycosaminoglycan production: current efforts and bottlenecks

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Animal-extraction, despite its limitations, continues to monopolize the fast-growing glycosaminoglycan (GAG) industry. The past few years have seen an increased interest in the development of alternative GAG production methods. Chemical and chemo-enzymatic synthesis and biosynthesis from GAG producing cells, including engineered recombinant strains, are currently under investigation. Despite achieving considerable successes, these alternate approaches cannot yet meet worldwide demands for these important polysaccharides. Bottlenecks associated with achieving high-titers need to be addressed using newly developed tools. Several parameters including chassis choice, analytics, intracellular precursor synthesis, enzyme engineering and use of synthetic biology tools need to be optimized. We envision that new engineering approaches together with advances in the basic biology and chemistry of GAGs will move GAG production beyond its currently limited supply chain.

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Introduction

Glycosaminoglycans (GAGs) are linear hetero-polysaccharides which interact with many cellular proteins. They contain amino-sugars such as glucosamine and galactosamine, and acidic sugars such as glucuronic acid and iduronic acid. Their specific biological roles are related to their structure, backbone polysaccharide and post-polymerization modification, and to their localization [1]. The vast potential chemical space occupied by GAGs results in a large protein-interactome and a wide variety of biological roles. As a result, they have attracted the attention of scientists world-wide. Every year, more publications report different facets of GAG research, ranging from newly discovered biological roles to cutting-edge analytics for deciphering their complex structures. In this review, we highlight some noteworthy recent studies that have led to research milestones on the path to biotechnological GAG production. The different bottlenecks associated with large-scale GAG production are also discussed.

Structure and function

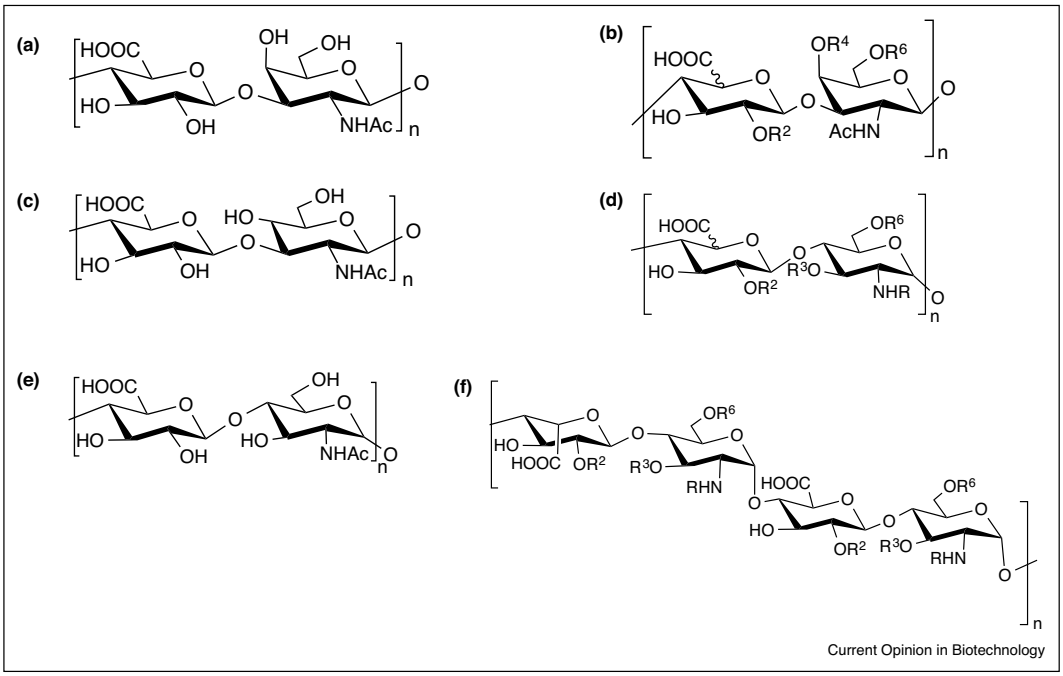
The repeating saccharide units of GAG polysaccharide chains are coupled through glycosidic linkages. The structures of the major commercial GAGs, hyaluronic acid (HA), heparan sulfate (HS) and chondroitin and dermatan sulfate (CS/DS) are shown in Figure 1. GAGs play many important roles in cells [2,3]. They are a major component of the extracellular matrix and contribute to its biomechanical properties. With the exception of bacterial GAGs and HA (the GAG having the simplest structure), most GAG chains are attached to the serine residues of core proteins in the form a proteoglycan. GAGs are commercially used as pharmaceuticals (such as the anticoagulant heparin (HP), a highly sulfated HS), nutraceuticals and functional foods, surgical aids, drug delivery vehicles, in cosmetics and in tissue engineering [4].

Current GAG sources and market

As more studies reveal the association of GAGs with critical biological processes, their applications and the need for sustainable, large-scale production have increased. Currently, GAGs are commercially extracted from animal sources, such as HP from porcine intestines. In 2016, China, the largest supplier of HP, met more than half the world's demand for this critical anticoagulant drug [5]. Global HP sodium sales have increased from 133698 kg in 2011 to 189515 kg in 2015, with an average increase rate of over 9% [6].

CS, extracted from bovine or porcine trachea and shark cartilage, is a sizable market that is expected to grow at the rate of 15% reaching 3 million kg by 2021 [7]. The lack of a sustainable, risk-free (free from virus, prions, adulteration, economic boycott) and steady source of CS

Figure 1



Typical repeating disaccharide units of some common GAGs. **(a)** Chondroitin, **(b)** chondroitin sulfate (CS) (a, b, c, d, e); CS-A: GlcA, R^2 = H, R^4 = SO_3H, R^6 = H; CS-B (DS): IdoA, R^2 = H, R^4 = SO_3H, R^6 = H; CS-C: GlcA, R^2 = H, R^4 = H. R^6 = SO_3H; CS-D: GlcA, R^2 = SO_3H, R^4 = H, R^6 = SO_3H; CS-E: GlcA, R^2 = H, R^4 = SO_3H, R^6 = SO_3H, **(c)** hyaluronan, **(d)** heparan sulfate (HS) (90%GlcA; 10%IdoA; avg 1 SO_3H/disaccharide) R^2 = OH or SO_3H, R^3 = OH or SO_3H, R^6 = OH or SO_3H, R = Ac (Ac = $COCH_3$), H or SO_3H, **(e)** heparosan and **(f)** Heparin (HP) (80%IdoA; 20%GlcA; avg 2.5 SO_3H/disaccharide) R = SO_3H/Ac/H, R^2 = SO_3H/H, R^3 = H/SO_3H, R^6 = SO_3H/H.

is impeding this industry from meeting growing nutraceutical and medical demands.

HA, in contrast, is the only GAG under biotechnological production, relying on microbial fermentation (mostly *Streptococci*), which has largely replaced extraction from rooster-comb tissues. The global HA market is predicted to reach $10.8 B by 2020 [8]. The global demand for medical grade HA alone was approximately 10 million kg in 2010 [9]. Currently, research groups worldwide are working towards developing biotechnological methods to synthesize GAGs that are structurally more complex than HA, the details of which are discussed in this review.

Alternate methods for GAG production

Animal extraction, despite meeting current worldwide demands, is not a sustainable option due to limited availability of source tissues (mostly from food animals), scale-up issues, adverse environmental impact and quality control issues. Research has increased our understanding of GAG biosynthesis and GAG structure. This is an ideal time for biochemical and metabolic engineers to provide the needed expertise, to employ new synthetic biology tools and create successful alternate production methods.

Chemical synthesis, chemoenzymatic synthesis and bioengineering of GAGs

Chemical and chemoenzymatic syntheses of complex GAG polysaccharides and oligosaccharides are rapidly advancing as substitutes to conventional extraction methods. Chemical techniques use organic reactions for the *de novo* synthesis of GAG oligosaccharides from monosaccharides [10]. While this approach has been commercially successful in the chemical synthesis of Arixtra^{TM}, a homogeneous ultra-low molecular weight heparin pentasaccharide, it is time consuming, requiring many steps, extremely costly, not easily scalable, and cannot be used for the synthesis of GAG polysaccharides [11].

Chemoenzymatic synthesis and bioengineering involve block synthesis or bacterial fermentation of the polysaccharide backbone and employ recombinantly expressed sulfotransferases and epimerases to catalyze post-polymerization modifications [12]. This eases operating conditions and reduces the time, number of steps and cost,

while improving the scalability and applicability for the synthesis of larger GAG oligosaccharides structurally equivalent to animal derived products. Recent successes have resulted in the chemoenzymatic synthesis of GAG libraries [13••] and low molecular weight heparins (e.g. a dodecasaccharide ready for preclinical studies) [14••].

Harnessing natural GAG producers

GAGs have been identified in most metazoans and many pathogenic bacteria but have not been reported in plants [15]. Consequently, bacterial producers and amenable cell-lines have been explored for GAG production. Before giving an overview of those efforts, this section introduces the GAG biosynthetic pathways in these producing organisms.

Biosynthesis of GAGs

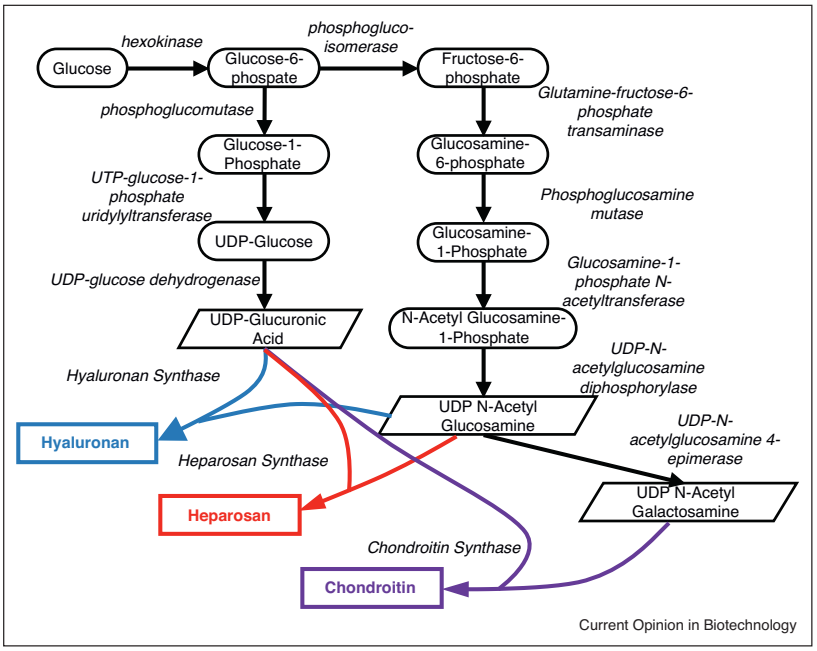
GAG biosynthesis consists of three basic steps: precursor synthesis, secondly, polymerization; and thirdly, post-polymerization modification. Each precursor is synthesized from a simple sugar (typically glucose) through a series of enzyme-catalyzed steps. The synthesized precursors are sequentially added to the growing GAG chain through the catalytic action of GAG synthases or glycosyltransferases (Figure 2). On release of the polymer backbone from the GAG synthase, sulfotransferases and epimerases modify the saccharide residues resulting in the complex GAG structure equipped with multiple specific functional roles (Figure 3).

Alternate GAG sources

Bacteria, such as *E. coli* K5, *E. coli* K4, Group A and C *Streptococci* and *Pastuerella multicida* naturally produce non-sulfated GAGs as part of their capsule to evade immunogenic response in infected host tissues [2]. Among these, *Streptococci* have been used commercially to obtain very high HA titers over the last few decades. Since HA does not undergo post-polymerization modifications like the other GAGs, the fermentation product is directly isolated, purified and sold. Recent studies have addressed the economic concerns of HA production by using inexpensive media like cheese-whey [16] or agro-industrial by-products [17,18].

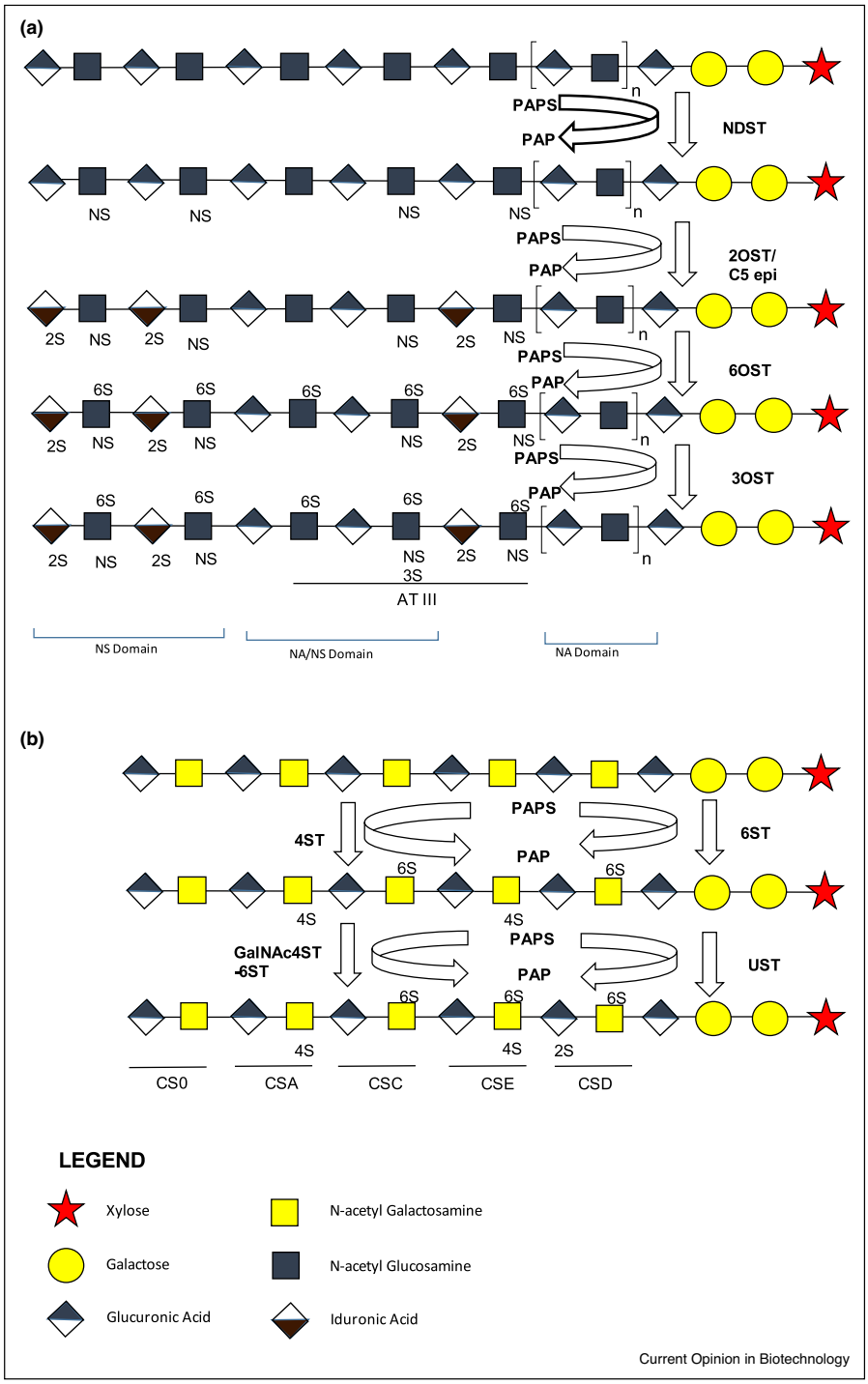
The first reports of high titer heparosan [19] and chondroitin [20] from *E. coli* K5 and K4, respectively, were published around 6 years ago. The main bottleneck in producing HS and CS using these microorganisms is that post-polymerization modification machinery, that is, sulfotransferases and epimerases, is absent in these bacterial systems. *In vitro* modification using recombinant enzymes prepared from *E. coli* BL21, *Pichia pastoris*, etc., in a biotransformation step, represents a potential solution for this bottleneck [21].

Figure 2



Biosynthetic pathway for GAG precursor and polysaccharide production. Enzymes are styled in italicized font; Intermediate metabolites in rounded rectangles; GAG precursors in parallelograms and polysaccharides in rectangles (hyaluronan — blue; heparosan — red; chondroitin — purple).

Figure 3



Biosynthesis of heparin/heparan sulfate and chondroitin sulfates. Modification pathway of **(a)** heparin/heparan sulfates. **(b)** Chondroitin sulfates.

Animal cell-lines, such as CHO, HEK and mastocytoma, have both polymerization and modification enzymes and can synthesize sulfated GAGs [22–24]. Recent studies report optimum reactor conditions [25] and process parameters [26] that improve HP or CS yield in these cell-lines. Some studies have also examined newly discovered polysaccharides from vastly abundant sources like seaweeds and marine bacteria as possible alternatives to commercial GAGs [27,28].

Metabolic engineering for GAG production

Several well-characterized microorganisms and cell-lines have been engineered for the production of GAGs. Due to ease of production from microbial systems (no post-polymerization modification), remarkable progress has been made in metabolic engineering for HA production.

Recent developments in metabolic engineering for HA production

Many microbes including *E. coli*, *Lactococcus lactis*, *Bacillus subtilis*, *Corynebacterium glutamicum* and *P. pastoris* have been engineered to make HA. The past couple of years has witnessed several patents on HA production methods. One such work patented a superior *B. subtilis* strain engineered with four strategies [29••]: firstly, up-regulation of pathway genes; secondly, down-regulation of competing glycolytic pathway; thirdly, expression of leech hyaluronidase to overcome dissolved oxygen limitation; and fourthly, ribosome binding site engineering of the hyaluronidase to achieve very specific molecular weights [30]. Other patented technology primarily manipulates regulation (e.g. altered and artificial promoters for pathway genes [31,32]). Another recent study systematically developed *C. glutamicum* for HA production by simultaneously optimizing operon organization, promoter strengths, as well as nutrient source and process parameters [33]. Other similar studies have been highlighted elsewhere [34].

Metabolic engineering for HP and CS production

Like native bacterial GAG producers, engineered systems (like *E. coli* BL21 [35•,36], *B. subtilis* [37•,38] and *Komagataella pastoris* [39]) also synthesize heparosan and chondroitin, which are later enzymatically modified to form HP, HS, CS or DS. The studies in *E. coli* explore different combinations of over-expressed genes to produce heparosan [36] and chondroitin [35•]. The use of bifunctional heparosan synthase from *P. multocida* instead of the two genes from *E. coli* K5 can produce higher yields and molecular weights for heparosan from *E. coli* [40] and *B. subtilis* [38]. Another study integrated heparosan and chondroitin synthase genes from K5 and K4 into the *B. subtilis* chromosome. UDP-glucose dehydrogenase was over-expressed and fed-batch was used to achieve high yields of both chondroitin (5.22 g/L) and heparosan (5.82 g/L) [37•]. These strategies appear to have been inspired from previous studies on HA [41,42].

Engineering improved GAG production

The increased interest in using synthetic biology and modern biotechnological approaches for GAG production has uncovered several bottlenecks that lie along the road to successful alternate GAG sources. Some of those major bottlenecks are outlined here.

Choice of chassis

Bacterial and mammalian cells are being developed as GAG production chasses [12]. Several *E. coli* strains have proven to be attractive and efficient hosts for producing metabolically engineered chondroitin [35•], heparosan [43] and HA [44]. *B. subtilis* is another well-characterized host that has been successfully harnessed for chondroitin and heparosan production, with yields of 2.54 g/L and 2.65 g/L respectively [37•] and also industrially established for HA production [30,45]. Several other bacteria, including *Agrobacterium*, *Lactococcus*, and *C. glutamicum*, have been engineered to produce HA [46]. All these efforts also clearly point to the requirement of substantial metabolic engineering for the development of economically viable producers.

Analytics and estimation

GAGs have complex structure and sulfation patterns that are closely linked to their function. This makes it necessary to develop robust and appropriate analytics to compare synthetic and biotechnological preparations to that of commercial GAGs from animal tissues. Powerful analytical techniques such as liquid chromatography (LC)-fluorescence and LC-mass spectrometry (MS) have been developed for the analysis of fluorescently tagged disaccharides prepared from GAGs, after their enzymatic breakdown [47]. There has also been a recent, sudden inflow of reports on the development of powerful NMR spectrometric and MS chemometric techniques [48–50]. These methods help provide better resolution for these complex structures and can reduce the cost and complexity of data analysis.

Making the intracellular precursors

GAG biosynthesis is regulated by the availability of UDP-sugar precursors [30,51,52•] which are added alternately at the non-reducing end of the polysaccharide chain [53•]. However, a high availability of UDP-sugars alone does not increase GAG titers unless the overall balance of intracellular precursors and carbon flux orientation towards competing pathways are considered [35•]. Sulfotransferases require the activated sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) [54]. While *in vitro* enzymatic sulfation uses enzymatically regenerated PAPS that can be obtained in high titers [12], the primary obstacle to *in vivo* GAG production in bacteria is the low PAPS biosynthesis compared with the high activity of the sulfotransferases. With the recent successful expression of the CS 4-*O*-sulfotransferase in *E. coli* [21], one can now envision the *in vivo* production of CS in *E. coli*.

Protein engineering

Potential interest in the modulation of functional group patterns, chain lengths and linkages in GAGs generate enzyme-engineering possibilities. For example, GAG lyases are useful for producing desired GAG oligosaccharides from GAG polysaccharides. Reducing their substrate specificity can be useful for their general applicability. Along these lines, a mutant of the heparinase enzyme from *Pedobacter heparinus*, which can degrade both HP and HA, has been developed [55]. A recombinant chondroitinase ACII lyase, which is applicable for the general structural determination of most standard chondroitin sulfates has also been developed [56••]. Sulfotransferases and glycosyltransferases are also top targets for protein engineering. A recently engineered HA synthase shows higher HA titer and molecular weight [57]. However, any further improvement in this sphere also requires unravelling structure–activity relationships of the concerned enzymes.

Employing synthetic biology tools

Developing synthetic biology tools to exert deeper levels of control and modularization is currently in vogue in biological engineering. Although GAG production research is not at the same stage of development as other natural products, this section points to some recent advances in tangential areas. For example, enhanced *N*-acetylglucosamine levels by employing DNA scaffolds for spatial modulation of pathway enzymes [58] might also facilitate enhanced GAG production. Recent synthetic biology and protein engineering studies have engineered superior control elements [31,32,59], such as a combinatorial directed evolution, to improve both regulatory and pathway elements [60]. This approach was applied to a highly specific leech hyaluronidase to generate several variants after a single round of evolution that exhibited higher hyaluronidase activities and/or heparinase activities.

Concluding remarks

GAG production has a huge learning curve that we are coping to adapt to and learn from. As GAGs are large molecular weight natural products, their recombinant production poses unique challenges compared to small-molecular weight natural products [61]. Health crises, such as the issue of adulterated HP containing oversulfated CS, have steered recent focus on developing robust analytics and safety assessment protocols [50,62]. The studies described should facilitate the movement to non-animal GAG production methods. Many areas highlighted in this review are already actively being explored. Chemoenzymatic synthesis of synthetic HP oligosaccharides has recently been scaled up to multigram titers leading to possibility of pre-clinical studies in mouse models for the very first time [14••]. CS synthase has been identified in an unexpected photosynthetic, phylogenetically differentiated green sulfur bacterium, thus expanding the chassis spectrum [63•]. A future with animal-free GAGs, possibly tailored for superior activities is not far from us.

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