Glossary

cGMP  cGMP refers to "current good manufacturing practice", a set of regulatory principles designed for managing pharmaceutical manufacturing processes and facilities.
Fed-batch fermentation  Fed-batch fermentation is used for high cell-density cultivation of recombinant cells. The fermentation begins with seeding cells in a fermentation medium in a bioreactor. The fermentation medium could be complex, enriched media (e.g., terrific broth) or defined media (e.g., phosphate-citrate broth, supplemented with chemicals such as carbon source and trace metals). Throughout the culture, the cells are fed with various nutrients, including a carbon source and nitrogen sources (such as amino acids). Usually, the feeds are concentrated so that the fermentation volume does not increase excessively.

Introduction

Glycosaminoglycans (GAGs) are naturally occurring polysaccharides, composed of alternating sugar units; these include an amino sugar (e.g., N-acetyl glucosamine, GlcNAc or N-acetyl galactosamine, GalNAc) and either a galactose (Gal) or an uronic acid (GlcA or IdoA) (Fig. 1). The carbon backbone of the GAG chain may undergo no further modifications (e.g., hyaluronan) or may be further modified through sulfation, de-acetylation, and/or epimerization (e.g., heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate). Simple non-sulfated GAGs (e.g., hyaluronan) and the precursors of highly sulfated GAGs (e.g., heparosan and chondroitin) are produced in certain bacteria as well as in animals (Cress et al., 2014). These simple GAGs are thought to contribute to pathogenicity of the host bacteria. Simple, non-sulfated GAGs as well as the highly modified sulfated GAGs (e.g., heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate) are produced in all animals, including humans. In humans, hyaluronan is part of the connective tissue extracellular matrix (ECM) and is involved in biological functions such as lubrication of joints. Highly sulfated GAGs have tissue-specific sulfated domains that are binding sites for various proteins (Capila and Linhardt, 2002) and, therefore, play critical roles in biological functions such as homeostasis, cell growth, cell migration, development, morphogenesis, tissue repair, and angiogenesis (Linhardt and Toida, 2004; Linhardt, 2003).

The current industrial production of GAGs can be broadly classified into three categories: (1) industrial production from animal source, (2) industrial production using microbial cells, and (3) industrial production using eukaryotes. Various GAGs have been routinely extracted and purified from animal sources, for example, hyaluronan from rooster combs, and heparin from pig intestines or bovine lung. An increased knowledge of GAG biosynthesis, as well as the advances in metabolic engineering strategies, bioprocess optimization, downstream processing approaches and analytical tools have led to the production of GAGs from microbial cells and exploration of eukaryotic cells, for example, CHO cells, toward heparin production.

Fig. 1  Building blocks of glycosaminoglycans (a) N-acetyl glucosamine, (b) N-acetyl D-galactosamine, (c) D-galactose, (d) D-glucuronic acid, and (e) L-iduronic acid. These sugar moieties may be further modified through the action of specific enzymes, such as N-deacetylases, sulfotransferases, and epimerases.
Glycosaminoglycans

The glycosaminoglycan family includes: (1) hyaluronan (hyaluronic acid); (2) heparin/heparan sulfate; (3) chondroitin/dermatan sulfate; and (4) keratan sulfate. Each GAG family has a common carbon backbone that can be variously modified (Table 1, Fig. 2) and may also have a variety of different molecular weight properties. The structural characterization of GAGs has been possible due to advances in biochemical and analytical tools, such as gel permeation chromatography, high-performance liquid chromatography (HPLC), ion-exchange chromatography, capillary electrophoresis, mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy (Sun et al., 2016; Schlesinger et al., 2000; Xiao et al., 2010; Cai et al., 2013, 2012; Laremore et al., 2007; Fu et al., 2016; Zhang et al., 2013; Volpi et al., 2009; Moon et al., 2012; Desai et al., 1993; Yang et al., 2011; Kailemia et al., 2013; Li et al., 2014; Thanawiroon and Linhardt, 2003; Liu et al., 2014; Chen et al., 2017; Volpi et al., 2012; Thanawiroon et al., 2004; Zhao et al., 2013; Lohse and Linhardt, 1992; Leach et al., 2012; Guerrini et al., 2009; Wang et al., 1991; Milhailov et al., 1997; Li et al., 2012). These analytical tools have aided GAG structural analysis and improved insight into the GAG structure-function relationships. In parallel, metabolic engineering tools, knock-in/out animal models and mammalian cell culture models, have also contributed to deciphering the GAG biosynthetic pathway and GAG functions (Lin et al., 2000; Sarrazin et al., 2011; Pönighaus et al., 2007; Xu and Esko, 2014; Esko and Selleck, 2002; Thacker et al., 2014; Bame and Esko, 1989; Bai and Esko, 1996; Aikawa et al., 2001; Fuster et al., 2007; Grobe et al., 2002; Bame et al., 1991). For example, metabolic engineering of suspension CHO cells through the up-regulation of exogenous Golgi-targeted SOST1 revealed formation of distinct antithrombin binding sites; antithrombin binding sites directly correlate to anticoagulant activity of CHO derived engineered HS (Datta et al., 2013). Metabolic engineering tools together with analytical tools synergistically contribute to GAG research.

Hyaluronic Acid

Hyaluronan (hyaluronic acid; hyaluronate) is a naturally occurring non-sulfated glycosaminoglycan (GAG), composed entirely of linear polymer of alternating GlcA and GlcNAc moieties (Table 1, Fig. 2) (Vigetti et al., 2014). Hyaluronan synthase (HAS) polymerizes linear HA polysaccharide from UDP-sugars by adding alternating GlcNAc and GlcA moieties to the growing HA carbon chain (Vigetti et al., 2014; Bodevin-Authelet et al., 2005; Fraser et al., 1997; O’Regan et al., 1994; Itano and Kimata, 2002; Itano et al., 1999). HAS enzymes occur in certain bacteria as well as mammals. Streptococcus sp., such as S. zooepidemicus express bacterial HAS and biosynthesize hyaluronan (O’Regan et al., 1994; Hascall et al., 2016; DeAngelis, 2015) which aids in bacterial pathogenesis.

Cellular biosynthesis of hyaluronan in mammalian cells occurs through the action of a family of hyaluronan synthases (HAS1-3), which are found in the plasma membrane (Itano and Kimata, 2002). In humans, hyaluronan is synthesized primarily in mesenchymal cells (e.g., fibroblasts, endothelial cells); however, it is found on the cell surfaces in many tissues. In humans, hyaluronan has several important roles (Hascall et al., 2016). It forms a vital constituent of the extracellular matrix and connective tissue systems, including, skin, synovial fluid in the joints, vitreous humor of the eye, heart valve, lungs, and umbilical cord (Vigetti et al., 2014; Bodevin-Authelet et al., 2005; Fraser et al., 1997; Itano and Kimata, 2002; Hascall et al., 2016; Robert et al., 2010; Inatani and Tanihara, 2002; Carrino et al., 2000; Oksala et al., 1995; Temple-Wong et al., 2016). Due to its physicochemical properties, hyaluronan can provide a framework for cell migration and is capable of retaining moisture (Papakonstantinou et al., 2012). Aging skin shows reduced expression of GAGs including hyaluronan and reduced moisture retention (Papakonstantinou et al., 2012). Reduced hyaluronan expression and activity in part correlates with decreased HAS1, increased hyaluronan lyases (HYAL1-3), and decreased hyaluronan receptors (CD44 and RHAMM).

A number of medical and personal care applications for exogenous hyaluronan have been developed in recent years with research ongoing, many to address physiological and pathological changes that occur with aging. Hyaluronan has been used in skin-cosmetics (Carrino et al., 2000; Naylor et al., 2011; Tzelloes et al., 2009; Anderegg et al., 2014; Oh et al., 2011). Due to its mucoclesive behavior and contribution to the elastoviscous behavior of soft connective tissues, for example, in synovial fluid in

<table>
<thead>
<tr>
<th>Type</th>
<th>Composition</th>
<th>Chemical Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronan</td>
<td>Linear polymer of alternating α-glucuronic acid (GlcA) and N-acetylglicosamine (GlcNAc).</td>
<td></td>
</tr>
<tr>
<td>Family of heparin, heparan sulfate and heparosan</td>
<td>Linear polymer of alternating α-glucuronic acid (GlcA) and N-acetylglicosamine (GlcNAc). Heparosan is modified through the actions of tissue specific sulfotransferases, epimerases to form heparan sulfate and heparin.</td>
<td></td>
</tr>
<tr>
<td>Family of chondroitin sulfate and dermatan sulfate</td>
<td>Linear polymer of alternating α-glucuronic acid (GlcA) and N-acetylglicosamine (GlcNAc).</td>
<td></td>
</tr>
<tr>
<td>Family of keratan sulfate</td>
<td>Linear polymer of alternating galactose (Gal) and N-acetylglicosamine (GalNAc), with sulfation, for example, sulfation at the carbon 6 of galactose and N-acetylglicosamine.</td>
<td></td>
</tr>
</tbody>
</table>
the joints and vitreous humor of the eye (Robert et al., 2010; Nishida et al., 1991; Yokoi et al., 1997), hyaluronan has been used in eye drops to lengthen the pre-corneal retention time (Salzillo et al., 2016). The synovial fluid of the joints is majorly composed of hyaluronan and functions in lubrication and knee movement. During osteoporosis, cartilage degradation in joint and reduced synovial fluid results in pain and reduced movement. High molecular weight (1.0–2.9 million Dalton) ultra-pure hyaluronan has

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**Fig. 2** Sugar moiety backbone of glycosaminoglycans (A) hyaluronan, (B) family of heparin, heparan sulfate and heparosan, (C) family of chondroitin and chondroitin sulfate, (D) dermatan sulfate, (E) family of keratan sulfate, and (F) sugar moieties, namely, glucuronic acid (GlcA), N-acetylg glucosamine (GlcNAc), N-acetylglucosamine (GlcNAc), galactose (Gal), and Iduronic acid (IdoA). Hyaluronan (A), heparosan (B), and chondroitin (C) are unmodified linear sugar moiety backbone chains. Keratan sulfates (E) have O-sulfated galactose and N-acetylg glucosamine moieties. The sugar moieties of heparosan (GlcA and GlcNAc) may be further modified through actions of tissue-specific N-sulfotransferases, C5 epimerases and O-sulfotransferases, to form sulfated heparan and heparan sulfate. The sugar moieties of chondroitin (GlcA and GalNAc) may be further modified through actions of tissue-specific O-sulfotransferases, to form sulfated chondroitin sulfate. The sugar moieties of chondroitin (GlcA and GalNAc) may be further modified through actions of tissue-specific epimerases (that transform GlcA to IdoA), and O-sulfotransferases, to form sulfated dermatan sulfate. Figures were drawn from Cheng, K., Zhou, Y., Neelamegham, S., 2017. DrawGlycan-SNFG: A robust tool to render glycans and glycopeptides with fragmentation information. Glycobiology 27 (3), 200–205.
been utilized to treat pain in osteoarthritis of the knee. Other applications of hyaluronan include wound healing (Oksala et al., 1995), a potential marker for tumors or cancers (Stern, 2009), a drug delivery vehicle (Brown and Jones, 2005), and a biomaterial scaffold for tissue engineering (Dicker et al., 2014; Shu et al., 2004). Currently, hyaluronan is used in a variety of pharmaceutical products including Euflexxa, Healon, Synvisc, Orthovisc, Seprafilm, and Restylane (Hascall et al., 2016; DeAngelis, 2015; Schiraldi et al., 2010; Boeri et al., 2013; Cimini et al., 2017).

Heparin and Heparan Sulfate

Heparin and heparan sulfate are naturally occurring, sulfated GAGs composed of alternating GlcA and GlcNAc moieties (Table 1, Fig. 2). During their biosynthesis, these GAGs are modified through the action of specific enzymes, N-deacetylases, sulfotransferases, and epimerases (Carlsson and Kjellén, 2012; Garg et al., 2005). Heparan sulfate is present on cell surfaces and binds to various protein ligands, resulting in the regulation of a variety of biological processes such as cell growth and angiogenesis. Heparin is predominantly present in mast cells and is commercially used as a biopharmaceutical anticoagulant drug (Carlsson and Kjellén, 2012; Garg et al., 2005).

In humans, heparan sulfate/heparin are biosynthesized as proteoglycans, attached to specific core proteins, such as syndecan, glypican, and serglycin. Heparan sulfate/heparin share a similar biosynthetic pathway. Biosynthesis begins with the formation of a tetrasaccharide linker, a disaccharide GalNAc-GlcA on serine residues of the core protein. Next, N-acetyl glucosaminyl transferase adds a GlcNAc to the non-reducing terminal GlcA of this tetrasaccharide linker. The family of EXT enzymes (Exostoses, EXT1-2) sequentially add GlcNAc and GlcA, forming a linear non-sulfated heparosan polysaccharide (Lin et al., 2000; Busse et al., 2007; Wuyts et al., 1998; Okada et al., 2010). The heparosan chain is modified through the action of various sulfotransferases and epimerases to produce sulfated heparan sulfate and highly sulfated heparin. Briefly, the GlcNAc residues are N-deacetylated and N-sulfated by a family of N-deacetylase/N-sulfotransferase (NDST) enzymes to form GlcNS (Saribas et al., 2004). The GlcA residues may be epimerized into Idoa by CS-epimerase (GLCE) enzymes (Feverabend et al., 2006) and the uronic acid residue O-sulfated by 2-O-sulfotransferase; the GlcNS moieties may be O-sulfated by families of 6-O- and 3-O-sulfotransferases (Moon et al., 2012; Carlsson and Kjellén, 2012; Garg et al., 2005; Anower-E-Khuda et al., 2013; Sasisekharan and Venkataraman, 2000). There are tissue-specific isoforms of the NDSTs and O-sulfotransferases, and these generate tissue-specific sulfated domains. The 3-O-sulfotransferase isoform-1 (3OST-1), for example, is critical for the production of anticoagulant heparin and is present in mast cells, which chiefly biosynthesize anticoagulant heparin (Forsberg et al., 1999). Heparin sulfate is characterized by partially sulfated domains, and these domains provide the specific ligand binding characteristic to heparan sulfate, for example, cell growth, cell development, angiogenesis, and metastasis (Sarrazin et al., 2011). Heparin is characterized by completely or nearly completely sulfated domains, and has specific antithrombin III (ATIII)-binding sites. The ATIII-binding site of heparin interacts with antithrombin (AT, a serine protease inhibitor). This interaction leads to a conformational change in the AT and amplifies inhibition of thrombin (factor IIa) and factor Xa via the action of AT. This results in anticoagulation and use of heparin as an anticoagulant drug (Onishi et al., 2015).

Chondroitin Sulfate and Dermatan Sulfate

Chondroitin/deermatan sulfate belong to a family of naturally occurring linear, sulfated GAGs made of repeating sugar moieties, namely GlcA and GalNAc residues (Tables 1 and 2, Fig. 2). Chondroitin sulfate/dermatan sulfate in higher animals are highly sulfated, and the sulfated domains act as ligands for proteins and mediate biological functions.

In humans, chondroitin sulfate-type A (e.g., CSA) is predominant in connective tissues and ECM, for example, cartilage, cornea, bone, skin, arterial walls. Chondroitin sulfate provides a framework, and promotes elasticity in cartilage. These properties have made chondroitin sulfate-type A a popular dietary supplement. Dermatan sulfate is predominant in skin, heart valve, tendons, and blood vessels. Chondroitin sulfate/dermatan sulfate also play regulatory roles in cell proliferation, cell development, cell adhesion, homeostasis, cardiovascular disease, tumorigenesis, infection, wound repair and fibrosis (Hardingham, 1998; Maeda et al., 2011; Stern, 2009).

Table 2  Disaccharide units of chondroitin sulfate and dermatan sulfate

<table>
<thead>
<tr>
<th>CS/DS</th>
<th>Major disaccharide units and their sulfation pattern</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin (CS-DS)</td>
<td>Unsulfated glucuronic acid and unsulfated N-acetylgalactosamine</td>
<td>→4) β-α-GlcA1→3β-α-GalNAc1→</td>
</tr>
<tr>
<td>Chondroitin sulfate A (CS-A); chondroitin – 4-sulfate</td>
<td>Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 4</td>
<td>→4) β-α-GlcA1→3β-α-GalNAc4S1→</td>
</tr>
<tr>
<td>Dermatan sulfate (CS-B)</td>
<td>Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 4</td>
<td>→4) α-β-IdoA1→3β-α-GalNAc4S1→</td>
</tr>
<tr>
<td>Chondroitin sulfate C (CS-C); chondroitin – 6-sulfate</td>
<td>Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 6</td>
<td>→4) β-α-GlcA1→3β-α-GalNAc6S1→</td>
</tr>
<tr>
<td>Chondroitin sulfate D (CS-D); chondroitin – 2,6-sulfate</td>
<td>Glucuronic acid sulfated at carbon 2 and N-acetylgalactosamine sulfated at carbon 6</td>
<td>→4) β-α-GlcA2S1→3β-α-GalNAc6S1→</td>
</tr>
<tr>
<td>Chondroitin sulfate E (CS-E); chondroitin – 4,6-sulfate</td>
<td>Unsulfated glucuronic acid and N-acetyl galactosamine sulfated at carbon 4 and 6</td>
<td>→4) β-α-GlcA1→3β-α-GalNAc4S6S1→</td>
</tr>
</tbody>
</table>
Linhardt and Hileman, 1995; Sugahara and Mikami, 2007; Uebelhart, 2008; Vallières and du Souich, 2010). In addition, dermatan sulfate may form complexes with heparin cofactor II, and may act as an anticoagulant. Chondroitin sulfate proteoglycans are potential targets for malaria vaccines (Dinglasan et al., 2007) as Plasmodium falciparum (the dominant African malaria parasite)-infected erythrocytes express proteins, such as variant surface antigen 2-CSA that bind to chondroitin sulfate A and mediate malarial pathogenesis. Commercial applications for both chondroitin sulfate/dermatan sulfate have been growing, such as the use of chondroitin sulfate in bone repair and dermatan sulfate in wound repair. Other applications of chondroitin sulfate, with other biopolymers such as hyaluronan and collagen, include designing slow and controlled biodegradable scaffolds for wound healing.

In humans, chondroitin sulfate also exists as proteoglycans, synthesized and attached to specific core proteins (Mikami and Kitagawa, 2013). Like heparan sulfate/heparin, the biosynthesis of chondroitin sulfate begins with the formation of the tetrasaccharide linker xylose-galactose-galactose-GlcA on serine residues of the core protein. Next, a b1–4 linked GalNAc is added to the non-reducing GlcA. Chondroitin synthase sequentially adds GalNAc and GlcA, forming a linear non-sulfated chondroitin. The polysaccharide-backbone is further modified through the action of various sulfotransferases to produce sulfated CS. Epimerization of GlcA into IdoA and subsequent sulfation of sugar moieties results in dermatan sulfate (Table 2).

**Keratan Sulfate**

Keratan sulfates are naturally occurring linear sulfated GAGs made of repeating Gal and GlcNAc residues (Pomin, 2015). The polysaccharide chain can be further modified through the action of 6-O-sulfotransferases (GlcNAc6ST) that act on both the GlcNAc and Gal moieties (Uchimura, 2015). Keratan sulfate is an important ECM component and has been found in cornea, cartilage, and brain (Funderburgh, 2000).

**Industrial Production of Glycosaminoglycans: Current State and Future Directions**

Industrial scale production of commercially relevant GAGs can be broadly grouped as (1) production from animal sources, (2) production from cultured microbial cells, and (3) production from cultured eukaryotic cells (Schiraldi et al., 2010; Boeriu et al., 2013; Cimini et al., 2017).

**Industrial Production of GAGs From Animal Sources**

Industrial production of GAGs from animal sources is performed for industrial production of hyaluronan, heparin, and chondroitin sulfate. GAGs are abundant in animal tissue. Specific animal tissues have been used for GAG extraction, for example, (1) hyaluronan from rooster combs and bovine/porcine eyes, (2) chondroitin from animal cartilage, (3) dermatan sulfate from mucosal tissue and animal hide/skin, and (4) heparin from porcine/marine mast cells that are found in intestine and lung tissues. The extraction of GAGs is performed in non-cGMP slaughter houses (Boeriu et al., 2013). The extracted GAGs are then purified in cGMP facilities. However, concerns such as the presence of undesirable animal products and contamination risks (viruses, prions) have motivated development of methods for industrial production of GAGs through fermentation or cell culture (Fu et al., 2016). In addition, the ability to create tailored GAGs with specific or novel functionality creates another impetus for controlled production (Oduah et al., 2016).

**Industrial Production of GAGs From Microbial Sources**

Certain bacteria have the necessary enzyme machinery for producing simple, non-sulfated GAGs, such as hyaluronan, heparosan, and chondroitin (Fig. 3). Industrial production of GAGs from microbial sources is performed for production of hyaluronan from Streptococcus sp. and chondroitin sulfate from E. coli K4.

Hyaluronan is a simple non-sulfated GAG. Bacteria such as the gram-positive Streptococcus sp. and gram-negative bacteria Pasteurella multocida, can naturally produce hyaluronan (Schiraldi et al., 2010; Cimini et al., 2017). The hyaluronan exists in the mucoid capsule of these bacteria and aids in the pathogenicity. In S. zooepidemicus, genes required for the hyaluronan biosynthesis are encoded in the HAS operon (hasA, hasB, hasC, hasD, and hasE) (Table 3). In the presence of magnesium ions (e.g., MgCl2), bacterial HAS enzymes polymerize the hyaluronan chain using UDP-GlcNAc and UDP-GlcA as substrates. Under certain conditions, the S. zooepidemicus may produce 6–7 g/L of hyaluronan. The hyaluronan from the fermentation broth is recovered using ultrafiltration, and precipitation with chemicals such as cetylpyridinium chloride. The final product is sterilized and tested for activity and endotoxin levels. Recent advances in exploring alternative bioengineered microbial hosts toward hyaluronan production include E. coli, Bacillus sp., Agrobacterium sp., and Lactobacillus lactis. Escherichia coli JM109 co-expressing two genes, pmHas and KdD, from E. coli K5 strain, was able to produce 0.5 g/L hyaluronan in shake-flask culture. The fed-batch fermentation and optimization of fermentation parameters, such as the addition of glucosamine supplements, led to the production of 2.0–3.8 g/L hyaluronan in fed-batch fermentation using the bioengineered E. coli JM109 strain. L. lactis, co-expressing genes hasA, hasB, and hasC from S. zooepidemicus, was able to produce 1.8 g/L hyaluronan in a batch bioreactor.
Industrial Production of Glycosaminoglycans


Table 3  Hyaluronan biosynthesis genes in bacteria Streptococcus zooepidemicus

<table>
<thead>
<tr>
<th>Hyaluronan genes</th>
<th>Gene (product) and function</th>
</tr>
</thead>
<tbody>
<tr>
<td>hasC</td>
<td>hasC (gtaB, UDP-glucose pyrophosphorylase) is required for conversion of glucose – 1-phosphate to UDP-glucose</td>
</tr>
<tr>
<td>hasB</td>
<td>hasB (tuAD, UDP-glucose dehydrogenase) is required for conversion of UDP-glucose to UDP-glucuronic acid</td>
</tr>
<tr>
<td>hasE</td>
<td>hasE (pgl, phosphoglucosaminate) is required for reversible conversion of glucose – 6-phosphate to fructose – 6-phosphate</td>
</tr>
<tr>
<td>hasD</td>
<td>hasD (gcAD, Acetyl transferase/UDP-GlcNAc pyrophosphorylase) is required for conversion of GlcN – 1-Pto UDP-GlcNAc</td>
</tr>
<tr>
<td>hasA</td>
<td>hasA (hyaluronan synthase) is required for polymerization of UDP-GlcA and UDP-GlcNAc</td>
</tr>
</tbody>
</table>
Agrobacterium sp. ATCC 31749, co-expressing pmHAS gene from *P. multocida* and the kfrd gene (encodes UDP-glucose dehydrogenase) from *E. coli* K5 strain, was able to produce 0.3 g/L hyaluronan in shake-flask cultures.

Industrial production of sulfated GAGs, such as chondroitin sulfate and heparin, would require production of chondroitin or heparan, respectively, in bacteria, followed by purification and subsequent chemoenzymatic synthesis of chondroitin sulfate and heparin. Certain bacteria, for example, *E. coli* K4 and *E. coli* K5, are able to synthesize chondroitin and heparan, respectively (Cress et al., 2013a,b). Bacterial chondroitin and heparan are part of their capsular polysaccharides.

Recently, industrial production of food grade chondroitin sulfate from microbial fermentation of *E. coli* K4 has been developed (see Relevant Websites section). Briefly, *E. coli* K4 fermentation and subsequent purification produced crude chondroitin sodium. Regioselective sulfation of the chondroitin sodium, followed by purification produced chondroitin sulfate sodium (Mythochondro™). In a parallel experiment, metabolic engineering of *E. coli* BL21 StarTM (DE3) strain with ePathBrick vector, encoding K4 CS-O genes, namely, KfoF, KfoA and KfoC, resulted in 2.4 g/L in a fed batch fermentation (He et al., 2015).

Production of pharmaceutical heparin is estimated at almost 100 metric tons annually, with an annual sale of over $3 billion (Fu et al., 2016; Onishi et al., 2015). Currently, pharmaceutical heparin is extracted and purified from pig or cow mucosal tissues. Animal-sourced heparin production begins in non-cGMP animal slaughterhouses, followed by extraction and purification in cGMP facilities. However, concerns such as extraction of heparin in non-cGMP slaughterhouses, contamination risks (virus, prions), and the possibility of adulteration in sites not under USFDA or EMEA supervision, have necessitated alternate routes for heparin production (Fu et al., 2016). Understanding the heparin biosynthetic pathway and the key enzymes required for biosynthesis of heparin has provided strategies for industrial production of heparin from non-animal sources, such as chemoenzymatic synthesis of heparin from microbial heparan and metabolic engineering of mammalian cells (Cai et al., 2013; Fu et al., 2016; Bhaskar et al., 2015, 2012; Masuko and Linhardt, 2012; Higashi et al., 2011; Wang et al., 2010, 2011; Jin et al., 2016). Knock-in and knock-out animal- and cell-culture studies demonstrated that HS/HP enzymes, NDST2, 2OST, C5epi (GLCE), 6OSTs and 3OST1 are important for the production of antithrombin binding sites on heparin (Fig. 4) (Lin et al., 2000; Pönighaus et al., 2007; Esko and Selleck, 2002; Bame and Esko, 1989; Bai and Esko, 1996; Aikawa et al., 2001; Bame et al., 1991; Gasimli et al., 2014). The chemoenzymatic synthesis of heparin uses microbial systems for the production of heparin precursors (i.e., heparan) followed by chemoenzymatic modification using chemicals and/or the critical enzymes NDST2, 2OST, GLCE, 6OSTs and 3OST1. For example, bacteria such as *Pasteurella multocida* type D strain and *E. coli* K5 naturally produce capsular heparosan (Wang et al., 2010, 2011; Chavaroche et al., 2012). Production of heparosan in *P. multocida* type D strain involves sequential addition of GlcNAc and GlcA, through the actions of glucosyltransferases (PmHS1) (Chavaroche et al., 2012). Production of heparan in *E. coli* K5 involves sequential addition of GlcNAc and GlcA, through the actions of glucosyltransferases (KfrA and KfrC) (Wang et al., 2010, 2011). Fermentation process optimization in *E. coli* K5 in a fed-batch fermentation in a glucose-based defined medium at 37°C, with 30% dissolved oxygen, resulted in 15 g/L crude heparosan (Wang et al., 2010, 2011). Recently, researchers have metabolically

engineered an *E. coli* BL21 strain with *E. coli* K5 glucosyltransferases and demonstrated heparosan production in the engineered BL21 strain (Zhang et al., 2012). The heparosan thus obtained from microbial sources, could be modified chemoenzymatically to produce anticoagulant heparin (Bhaskar et al., 2015). In another approach, mammalian cells, such as CHO cells which naturally produce heparan sulfate, could be metabolically engineered toward the production of heparin (Baik et al., 2012).

Optimization of GAG production in bacteria requires availability of genome sequence combined with advances in analytical and metabolic engineering tools. Genome sequencing has revealed gram-positive and gram-negative bacteria capable of producing simple non-sulfated GAGs, such as hyaluronan, chondroitin and heparanosan. The biosynthesis of hyaluronan, heparan sulfate and chondroitin share a carbon source that is also required for maintaining cell growth and biosynthesis of cell wall components. For example, glucose, the precursor for of GlcNAc and Glc, is utilized for the biosynthesis of glucose-6-phosphate (Fig. 3). The glucose-6-phosphate is utilized for cell growth via glycolysis and the pentose phosphate pathway, as well as biosynthesis of cell wall components (Wang et al., 2011). Commercial production of GAGs from bacterial sources will require studies on metabolic control analysis and metabolic flux analysis. These methods may be utilized to evaluate correlations between cell metabolism and GAG production, in response to internal cues (e.g., genetics) and external cues (e.g., media composition). For example, it has been postulated that UDP-glucose 6-dehydrogenase and production of UDP-GlcA could be limiting factors for GAG biosynthesis. High throughput gene screening may reveal genes that steer metabolites toward production of GAGs without affecting cell growth.

ePathbrick (Xu et al., 2012) vectors have been used to metabolically engineer a chondroitin pathway in BL21, resulting in CS yield comparable to CS produced from *E. coli* K4 (2.9 g/l) (He et al., 2015; Xu et al., 2012; Zhao et al., 2015; Xu and Koffas, 2013). In parallel, CRISPathbrick (Cress et al., 2015) can be utilized to tune expression of exogenous genes and repress endogenous genes in *E. coli* (Cress et al., 2015).

**Conclusion**

The medical and non-medical uses of GAGs (e.g., heparin, hyaluronan, chondroitin sulfate, dermatan sulfate) have led to a need for industrial-scale production of GAGs from Generally recognized as safe (GRAS) organisms. Collaborative research toward the improvement of current strains that produce simple GAGs, exploring new organisms (bacterial, simple eukaryotes, and mammalian systems, such as CHO cells), as well as improvement and development of analytical and metabolic engineering tools will play significant roles in the industrial production of simple and complex GAGs.

**References**


Relevant Websites


Amin Talati & Upadhye (Attorneys at Law).

https://www.fda.gov/drugs/developmentapprovalprocess/manufacturingucm169105.htm

US Food and Drug Administration.