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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

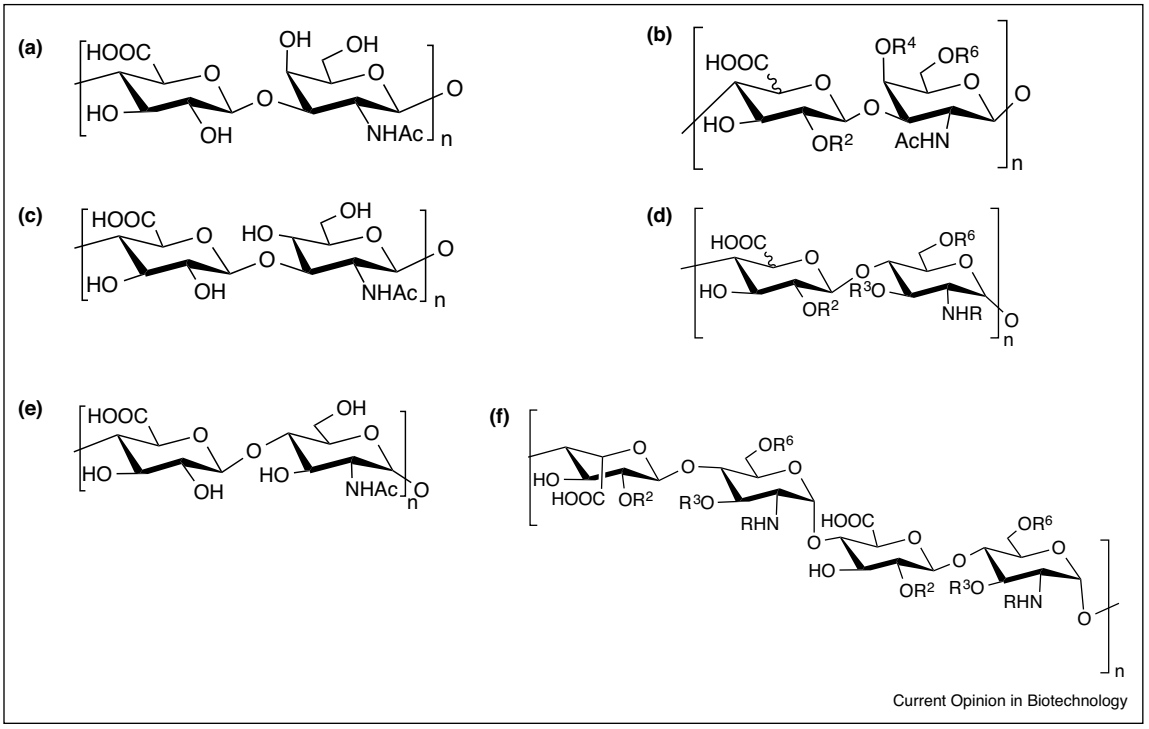
The repeating saccharide units of GAG polysaccharide chains are coupled through glycosidic linkages. The structures of the major commercial GAGs, hyaluronic acid (HA), heparan sulfate (HS) and chondroitin and dermatan sulfate (CS/DS) are shown in Figure 1. GAGs play many important roles in cells [2,3]. 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,