Industrial Production of Glycosaminoglycans

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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.

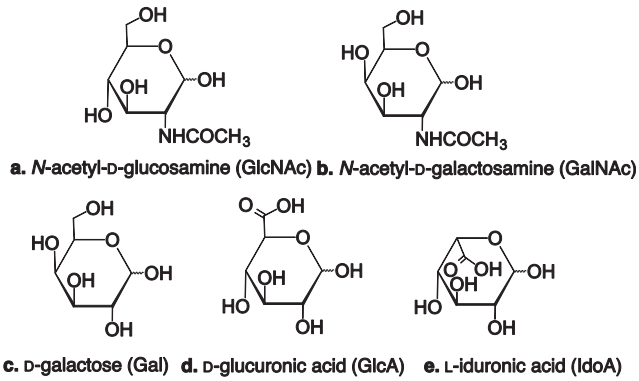

Fig. 1 Building blocks of glycosaminoglycans (a) *N*-acetyl glucosamine, (b) *N*-acetyl D-galactosamine, (c) D-galactose, (d) D-glucuronic acid, and (e) L-iduronic acid. These sugar moieties may be further modified through the action of specific enzymes, such as *N*-deacetylases, sulfotransferases, and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

Glycosaminoglycans

The glycosaminoglycan family includes: (1) hyaluronan (hyaluronate, 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 spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy (Sun *et al.*, 2016; Schlessinger *et al.*, 2000; Xiao *et al.*, 2010; Cai *et al.*, 2013, 2012; Laremore *et al.*, 2007; Fu *et al.*, 2016; Zhang *et al.*, 2015; Volpi *et al.*, 2009; Moon *et al.*, 2012; Edens *et al.*, 1992; 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; Mikhailov *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 3OST1 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 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; Tzellos *et al.*, 2009; Anderegg *et al.*, 2014; Oh *et al.*, 2011). Due to its mucoadhesive behavior and contribution to the elastoviscous behavior of soft connective tissues, for example, in synovial fluid in

Table 1 Glycosaminoglycans

Type	*Composition*	*Chemical Formula*
Hyaluronan	Linear polymer of alternating D-glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc).	→4)β-D-GlcA (1→3) β-D-GlcNAc (1→, with no sulfation
Family of heparin, heparan sulfate and heparosan	Linear polymer of alternating D-glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc). Heparosan is modified through the actions of tissue specific sulfotransferases, epimerases to form heparan sulfate and heparin.	→4) β-D-GlcA or α-L IdoA(1→4) β-D-GlcNAc/S(1→, with sulfation
Family of chondroitin sulfate and dermatan sulfate	Linear polymer of alternating D-glucuronic acid (GlcA) and N-acetylglucosamine (GalNAc). Chondroitin modified through the actions of tissue specific sulfotransferases to form chondroitin sulfate; dermatan sulfate is sulfated as well as the GlcUAGlcA moiety of dermatan sulfate is epimerized into iduronic acid.	→4) β-D-GlcA or α-L-IdoA(1→3) β-D-GalNAc(1→, with sulfation
Family of keratan sulfate	Linear polymer of alternating galactose (Gal) and N-acetylglucosamine (GalNAc), with sulfation, for example, sulfation at the carbon 6 of galactose and N-acetylglucosamine.	→3) β-D-Gal (1→4) β-D-GalNAc (1→, with sulfation

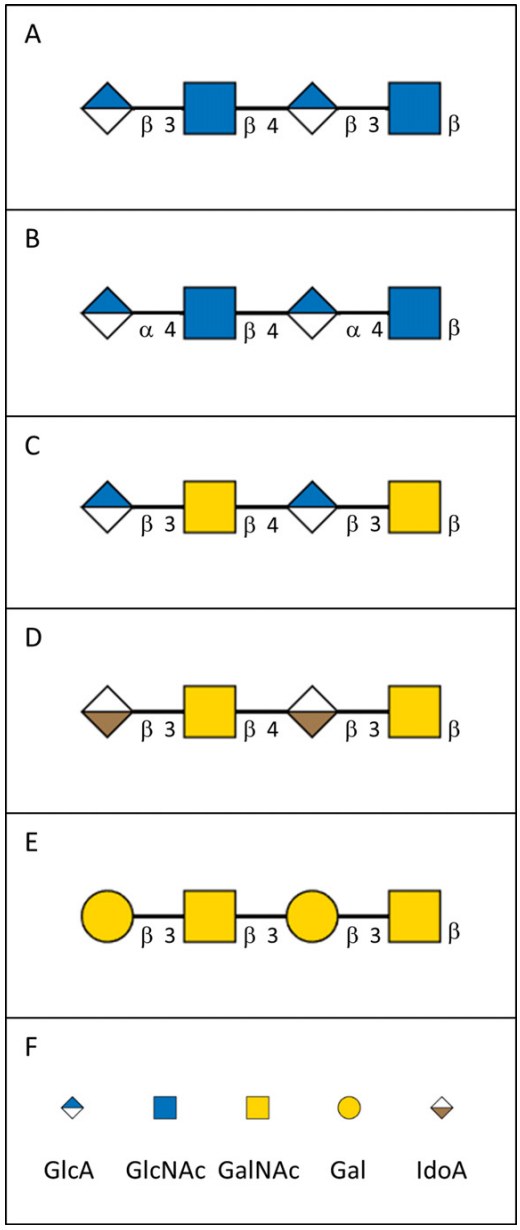

Fig. 2 Sugar moiety backbone of glycosaminoglycans (A) hyaluronan, (B) family of heparin, heparan sulfate and heparosan, (C) family of chondroitin and chondroitin sulfate, (D) dermatan sulfate, (E) family of keratan sulfate, and (F) sugar moieties, namely, glucuronic acid (GlcA), N-acetylglucosamine (GlcNAc), N-acetylglucosamine (GalNAc), 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-acetylglucosamine 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 heparin 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.

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

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; Boeriu *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 -xylose-galactose-galactose-GlcA on serine residues of the core protein. Next, α-*N*-acetyl glucosaminyl transferase adds α-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 (Saribaş *et al.*, 2004). The GlcA residues may be epimerized into IdoA by C5-epimerase (GLCE) enzymes (Feyerabend *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*-sulfotransferse 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). Heparan 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 the 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/dermatan 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;

Table 2 Disaccharide units of chondroitin sulfate and dermatan sulfate

CS/DS	*Major disaccharide units and their sulfation pattern*	*Chemical formula*
Chondroitin (CS-DS)	Unsulfated glucuronic acid and unsulfated N-acetylgalactosamine	→4) β-D-GlcA(1→3)- β-D-GalNAc(1→
Chondroitin sulfate A (CS-A); chondroitin – 4-sulfate	Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 4	→4) β-D-GlcA(1→3)- β-D-GalNAc4S(1→
Dermatan sulfate (CS-B)	L-iduronic acid and N-acetylgalactosamine sulfated at carbon 4	→4) α-L-IdoA(1→3)- β-D-GalNAc4S(1→
Chondroitin sulfate C (CS-C); chondroitin – 6-sulfate	Unsulfated glucuronic acid and N-acetylgalactosamine sulfated at carbon 6	→4) β-D-GlcA(1→3)- β-D-GalNAc6S(1→
Chondroitin sulfate D (CS-D); chondroitin – 2,6-sulfate	Glucuronic acid sulfated at carbon 2 and N-acetylgalactosamine sulfated at carbon 6	→4) β-D-GlcA2S(1→3)- β-D-GalNAc6S(1→
Chondroitin sulfate E (CS-E); chondroitin – 4,6-sulfate	Unsulfated glucuronic acid and N-acetyl galactosamine sulfated at carbon 4 and 6	→4) β-D-GlcA(1→3)- β-D-GalNAc4S6S(1→

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 β1–4 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 (**Table 1**, **Fig. 2**) (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/bovine 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., $MgCl_2$), 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 gene from *P. multocida* and kfiD gene 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.

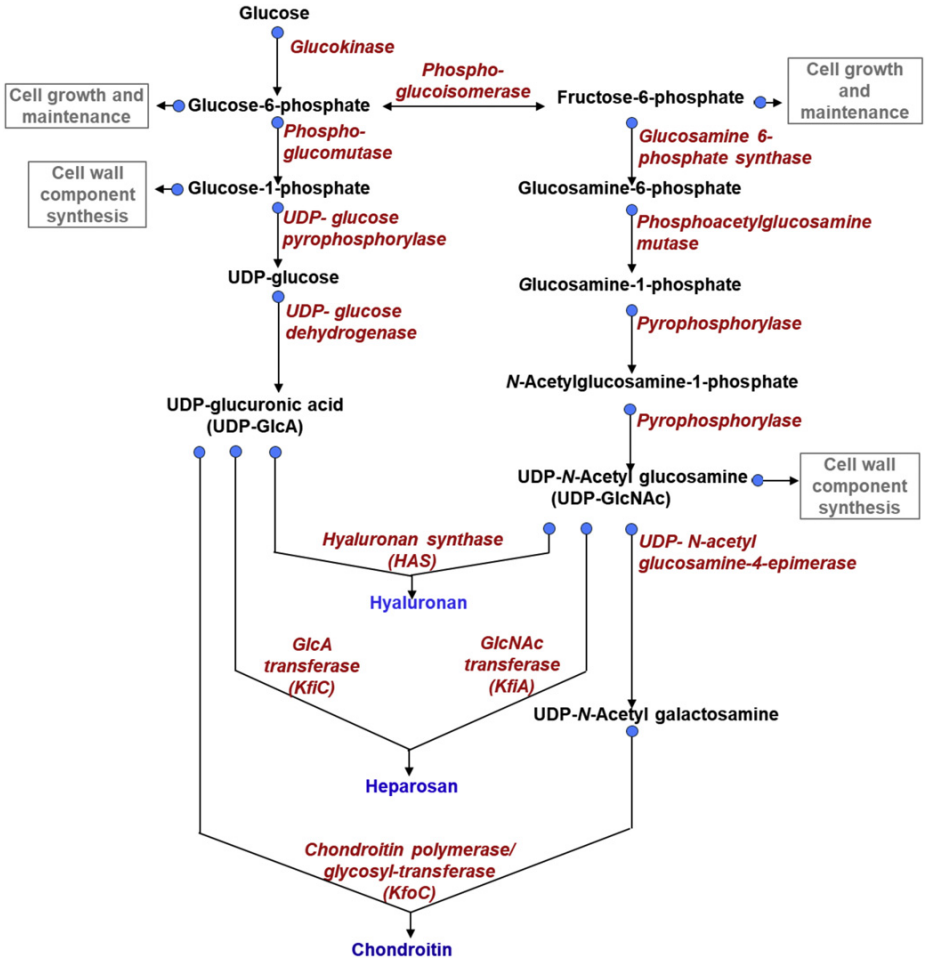

Fig. 3 Biosynthetic pathways for hyaluronan, heparosan and chondroitin in microbial species Reproduced from Schiraldi, C., Gatta, A. La, Rosa, M. De, 2010. Biotechnological production and application of hyaluronan. Biopolymers. 387–412; Cimini, D., Iacono, I. Dello, Carlino, E., *et al.*, 2017. Engineering S. equi subsp. zooepidemicus towards concurrent production of hyaluronic acid and chondroitin biopolymersof biomedical interest. AMB Express 7 (1), 61; Cress, B.F., Greene, Z.R., Linhardt, R.J., Koffas, M.A.G., 2013a. Draft genome sequence of Escherichia coli strain ATCC 23502, serovar O5:K4:H4. GenomeAnnouncements 1 (2); Cress, B.F., Greene, Z.R., Linhardt, R.J., Koffas, M.A.G., 2013b. Draft genome sequence of Escherichia coli strain ATCC 23506 (Serovar O10:K5:H4). GenomeAnnouncements 1 (2), 1–2; He, W., Fu, L., Li, G., *et al.*, 2015. Production of chondroitin in metabolically engineered *E. coli.* Metabolic Engineering 27, 92–100; Wang, Z., Ly, M., Zhang, F., *et al.*, 2010. *E. coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. Biotechnology and Bioengineering 107 (6), 964–973; Wang, Z., Dordick, J.S., Linhardt, R.J., 2011. *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. Bioengineered Bugs 2 (1), 63–67; Chavaroche, A.A.E., Van Den Broek, L.A.M., Boeriu, C., Eggink, G., 2012. Synthesis of heparosan oligosaccharides by *Pasteurella multocida* PmHS2 single-action transferases. Applied Microbiology and Biotechnology 95 (5), 1199–1210; Zhang, F., Lee, K.B., Linhardt, R.J., 2015. SPR biosensor probing the interactions between TIMP-3 and heparin/GAGs. Biosensors 5 (3), 500–512; Xu, P., Vansiri, A., Bhan, N., Koffas, M.A.G., 2012. EPathBrick: A synthetic biology platform for engineering metabolic pathways in *E. coli.* ACS Synthetic Biology 1 (7), 256–266; Zhao, S., Jones, J.A., Lachance, D.M., *et al.*, 2015. Improvement of catechin production in Escherichia coli through combinatorial metabolic engineering. Metabolic Engineering. 28, 43–53; Xu, P., Koffas, M.A.G., 2013. Assembly of multi-gene pathways and combinatorial pathway libraries through ePathBrick vectors. Methods in Molecular Biology 1073,107–129.

Table 3 Hyaluronan biosynthesis genes in bacteria *Streptococcus zooepidemicus*

Hyaluronan genes	*Gene (product) and function*
hasC	*hasC* (gtaB, UDP-glucose pyrophosphorylase) is required for conversion of glucose – 1-phosphate to UDP-glucose
hasB	*hasB* (tuaD, UDP-glucose dehydrogenase) is required for conversion of UDP-glucose to UDP-glucuronic acid
hasE	*hasE* (pgI, phosphoglucoisomerase) is required for reversible conversion of glucose – 6-phosphate to fructose – 6-phosphate
hasD	*hasD* (gcaD, Acetyl transferase/UDP-GlcNAc pyrophosphorylase) is required for conversion of GlcN – 1-Pto UDP-GlcNAc
hasA	*hasA* (hyaluronan synthase) is required for polymerization of UDP-GlcA and UDP-GlcNAc

Agrobacterium sp. ATCC 31749, co-expressing *pmHAS* gene from *P. multocida* and the kfiD 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 heparosan, 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 heparosan, respectively (Cress *et al.*, 2013a,b). Bacterial chondroitin and heparosan 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 heparosan 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., heparosan) 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 heparosan in *E. coli* K5 involves sequential addition of GlcNAc and GlcA, through the actions of glucosyltransferases (KfiA and KfiC) (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

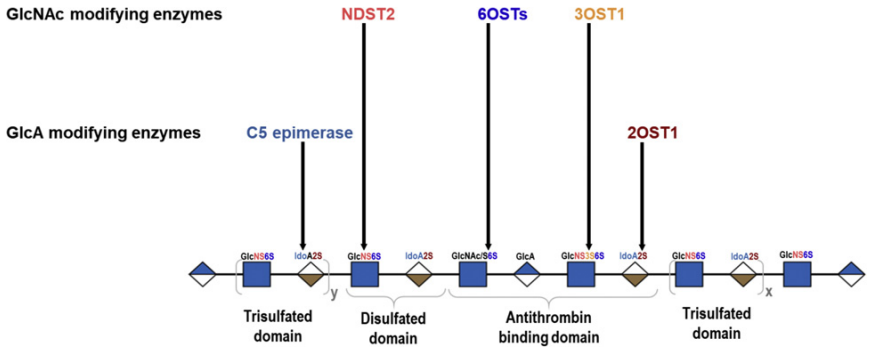

Fig. 4 HS/HP enzymes, NDST2, C5epi, 2OST1, 6OSTs, 3OST1 are important for biosynthesis of antithrombin site on anticoagulant heparin. Lin, X., Wei, G., Shi, Z., *et al.*, 2000. Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice. Developmental Biology 224 (2), 299–311; Pönighaus, C., Ambrosius, M., Casanova, J.C., *et al.*, 2007. Human xylosyltransferase II is involved in the biosynthesis of the uniform tetrasaccharide linkage region inchondroitin sulfate and heparan sulfate proteoglycans. Journal of Biological Chemistry 282 (8), 5201–5206; Esko, J.D., Selleck, S.B., 2002. Order out of chaos: Assembly of ligand binding sites in heparan sulfate. Annual Review of Biochemistry 71, 435–471. Available from: papers http://626fef3a-d60d-49b2-89f3-1999b93e20cf/Paper/p2885; Bame, K.J., Esko, J.D., 1989. Undersulfated heparan sulfate in a Chinese hamster ovary cell mutant defective in heparan sulfate N-sulfotransferase. The Journal ofbiological chemistry 264 (14), 8059–8065. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2524478; Bai, X., Esko, J.D., 1996. An animal cell mutant defective in heparan sulfate hexuronic acid 2-O- sulfation. Journal of Biological Chemistry 271 (30), 17711–17717; Aikawa, J.I., Grobe, K., Tsujimoto, M., Esko, J.D., 2001. Multiple isozymes of heparan sulfate/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase. Structure and activity ofthe fourth member, NDST4. Journal of Biological Chemistry 276 (8), 5876–5882; Bame, K.J., Lidholt, K., Lindahl, U., Esko, J.D., 1991. Biosynthesis of heparan sulfate: Coordination of polymer-modification reactions in a Chinese hamster ovary cell mutantdefective in N-sulfotransferase. Journal of Biological Chemistry 266 (16), 10287–10293; Datta, P., Li, G., Yang, B., *et al.*, 2013. Bioengineered Chinese hamster ovary cells with golgi-targeted 3-O-sulfotransferase-1 biosynthesize heparan sulfate with anantithrombin-binding site. Journal of Biological Chemistry 288 (52), 37308–37318; Gasimli, L., Glass, C.A., Datta, P., *et al.*, 2014. Bioengineering murine mastocytoma cells to produce anticoagulant heparin. Glycobiology 24 (3), 272–280.

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 heparosan. The biosynthesis of hyaluronan, heparosan 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 GlcA, 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.

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