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Production methods for heparosan, a precursor of heparin and heparan sulfate

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ABSTRACT

Heparin and heparan sulfate belong to the glycosaminoglycan family. Heparin which is known as a powerful anticoagulant has been also described to have potential in therapeutic applications such as in the treatment against cancer and prevention of virus infections. Heparan sulfate, an analog of heparin, which is not used for medical purposes yet, was reported to have the same pharmaceutical potential as heparin. Both heparin and heparan sulfate share a common precursor molecule known as heparosan. Heparosan determines the polymer chain length and the sugar unit backbone composition, which are determinant structural parameters for the biological activity of heparin and heparan sulfate. In this review we give an overview of the different methods used to synthesize heparosan, and we highlight the pro and cons of each method in respect to the synthesis of bioengineered heparin-like molecules. Advancements in the field of the synthesis of bioengineered heparin are also reported.

1. Introduction

Glycosaminoglycans (GAGs) which are un-branched and negatively charged polysaccharides are made of a repetition of disaccharide units (Bishop, Schuksz, & Esko, 2007; Linhardt & Toida, 2004). This group of carbohydrates play a key role in the cells by being involved in cell adhesion, chemokine signaling, biochemical cascades, signal transduction, and even pathogen recognition (Bishop et al., 2007; Linhardt & Toida, 2004). Due to their physiological functions, GAGs constitute a class of compounds with a large potential for therapeutic applications. Some GAGs, such as hyaluronic acid, chondroitin, and heparin, either obtained from animal derivatives, produced by chemical synthesis or by genetically modified microorganisms, are already used in medical applications. These polysaccharides differ by their level of structural complexity (Esko, Koji, & Lindahl, 2009, chap. 16). Among them heparin, which is one of the most complex GAGs, is used since the middle of the 1930s as an anticoagulant compound. It is mainly used to prevent blood clotting during surgery and it is also administrated in kidney dialysis and for the treatment of acute coronary syndromes (Rabenstein, 2002). Worldwide, about 100 t of pharmaceutically grade heparin products are annually produced and used (Bhaskar et al., 2012; Liu, Zhang, & Linhardt, 2009). Heparin products are commercialized as unfractionated (UF) heparin (Mw. 14,000), low molecular weight (LMW) heparin (Mw. 6000) and synthetic ultra-low molecular weight (ULMW) heparin (Mw. 1508.3) (Xu et al., 2011). Most of this anticoagulant heparin is isolated from pig intestines and bovine lungs (UF and LMW heparin) (Liu, Zhang, et al., 2009; Xu et al., 2011), and only a small fraction of it is obtained by chemical synthesis (ULMW heparin) (Choay et al., 1983; Petitou & Van Boeckel, 2004). The production of anticoagulant heparin-based products using genetically modified microorganisms and/or recombinant enzymes is still under investigation.

In addition to its antithrombotic activity, heparin (Hep) but also the structurally related polysaccharide heparan sulfate (HS) have been described as having a therapeutic potential in the treatment against cancer (Yip, Smollich, & Götte, 2006) and in the prevention of virus infections (Bishop et al., 2007; Rusnati et al., 2009). The biological activity of these polysaccharides was found to be influenced by the polymer chain length, the sugar unit composition, and the sulfation patterns (Chen et al., 1997; Rusnati et al., 2009; Yip et al., 2006). Thus, to study the potential of a variety of heparin and heparan sulfate as new drugs and their utilization in new therapeutic settings, the availability of well-defined heparin and heparin-like molecules is required.

Both currently used heparin production methods have some drawbacks. Indeed the traditional production systems using animal derivatives do not yield homogenous and well defined products and can represent a potential safety risk (Guerrini et al., 2008). The chemical synthesis of heparin oligomers is laborious and not economically feasible for the synthesis of heparin longer than hexasaccharides. Therefore, in order to guaranty cost effective and safe anticoagulant products and to synthesize well defined heparin-based drugs, there is a need in developing alternative systems that control tightly each synthesis step of Hep/HS.

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In this review an overview is given of the different ways enabling to design specific heparin-like polymers. Here, the main focus is on the production of heparosan, the precursor of heparin and heparan sulfate, which determine the backbone structure and the polymer length.

2. Hep/HS biosynthesis in mammalian cells

Heparin and heparan sulfate have closely related structures. In mammalian cells, their respective biosynthesis takes place in the Golgi apparatus and involves many enzymatic steps which only differ by the presence of various enzyme isoforms (Gorsi & Stringer, 2007; Rabenstein, 2002). As shown in Fig. 1, Hep/HS chains are initiated by the synthesis of a tetrasaccharide linker, composed of one glucuronic acid (GlcA), two galactose (Gal), and one xylose (Xyl) moieties, which binds to the serine residue of a core protein and leads to the production of proteoglycans (Esko et al., 2009; Kjellen & Lindahl, 1991; Silbert & Sugumaran, 2002). Among the 30 different proteoglycan protein cores (Iozzo, 1998), three major families of proteoglycans have been reported; membrane-spanning syndecans, the glycosylphosphatidylinositol-linked glypicans, and the basement membrane proteoglycans perlecans and agrin (Esko & Selleck, 2002). Heparin has only been reported as serglycin proteoglycans (Esko et al., 2009; Rabenstein, 2002). However, unlike heparin, heparan sulfate can be encountered with different core proteins. The core proteins are cell-type specific, but they are not specific for a defined heparan sulfate structure. Indeed, a same core protein can be found with different heparan sulfate structures (Coombe & Kett, 2005). The saccharide linker serves as template for the synthesis of the unsulfated precursor of both the Hep- and HS-proteoglycans known as heparosan. Heparosan is polymerized by glycosyltransferases belonging to the tumor suppressor *EXT* gene family and *EXT*-like genes (EXTL) family (Gorsi & Stringer, 2007; Mccormick, Duncan, Goutsos, & Tufaro, 2000). The glycosyltransferase EXT1 and EXT2 transfer GlcA and GlcNAc from activated sugar (UDP-α-sugar), using alternating inverting and retaining mechanisms and, thus forming a repetition of GlcA and GlcNAc units (β-D-1,4-GlcA-α-D-1,4-GlcNAc) (Esko & Selleck, 2002). Once heparosan elongation is terminated (step 1), the *N*-acetylglucosamine (GlcNAc) residues are randomly *N*-deacetylated into *N*-glucosamine (GlcN), prior to be *N*-sulfated into GlcNS by the dual action of the *N*-deacetylase/*N*-sulfatase (NDST) enzyme (step 2) (Bame, Lidholt, Lindahl, & Esko, 1991; Bame, Reddy, & Esko, 1991). Four NDST isoforms have been reported; their level of expression is tissue dependent and each isoform catalyze different ratio of deacetylation/sulfation (Raman, Nguyen, & Kuberan, 2011). It was reported that the *N*-sulfation orchestrated by NDST determines the occurrence of the following modification steps and thus is a critical step for generating diverse structures (Sheng, Liu, Xu, & Liu, 2011). It was indeed found that NDST2 is required for the synthesis of heparin in mast cells (Forsberg, 1999; Humphries, 1999), while NDST1 seems to be critical for the synthesis of heparan sulfate (Dagälv, Holmborn, Kjellén, & Åbrink, 2011). Following this step, some of the GlcA residues are converted into iduronic acid (IdoA) residues by the action of the glucuronyl C5-epimerase (Hepsi) (step 3). Up to date, the same glucuronyl C5-epimerase isoform has been identified to catalyze both heparin and heparan sulfate. Only the GlcA residues present in GlcNS–GlcA–GlcNS and GlcNS–GlcA–GlcNAc sequences can be converted into IdoA (Rabenstein, 2002). The disaccharide units in which the C5-epimerization catalyzes the conversion of GlcA adopts the α-L-1,4-IdoA-α-D-1,4-GlcNAc conformation (Hileman, Smith, Toida, & Linhardt, 1997; Rabenstein, 2002). Hereafter, the polysaccharide chain is *O*-sulfated by three *O*-sulfotransferases (*OST*): 2-*OST* (step 4), followed by 6-*OST* (step 5) and subsequently 3-*OST* (step 6) (Bhaskar et al., 2012). These enzymes transfer a sulfate group to the hydroxyl oxygen atom of distinct saccharide residues (Rabenstein, 2002). The *O*-sulfotransferases (*OST*) are not the only players in the sulfation pattern of the HS-proteoglycan chains. It was found that endosulfatase (SULF) participate also in the heparan sulfate sulfation pattern by reducing the amount of *O*-sulfate groups, mainly present in position C6 (step 7a) (Gorsi & Stringer, 2007; Lamanna et al., 2006, 2007; Lamanna, Frese, Balleininger, & Dierks, 2008).

Heparin and heparan sulfate synthesis involves the same cascade of enzymatic steps, nevertheless, as reported above, the participation of different enzyme isoforms results in distinct structures. While heparin is organized in one domain, heparan sulfate is composed of three domains in which the disaccharide unit composition and sulfation pattern differ. The non-sulfated domain (NA) is made of GlcA–GlcNAc repeats, the intermediate domain (NA/NS) is more sulfated than NA and composed of GlcNAc and GlcNS in combination with GlcA, and the other domain contains the highly sulfated GlcNS residues (NS). In heparan sulfate the NA domain is the most abundant one, the number of GlcNAc and GlcNS are about the same, and the number of IdoA residue is lower than the GlcA residues (Coombe & Kett, 2005; Rabenstein, 2002). The different ratios of NA, NS, and NA/NS domains appear to be determined by the cell-type in which the synthesis occurs (Esko & Selleck, 2002). Heparin, however, is only composed of the NS-like domain and is therefore highly sulfated. In addition, unlike heparan sulfate which is an ubiquitous component of cell surfaces and extracellular matrix (Esko et al., 2009; Rabenstein, 2002), and remains as a proteoglycan in the cells, heparin chains (60–100 kDa) are found exclusively in the mast cells granules and are randomly cleaved by endo-β-D-glucuronidase at the GlcA residues at the end of the synthesis process (step 7b) (Liu, Zhang, et al., 2009; Rabenstein, 2002). The uneven distribution of GlcA residues along the chain results in a polydisperse mixture of heparin chains (5–25 kDa) (Lindahl, Feingold, & Roden, 1986; Rabenstein, 2002).

3. Production of pharmaceutical Hep/HS compounds

3.1. Recovery of active compounds from animal derivatives

Most of the commercialized anticoagulant heparin products are obtained from pig intestine mucosa as starting material (Liu, Zhang, et al., 2009). The raw material is digested by proteolytic enzymes. The first step of the procedure is the pre-hydrolysis of the mixture at ambient temperature; it is followed by the hydrolysis step at 50–75 °C for about 6 h. Heparin, which is a polyanionic molecule, is extracted from the hydrolysate using an anion exchange resin. The adsorbed heparin is eluted with a high salt solution (Van Houdenhoven, 1999), and recovered from the eluent by ethanol precipitation. From there additional purification steps are carried on in facilities following good manufacturing practices (Bhaskar et al., 2012). From pig mucosa, a mixture of polydisperse heparin polysaccharides of 5–30 kDa is recovered (Rabenstein, 2002). In order to suit the medical requirements, low molecular weight heparin 4–6 kDa is produced from the native unfractionated heparin. Heparin fractionation is performed by chemical cleavage using nitrous acid or by enzymatic cleavage using heparinase (Gray, Mulloy, & Barrowcliffe, 2008; Linhardt, 2001, chap. 17). The low molecular weight heparin products represent the largest part of the heparin product sales. In the US market, the low molecular weight heparin product Lovenox (Adventis) corresponds to 70% of the heparin sales (Liu, Zhang, et al., 2009).

Although this traditional method is suitable to produce large quantity of anticoagulant heparin, it is important to keep in mind that structural variations can occur from one batch to another due

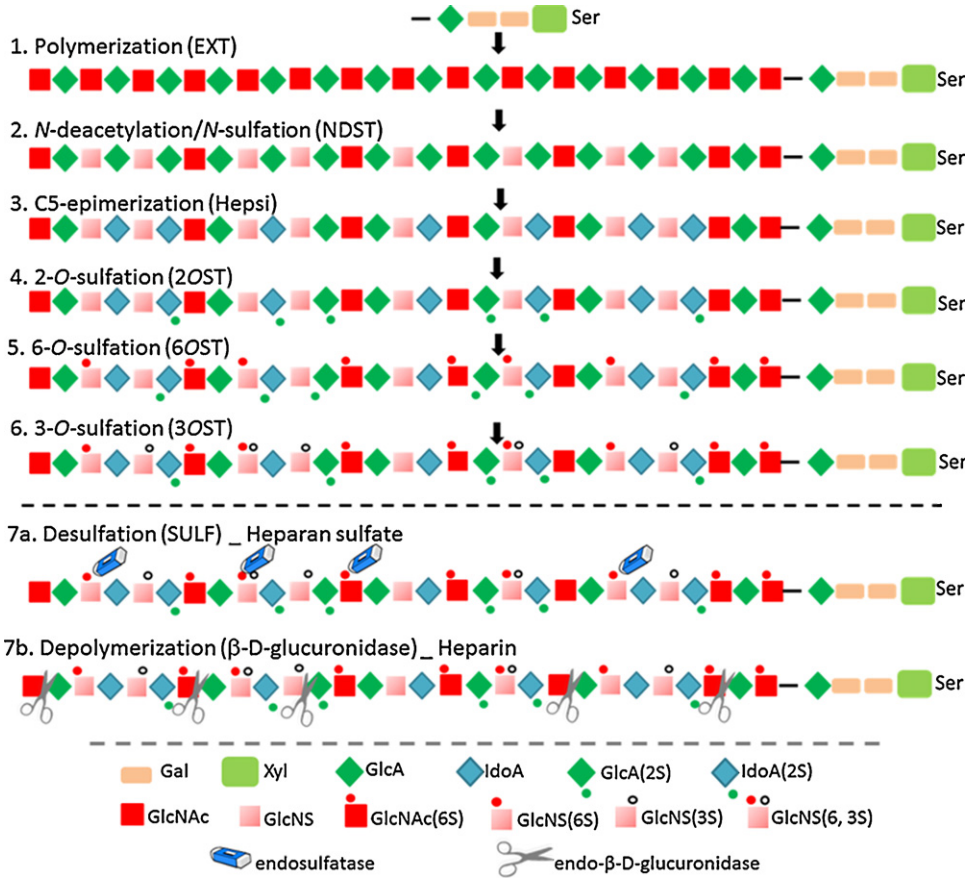

Fig. 1. Multi-step synthesis of heparin (Hep) and heparan sulfate (HS). The synthesis of Hep/HS is initiated by the polymerization of a tetrasaccharide linker: β-GlcA-1,3-β-Gal-1,3-β-Gal-1,4-β-Xyl-1-*O*-Ser. Subsequently, heparin and heparan sulfate are synthesized by a cascade of catalytic steps. Differences in the synthesis of heparin and heparan sulfate occur in the last step (step 7a: desulfation (endosulfatase SULF) and 7b: depolymerization by cleavage at the GlcA residues (endo-β-D-glucuronidase)). Adapted from Rabenstein (2002).

to the influence of environmental parameters such as variations in animal breeding. In addition to this fluctuation in quality, the use of animal tissue as primary material makes it impossible to tailor the chain composition and only the chain length can be controlled. The capacity to tailor make heparan sulfate structures is very important in order to explore the therapeutic potential of this GAG category.

3.2. Chemical synthesis of heparin products

Chemical synthesis of Hep/HS tetra- and pentasaccharide was introduced for the first time by Choay et al. (1983). In the 1980s, the collaboration between Sanofi (now Sanofi-Synthelabo) and Organon (currently MSD-Oss) resulted in the synthesis of a heparin synthetic analog pentasaccharide (Choay et al., 1983; Petitou & Van Boeckel, 2004). From this work, and after about ten years of a successful clinical development, Sanofi commercialized in 2002 a specific heparin pentasaccharide known as fondaparinux (brand name Arixtra, Sanofi-Synthelabo), and recognized to have strong anticoagulant properties. The right for Arixtra was sold to GSK in 2004 (Liu, Zhang, et al., 2009). Despite the fact that the production of this heparin pentasaccharide requires many steps (about 50 steps), synthesis of highly pure compound is performed at industrial scale (Petitou & Van Boeckel, 2004). Nowadays, the sales of the chemically synthesized Arixtra represent 3% of the heparin product market (Liu, Zhang, et al., 2009), while the low molecular weight heparin product Lovenox (Adventis) obtained from animal derivatives holds 70% of the heparin sales.

The chemical synthesis of longer chains is not possible due to the laborious work that represents the synthesis of the polysaccharide backbone and the epimerization of GlcA. Longer molecules cannot be synthesized using only chemical synthesis, and therefore combinations of enzymatic and chemical steps need to be applied for that purpose.

3.3. Enzymatic synthesis for the production of bioengineered heparin-based drugs

As an alternative to the extraction of heparin from animal derivatives and the chemical synthesis, (chemo)enzymatic catalysis can be used to produce well defined Hep/HS chains (Lindahl et al., 2005). The enzymes involved in each step of the Hep/HS synthesis (Fig. 1) such as heparosan synthases, *N*-deacetylase/*N*-sulfatase (NDST), glucuronyl C5-epimerase, and *O*-sulfotranferases (*OST*) have been isolated from mammalians cells or microorganisms, and expressed as recombinant proteins in *Escherichia coli* (Peterson, Frick, & Liu, 2009). They have been characterized in order to be used for the production of well-defined Hep/HS polymers.

Important advancements in the field of (chemo)enzymatic synthesis of anticoagulant heparin and heparin-based molecules have been done during the last decades and have been reviewed (Laremore, Zhang, Dordick, Liu, & Linhardt, 2009; Liu, Zhang, et al., 2009). A successful example of this is the synthesis of "neoheparin" (Lindahl et al., 2005). In addition, Kuberan et al., reported the enzymatic synthesis of a powerful anticoagulant molecule, Mitrin, using heparosan K5 as template (Chen et al., 2005; Kuberan, Beeler, Lawrence, Lech, & Rosenberg, 2003; Kuberan, Beeler, Lech, Wu, &

Rosenberg, 2003), and Xu et al. (2011), reported the synthesis of homogeneous ultra-low molecular weight heparin using recombinant heparosan synthase to synthesize the heparosan template.

Up to now, example of alternative production methods involving only enzymatic steps are still rare and do not allow the synthesis of a large diversity and molecules range of heparin-based polymers. In addition, currently the tailored enzymatic synthesis of heparin like compounds is only at gram scale (Xu et al., 2011). A better understanding and a tight control of each step is needed to achieve a higher production.

4. Heparosan production strategies

In this review the main focus is on the controlled production of heparosan (Fig. 2), the common precursor of heparin and heparan sulfate.

Since it was observed that the molecular weight and the unit composition are important for the Hep/HS biological activity, there is a general interest in controlling the length of the starting material and modifying the incorporated sugars. Heparosan, which can be obtained by different methods, determines the chain length and the size distribution of the Hep/HS polymers.

Thus a good method to control heparosan molecular weight is primordial since it is the most important "template" in the synthesis process.

4.1. Extraction of heparosan from microorganisms

GAGs are present in the polysaccharide capsule of some microorganisms in order to mimic the host polysaccharides and to attenuate the immune response during infection (Roberts, 1996). Until now, heparosan polymer has been found in the capsule of the pathogenic bacteria *E. coli* K5 (Vann, Schmidt, Jann, & Jann, 1981), *Pasteurella multocida* Type D (Pandit & Smith, 1993; Rimler, 1994), and *Avibacterium paragallinarum* (Wu et al., 2010). Heparosan production by fermentation of microorganisms has only been reported for *E. coli* K5 (Fig. 5A), and large scale fermentation enabled the recovery of 15 g of heparosan per liter culture. The heparosan molecular weight obtained after fermentation was found to be influenced by the medium composition and the culture conditions. Indeed Manzoni et al., reported after *E. coli* K5 fermentation, heparosan of about 16 and 1.5 kDa due to the presence of lyase in the culture medium (Manzoni, Bergomi, & Cavazzoni, 1996; Manzoni, Rollini, Piran, & Parini, 2004); while Wang, Dordick, and Linhardt (2011), reported higher molecular weight heparosan from 50 to 80 kDa (PDI > 1.5).

Up to date, heparosan templates used for (chemo)enzymatic synthesis are obtained from *E. coli* K5 capsule and fractionated to the desired length (Kuberan, Beeler, Lawrence, et al., 2003; Kuberan, Beeler, Lech, et al., 2003). Fractionation of heparosan is usually done using heparin lyase from *Flavobacterium heparinum*, however, this method produces unsaturated oligosaccharides. The double bond of the unsaturated uronic acid residue can be removed using mercuric salts (Ludwigs, Elgavish, Esko, Meezan, & Rodén, 1987) or ozonolysis (Masuko et al., 2011). Recently, a titanium dioxide-catalyzed photochemical reaction was used to control heparosan depolymerization (up to 8 kDa) without generating unsaturated oligosaccharides (Higashi et al., 2011). Although promising, this method needs to be further investigated to be able to produce pharmaceutical heparin. Despite the fact that the production of heparosan by microorganisms is cost effective and that efforts are made to depolymerize bacterial heparosan, neither the heparosan chain length can be tightly controlled and the sugar residue composition can be regulated. Indeed GlcA and GlcNAc present in the heparosan chain can only be modified after the polymer chain synthesis. However, due to the difficulty to control some post-polymerization modifications, it would be of interest to control more precisely the structure of the chain by adding analog sugars, such as IdoA instead of GlcA, during the elongation of heparosan chains (Liu et al., 2010; Xu et al., 2011).

4.2. Synthesis of heparosan using recombinant enzymes

In mammalian cells and in microorganisms, heparosan is synthesized by glycosyltransferases that catalyze alternately the transfer of the GlcNAc and GlcA residues from UDP-sugars to the growing polymer chain.

4.2.1. Recombinant heparosan synthases from mammalians and drosophila

The mammalians glycosyltransferases EXT1 and EXT2 have been reported to catalyze the elongation of heparosan chains. EXT1 and EXT2 form together an active hetero-complex, and their simultaneous expression in recombinant cells resulted in their full catalytic activity (Senay et al., 2000). The complex EXT1/2, as well as only EXT1 were able to elongate *in vitro* K5 heparosan acceptors by adding some extra sugar units (10–20 sugar units) (Busse & Kusche-Gullberg, 2003). Unlike EXT1, no significant transferase activity was observed when EXT2 was incubated in the absence of EXT1 (Mccormick et al., 2000). Kim et al. observed that the purified EXT1/2 complex synthesized heparosan polymers of about 170 kDa in the presence of GlcA–Gal–O–C_2H_4NH–benzyloxycarbonyl as template. If glypican-I core protein or α-trombomodulin proteoglycan were used as template molecules then heparosan polymers of 200 kDa were synthesized (Kim, Kitagawa, Tanaka, Tamura, & Sugahara, 2003). Detailed analysis of the mammalian glycosyltransferases in terms of activity and regulation of the elongation of polymer chains have not been reported yet.

In drosophila, a family of homolog proteins to the mammalians EXT is involved in the synthesis of heparosan: TTV, SOTV, and BOTV (Bellaiche, The, & Perrimon, 1998; Izumikawa, Egusa, Taniguchi, Sugahara, & Kitagawa, 2006).

4.2.2. Recombinant heparosan synthases from E. coli K5

In *E. coli* K5, the synthesis of heparosan is mainly regulated by the glucosaminyl transferase KfiA (Chen, Bridges, & Liu, 2006; Hodson et al., 2000) and the glucuronyl transferase KfiC (Griffiths et al., 1998), which transfer activated monosaccharide units to the non-reducing end of the heparosan growing chain. Sugiura et al. (2010) expressed KfiC and KfiA in *E. coli* BL21 (DE3) and showed that KfiC does not exhibit a transferase activity when incubated without KfiA in the presence of substrate and template molecules. In contrast, KfiA exhibited acetylglucosaminyl transferase activity when incubated in the absence of KfiC. In addition, they observed that the presence of an excess of KfiA increased the polymerization activity, while an excess of KfiC had no effect on the GlcNAc transferase activity. Heparosan chains of about 10 and 20 kDa were synthesized by the complex KfiA/KfiC after 8 and 18 h of incubation in the presence of heparosan oligosaccharide templates (7-mer), respectively. Recently, KfiA in combination to PmHS2 (described below) was used to control the elongation of heparosan oligosaccharides (Liu et al., 2010).

4.2.3. Recombinant heparosan synthases from P. multocida Type D

Unlike in *E. coli* K5, in *P. multocida* Type D the synthesis of heparosan is controlled by a bifunctional glycosyltransferase; the heparosan synthase PmHS1. Also a cryptic homolog to PmHS1 entitled PmHS2 has been identified, but its function was not known at the moment of identification. Nevertheless, when PmHS1 and PmHS2 were expressed as recombinant proteins in *E. coli*

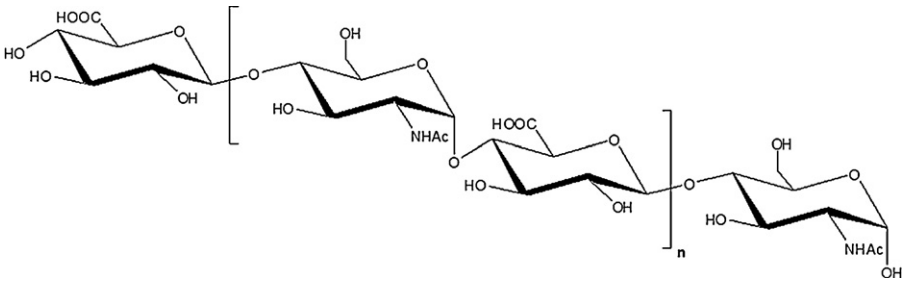

Fig. 2. Heparosan structure. Heparosan is the unsulfated precursor of heparin (Hep) and heparan sulfate (HS) and is constituted of a repetition of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) – unit (β-D-1,4-GlcA-α-D-1,4-GlcNAc)$_n$.

they both showed activity (Deangelis & White, 2002; May et al., 2001). Despite their high amino acid level homology (73%), the recombinant PmHS1 and PmHS2 exhibit different polymerization properties (Deangelis & White, 2004; Sismey-Ragatz et al., 2007). Polymerization in the absence or presence of templates has been investigated for *P. multocida* GAG synthases. Similarities between the GAG synthases PmHS1 (heparosan synthase) (Sismey-Ragatz et al., 2007) and PmHAS (hyaluronan synthase) (Jing & Deangelis, 2004) have been observed (Fig. 3A). Indeed, both PmHS1 and PmHAS exhibit a higher affinity at the acceptor site for short oligosaccharides than for UDP-sugars. The addition of oligosaccharide templates in the reaction mixture enable to avoid the polymer chain initiation step, and favor the synthesis of chains with a more narrow length distribution (Jing & Deangelis, 2004; Sismey-Ragatz et al., 2007). By controlling the amount of PmHAS and the UDP-sugar/hyaluronan templates, defined molecular weight hyaluronan polymers (16 kDa to 2 MDa) with a narrow polydispersity (PDI = 1.0–1.2) were obtained (Jing & Deangelis, 2004). Thus, the controlled elongation of heparosan by PmHS1 may be possible using similar conditions as PmHAS.

In contrast with PmHS1 and PmHAS, it was observed that PmHS2 did not exhibit a higher affinity for the heparosan templates than for the UDP-sugars (Chavaroche, Van Den Broek, Springer, Boeriu, & Eggink, 2011; Sismey-Ragatz et al., 2007). It was observed that

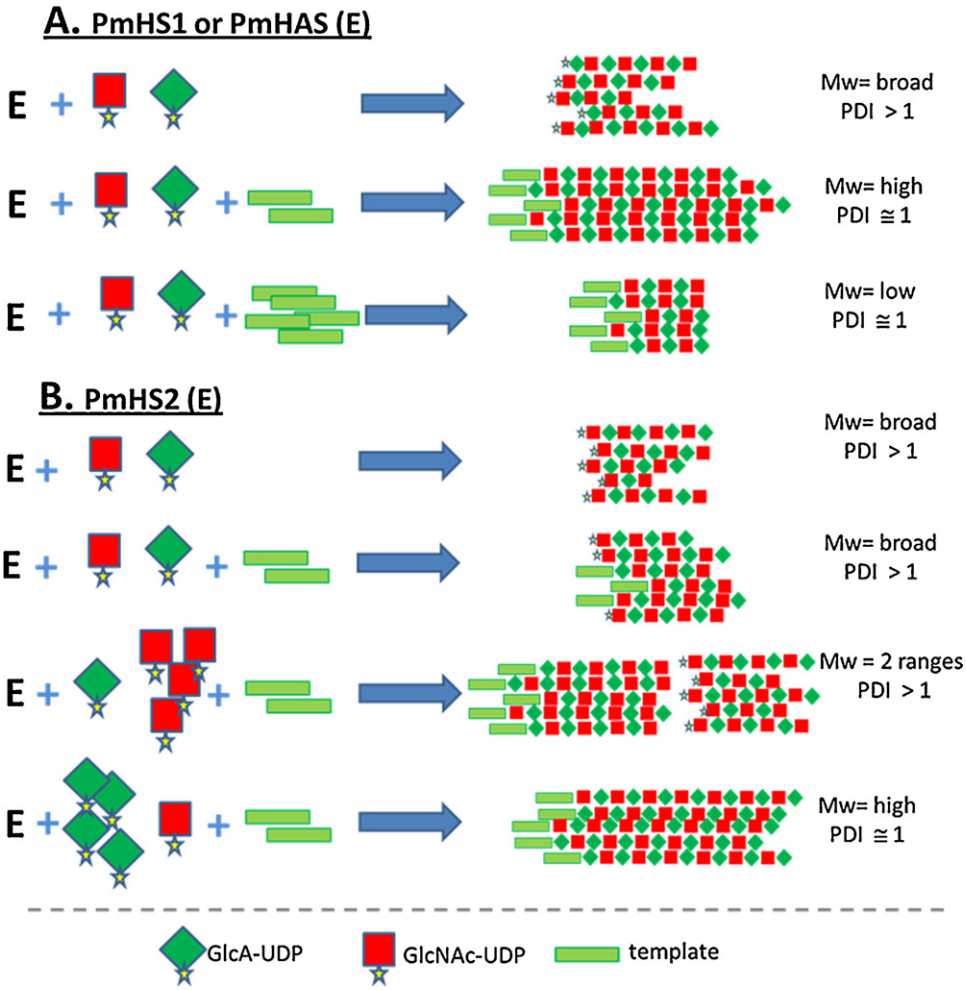

Fig. 3. Comparison between *P. multocida* GAG's synthase. Polymerization mechanism in the presence or the absence of oligosaccharide templates on the polymer elongation. (A) Comparison between PmHS1 and PmHAS. (B) PmHS2 polymerization mechanism (Chavaroche et al., 2010, 2011).
Adapted from Jing and Deangelis (2004).

the polymer chain initiation is not controlled by the affinity of the acceptor binding site for short oligosaccharides, but by the affinity for UDP–GlcNAc present in the polymerization reaction (Chavaroche et al., 2011). In that case narrower size distribution can be obtained when heparosan templates are incubated in the presence of an excess of UDP–GlcA (Fig. 3B).

PmHS bifunctional glycosyltransferases can be used to control heparosan elongation; nevertheless the control of heparosan elongation using *P. multocida* glycosyltransferases can also be achieved in another way. For the GTs belonging to the GT-A superfamily (Coutinho, Deleury, Davies, & Henrissat, 2003), such as the *P. multocida* glycosyltransferases, it has been shown that the substitution of both aspartic acids (D) by asparagines (N) in the DXD amino acid motif present in the catalytic center, results in the inactivation of the catalytic domains. PmHS1 (Kane, White, & Deangelis, 2006) and PmHS2 (Chavaroche et al., 2011) single action transferase mutants were obtained by applying accordingly site directed mutagenesis on the DXD motifs. Both the PmHS1 and PmHS2 glucuronyl transferases (PmHS-GlcA$^+$) and acetylglucosaminyl transferases (PmHS-GlcNAc$^+$) when incubated together were capable to elongate heparosan by transferring a GlcA and a GlcNAc residue. PmHS2 was found to initiate and elongate heparosan chain in the absence of oligosaccharide template. Important is that, in the case of PmHS2, only the PmHS2-GlcA$^+$ is able to initiate heparosan synthesis (Fig. 4) (Chavaroche et al., 2011).

PmHS2 single action transferases have been used for step by step synthesis of heparosan oligosaccharides (Fig. 5B) (Chavaroche, Van Den Broek, Boeriu, & Eggink, 2012). Analysis of the reaction mixtures confirmed that heparosan oligomers (disaccharides to octasaccharides) can be synthesized in a controlled manner and in the absence of template molecules by PmHS2 single action transferases. Since PmHS2-GlcNAc$^+$ cannot initiate heparosan chains (Chavaroche et al., 2011), the step by step synthesis using alternatively PmHS2-GlcA$^+$ and PmHS2-GlcNAc$^+$ resulted in a mixture of only even numbered or only odd numbered oligosaccharides depending on the last enzyme used (Chavaroche et al., 2012).

In conclusion, PmHS2-GlcA$^+$ and PmHS2-GlcNAc$^+$ do not need to form a complex together to exhibit catalytic activity. In this respect, the *P. multocida* heparosan synthases are an exception among the known heparosan synthases, and this represents a great advantage for the control synthesis of heparosan.

The step by step elongation was also reported in the presence of the glycosyltransferases KfiA (*N*-acetyl-D-glucosaminyl transferase) and PmHS2 (*N*-acetylglucosaminyl transferase and glucuronyl transferase) in the presence of a disaccharide acceptor (GlcA-AnMannose). Nevertheless, due to the dual action of PmHS2, the synthesis had to be carefully monitored (Liu et al., 2010). In addition, PmHS2 exhibits interesting polymerization characteristic by transferring modified sugars such as GlcNAc residues having different acyl chain length at the C2 position (Sismey-Ragatz et al., 2007).

The fact that, PmHS2 is composed of two functional single action transferases, and that it can elongate from UDP-sugar analogs, is of interest to control heparosan synthesis and per consequent can be applied to tailor heparin and heparan sulfate polymers with new biological properties.

4.3. Production of heparosan using genetically modified bacteria

The production of heparosan by genetically modified bacteria (Fig. 5C) has not been reported yet, but it was shown to be a successful method to produce hyaluronan. Hyaluronan or hyaluronic acid, is composed of the same sugar units as heparosan but with different glycoside linkages (-β-D-1,3-GlcNAc-β-D-1,4-GlcA)$_n$.

The expression of the *Streptococcus equisimilis* hyaluronan synthase *has*A gene and the UDP-glucose dehydrogenase gene,

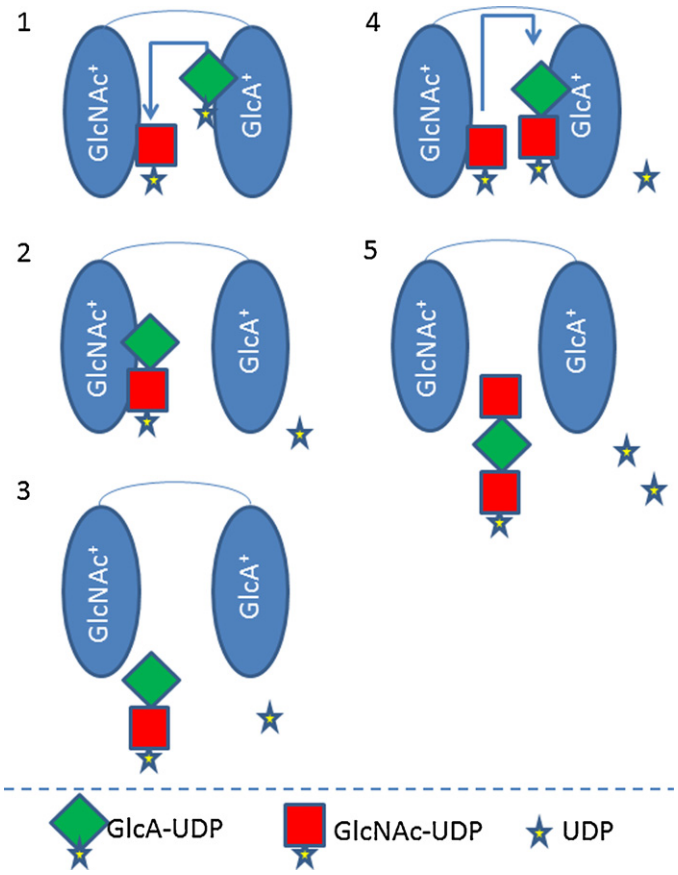

Fig. 4. Initiation of a heparosan chain by PmHS2. Step 1: UDP–GlcA binds to the GlcA$^+$-donor site and UDP–GlcNAc binds to the GlcNAc$^+$-acceptor site. Step 2: GlcA is transferred from the donor site to the UDP–GlcNAc acceptor molecule. Step 3: the newly synthesized GlcA–GlcNAc–UDP is released from the acceptor site. Step 4: the GlcA–GlcNAc binds to the GlcA$^+$-acceptor site, then the GlcNAc of the UDP–GlcNAc present on the GlcNAc$^+$-donor site is transferred. Step 5: the GlcNAc–GlcA–GlcNAc–UDP (DP3) is released. The heparosan elongation continues by adding alternately GlcNAc and GlcA to the growing chain. Important, it is still unknown if PmHS2 contains 2 acceptors sites in addition to the 2 donors sites.

in *Bacillus subtilis* permitted to recover from the supernatant multi-grams of hyaluronan (1.1–1.2 MDa) per liter culture (Widner et al., 2005). The production yield was comparable to the yield observed with *S. equisimilis* strains (7 g/L) a natural producer of hyaluronan (Kim et al., 1996). In addition, hyaluronan synthase from *P. multocida* has been expressed into recombinant *E. coli* (Mao, Shin, & Chen, 2009; Yu & Stephanopoulos, 2008) and succeeded in the production of hyaluronan. This finding is promising for the production of heparosan using *P. multocida* heparosan synthase into recombinant *E. coli*.

5. From heparosan to heparin-like molecules

Controlling heparosan elongation represents the first step in the design of specific heparin-based oligo and polysaccharides since it determines the polymer length and the sugar unit backbone. Nevertheless due to the complexity of heparin and heparan sulfate GAGs, numerous steps are also important to ensure the biological activity of the molecules and therefore need to be controlled as well. Here, a brief overview for each of the catalytic step needed to convert heparosan into tailored heparin-based molecules is presented.

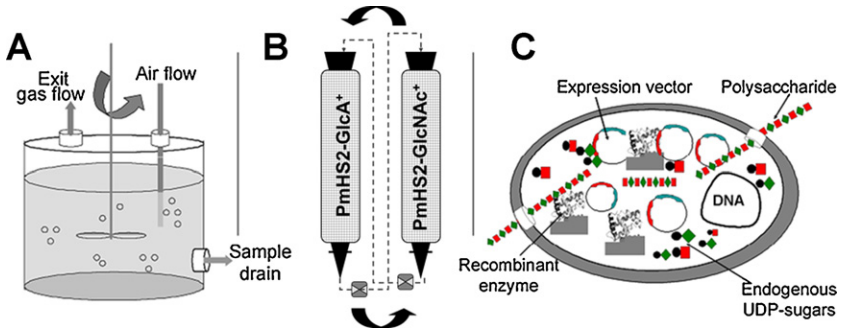

Fig. 5. Overview of heparosan production strategies. (A) *E. coli* fermentation for the extraction of capsular heparosan. (B) Synthesis of heparosan by recombinant heparosan synthases (controlled synthesis of heparosan oligosaccharides using immobilized single action transferases). (C) Production of heparosan polysaccharides using genetically modified bacteria.

5.1. N-Deacetylation and N-sulfation of heparosan

The dual action *N*-deacetylase/*N*-sulfatase (NDST) enzyme catalyzes the *N*-deacetylation/*N*-sulfation of GlcNAc groups in the presence of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as sulfate donor. Single action *N*-deacetylase (NDase) (Duncan, Liu, Fox, & Liu, 2006; Kakuta, Sueyoshi, Negishi, & Pedersen, 1999) and single action *N*-sulfotransferase (NST) (Berninsone & Hirschberg, 1998) obtained by site directed mutagenesis were reported to have the potential to be used *in vitro* to modulate the *N*-deacetylation/*N*-sulfation of the polymer.

In addition, the PAPS concentration was found to determine the *N*-deacetylation and *N*-sulfation pattern of the Hep/HS polymer (Carlsson, Presto, Spillmann, Lindahl, & Kjellén, 2008). This finding is important to control the deacetylation/sulfation pattern. Nevertheless, due to the high cost of PAPS, incubation conditions in which the NDST step and the regeneration of PAPS can simultaneously be performed have to be optimized (Saribas et al., 2004; Zhao & Van Der Donk, 2003). The *N*-deacetylation/*N*-sulfation can also be chemically catalyzed (Kuberan, Beeler, Lech, et al., 2003; Lindahl et al., 2005). The use of a factorial design approach in which: heparosan chain length, reactant concentration, reaction time and reaction temperature were the variant, was used and it showed that it is possible to control the *N*-deacetylation of heparosan (Wang, Li, et al., 2011).

5.2. Glucuronyl C5-epimerization

The glucuronyl C5-epimerase converts some of the GlcA residues into IdoA. The enzyme recognition for the substrate is highly dependent on the GlcA neighboring residues, and it is still unclear what the substrate prerequisites are for C5-epimerase in order to convert GlcA into IdoA (Li & Lijuan, 2010). Due to the importance of the presence of IdoA to ensure biological activity, the discovery or the protein engineering of glucuronyl C5-epimerases capable to catalyze a larger substrate range, such as observed for alginate epimerase (Valla, Li, Ertesvåg, Barbeyron, & Lindahl, 2001), is of interest. On the other hand, an alternative is to use chemically synthesized UDP-IdoA as a substrate in the synthesis of heparosan and in this way controlling its position in the polysaccharide chain (Weïwer et al., 2008).

5.3. O-Sulfation of the epimerized heparosan

The *O*-sulfotranferases require the sulfate donor PAPS to catalyze the sulfation of hydroxyl groups. It was shown that immobilized *O*-sulfotransferases catalyze successfully the *O*-sulfation of the Hep/HS polymers when incubated in the presence of the PAPS regeneration system (aryl sulfotransferase-IV and *p*-nitrophenyl sulfate) (Burkart, Izumi, Chapman, Lin, & Wong, 2000; Chen et al., 2005). Although the *O*-sulfation can also be performed chemically (Naggi, De Cristofano, Bisio, Torri, & Casu, 2001), the use of *O*-sulfotransferases to catalyze this step is favored to obtain defined sulfation patterns (Chen, Jones, & Liu, 2007). Indeed, a large number of *O*-sulfotransferase isoforms is available; they all catalyze different substrates resulting in the synthesis of a variety of defined sulfation patterns.

6. Perspectives

6.1. Heparosan

Controlling heparosan synthesis is an important milestone in tailoring heparin and heparan sulfate polysaccharides.

Different methods can be used to synthesize heparosan oligo- and polymers. The pro and cons of heparosan production are summarized in Table 1. Despite the fact that the isolation of heparosan from bacterial capsule is economically advantageous, *E. coli* K5 is a human pathogen. Thus, rounds of random mutagenesis should be applied to decrease its virulence (Kim et al., 1996) in order to use it for the production of pharmaceutical compounds. In addition, the production of heparosan from bacterial capsule does not enable to control the chain elongation and disaccharide unit composition (Roman, Roberts, Lidholt, & Kusche-Gullberg, 2003).

The use of genetically modified bacteria is interesting since non-pathogenic microorganisms can be used, and regulated expression of engineered heparosan synthases could enable a partial control of the polymer synthesis. However, with the current knowledge the polymer chain length cannot be controlled yet (Chen, Marcellin, Hung, & Nielsen, 2009).

The *P. multocida* heparosan synthase PmHS2 appears to be the most adequate biocatalyst to tailor Hep/HS chains due to its two independent single action transferase activity, its capacity to initiate heparosan chain in the absence of template, and its property to elongate from unusual UDP-sugars. In our opinion, to produce defined heparosan polymers with respect to the control of the polymer molecular weight (Chavaroche, Springer, Kooy, Boeriu, & Eggink, 2010; Chavaroche et al., 2011) and the incorporation of modified UDP-sugars (Liu et al., 2010; Sismey-Ragatz et al., 2007), the use of biocatalysts *in vitro* is the most promising method although a UPD-sugar regeneration method is needed.

6.2. Heparin and heparan sulfate

In the context of the production of anticoagulant heparin the use of (chemo)enzymatic and strictly enzymatic systems, instead of using the traditional production method, is an interesting approach and has to be developed further for production at industrial scale.

Table 1
Advantages and disadvantages of heparosan production methods.

Method	A Extraction from *E. coli* K5	B Recombinant heparosan synthase	C Recombinant bacteria
Pro	No UDP-sugar cost High yield	Control polymer Mw (from disaccharide to 800 kDa) Control sugar incorporation Free of contaminant	No UDP-sugar cost (intrinsic production) Non pathogenic bacteria
Cons	No control on polymer Mw No control on sugar incorporation *E. coli* K5 pathogenicity	Need for UDP-sugars Expensive process	No control on polymer Mw Not yet investigated for heparosan synthesis
In the future	Strain improvement	Production of UDP-sugars	Investigation for heparosan production

Recently, the (chemo)enzymatic synthesis of homogeneous ultra-low molecular weight heparin has been achieved at a gram scale suggesting that anticoagulant heparin (chemo)enzymatically synthesized will be soon available for medical applications (Xu et al., 2011).

For the production of tailored Hep/HS-based molecules to be used in novel therapeutic applications, both the elucidation of interesting Hep/HS structures and the optimization of each (chemo)enzymatic synthesis step is needed. The main challenge for the controlled (chemo)enzymatic synthesis of heparin-like polymers is to synthesize cost effectively a large variety of oligo/polysaccharides. To achieve this goal the priority is to reduce the production cost of heparosan inherent to the use of recombinant heparosan synthases. Indeed, UDP-sugars are expensive and research is needed to make the heparosan synthesis cost effective. This could be done either by recovering UDP for regenerating it in UDP-sugars or by producing UDP-sugars at low price. Furthermore, it is critical to optimize the step by step elongation of heparosan oligosaccharides in order to synthesize monodisperse products. A special effort should be made to understand and control the *N*-deacetylation/*N*-sulfation step (Saribas et al., 2004; Zhao & Van Der Donk, 2003), as well as the C5-epimerization step of heparosan (Li & Lijuan, 2010; Weïwer et al., 2008). The *O*-sulfation step has been investigated in detail and according to the large diversity of *O*-sulfotransferase isoforms available the synthesis of a variety of defined sulfation patterns is possible (Burkart et al., 2000; Chen et al., 2005; Habuchi et al., 2000; Zhang et al., 2008).

The Hep/HS structures involved in physiological pathways are being identified and elucidated in order to set up new therapeutic strategies for the treatment of cancer and the prevention against virus infections. For this purpose, large libraries of unusual Hep/HS-like molecules are screened for their biological activities using newly developed microarray devices (De Paz, Noti, & Seeberger, 2006; De Paz & Seeberger, 2008; Liu, Palma, & Feizi, 2009b; Park, Lee, Dordick, & Linhardt, 2008; Powell, Zhi, & Turnbull, 2009; Yin & Seeberger, 2010). Chinese hamster ovary (CHO) cells are also used to synthesize and study the biological interactions of heparan sulfate in physiological processes (Zhang, Lawrence, Frazier, Esko, & Minoru, 2006). Moreover, an artificial Golgi apparatus on a digital microfluidic chip has been developed for nano-scale production of unusual Hep/HS molecules (Martin et al., 2009) to be used in small scale trials (Xu & Esko, 2009).

7. Conclusion

Further developments of the (chemo)enzymatic catalysis for the synthesis of heparin-based molecules will result in the synthesis of well-defined polysaccharides. This will give opportunities to elucidate the relationship between structure and biological activity, which is important to be able to understand the structures needed in the treatment of cancer and prevention against virus infections.

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References

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 mutant defective in N-sulfotransferase. *Journal of Biological Chemistry*, *266*, 10287–10293.

Bame, K. J., Reddy, R. V., & Esko, J. D. (1991). Coupling of N-deacetylation and N-sulfation in a Chinese hamster ovary cell mutant defective in heparan sulfate N-sulfotransferase. *Journal of Biological Chemistry*, *266*, 12461–12468.

Bellaiche, Y., The, I., & Perrimon, N. (1998). Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature*, *394*, 85–88.

Berninsone, P., & Hirschberg, C. B. (1998). Heparan sulfate/heparin N-deacetylase/N-sulfotransferase. *Journal of Biological Chemistry*, *273*, 25556–25559.

Bhaskar, U., Sterner, E., Hickey, A., Onishi, A., Zhang, F., Dordick, J., et al. (2012). Engineering of routes to heparin and related polysaccharides. *Applied Microbiology and Biotechnology*, *93*, 1–16.

Bishop, J. R., Schuksz, M., & Esko, J. D. (2007). Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*, *446*, 1030–1037.

Burkart, M. D., Izumi, M., Chapman, E., Lin, C.-H., & Wong, C.-H. (2000). Regeneration of PAPS for the enzymatic synthesis of sulfated oligosaccharides. *The Journal of Organic Chemistry*, *65*, 5565–5574.

Busse, M., & Kusche-Gullberg, M. (2003). In vitro polymerization of heparan sulfate backbone by the EXT proteins. *Journal of Biological Chemistry*, *278*, 41333–41337.

Carlsson, P., Presto, J., Spillmann, D., Lindahl, U., & Kjellén, L. (2008). Heparin/heparan sulfate biosynthesis. *Journal of Biological Chemistry*, *283*, 20008–20014.

Chavaroche, A. A. E., Springer, J., Kooy, F., Boeriu, C., & Eggink, G. (2010). In vitro synthesis of heparosan using recombinant *Pasteurella multocida* heparosan synthase PmHS2. *Applied Microbiology and Biotechnology*, *85*, 1881–1891.

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*, http://dx.doi.org/10.1007/s00253-011-3813-2

Chavaroche, A. A. E., Van Den Broek, L. A. M., Springer, J., Boeriu, C., & Eggink, G. (2011). Analysis of the polymerization initiation and activity of *Pasteurella multocida* heparosan synthase PmHS2 an enzyme with glycosyltransferase and UDP-sugar hydrolase activity. *Journal of Biological Chemistry*, *286*, 1777–1785.

Chen, J., Avci, F. Y., Munoz, E. M., Mcdowell, L. M., Chen, M., Pedersen, L. C., et al. (2005). Enzymatic redesigning of biologically active heparan sulfate. *Journal of Biological Chemistry*, *280*, 42817–42825.

Chen, J., Jones, C. L., & Liu, J. (2007). Using an enzymatic combinatorial approach to identify anticoagulant heparan sulfate structures. *Chemical Biology*, *14*, 986–993.

Chen, M., Bridges, A., & Liu, J. (2006). Determination of the substrate specificities of N-acetyl-D-glucosaminyl transferase. *Biochemistry*, *45*, 12358–12365.

Chen, W. Y., Marcellin, E., Hung, J., & Nielsen, L. K. (2009). Hyaluronan molecular weight is controlled by UDP-N-acetylglucosamine concentration in *Streptococcus zooepidemicus*. *Journal of Biological Chemistry*, *284*, 18007–18014.

Chen, Y., Maguire, T., Hileman, R., Esko, J., Linhardt, R., & Marks, R. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nature Medicine*, *3*, 866–871.

Choay, J., Petitou, M., Lormeau, J. C., Sinaÿ, P., Casu, B., & Gatti, G. (1983). Structure–activity relationship in heparin: A synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. *Biochemical and Biophysical Research Communications*, *116*, 492–499.

Coombe, D. R., & Kett, W. C. (2005). Heparan sulfate-protein interactions: Therapeutic potential through structure–function insights. *Cellular and Molecular Life Sciences*, *62*, 410–424.

Coutinho, P. M., Deleury, E., Davies, G. J., & Henrissat, B. (2003). An evolving hierarchical family classification for glycosyltransferases. *Journal of Molecular Biology*, *328*, 307–317.

Dagälv, A., Holmborn, K., Kjellén, L., & Åbrink, M. (2011). Lowered expression of heparan sulfate/heparin biosynthesis enzyme N-deacetylase/N-sulfotransferase 1. Results in increased sulfation of mast cell heparin. *Journal of Biological Chemistry*, *286*, 44433–44440.

De Paz, J. L., Noti, C., & Seeberger, P. H. (2006). Microarrays of synthetic heparin oligosaccharides. *Journal of the American Chemical Society*, *128*, 2766–2767.

De Paz, J. L., & Seeberger, P. H. (2008). Deciphering the glycosaminoglycan code with the help of microarrays. *Molecular BioSystems*, *4*, 707–711.

Deangelis, P. L., & White, C. L. (2002). Identification and molecular cloning of a heparosan synthase from *Pasteurella multocida* Type D. *Journal of Biological Chemistry*, *277*, 7209–7213.

Deangelis, P. L., & White, C. L. (2004). Identification of a distinct, cryptic heparosan synthase from *Pasteurella multocida* Types A, D and F. *Journal of Bacteriology*, *186*, 8529–8532.

Duncan, M. B., Liu, M., Fox, C., & Liu, J. (2006). Characterization of the N-deacetylase domain from the heparan sulfate N-deacetylase/N-sulfotransferase 2. *Biochemical and Biophysical Research Communications*, *339*, 1232–1237.

Esko, J. D., Koji, K., & Lindahl, U. (2009). *Proteoglycans and sulfated glycosaminoglycans* (2nd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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.

Forsberg, E. (1999). Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. *Nature*, *400*, 773–776.

Gorsi, B., & Stringer, S. E. (2007). Tinkering with heparan sulfate sulfation to steer development. *Trends in Cell Biology*, *17*, 173–177.

Gray, E., Mulloy, B., & Barrowcliffe, T. W. (2008). Heparin and low-molecular-weight heparin. *Thrombosis and Haemostasis*, *99*, 799–983.

Griffiths, G., Cook, N. J., Gottfridson, E., Lind, T., Lidholt, K., & Roberts, I. S. (1998). Characterization of the glycosyltransferase enzyme from the *Escherichia coli* K5 capsule gene cluster and identification and characterization of the glucuronyl active site. *Journal of Biological Chemistry*, *273*, 11752–11757.

Guerrini, M., Beccati, D., Shriver, Z., Naggi, A., Viswanathan, K., Bisio, A., et al. (2008). Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nature Biotechnology*, *26*, 669–675.

Habuchi, H., Tanaka, M., Habuchi, O., Yoshida, K., Suzuki, H., Ban, K., et al. (2000). The occurrence of three isoforms of heparan sulfate 6-O-sulfotransferase having different specificities for hexuronic acid adjacent to the targeted N-sulfoglucosamine. *Journal of Biological Chemistry*, *275*, 2859–2868.

Higashi, K., Ly, M., Wang, Z., Masuko, S., Bhaskar, U., Sterner, E., et al. (2011). Controlled photochemical depolymerization of K5 heparosan a bioengineered heparin precursor. *Carbohydrate Polymers*, *86*, 1365–1370.

Hileman, R. E., Smith, A. E., Toida, T., & Linhardt, R. J. (1997). Preparation and structure of heparin lyase-derived heparan sulfate oligosaccharides. *Glycobiology*, *7*, 231–239.

Hodson, N., Griffiths, G., Cook, N., Pourhossein, M., Gottfridson, E., Lind, T., et al. (2000). Identification that KfiA, a protein essential for the biosynthesis of the *Escherichia coli* K5 capsular polysaccharide, is an alpha-UDP-GlcNAc glycosyltransferase. The formation of a membrane-associated K5 biosynthetic complex requires KfiA, KfiB and KfiC. *Journal of Biological Chemistry*, *275*, 27311–27315.

Humphries, D. E. (1999). Heparin is essential for the storage of specific granule proteases in mast cells. *Nature*, *400*, 769–772.

Iozzo, R. V. (1998). Matrix proteoglycans: From molecular design to cellular function. *Annual Review of Biochemistry*, *67*, 609–652.

Izumikawa, T., Egusa, N., Taniguchi, F., Sugahara, K., & Kitagawa, H. (2006). Heparan sulfate polymerization in drosophila. *Journal of Biological Chemistry*, *281*, 1929–1934.

Jing, W., & Deangelis, P. L. (2004). Synchronized chemoenzymatic synthesis of monodisperse hyaluronan polymers. *Journal of Biological Chemistry*, *279*, 42345–42349.

Kakuta, Y., Sueyoshi, T., Negishi, M., & Pedersen, L. C. (1999). Crystal structure of the sulfotransferase domain of human heparan sulfate N-deacetylase/N-sulfotransferase 1. *Journal of Biological Chemistry*, *274*, 10673–10676.

Kane, T. A., White, C. L., & Deangelis, P. L. (2006). Functional characterization of PmHS1 a *Pasteurella multocida* heparosan synthase. *Journal of Biological Chemistry*, *281*, 33192–33197.

Kim, B.-T., Kitagawa, H., Tanaka, J., Tamura, J.-I., & Sugahara, K. (2003). In vitro heparan sulfate polymerization. *Journal of Biological Chemistry*, *278*, 41618–41623.

Kim, J.-H., Yoo, S.-J., Oh, D.-K., Kweon, Y.-G., Park, D.-W., Lee, C.-H., et al. (1996). Selection of a Streptococcus equi mutant and optimization of culture conditions for the production of high molecular weight hyaluronic acid. *Enzyme and Microbial Technology*, *19*, 440–445.

Kjellen, L., & Lindahl, U. (1991). Proteoglycans: Structures and interactions. *Annual Review of Biochemistry*, *60*, 443–475.

Kuberan, B., Beeler, D. L., Lawrence, R., Lech, M., & Rosenberg, R. D. (2003). Rapid two-step synthesis of mitrin from heparosan: A replacement for heparin. *Journal of the American Chemical Society*, *125*, 12424–12425.

Kuberan, B., Beeler, D. L., Lech, M., Wu, Z. L., & Rosenberg, R. D. (2003). Chemoenzymatic synthesis of classical and non-classical anticoagulant heparan sulfate polysaccharides. *Journal of Biological Chemistry*, *278*, 52613–52621.

Lamanna, W. C., Baldwin, R. J., Padva, M., Kalus, I., Ten Dam, G., Van Kuppevelt, T. H., et al. (2006). Heparan sulfate 6-O-endosulfatases: Discrete in vivo activities and functional co-operativity. *Biochemical Journal*, *400*, 63–73.

Lamanna, W. C., Frese, M.-A., Balleininger, M., & Dierks, T. (2008). Sulf loss influences N-, 2-O- and 6-O-sulfation of multiple heparan sulfate proteoglycans and modulates fibroblast growth factor signaling. *Journal of Biological Chemistry*, *283*, 27724–27735.

Lamanna, W. C., Kalus, I., Padva, M., Baldwin, R. J., Merry, C. L. R., & Dierks, T. (2007). The heparanome—The enigma of encoding and decoding heparan sulfate sulfation. *Journal of Biotechnology*, *129*, 290–307.

Laremore, T. N., Zhang, F., Dordick, J. S., Liu, J., & Linhardt, R. J. (2009). Recent progress and applications in glycosaminoglycan and heparin research. *Current Opinion in Chemical Biology*, *13*, 633–640.

Li, J.-P., & Lijuan, Z. (2010). Glucuronyl C5-epimerase: An enzyme converting glucuronic acid to iduronic acid in heparan sulfate/heparin biosynthesis. *Progress in Molecular Biology and Translational Science*, *93*, 59–78.

Lindahl, U., Feingold, D. S., & Roden, L. (1986). Biosynthesis of heparin. *Trends in Biochemical Sciences*, *11*, 221–225.

Lindahl, U., Li, J., Kusche-Gullberg, M., Salmivirta, M., Alaranta, S., Veromaa, T., et al. (2005). Generation of neoheparin from *E. coli* K5 capsular polysaccharide. *Journal of Medicinal Chemistry*, *48*, 349–352.

Linhardt, R. J. (2001). *Analysis of glycosaminoglycans with polysaccharide lyases*. John Wiley & Sons, Inc.

Linhardt, R. J., & Toida, T. (2004). Role of glycosaminoglycans in cellular communication. *Account of Chemical Research*, *37*, 431–438.

Liu, H., Zhang, Z., & Linhardt, R. J. (2009). Lessons learned from the contamination of heparin. *Natural Product Reports*, *26*, 313–321.

Liu, R., Xu, Y., Chen, M., Weïwer, M., Zhou, X., Bridges, A. S., et al. (2010). Chemoenzymatic design of heparan sulfate oligosaccharides. *Journal of Biological Chemistry*, *285*, 34240–34249.

Liu, Y., Palma, A. S., & Feizi, T. (2009). Carbohydrate microarrays: Key developments in glycobiology. *Biological Chemistry*, *390*, 647–656.

Ludwigs, U., Elgavish, A., Esko, J. D., Meezan, E., & Rodén, L. (1987). Reaction of unsaturated uronic acid residues with mercuric salts Cleavage of the hyaluronic acid disaccharide 2-acetamido-2-deoxy-3-O-(beta-D-gluco-4-enepyranosyluronic acid)-D-glucose. *Biochemical Journal*, *245*, 795–804.

Manzoni, M., Bergomi, S., & Cavazzoni, V. (1996). Production of K5 polysaccharides of different molecular weight by *Escherichia coli*. *Journal of Bioactive and Compatible Polymers*, *11*, 301–311.

Manzoni, M., Rollini, M., Piran, E., & Parini, C. (2004). Preliminary characterisation of an *Escherichia coli* K5 lyase-deficient strain producing the K5 polysaccharide. *Biotechnology Letters*, *26*, 351–356.

Mao, Z., Shin, H.-D., & Chen, R. (2009). A recombinant *Escherichia coli* bioprocess for hyaluronan synthesis. *Applied Microbiology and Biotechnology*, *84*, 63–69.

Martin, J. G., Gupta, M., Xu, Y., Akella, S., Liu, J., Dordick, J. S., et al. (2009). Toward an artificial Golgi: Redesigning the biological activities of heparan sulfate on a digital microfluidic chip. *Journal of the American Chemical Society*, *131*, 11041–11048.

Masuko, S., Higashi, K., Wang, Z., Bhaskar, U., Hickey, A. M., Zhang, F., et al. (2011). Ozonolysis of the double bond of the unsaturated uronate residue in low-molecular-weight heparin and K5 heparosan. *Carbohydrate Research*, *346*, 1962–1966.

May, B. J., Zhang, Q., Li, L. L., Paustian, M. L., Whittam, T. S., & Kapur, V. (2001). Complete genomic sequence of *Pasteurella multocida*, Pm70. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 3460–3465.

Mccormick, C., Duncan, G., Goutsos, K. T., & Tufaro, F. (2000). The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the Golgi apparatus and catalyzes the synthesis of heparan sulfate. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 668–673.

Naggi, A., De Cristofano, B., Bisio, A., Torri, G., & Casu, B. (2001). Generation of anti-factor Xa active 3-O-sulfated glucosamine-rich sequences by controlled desulfation of oversulfated heparins. *Carbohydrate Research*, *336*, 283–290.

Pandit, K. K., & Smith, J. E. (1993). Capsular hyaluronic-acid in *Pasteurella multocida* Type-A and its counterpart in Type-D. *Research in Veterinary Science*, *54*, 20–24.

Park, T.-J., Lee, M.-Y., Dordick, J. S., & Linhardt, R. J. (2008). Signal amplification of target protein on heparin glycan microarray. *Analytical Biochemistry*, *383*, 116–121.

Peterson, S., Frick, A., & Liu, J. (2009). Design of biologically active heparan sulfate and heparin using an enzyme-based approach. *Natural Product Reports*, *26*, 610–627.

Petitou, M., & Van Boeckel, C. A. (2004). A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? *Angewandte Chemie International Edition*, *43*, 3118–3133.

Powell, A. K., Zhi, Z.-L., & Turnbull, J. E. (2009). Methods in molecular biology (glycomics). In NH. Packer, & NG. Karlsson (Eds.), *Saccharide microarrays for high-throughput interrogation of glycan–protein binding interactions* (pp. 312–329). New Jersey: Humana Press.

Rabenstein, D. L. (2002). Heparin and heparan sulfate: Structure and function. *Natural Product Reports*, *19*, 312–331.

Raman, K., Nguyen, T. K. N., & Kuberan, B. (2011). Is N-sulfation just a gateway modification during heparan sulfate biosynthesis? *FEBS Letters*, *585*, 3420–3423.

Rimler, R. B. (1994). Presumptive identification of *Pasteurella multocida* serogroups A, D and F by capsule depolymerisation with mucopolysaccharidases. *Veterinary Record*, *134*, 191–192.

Roberts, I. S. (1996). The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology*, *50*, 285–315.

Roman, E., Roberts, I., Lidholt, K., & Kusche-Gullberg, M. (2003). Overexpression of UDP-glucose dehydrogenase in *Escherichia coli* results in decreased biosynthesis of K5 polysaccharide. *Biochemical Journal*, *374*, 767–772.

Rusnati, M., Vicenzi, E., Donalisio, M., Oreste, P., Landolfo, S., & Lembo, D. (2009). Sulfated K5 *Escherichia coli* polysaccharide derivatives: A novel class of candidate antiviral microbicides. *Pharmacology & Therapeutics*, *123*, 310–322.

Saribas, A. S., Mobasseri, A., Pristatsky, P., Chen, X., Barthelson, R., Hakes, D., et al. (2004). Production of N-sulfated polysaccharides using yeast-expressed N-deacetylase/N-sulfotransferase-1 (NDST-1). *Glycobiology*, *14*, 1217–1228.

Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., et al. (2000). The EXT1/EXT2 tumor suppressors: Catalytic activities and role in heparan sulfate biosynthesis. *EMBO Reports*, *1*, 282–286.

Sheng, J., Liu, R., Xu, Y., & Liu, J. (2011). The dominating role of N-deacetylase/N-sulfotransferase 1 in forming domain structures in heparan sulfate. *Journal of Biological Chemistry*, *286*, 19768–19776.

Silbert, J. E., & Sugumaran, G. (2002). A starting place for the road to function. *Glycoconjugate Journal*, *19*, 227–237.

Sismey-Ragatz, A. E., Green, D. E., Otto, N. J., Rejzek, M., Field, R. A., & Deangelis, P. L. (2007). Chemoenzymatic synthesis with distinct pasteurella heparosan synthases: Monodisperse polymers and unnatural structures. *Journal of Biological Chemistry*, *282*, 28321–28327.

Sugiura, N., Baba, Y., Kawaguchi, Y., Iwatani, T., Suzuki, K., Kusakabe, T., et al. (2010). Glucuronyltransferase activity of KfiC from *Escherichia coli* strain K5 requires association of KfiA. *Journal of Biological Chemistry*, *285*, 1597–1606.

Valla, S., Li, J.-P., Ertesvåg, H., Barbeyron, T., & Lindahl, U. (2001). Hexuronyl C5-epimerases in alginate and glycosaminoglycan biosynthesis. *Biochimie*, *83*, 819–830.

Van Houdenhoven, F. (1999). *Process for the production of heparin*. Patent 1999WO/1999/003893.

Vann, W. F., Schmidt, M. A., Jann, B., & Jann, K. (1981). The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective *Escherichia coli* 010:K5:H4. A polymer similar to desulfo-heparin. *European Journal of Biochemistry*, *116*, 359–364.

Wang, Z., Dordick, J. S., & Linhardt, R. J. (2011). *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. *Bioengineered Bugs*, *2*, 63–67.

Wang, Z., Li, J., Cheong, S., Bhaskar, U., Akihiro, O., Zhang, F., et al. (2011). Response surface optimization of the heparosan N-deacetylation in producing bioengineered heparin. *Journal of Biotechnology*, *156*, 188–196.

Weïwer, M., Sherwood, T., Green, D. E., Chen, M., Deangelis, P. L., Liu, J., et al. (2008). Synthesis of uridine-diphosphoiduronic acid: A potential substrate for the chemoenzymatic synthesis of heparin. *The Journal of Organic Chemistry*, *73*, 7631–7637.

Widner, B., Behr, R., Von Dollen, S., Tang, M., Heu, T., Sloma, A., et al. (2005). Hyaluronic acid production in *Bacillus subtilis*. *Applied and Environmental Microbiology*, *71*, 3747–3752.

Wu, J.-R., Chen, P.-Y., Shien, J.-H., Shyu, C.-L., Shieh, H. K., Chang, F., et al. (2010). Analysis of the biosynthesis genes and chemical components of the capsule of *Avibacterium paragallinarum*. *Veterinary Microbiology*, *145*, 90–99.

Xu, D., & Esko, J. D. (2009). A Golgi-on-a-chip for glycan synthesis. *Nature Chemical Biology*, *5*, 612–613.

Xu, Y., Masuko, S., Takieddin, M., Xu, H., Liu, R., Jing, J., et al. (2011). Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins. *Science*, *334*, 498–501.

Yin, J., & Seeberger, P. H. (2010). Methods in enzymology. In F. Minoru (Ed.), *Applications of heparin and heparan sulfate microarrays* (pp. 197–218). Academic Press.

Yip, G. W., Smollich, M., & Götte, M. (2006). Therapeutic value of glycosaminoglycans in cancer. *Molecular Cancer Therapeutics*, *5*, 2139–2148.

Yu, H., & Stephanopoulos, G. (2008). Metabolic engineering of *Escherichia coli* for biosynthesis of hyaluronic acid. *Metabolic Engineering*, *10*, 24–32.

Zhang, L., Lawrence, R., Frazier, B. A., Esko, J. D., & Minoru, F. (2006). Methods enzymology. In *CHO glycosylation mutants: Proteoglycans*. Academic Press., pp. 205–221

Zhang, Z., Mccallum, S. A., Xie, J., Nieto, L., Corzana, F., Jiménez-Barbero, J., et al. (2008). Solution structures of chemoenzymatically synthesized heparin and its precursors. *Journal of American Chemistry Society*, *130*, 12998–13007.

Zhao, H., & Van Der Donk, W. A. (2003). Regeneration of cofactors for use in biocatalysis. *Current Opinion in Biotechnology*, *14*, 583–589.