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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