# Chapter 5
# Chondroitin, Dermatan, Heparan, and Keratan Sulfate: Structure and Functions

**Emiliano Bedini, Maria Michela Corsaro, Alfonso Fernández-Mayoralas, and Alfonso Iadonisi**

**Abstract** Sulfated glycosaminoglycans (heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate) are a family of complex polysaccharides ubiquitously, but not exclusively, distributed among mammals, found both in extracellular matrices and on cell surfaces. They play key roles in a myriad of physiological and pathological processes, including, among others, angiogenesis, cancer, immunity, and infectious diseases. Here the main issues concerning their chemical structure, biosynthesis, extraction, and purification from natural sources, structural characterization, as well as their most important biological functions are discussed.

## 5.1 Introduction

The class of proteoglycans (PGs) is one of the major and critically important components of the extracellular matrix (ECM) as well as of cell surfaces. PGs play several essential roles in a variety of biological events. They are involved both in ECM structure, by governing ECM assembly and its physical properties, and in regulating signaling pathways by interacting with other ECM components, directing tissue growth and development, cell proliferation, adhesion, and motility. This leads to key roles for PGs in several physiological and pathological processes, for example, angiogenesis, cancer, immunity, and infectious diseases (Iozzo and Karamanos 2010).

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E. Cohen, H. Merzendorfer (eds.), *Extracellular Sugar-Based Biopolymers Matrices*, Biologically-Inspired Systems 12, https://doi.org/10.1007/978-3-030-12919-4_5

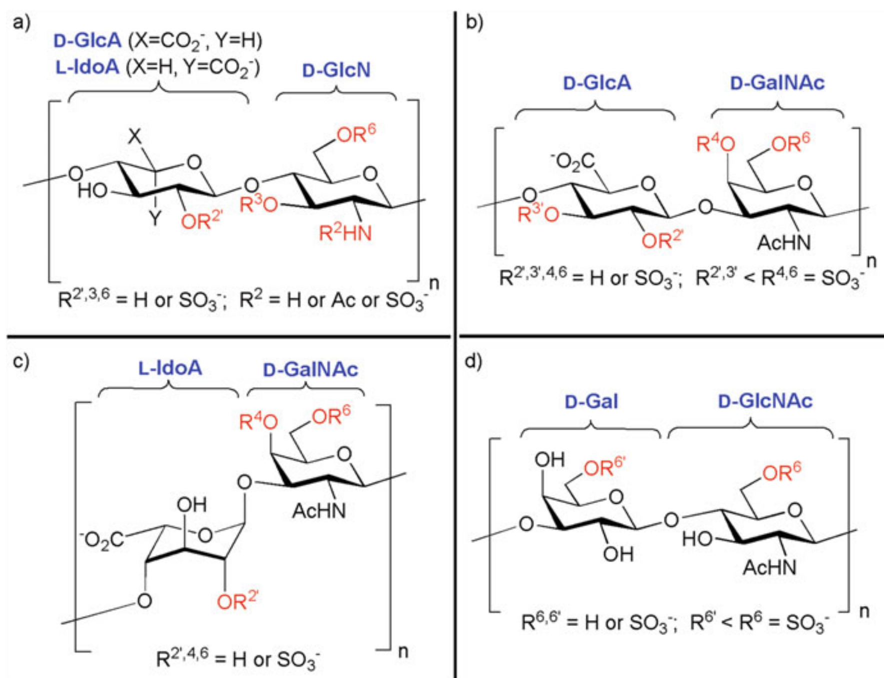



**Fig. 5.1** Structure of sulfated GAGs: (**a**) heparan sulfate, (**b**) chondroitin sulfate, (**c**) dermatan sulfate, and (**d**) keratan sulfate

From a structural point of view, PGs are very complex macromolecular glycoconjugates, composed of a protein core and highly negatively charged, linear polysaccharide chains, termed glycosaminoglycans (GAG), which are covalently linked through their reducing ends to the side chains of serine – or more rarely threonine or asparagine – residues in the protein. GAGs can be distinguished into five main different types: hyaluronic acid (HA), heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS). Except for HA, the only GAG that is non-sulfated and non-covalently linked to a protein core, all the other GAG polysaccharides of PGs are extensively decorated with sulfate groups (Fig. 5.1). The distribution of such sulfate groups along the polymeric chain (sulfation pattern) can be theoretically arranged in an extremely huge number of different combinations. Together with the different possibilities of GAG chains anchoring on the protein core of PGs, it creates an enormous structural diversity and potential variation in biological activity (Lindahl et al. 2017). Nonetheless, sulfation pattern seems to be strictly regulated in vivo and able to encode a variety of information, in line with the central role played by sulfated GAGs in many biological events. This “GAG sulfation code” is still very poorly understood in detail, even if in the last years, some of its roles have been deciphered (Gama et al. 2006; Swarup et al. 2013).

## 5.2 Structural Features and Biosynthesis of Sulfated Glycosaminoglycans

### *5.2.1 Heparan Sulfate*

Heparan sulfate (HS) is a type of glycosaminoglycan composed of repeating units of D-glucuronic (GlcA) or L-iduronic (IdoA) acid linked to 2-amino-2-deoxy-D-glucopyranose (glucosamine, GlcN) (Fig. 5.1a). These disaccharide units may be sulfated at *C*-3 and *C*-6 of GlcN and at *C*-2 of the uronic acid, and the GlcN amine function may be changed to sulfamate, acetamide, or unsubstituted. HS polysaccharides do not usually occur as free molecules but rather form covalent species with proteins presenting the so-called heparan sulfate proteoglycans (HSPG, Fig. 5.2). HSPGs are present in the ECM or on the cell surface of essentially all animals, from simple invertebrates to humans. Syndecans and glypicans are the two main cell surface HSPGs, whereas agrin and perlecan are found in the extracellular matrix. The anticoagulant heparin is commonly considered as a highly sulfated variant of HS, which was first discovered in 1916 and derives its name from its abundance in hepatic tissue (Howell and Holt 1918). In fact, HS was originally called heparitin sulfate since it was initially identified as an impurity of heparin (Linker et al. 1958). Actually, the higher sulfate content of heparin with respect to HS is true for most but not all cases. Recently, it was demonstrated that HS extracted from rabbit cartilage chondrocytes in rabbits has a sulfation degree very similar to heparin (Parra et al. 2012). The major difference between HS and heparin rather lies in a biosynthetic aspect: heparin is produced exclusively in the Golgi of vertebrate mast cells, whereas HS is found ubiquitously to the cell surface of both vertebrate and invertebrate species (Sampaio et al. 2006).

Although a huge number of combinations could derive from the abovementioned modifications at the different positions of the disaccharide unit, the biosynthetic process imposes some restrictions, and the fine structure of the chains is eventually

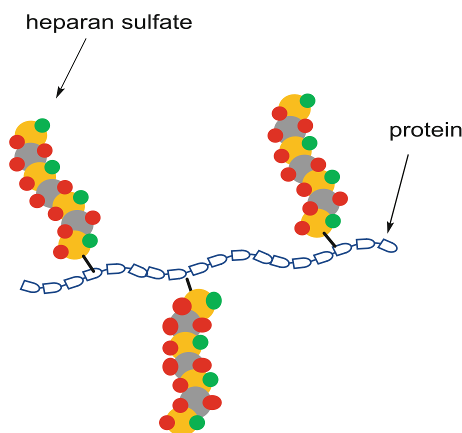



**Fig. 5.2** Typical assembly of heparan sulfate proteoglycans

regulated by the expression and action of a variety of enzymes. HS biosynthesis is a multi-step process that takes place in the Golgi (Esko and Selleck 2002; Johan and Lena 2012). The process is initiated with the xylosylation of specific serine residues of the triplet sequence (serine-glycine-acidic amino acid) of the core protein, followed by the formation of a linkage tetrasaccharide, glucuronic acid-galactose-galactose-xylose (Fig. 5.3). This fragment may be phosphorylated at the xylose moiety and sulfated at the two galactose units. These modifications seem to influence the catalytic activity of the enzyme glucuronyltransferase-I (GlcAT-1) that completes the synthesis of this linkage region (Tone et al. 2008). This part of the biosynthesis is shared by HS and chondroitin sulfate (CS), but the pathways diverge after formation of the tetrasaccharide linkage. The addition of a 2-acetamido-2-deoxy-D-glucopyranose (*N*-acetylglucosamine, GlcNAc) unit to the chain by the action of EXTL family of glycosyltransferases initiates HS chain formation. There is a competition between this reaction and the addition of a 2-acetamido-2-deoxy-D-galactopyranose (*N*-acetylgalactosamine, GalNAc) unit catalyzed by a different enzyme, and when the latter glycosidation occurs, the formation of CS takes place. In a study using cultures of genetically engineered cells unable to produce HS, it was shown that the cells gave instead a twofold increase in CS biosynthesis as compared to wild-type ones (Jan et al. 2012). Since HS and CS biosynthesis competes for the same tetrasaccharide intermediate, the reduction of HS biosynthesis results in more available substrate for CS biosynthesis, which explains the increase in CS levels. A different local environment around the glycosylated serine residue of the core protein could determine the presence of HS or CS chain in the proteoglycan. In particular, a series of serine-glycine dipeptides flanked by a cluster of acidic amino acids allows HS chain assembly, whereas CS formation requires a serine-glycine-acidic amino acid-glycine tetrapeptide sequence (Kokenyesi and Bernfield 1994). Nonetheless, also portions of the core protein far away from the glycosylation sites can influence HS vs. CS chain formation (Chen and Lander 2001).

After the EXTL-mediated attachment of the first GlcNAc unit, HS polymer formation proceeds by the stepwise alternating addition of GlcA and GlcNAc residues to the growing polymer (chain polymerization step, Fig. 5.3). During or immediately following the assembly process, the chains are subjected to a series of structural modifications in which a number of GlcNAc units become *N*-deacetylated and sulfamated at *C*-2, the adjacent glucuronic acids are epimerized to IdoA, and finally sulfation of uronic acid residues at *C*-2 and of GlcNAc and GlcNS units at *C*-6 takes place with 3′-phosphoadenosine-5′-phosphosulfate (PAPS) cofactor as sulfate donor (El Masri et al. 2017). Occasionally, the *C*-3 of GlcN residues may also be sulfated. These modifications are catalyzed by the enzymes NDST (*N*-deacetylase *N*-sulfotransferase), epimerase, and 2OST, 6OST, and 3OST (2-*O*-, 6-*O*-, and 3-*O*-sulfotransferases), respectively. Since these modification reactions do not reach completion, the mature HS chain contains three types of domains that give rise to another way to describe its structure: contiguous sulfamated regions (NS domains), regions of alternating *N*-acetylated and sulfamated disaccharide units (NA/NS domains), and unmodified *N*-acetylated regions (NA domains). The degrees of modification and the sulfation pattern can vary widely between different tissues

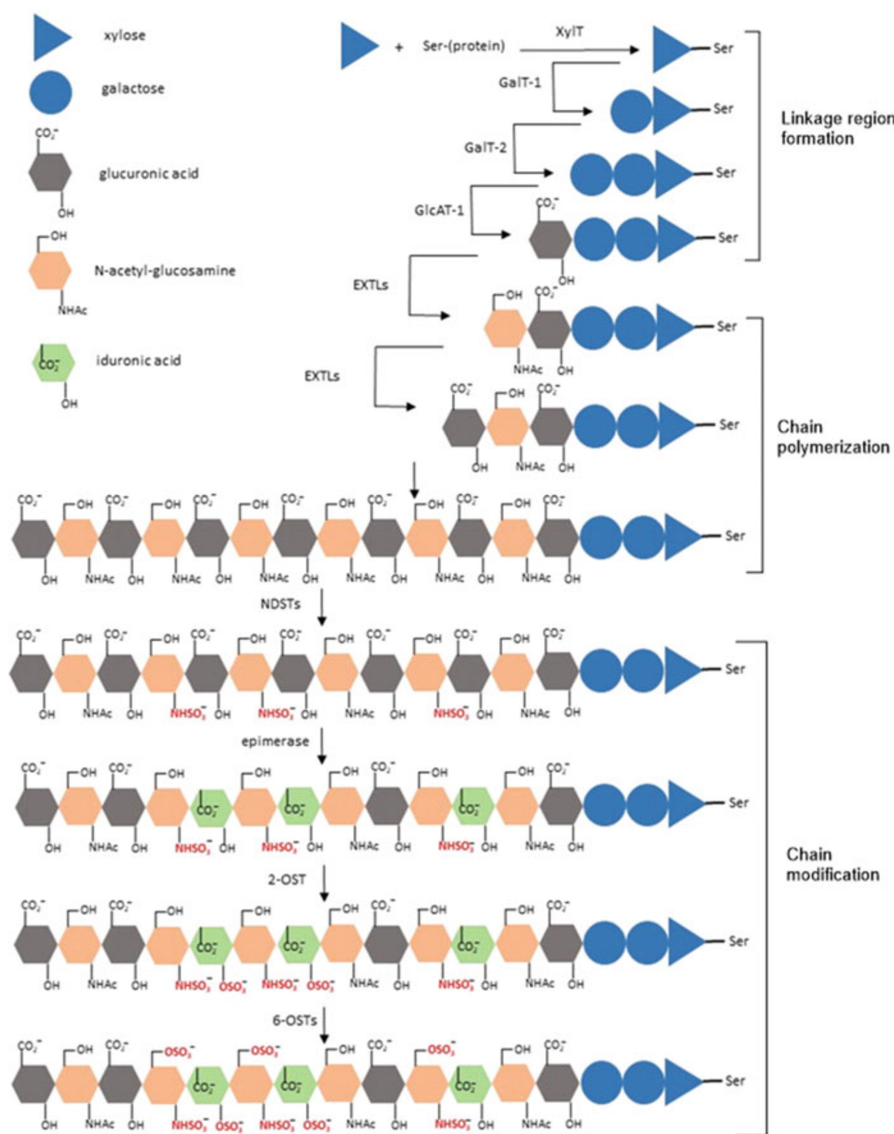



**Fig. 5.3** Scheme of HS biosynthesis. The different steps and enzymes involved are shown. The nascent polysaccharide chains are partially modified by GlcNAc *N*-deacetylase/*N*-sulfotransferase (NDST). Some GlcA units are epimerized to IdoA, and sulfation occurs at different positions. Please note that, for sake of figure simplicity, chain modification reactions are depicted as occurring exclusively following the polymerization process, even if the events can also be simultaneous

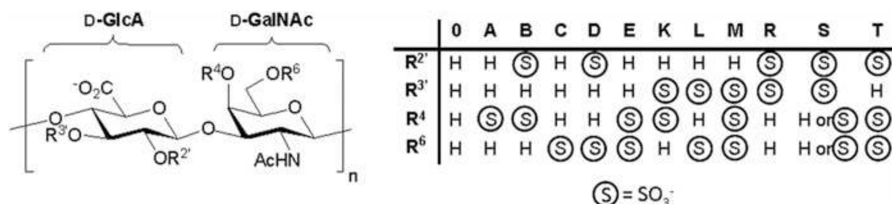
and during developmental stages due to specific expression of enzyme isoforms having different specificities (Safaiyan et al. 2000; Esko and Lindahl 2001; Yabe et al. 2005; Warda et al. 2006). Furthermore, it has been also hypothesized that

differently composed macromolecular assemblies of HS (as well as other sulfated GAGs) biosynthetic enzymes (termed GAGOSOMEs) in different regions of Golgi apparatus are able to finely coordinate and tune polymerization and structural modifications of the nascent polysaccharide chain (Chua and Kuberan 2017). In addition to the structural variations produced during biosynthesis, the sulfation and sulfamation pattern of HS may be modified extracellularly by the action of sulfatases (Nagamine et al. 2012). This structural variability generates different protein-binding HS sequences that regulate a diversity of biological processes (see Paragraph 5.4.1).

### 5.2.2 *Chondroitin Sulfate*

CS is a type of glycosaminoglycan found in both vertebrates and invertebrates, composed of GlcA and GalNAc linked together through alternating β-1→3 and β-1→4 glycosidic bond. The resulting →4)-β-GlcA-(1→3)-β-GalNAc-(1→ disaccharide repeating unit can be sulfated to various extents (Fig. 5.1b). Interestingly, sulfation decoration of chondroitin polysaccharides seems to be a result of evolution, in order to let CS play key roles in some special functions typical of higher animals (e.g., central nervous system development: see Paragraph 5.4.2). Indeed, chondroitin isolated from simple and evolutionary more ancient organisms such as bacteria and nematodes shows only or mostly unsulfated GlcA and GalNAc units (Yamada et al. 2007), whereas CS with different sulfation patterns can be extracted from arthropods up to mammals. A list of the sulfation patterns found in CSs is depicted in Fig. 5.4. The differently sulfated disaccharide subunits are commonly identified with a letter (e.g., CS-A means a →4)-β-GlcA-(1→3)-β-GalNAc4S-(1→ disaccharide with a single sulfate group on *C*-4 hydroxy of GalNAc).

Interestingly, sulfate groups are almost exclusively found at position *C*-4 and/or *C*-6 of GalNAc units in CS from terrestrial animal sources, whereas CS from marine species usually shows oversulfated disaccharide units along the polysaccharide backbone, with additional sulfate groups on GlcA units at *C*-2 (shark, ray) (Nadanaka and Sugahara 1997; Takeda et al. 2016) or *C*-3 (invertebrates such as crab, squid, octopus) (Sugahara et al. 1996; Kumar Shetty et al. 2009; Higashi et al. 2015), or, very rarely (shrimp), at both *C*-2 and *C*-3 positions (Cavalcante et al.




|  | 0 | A | B | C | D | E | K | L | M | R | S | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $R^{2'}$ | H | H | Ⓢ | H | Ⓢ | H | H | H | H | Ⓢ | Ⓢ | Ⓢ |
| $R^{3'}$ | H | H | H | H | H | H | Ⓢ | Ⓢ | Ⓢ | Ⓢ | Ⓢ | H |
| $R^4$ | H | Ⓢ | Ⓢ | H | H | Ⓢ | Ⓢ | H | Ⓢ | H | H or Ⓢ | Ⓢ |
| $R^6$ | H | H | H | Ⓢ | Ⓢ | Ⓢ | H | Ⓢ | Ⓢ | H | H or Ⓢ | Ⓢ |

Ⓢ = $SO_3^-$

**Fig. 5.4** Distribution of sulfation pattern in CS

2018). However, it is worth noting that sulfation pattern is not only varying with the animal species but also with the tissue source. Furthermore, in higher animals it can be subjected to variations also due to different physiopathological conditions, such as aging, inflammation, and tumor formation (Collin et al. 2017).

Some marine invertebrates possess unique CS polysaccharides, with neutral or negatively charged mono- or disaccharide branches, linked predominantly but not exclusively to *C*-3 of GlcA units (Kinoshita-Toyoda et al. 2004; Higashi et al. 2016). Among these branched CSs, fucosylated chondroitin sulfate (fCS) has been the focus of much attention in the last decade, due to its highly interesting potential application as orally deliverable anticoagulant drug alternative to heparin (Pomin 2014). fCS has been found up to now exclusively in sea cucumbers; its structure usually shows variously sulfated L-fucose monosaccharides or, much more rarely, oligosaccharide branches (Chen et al. 2011; Myron et al. 2014; Li et al. 2018; Soares et al. 2018) linked through an α-configured glycosidic bond at *C*-3 of GlcA units or, in very few cases, at *C*-6 of GalNAc residues (Yang et al. 2015; Ustyuzhanina et al. 2016; Li et al. 2018). Figure 5.5 summarizes the variability of fCS structures.

CS in animals is ubiquitously found in the ECM and on cell surfaces as CS proteoglycans (CSPGs), in which the CS polysaccharide is covalently anchored to a core protein through the tetrasaccharide linker region common to both HSPGs and CSPGs, as discussed in Paragraph 5.2.1. CSPGs are usually large biomacromolecules containing from 1 up to 100 CS side chains departing from the core protein. The main CSPG found in cartilaginous tissues is aggrecan, a giant biomacromolecule with about 100 CS polysaccharide branches anchored on the central domain of a 220 kDa protein core, which carries also some keratan sulfate

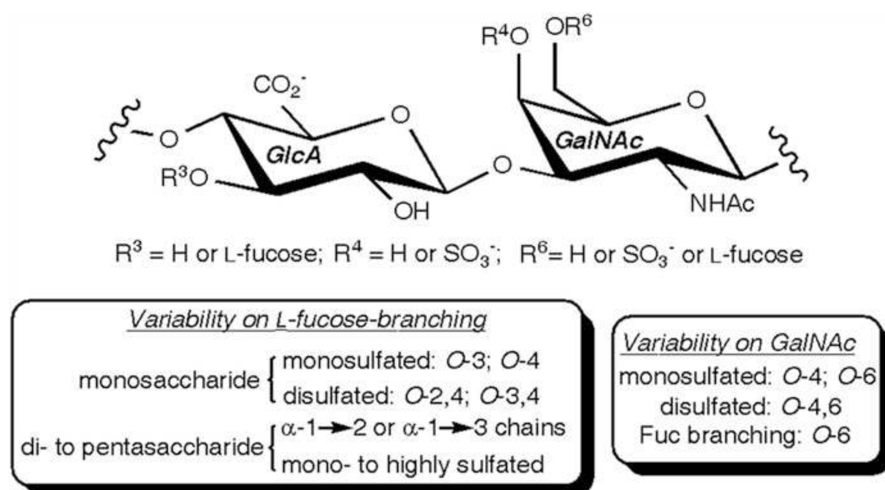



**Fig. 5.5** Structural variability of fCS from sea cucumbers

chains (see also Paragraph 5.2.4) (Iozzo 1998). CS chains are similar in length and so closely packed to each other (their spacing is approx. 3–4 nm) that they have almost no conformational freedom, and therefore the whole PG is forced to adopt a highly extended 3D structure. Interestingly, it has been shown that aggrecans from different cartilaginous tissues can differ in the length and number of CS chains, in order to bestow a higher or lower stiffness to its brush-like structure affording different levels of mechanical strength to, for example, load-bearing or non-load-bearing cartilages (Ng et al. 2003).

Aggrecan and three other CSPGs with lower numbers of CS chains (versican, neurocan, and brevican with 10–30, 3–7, and 1–3 polysaccharide branches, respectively) are together named as hyalectans (hyaluronic- and lectin-binding proteoglycans), as the *N*-terminal domain of the core protein binds to hyaluronic acid non-covalently, and at the same time, the *C*-terminal domain can bind lectins. The so-formed huge aggregates are considered to serve as frameworks for construction of the ECM in the brain (Maeda 2015). Decorin and biglycan are instead examples of CSPGs (or more precisely CS/DS proteoglycans (CS/DSPGs): see also Paragraph 5.2.3), which are not aggregated on a hyaluronic acid backbone. They are also named as small leucine-rich CSPGs, with only 1–2 CS (or DS) chains attached on terminal domains of a leucine-rich protein. Other CSPGs displaying a low number of CS chains are phosphacan, bamacan, neuroglycan C, leprecan, thrombomodulin, epiphycan, and bikunin (Ly et al. 2010). The last one is the smallest PG discovered up to now, with a single CS chain on a small (approx. 16 kDa) protein. Interestingly, the nonreducing region of the CS chain is composed exclusively of non-sulfated GlcA-GalNAc subunits (see also Paragraph 5.3.3.2) (Ly et al. 2011) and is linked through ester bonds involving GalNAc *C*-6 positions to two proteins (HC1 and HC2) (Enghild et al. 1999). Such unique covalent assemblage with CS acting as bridging unit between three proteins seems to have an essential role in ECM formation in the context of inflammatory diseases and ovulation (Rugg et al. 2005).

The biosynthesis of CS has been already described in part in Paragraph 5.2.1, since the tetrasaccharide linkage to serine units of CSPGs core protein is shared with HS. By action of a GalNAc- instead of a GlcNAc-transferase, the biosynthesis then diverges to CS instead of HS assembly (see Paragraph 5.2.1 for CS vs. HS biosynthesis regulating factors). The polymerization process consists of a stepwise alternating transfer of GlcA and GalNAc residues from activated precursor to the nonreducing terminal of the nascent polymer chain through the action of GalNAc- and GlcA-transferases, respectively. Interestingly, the transfer of exclusively the first GalNAc unit on the tetrasaccharide linkage region and of all the other ones on the growing polymer chain is catalyzed by two different GalNAc-transferases (CS-GalNAcT-1 and CS-GalNAcT-2). The concomitant action of a variety of highly specific sulfotransferases is devoted to the decoration of the growing chain with sulfate groups at specific positions (Habuchi 2000). It is worth noting that the machinery involved in the biosynthesis of the branched CSs found in some invertebrates, such as fCS from sea cucumbers, is still completely unknown.

### 5.2.3 Dermatan Sulfate

Dermatan sulfate (DS) is a glycosaminoglycan composed of repeating disaccharide units of IdoA and GalNAc. The iduronic acid residues display an α-configuration and are attached to *O*-3 of the *N*-acetylgalactosamine units which in turn are linked, as β-glycosides, to *O*-4 of the subsequent IdoA residues (Fig. 5.1c). This polysaccharide is one of the most abundant GAGs in eukaryotes and is typically found conjugated to a core protein as a proteoglycan. It is present in the skin, cartilage, central nervous system, and blood vessels, mainly as a component of ECM.

DS shares with CS the first stages of the biosynthetic pathway where a tetrasaccharide primer is generated through *O*-glycosidation of lateral chains of serine residues within the core protein (see Paragraph 5.2.1); a chondroitin chain is created from the primer (see Paragraphs 5.2.2), and then the DS sequences are generated through epimerization of the D-glucuronic acid units into L-iduronic acid residues, with subsequent sulfation steps (Malmström et al. 2012). Unlike CS, DS is almost always sulfated at *C*-4 of the GalNAc units (Fig. 5.6); residues of GalNAc sulfated only at *C*-6 are quite unusual (iC subunit), whereas the *C*-6 sulfation of a residue previously sulfated at *C*-4 could be detected (iE subunit), in view of the existence of an enzyme devoted to this specific process (see below). Iduronic acid residues can also be sulfated at position *C*-2, to give iB and iD subunits. Interestingly, in spite of its low occurrence in DS (approximately 5%), iB subunit has an important biological role (see Paragraph 5.4.2.), as the repeat of three of such subunits gives a hexasaccharide with a high affinity for heparin cofactor II (HCII) (Tollefsen 1992).

As for the key epimerization step, two different epimerases, namely, dermatan sulfate epimerase 1 and 2 (DS-epi1 and DS-epi2), are known. They are differentiated in the chromosomal origin and in the relative distribution in the body (Pacheco et al. 2009a). The epimerization steps are not quantitative, and the final polysaccharide chain is indeed a hybrid of CS and DS tracts with the specific content being dependent on several factors. The distribution of DS motifs within the chain can also be variable, with the possible generation of IdoA blocks (relatively long DS

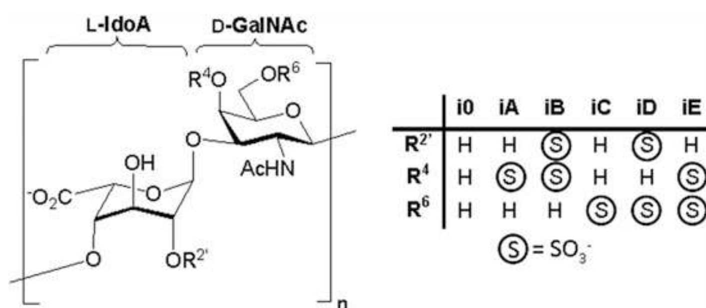



**Fig. 5.6** Sulfation pattern distribution in DS

sequences with consecutive L-iduronic acid residues) as found in CS/DSPGs such as decorin and biglycan, or sequences bearing isolated IdoA units (as in versican CS/DSPG), or tracts with alternating of D-gluco- and L-iduronic acid residues, as found in decorin and biglycan again. Experiments with mutants have shown that the epimerization process likely entails an initial β-elimination mechanism induced by deprotonation at *C*-5 of a GlcA residue and detachment of the GalNAc residue as an hemiacetal; the resulting α,β-unsaturated acid then undergoes an addition process ultimately leading to reattachment of the GalNAc hemiacetal to the *C*-4 position (with retention of the initial configuration at *C*-4), and reprotonation at *C*-5 from the top face of the ring, to cause the change of configuration at that position (Pacheco et al. 2009b). The initial elimination step in this mechanism is analogous to that occurring in the lyase catalyzed GAG chain fragmentation (see Paragraph 5.3.2) and not surprisingly DS-epimerases, and some lyases feature some structural similarities. It has been recently demonstrated that epimerization mechanism of DS-epi1 requires a sufficiently long oligosaccharide chain as the substrate, with the process starting from a random uronic acid residue and proceeding toward the nonreducing terminus (Tykesson et al. 2016).

As for CS and HS, sulfation of the DS skeleton entails PAPS as the sulfo-transfer activated reagent. *C*-4 sulfation is catalyzed by a single sulfotransferase, dermatan sulfate 4-*O*-sulfotransferase 1, indicated as D4ST1. Interestingly, this enzyme was found to critically influence the extent of generation of IdoA blocks in the GAG chain, in spite of not being directly involved in the epimerization process (the sulfation step catalyzed by this enzyme follows the epimerization) (Pacheco et al. 2009c). This is just one of the numerous examples of the regulatory aspects governing GAG biosynthesis which has yet to be unveiled.

Further sulfation events can occur in DS blocks; the enzyme GalNAc(4S)-6ST specifically catalyzes sulfation at GalNAc *C*-6 position of a residue previously sulfated at *C*-4; alternatively, IdoA *C*-2 sulfation can be catalyzed by uronosyl sulfotransferase (UST), the same enzyme involved in the *C*-2 sulfation of GlcA residues in CS.

### *5.2.4 Keratan Sulfate*

Keratan sulfate (KS) is a glycosaminoglycan consisting of the repeating disaccharide unit →3)-β-Gal-(1→4)-β-GlcNAc-(1→ (Fig. 5.1d). This is the only GAG that does not contain any acidic residue (Funderburgh 2000, 2002). The amount of substitution of the saccharide portion with sulfate groups is highly variable and depends on the localization of the KS proteoglycan (KSPG). Sulfate groups usually decorate the *C*-6 position of GlcNAc residues, although Gal units can also be sulfated at the same position.

A KSPG was firstly identified in the cornea by Suzuki (1939), who suggested only a compositional structure. Later, the structural analysis indicated the linkage of the polysaccharide to a protein moiety (Bray et al. 1967; Bhavanandan and Meyer

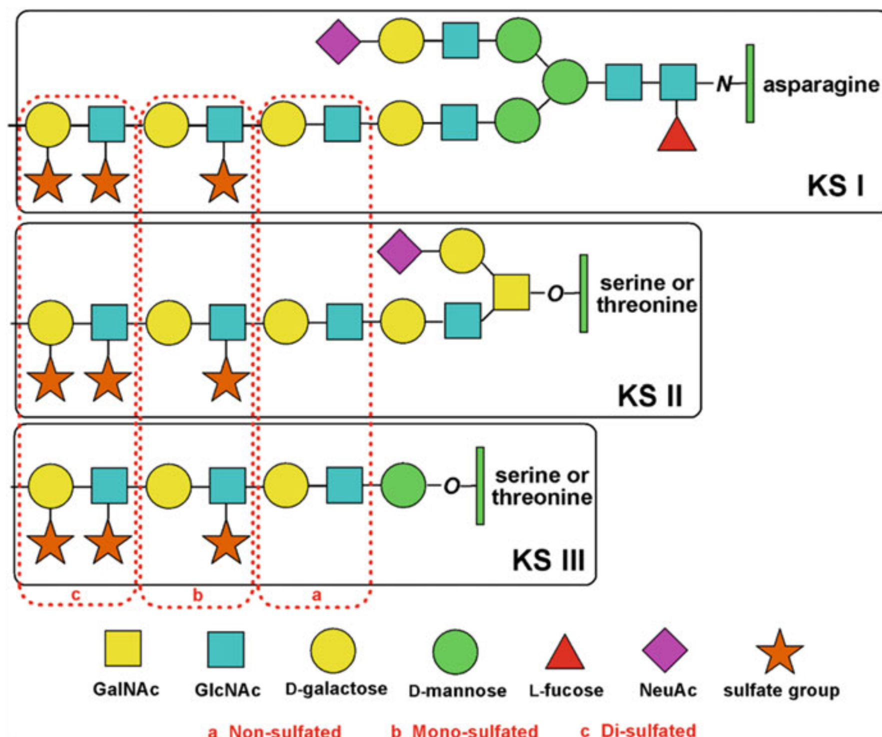



**Fig. 5.7** Schematic representation of KS-I, KS-II, and KS-III structure

1968; Choi and Meyer 1975; Brown et al. 1996), revealing that in the cornea, the KS chain is anchored to an asparagine residue of the protein through a *N*-glycosidic linkage. As for the linkage to the protein core, KS is the only sulfated GAG not attached via the tetrasaccharide linker found in PGs carrying CS, DS, and HS chains (see Paragraphs 5.2.1, 5.2.2, and 5.2.3). In particular, today we know that at least three types of KS, namely, types I, II, and III, exist in nature. The classification depends on the tissue where the KS was firstly isolated and on the kind of linkage to the protein core. All these KS structures comprise segments with unsulfated disaccharide subunits, as well as regions showing mono-sulfated and disulfated disaccharides. KS-I has been mainly isolated from the cornea, even if it has also been found from small leucine-rich proteoglycans in tissues different from the cornea. The linkage of KS-I to the core protein involves a complex-type biantennary oligosaccharide, which is linked in turn to an asparagine residue through a *N*-glycosidic bond (Fig. 5.7). The second core branch is substituted by a single lactosamine disaccharide (2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucopyranose) capped with a *N*-acetylneuraminic acid (NeuAc) (Fig. 5.7) (Funderburgh 2000). Corneal KSPGs such as lumican, keratocan, and osteoglycin/mimecan have 2–3 KS chains with a 10–15 kDa molecular weight each (Hassell et al. 1979; Funderburgh et al. 1997; Dunlevy et al. 1998). Instead, in KS-I from non-corneal tissues, such as in

fibromodulin KSPG from human articular cartilage, the polysaccharide chain is relatively shorter and more heavily sulfated with respect to that of the cornea (Lauder et al. 1997). Fibromodulin aside, KS-I can be found in cartilage tissues in lumican and keratocan KSPGs (Plaas et al. 1990; Lauder et al. 1996).

KS-II appears in aggrecan, the most abundant proteoglycan in cartilage, which mainly contains CS chains (see Paragraph 5.2.2). The linkage to the protein core in bovine articular cartilage is, for most of the KS chains, through an *O*-glycosidic bond. KS chains in aggrecan can be found in five different domains, namely, in the hyaluronan-binding region (HABR), in the interglobular domain (IGD), in the G2 globular domain, in the KS-rich region, and interspersed throughout the CS1 and CS2 domains of the CS-rich region (Caterson and Melrose 2018). The nonreducing ends of the chains are capped by NeuAc substituting the terminal GlcNAc at the *O*-3 or *O*-6 position. A residue of α-fucose can be also present at the position *O*-3 of sulfated GlcNAc (Brown et al. 1996). Conversely, KS-II chains from non-load-bearing nasal and tracheal cartilage are not fucosylated, nor do they contain a NeuAc linked at *O*-6 position of the terminal GlcNAc (Nieduszynski et al. 1990; Dickenson et al. 1991).

KS-III, first isolated from mouse brain tissues (Krusius et al. 1986), has been recognized to be *O*-linked to a Ser/Thr unit of the protein core through a single mannose residue. Proteoglycans such as phosphacan, synapse vesicle proteoglycan-2 (SV-2), and abakan in brain tissues are rich in KS-III chains (Caterson and Melrose 2018).

Although KS is found in many kinds of tissues, only a few proteins in each tissue type are modified with KS. Consequently, there should be a fine recognition of protein amino acid sequence to specifically insert the KS chains, and some studies report indeed on the relationships between the consensus sequence and KS decoration (Barry et al. 1994; Dunlevy et al. 1998). KS biosynthesis occurs through alternating addition of Gal and GlcNAc residues to the growing chain. The Gal unit is transferred by a β-(1-4)-galactosyltransferase (β4GalT-1) (Funderburgh 2002). Interestingly, two types of β4GalT-1 have been identified up to now, of which one is able to catalyze the transfer of Gal to a nonreducing terminal GlcNAc acceptor, producing the non-sulfated poly-*N*-acetyl-lactosamine (Brew et al. 1968; Schanbacher and Ebner 1970), whereas the other recognizes the nonreducing terminal GlcNAc-6-sulfate as acceptor (Seko et al. 2003) and is responsible for the production of mono- and disulfated disaccharide subunits in the KS chain.

As for the β-(1-3)-*N*-acetylglucosaminyl transferases, many enzymes capable to transfer GlcNAc to Gal/GalNAc residues have been identified (β3GnT-1, 2, 3, 4, 5, 6, 7), even if only β3GnT-7 is clearly reported to be involved in KS chain elongation (Seko and Yamashita 2004; Uchimura 2015). After the transfer of a residue of GlcNAc, a GlcNAc-6-*O*-sulfotransferase-1 (GlcNAc6ST1) transfers a sulfo group to the *C*-6 position of GlcNAc at the nonreducing end (Uchimura et al. 1998, 2002). Finally, sulfation of the Gal residue at position *C*-6 can occur through a KS galactose 6-*O*-sulfotransferase (KSGal6ST) after the formation of the polysaccharide chain (Uchimura 2015). The biosynthesis of KS can start either in the ER as for KS-I or directly in the Golgi, as for KS-II (Hassell et al. 1986).

## 5.3 Structural Characterization of Sulfated Glycosaminoglycans

### *5.3.1 Isolation and Purification from Natural Sources*

The isolation of sulfated GAGs from animal tissues firstly requires their detachment from the core protein of PGs. This can be done through a β-elimination reaction like for *O*-linked glycoproteins, nonetheless the requirement for strong alkaline conditions can result in polysaccharide structural modifications (Conrad 2001). A nonselective peptidase digestion with a proteolytic enzyme under denaturing conditions, or a stepwise treatment with different proteases, followed by purification by iterative precipitations (Garnjanagoonchorn et al. 2007; Vázquez et al. 2013), is often preferred to obtain the intact sulfated GAG chain, which in this case is still attached to the serine primer through the tetrasaccharide linkage region. Very often the polysaccharide purity achieved after the precipitations is not satisfying for a detailed structural characterization, and therefore sulfated GAG is further purified by ultrafiltration/diafiltration/dialysis techniques and/or chromatography (typically anion exchange or size exclusion techniques). Lyases – enzymes able to cleave the polysaccharide chain of GAGs (see Paragraph 5.3.2) – are also often employed in combination with molecular weight-based separation techniques, in order to avoid the contamination of the sample with other GAGs.

### *5.3.2 Chemical and Enzymatic Methods*

Several reactions have been developed as useful tools for the analysis of sulfated GAGs structure. For example, the reaction of sulfated GAGs with 1,9-dimethylmethylene blue (DMMB) dye produces a visible light absorption with a blue shift, which is more or less pronounced depending upon the type of GAG being analyzed, thus giving a preliminary and rapid clue to the identity of the investigated GAG (Stone et al. 1994). Degradative reactions cleaving the glycosidic bonds of GAG polysaccharides (typically acid-catalyzed hydrolysis or alcoholysis) are employed for obtaining a mixture of their monosaccharide components. They can be then separated and analyzed by several chromatographic techniques as themselves or after further derivatizations. It is worth noting that such unselective depolymerization methods cause also the cleavage of sulfate groups (Toida et al. 2009). For this reason, many efforts have been devoted toward the optimization of selective degradation reactions able to cleave only a specific kind of glycosidic bond within the GAG structure, leaving the other ones as well as sulfate esters untouched. Examples include β-elimination reaction at uronic acid sites (Gao et al. 2015), deaminative cleavage on the amino sugar residues (Bienkowski and Conrad 1985), free radical or photochemical-promoted oxidative depolymerization preferentially degrading IdoA units (Nagasawa et al. 1992; Ofman et al. 1997; Panagos et al.

2016), and mild acid hydrolysis selective for L-fucose branches in fCS (Santos et al. 2017a). After extensive and suitably optimized separation of the reaction mixtures, pure sulfated GAG oligosaccharides can be obtained in this way. They are useful not only to gain some insights into subtle details of the native GAG structure, such as the determination of short sequence (4–12 saccharides) of monosaccharide constituents, but also for precise structure-activity relationship investigations.

The mildest and most selective depolymerizations of sulfated GAG chains can be achieved through enzymatic reactions. For example, endolytic enzymes such as CS/DS lyases AC I, B and ABC, heparin lyases, and hyaluronan hydrolase have been used to obtain a mixture of differently sulfated disaccharides, which can be easily separated into its single components by liquid chromatographic techniques, for example, ion-pairing reversed-phase high-pressure liquid chromatography (IPRP-HPLC) (Grøndahl et al. 2011). The obtained data allow a qualitative-quantitative determination of the sulfation pattern in the original CS/DS polysaccharide. Investigators have also reported the possibility of obtaining sulfated CS and DS oligosaccharides longer than disaccharides through non-exhaustive depolymerizations under controlled lyase digestion conditions, followed by extensive multistep chromatographic separations (Yang et al. 2000; Deepa et al. 2007; Pomin et al. 2012b). The same approach was also used for sequencing heparin blocks through enzymatic reaction with heparin lyases (Liu et al. 1995; Mourier et al. 2015). It is worth noting that, even if enzymatic cleavage of sulfated GAG chains has a much higher specificity with respect to chemical reactions, some limitations have to be considered. Apart from the cost of such methods, some structural features of sulfated GAGs have been demonstrated to be resistant to enzymatic cleavage, as, for example, CS branching at GlcA *O*-3 position in fCS extracted from sea cucumbers. It has been proposed indeed that such branching serves to prevent digestion of the sea cucumber body wall, which is rich in fCS polysaccharide, by sea microorganisms (Vieira et al. 1991).

Another kind of enzymes useful for sulfated GAG structural characterization are sulfatases from microorganisms. They are able to cleave sulfate groups at selective positions and/or on specific units of the GAG polysaccharide chain (Ulmer et al. 2014; Wang et al. 2015). This can be exploited for confirming or refuting the presence of sulfate groups on specific sites of the studied GAG (Chi et al. 2009).

### *5.3.3 Physical Methods*

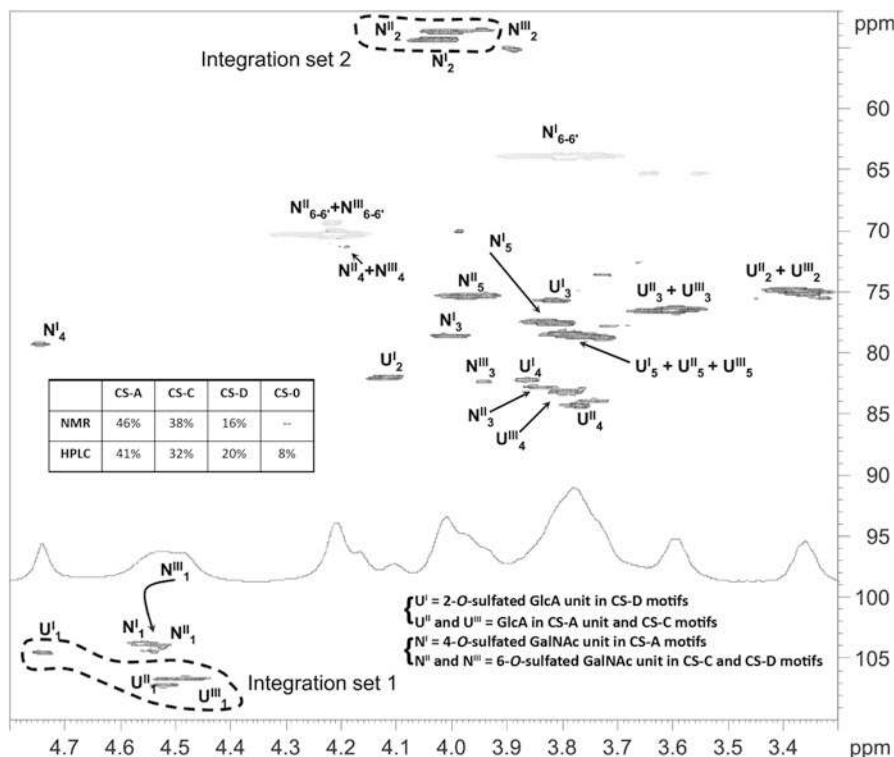
#### 5.3.3.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Solution NMR spectroscopy has become one of the preferred analytical techniques for structural characterization of sulfated GAGs in the last 25 years. Indeed, confirmation of data inferred from monosaccharide composition analysis (Paragraph 5.3.2) and, in addition, the determination of the positions involved in glycosidic bonds connecting the monosaccharide units and the stereochemistry of such linkages

as well as sulfation and other decoration (acetylation, fucosylation) patterns can be obtained. First, $^{1}$H and $^{13}$C-NMR chemical shift assignment is performed, usually exploiting homo-correlated (COSY, TOCSY, NOESY) and hetero-correlated (DEPT-HSQC, HSQC-TOCSY, HMBC) two-dimensional experiments, due to the crowd of superimposed signals in mono-dimensional spectra – especially $^{1}$H-NMR – of GAGs; thereafter, comparison between the observed chemical shift values and literature data (Mucci et al. 2000; Mulloy et al. 2000; Huckerby et al. 2005; Pomin 2015) allows the elucidation of many structural details. In particular, a 0.7–1.2 ppm downfield shift for $^{1}$H chemical shifts with respect to normal values, with a concomitant 5–10 ppm downfield shift for $^{13}$C carbon signals, is typically informative for sulfation at the related carbinolic position. Similarly, a 8–12 ppm downfield shift of the sole $^{13}$C chemical shift is indicative of a carbinolic group involved in a glycosylation linkage. $^{3}J_{H1,H2}$ and $^{1}J_{C1,H1}$ coupling constant values can be exploited for confirming the anomeric configuration expected for the monosaccharide constituents of sulfated GAGs.

The peaks of a 2D-NMR experiment such as HSQC or DEPT-HSQC can be integrated, if specific constraints are satisfied, in order to furnish a quantitative estimation of the GAG sulfation pattern in terms of the relative proportion of disaccharide subunit in the polysaccharide (Guerrini et al. 2005; Gargiulo et al. 2009; Mauri et al. 2017). It is worth noting that this is a nondestructive method for the quali-quantitative determination of sulfation pattern on GAG polysaccharide chains (Fig. 5.8), as an alternative to enzymatic hydrolysis followed by chromatographic separation of the obtained disaccharides and their comparison with standards (see Paragraph 5.3.2).

NMR spectroscopy was employed also for unveiling subtle but often crucial structural details of sulfated GAGs (e.g., the different conformations of IdoA that depend on the sulfation pattern of adjacent residues in HS chains: Ferro et al. 1990; Hsieh et al. 2016) and GAG mixtures. In the latter case, a very illustrative example is the use of NMR spectroscopy for determining the etiological agent of the “heparin contamination crisis” in 2008, with hundreds of serious adverse events, including 149 deaths, in the USA and Europe (Kishimoto et al. 2008). Indeed, a combination of $^{13}$C 1D-NMR and DEPT-HSQC and HMBC 2D-NMR techniques allowed the identification of a contamination of some heparin lots with per-sulfated chondroitin (Guerrini et al. 2008), a GAG polysaccharide able to induce strong allergic-type responses (Greinacher et al. 1992). Thereafter, such 1D- and 2D-NMR techniques were indicated as an effective routine method for determining the structural differences between pharmaceutical grade heparins from different animal sources as well as for assessing their purity (Tovar et al. 2016). Diffusion-Ordered Spectroscopy (DOSY), a very powerful NMR technique for analyzing mixtures of chemical species, was also proposed for unveiling the presence of polysaccharide impurities in heparin lots (Sitkovski et al. 2008). Analogously to the heparin case, 1D- and 2D-NMR spectroscopy was shown to be an effective analytical tool also for the determination of mono- and polysaccharide ingredients and contaminants (mainly KS: Pomin et al. 2012a) present in pharmaceutical grade CS formulations (Santos et al. 2017b).




| | CS-A | CS-C | CS-D | CS-0 |
|---|---|---|---|---|
| NMR | 46% | 38% | 16% | -- |
| HPLC | 41% | 32% | 20% | 8% |

**Fig. 5.8** Structural characterization of CS from dogfish and comparison between disaccharide subunit relative proportion evaluation by DEPT-HSQC 2D-NMR spectroscopy and by chondroitinase ABC chain cleavage followed by HPLC separation. (adapted with permission from Gargiulo et al. 2009; ©2009 Oxford University Press)

#### 5.3.3.2 Mass Spectrometry

Analytical methods based on mass spectrometry (MS) are in general highly desirable for applications involving low amounts of sample and/or heterogeneous mixtures, due to its very high sensitivity and the possibility, depending upon the kind of ionization method, to detect any single component in sample mixtures or to connect the mass spectrometer online with a liquid chromatography instrument. In the case of the analysis of a pure sulfated GAG oligosaccharide or a mixture thereof, coming from chemical or enzymatic hydrolysis of the polysaccharide (see Paragraph 5.3.2), MS methods involving a matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) were reported, often enhanced with tandem MS and sometimes also $MS^3$ techniques (Juhasz and Biemann 1995; Chai et al. 1998; Zaia and Costello 2003; Laremore et al. 2006; Nimptsch et al. 2009; Bielik and Zaia 2011). These methods were employed not only for the fine structural characterization of sulfated GAGs but also for the rapid detection, identification, and quantification of

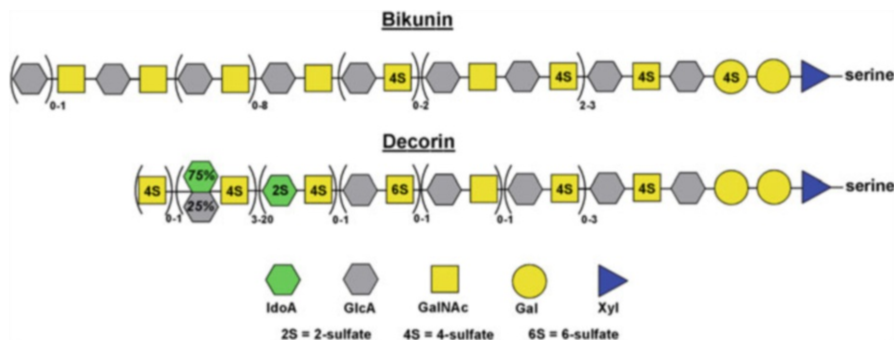



**Fig. 5.9** Sequence of monosaccharide components in sulfated GAG chain of bikunin and porcine skin decorin

impurities and adulterants in pharmaceutical grade heparin and CS lots (Saad et al. 2005). Nonetheless, there is a serious limitation to the analysis of complex sulfated GAG oligosaccharides by mass spectrometric methods. It is the difficulty to gain accurate identification of the sulfation pattern as sulfate groups are labile and therefore easily lost upon collisional activation. A strategy to overcome this limitation consists in subjecting the GAG oligosaccharide mixture, coming from a controlled chemical or enzymatic hydrolysis, to a standardized chemical protocol with the aim to convert the labile sulfate groups into stable acetyl esters, while maintaining the information on the original sulfation sites (Huang et al. 2011). Furthermore, some advanced MS techniques, such as negative electron transfer dissociation (NETD) for ion trap MS and Fourier transform ion cyclotron resonance mass spectrometers (FTICR-MS), as well as collision-induced detachment (CID) or electron detachment dissociation (EDD) for FTICR-MS, have been recently applied to sulfated GAGs. They demonstrated the possibility of analyzing intact sulfated GAG oligosaccharides, by minimizing sulfate group loss and contemporarily enhancing structurally relevant glycosidic and cross-ring fragment ions, to be revealed in tandem MS or $MS^n$ experiments (Wolff et al. 2007, 2010; Huang et al. 2013; Kailemia et al. 2014; Agyekum et al. 2018). The great potential of FTICR-MS techniques has been very well exemplified in the full sequence analysis of CS chain in bikunin, the smallest CSPG discovered up to now. Its molecular size-based fractionation through electrophoresis gave picomolar quantities of serine-linked polysaccharides with a degree of polymerization from 27 to 43. Then, the complete structure of each of them was determined by CID-FTICR-MS, and the integrated data analysis not only demonstrated unequivocally that CS chain of bikunin possesses a defined sequence but also unveiled it (Fig. 5.9) (Ly et al. 2011).

The same approach was successfully applied also on the CS/DS chain of a more complex proteoglycan such as decorin from porcine skin (Figs. 5.9 and 5.10), even if a slightly less precise sequencing output could be gained (Yu et al. 2017). Since the biosynthetic pathway is common to all natural proteoglycans, these results strongly suggest that all sulfated GAG chains of PGs, even the more structurally complex ones, such as HS, have a single or small number of sequences among the extremely huge amount of possible ones.

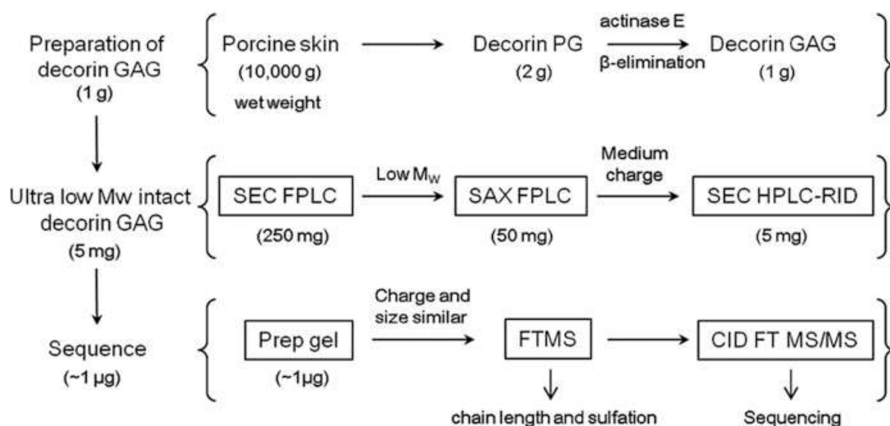



**Fig. 5.10** Protocol flowchart for solving GAG chain structure of decorin. (reprinted with permission from Yu et al. 2017; ©2017 American Chemical Society)

An alternative method for sequencing sulfated GAG chains was very recently reported through an original combination of mass spectrometry and gas-phase infrared (IR) ion spectroscopy. Even if this method has been applied up to now for discriminating between IdoA and GlcA as well as determining the sulfation pattern only on short sulfated GAG oligosaccharides (Schindler et al. 2017; Renois-Predelus et al. 2018), it appears very promising as a valuable alternative to MS/MS and $MS^n$ technique in the cases where no conditions can be found for collecting a satisfying amount of structurally informative fragment ions. The usually low resolution of IR spectra can be improved by performing the vibrational analysis on cryogenically cooled ions. Indeed, a combination of cryogenic IR spectroscopy and MS spectrometry, together with an ion mobility-based gas-phase separation, has been very recently proposed as a technique able to distinguish and sequence sulfated GAGs using very low quantity of biological samples (Khanal et al. 2017).

Recently, a MS technique has been reported that employs direct analysis in real-time (DART) pyrolysis ion source for the very rapid (approx. 30 s/sample), nanogram scale discrimination of sulfated GAGs from biological or pharmaceutical sample (Nemes et al. 2013).

## 5.4 Biological Functions of Sulfated Glycosaminoglycans

Sulfated proteoglycans are key molecules in a variety of physiological and pathological processes. This research field has expanded so far in the last two decades that here it is impossible to cover all aspects of sulfated GAG biology. Therefore, only a global overview of the most investigated biological processes in which HSPGs, CS/DSPGs, and KSPGs play a key role is presented here below. For more details, the reader is referred to the several, recently published, excellent reviews covering the

specific fields (Malmström et al. 2012; Xu and Esko 2014; Mizumoto et al. 2015; Mulloy et al. 2016; Caterson and Melrose 2018; Karamanos et al. 2018).

### 5.4.1 Heparan Sulfate

Heparan sulfate, normally in the form of heparan sulfate proteoglycans, is present in the ECM or on the cell surface of essentially all animal cells. HS has structural functions and is involved in numerous biological processes (Lindahl and Li 2009; Sarrazin et al. 2011; Xu and Esko 2014). As a component of ECM, HS contributes to its structural integrity through binding with other ECM components, such as collagen I and IV, fibronectin, and laminin. HS in the ECM can also bind cytokines, chemokines, growth factors, and morphogens, creating a protecting depot of these proteins from protease degradation and environmental damage. The proteins are released by selective degradation of the heparan sulfate chains. In addition, HS can modulate the function of the extracellular proteins acting to increase their range of diffusion and local concentration or to present the proteins to their high-affinity receptors. The highly sulfated chain of HS binds to proteins mainly through electrostatic interactions between its sulfate groups and positively charged groups of the protein, typically lysine/arginine residues aligned in "Cardin-Weintraub" sequences (Cardin and Weintraub 1989). Some nonionic interactions are also present and they do not occur randomly. The specificity and the affinity of a protein for HS chains depends largely on the sulfation profile and chain length of HS (Capila and Linhardt 2002; Lindahl and Li 2009). Some examples of HS-binding proteins with their biological activity are reported in Table 5.1. A comprehensive list can be found in Mulloy et al. 2016.

One of the most relevant aspects of HSPGs located in the ECM or inserted in the cell membrane is that they can act as scaffold bringing two proteins into close proximity to facilitate their interactions. A ternary complex is formed between the two proteins and the HS chain. This property of HSs is possible owing to their long-chain length, flexibility, and structural diversity. A very well-documented system in which HS serves as molecular scaffold is the antithrombin/thrombin interaction, which results in the inhibition of blood coagulation cascade (van Boeckel and Petitou 1993; Desai et al. 1998). Thrombin is an enzyme that converts fibrinogen into soluble strands of fibrins, which is a main step in the blood coagulation cascade. Antithrombin by itself is not an efficient inhibitor of thrombin. However, its inhibitory activity increases up to several thousand-fold in the presence of heparin, due to the ability of the polysaccharide to bring antithrombin and thrombin into close proximity. The binding of antithrombin to heparin induces a conformational change in the protein which results in the inhibitory activation of antithrombin. While a specific sulfated pentasaccharide fragment is sufficient for antithrombin and a sulfated hexasaccharide is required for thrombin binding (Desai et al. 1998), a longer octadecasaccharide containing the sulfated pentasaccharide fragment at the reducing

**Table 5.1** Examples of HS-binding proteins and their biological activity

| Class | Examples | Physiological function | Pathophysiology |
|---|---|---|---|
| Growth factors | FGF2, $VEGF_{165}$, neuropilin-1 | Mitogenesis, development, wound healing, angiogenesis, axon guidance | Cancer |
| ECM proteins | Collagen, fibrinogen, laminin | Cell adhesion, migration, differentiation, blood coagulation | Cancer, Knobloch syndrome |
| Cell adhesion proteins | P-selection, L-selectin, integrins | Cell adhesion, inflammation | Cancer |
| Morphogens | Activin, BMP-2, sonic hedgehog | Development, regeneration, bone formation | Multiple hereditary exostoses, osteoarthritis |
| Chemokines | Platelet factor 4, IL-8, TNF-α, CXCL12 | Chemotaxis, cell migration, immune response, angiogenesis | Inflammation, arteriosclerosis, cancer |
| Blood coagulation factors | Antithrombin III, factor Xa, leuserpin-2 | Regulation of clotting cascade | Heparin-induced thrombocytopenia |
| Lipoproteins | ApoE, ApoB, lipoprotein lipase | Lipid metabolism, cell membrane functions | Atherosclerosis, Alzheimer's disease, AA amyloidosis |
| Nuclear proteins | Histones, transcription factors | Unknown | Cancer |
| Amyloid proteins | App, Aβ, tau protein, α-synuclein, $PrP^{Sc}$ | Synapse organization, brain development, memory, circadian rhythm | Alzheimer's/Parkinson's disease, prion disease |
| Viral proteins | gB, gC, gD, gp120, Tat, E protein, L1 capsid protein | – | Viral attachment and invasion |
| Microbial proteins | M protein, PfEMP1, Opa, circumsporozoite protein | – | Bacterial/parasite infection, inflammation |

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end is the minimal sequence able to form the ternary antithrombin-thrombin-heparin complex (Lane et al. 1984; Petitou and van Boeckel 2004).

A large number of studies on the ternary complex model involving HS, growth factors, and their high-affinity receptors have also been reported. Commercial heparin and fragments derived from its partial enzymatic hydrolysis have been frequently used to investigate this model. Thus, it has long been known that fibroblast growth factor (FGF) signaling requires the formation of a FGF/FGFR/HS ternary complex (Rapraeger et al. 1991; Kan et al. 1993). The FGF/FGFR complexes tend to dissociate under size exclusion chromatography conditions. However, in the presence of heparin, the ternary complex is much more stable and can be purified by chromatography, indicating that heparin increases the affinity of FGF toward FGFR (Schlessinger et al. 2000). The binding to heparin might induce a conformational change in FGF to generate a molecular structure that better fits into

the FGFR binding site. Structural studies have shown different complex stoichiometries, and two ternary complex models have been suggested: a symmetrical 2:2:2 FGF/FGFR/HS complex with two saccharide chains and a 2:2:1 complex with a single saccharide (Goodger et al. 2008). An octasaccharide seemed to be the shortest heparin fragment that formed 2:2:1 complexes, while very short heparin fragments (tetra- and hexasaccharides) would form symmetrical 2:2:2 complexes (Robinson et al. 2005; Goodger et al. 2008). Similar mechanisms based on ternary complexes were also proposed for other signaling pathways, such as those for bone morphogenetic protein (BMP) (Ruppert et al. 1996), wingless-type MMTV integration site family (WNT) (Reichsman et al. 1996), and vascular endothelial growth factor (VEGF) (Gengrinovitch et al. 1999). As a result of these interactions with proteins, many of them involved in cell signaling pathways, HSs are able to modulate a variety of processes that are important in the regulation of cell, tissue, and organ development in health and disease (Whitelock and Iozzo 2005; Xu and Esko 2014), which is discussed next.

Several studies have shown that HSPGs are key regulators of stem cell biology (Mikami and Kitagawa 2017). Stem cells are defined by their ability to self-renew and their capacity for differentiation into specialized cells. The decision of stem cells to self-renew or undergo differentiation is regulated at multiple levels. Several growth factors that regulate signaling pathways in cellular proliferation and differentiation are HS-dependent. Because the interaction with these proteins depends on the structure and sulfation profile of the HS chain, the cellular biosynthetic machinery for HS production participates in the regulation of stem cell fate. Thus, it has been shown that the biosynthesis of HS increased and its composition changed during the transition of embryonic stem cells from self-renewal to differentiation (Johnson et al. 2007; Nairn et al. 2007; Smith et al. 2011). This switch seems to be dependent on specific sulfation motifs on HS chain, since differentiation is accompanied with expression of specific sulfotransferases that modified HS sulfation profile (Johnson et al. 2007; Nairn et al. 2007). On the other hand, when the stem cells are deficient in the HS polymerase EXT-1 and are unable to synthesize HS chains, these cells are maintained in a pluripotent state and are unable to differentiate.

Some relevant biological functions of HS are played in the central nervous system together with CS. In contrast to other organs and tissues, the ECM of the central nervous system (CNS) shows relatively low content of fibrous matrix proteins such as collagens and fibronectin. Instead, it is particularly rich in proteoglycans that are synthesized by neurons and astrocytes (Ruoslahti 1996). HS and CS proteoglycans of the ECM and on the surface of neural cells play relevant roles in brain development, axon growth and guidance, formation and function of synapses, and also response of the CNS to injury (Maeda 2015; Smith et al. 2015).

When the nervous system is forming, the neurons connect with other neurons by fibers, dendrites, and axons to form complex neuronal networks. During this process, the axons extend over long distances to connect with other brain cells. In order to reach their targeted brain regions, some chemical substances act as guidance cues, by attracting or repelling axons. These molecules with key roles in neural wiring are

classified into four major families: netrins, semaphorins, slits, and ephrins. A critical role for HS in axon guidance was demonstrated in mutant embryonic mouse brain cells that are unable to synthesize HS (Inatani et al. 2003). The EXT1-null brain displayed severe guidance errors in major commissural tracts. In another study with two HS sulfotransferase mutant embryos lacking the capacity to catalyze sulfation at *C*-2 of uronic acid and at *C*-6 of glucosamine, it was shown that the sulfation pattern of HS was important in the navigation of retinal ganglion cell (RGC) axons (Pratt et al. 2006). The author demonstrated that the response of RGC axons to slit2 depended on the 6-*O*-sulfotransferase synthesized by the RGC. Similarly, by manipulating extracellular sulfation pattern of HS, the axonal projection of motor neurons was redirected upon misexpression of the HS 6-*O*-sulfotransferase (Bülow et al. 2008). In another study that focused on the family of ephrins, which induce repulsive responses such as growth cone collapse, it was shown that in mutant cells, which are defective in HS synthesis, the ephrin-A3-dependent EphA receptor activation was reduced, which indicates that HS modulates this signaling pathway (Irie et al. 2008). These examples, illustrating the role of HS and the influence of their sulfation profile on axon guidance, use gene knockout experiments of HS biosynthetic enzymes. The results from these experiments, however, must be taken with caution since there may exist compensation mechanisms with an increase in other sulfation types (Kamimura et al. 2006). In order to study the structure-function relationships of HS, the availability of HS structures with defined sulfation motifs is of great value. Using a microarray-based approach, Shipp and Hsieh-Wilson showed the importance of the sulfation profile of HS in the interaction with a number of axon guidance proteins such as slit, netrin, ephrin, and semaphoring (Shipp and Hsieh-Wilson 2007). They used heparins that had been previously desulfated selectively at certain positions using chemical tools. A strong preference of slit2 for 6-sulfated heparin and 2-desulfated heparin was observed. They also found that netrin1 requires sulfation at *C*-2 and *C*-6 and sulfamation at *C*-2. In the case of ephrinA1 and ephrinA5, they exhibited preference for HS having both 2-sulfated and 2-sulfamated motifs.

HSPGs are strongly involved in cancer proliferation, invasion, and tumor metastasis (Afratis et al. 2012). Certain cancer cells show an increased expression of HSPG on the cell surface, which results in an enhancement of the proliferative response to growth factors (Knelson et al. 2014). In the ECM, on the other hand, HSPGs play a crucial role in cancer progression. For tumor cells invading surrounding normal tissue and spreading via the circulatory system, degradation by heparanase of HSPGs in the ECM is an important step. The growth factors that bind HS in the extracellular matrix include the proteins that regulate angiogenesis and vasculogenesis, such as the VEGF and FGF families. Therefore, the disruption of HS chains of the ECM catalyzed by heparanase will lead to the release of proteins that stimulate proliferation of tumor cells, angiogenesis, and vascularization of the tumor. Angiogenesis is a prerequisite for the growth of tumors, and hence the enzyme heparanase produced by tumor cells is considered a pro-angiogenic factor.

Heparanase is an endo-D-glucuronidase that cleaves heparin and HS. The enzyme catalyzes hydrolysis of the glycosidic bond between anomeric carbon of D-glucuronic acid and *O*-4 of D-glucosamine. The enzyme, however, does not

cleave all the glucuronide bonds, but there is a selectivity depending on the presence of sulfate groups around the glycosidic bond. Therefore, hydrolysis of HS by heparanase generates fragments of variable size, between 10 and 20 monosaccharide units. The existence of heparanase activity has been known for more than 30 years. However, the structures of free human heparanase and heparanase associated with several heparan sulfate ligands have recently been described (Wu et al. 2015). Expression of heparanase in normal tissues is relatively low. In contrast, the enzyme is overexpressed in many tumor cells, and some studies have confirmed a correlation between heparanase expression and increased mortality (Sato et al. 2004). Several causes for tumor aggressiveness have been proposed. In addition to releasing proteins that promote proliferation and angiogenesis, some fragments of HS generated by heparanase-catalyzed hydrolysis may also activate intracellular signaling pathways (Goodall et al. 2014). Also, when the structural integrity of the ECM and of the basement membrane is lost, mobility and invasion of neighboring tissues is facilitated; furthermore, tumor cells may enter the bloodstream and lead to metastasis. Destruction of blood vessels is another consequence of overexpression of heparanase, which potentiates metastasis. HSPGs are important components in the structural integrity of blood vessels. Excessive rupture of HS from blood vessels and capillaries by the action of heparanase weakens their structure and facilitates extravasation of tumor cells (Jin-Ping 2008).

There is only one heparanase enzyme activity in humans, which is expressed at very low levels in normal cells. Animals with knock-out of the gene encoding heparanase did not show any type of deficiency or phenotype change, which suggests that the inhibition of heparanase will cause minimal side effects in patients with cancer. Altogether, this makes the enzyme a highly attractive target for cancer therapy (Vlodavsky et al. 2016).

Heparin has been evaluated in clinical trials against various types of cancer. The properties of heparin as an anticancer agent were discovered indirectly in patients with cancer who developed venous thrombosis (Jin-Ping 2008). When these patients were treated with heparin, they showed a reduction in mortality compared to those not treated with the anticoagulant. Epidemiological studies and subsequent clinical trials with heparin and other anticoagulants demonstrated its protective and therapeutic effect in treatment of metastatic tumors (Tagalakis et al. 2007). Several factors may contribute to the antitumor activity of heparin. A main line of study focuses on its activity as a heparanase inhibitor. Although heparin can be a substrate for heparanase, since it is a large molecule, its hydrolysis can generate fragments that act as inhibitors of the enzyme. Heparin might also associate and sequester growth factors that stimulate proliferation of tumor cells. Concerning its antimetastatic activity, some studies have focused on its ability to inhibit the interaction of tumor cells with adhesion proteins (P- and L-selectins) expressed in the endothelial cells of blood vessels and involved in the extravasation process during metastasis (Stevenson et al. 2005). P- and L-selectin are also involved in inflammatory processes, and hence heparin has been studied as an anti-inflammatory agent.

The use of heparin as an anticancer drug, however, is restricted by its anticoagulant activity. In addition, its highly negative charge density (heparin is a highly

sulfated HS) allows its interaction with a variety of proteins in a non-specific way and therefore affects many other functions. For this reason, more specific heparin analogs without anticoagulant activity are being studied as anticancer agents (Afratis et al. 2012; Vlodavsky et al. 2016). Four compounds with anti-heparanase activity have been approved for clinical trials with cancer patients (PI-88, Roneparstat, M402, and PG545). All of them are heparin analogs/mimetics, which presumably block the active center of the enzyme by interacting with the heparin-binding domains flanking the catalytic center. PI-88, also called Muparfostat, is the most extensively studied HS mimetic in clinical trials. It has reached stage III in the treatment of patients with hepatocarcinoma (Vlodavsky et al. 2016). PI-88 is a sulfated derivative of a phosphomannopentaose, which inhibits heparanase activity with an $IC_{50}$ value of 1.7 μM and exhibits anti-angiogenic and antimetastatic activity. In addition to heparin and structural analogs, other non-sugar molecules have been studied to inhibit heparanase activity, including small molecules, anti-heparanase antibodies, or nucleic acid-based inhibitors (Rivara et al. 2016). In conclusion, a substantial amount of knowledge concerning heparanase function comes from studies on its role in cancer progression. The current interest in development of clinically relevant heparanase inhibitors for cancer therapy is, after all, a direct consequence of the important roles of heparan sulfate in the ECM.

Cell surface HSs are exploited by several bacteria, parasites, and viruses as mediators for infecting animals. HSPGs are used by microbial pathogens not only to achieve adherence and colonization but also in the invasion, internalization, dissemination, and toxicity processes. A comprehensive list of human pathogenic microorganisms that are known to recognize HS (and in some cases also CS and DS) as cell surface receptors can be found in García et al. (2016). The interactions have been inferred to be non-specific, driven by the highly negative charge density of HS chains, even if in some cases unique chain sequences appear to be involved in the adherence process, as demonstrated for the interaction of HS with herpes simplex virus type-1 (HSV-1) proteins (Shukla and Spear 2001) and suggested for the binding of HS with human immunodeficiency virus (HIV) envelope glycoprotein gp120 (Connell and Lortat-Jacob 2013).

### *5.4.2 Chondroitin and Dermatan Sulfate*

CS/DSPGs have been demonstrated to interact with a plethora of key proteins for important biological functions, such as cell proliferation, differentiation and migration, cytokinesis, tissue morphogenesis and wound repair, and infection. A schematic, non-comprehensive picture of CS/DSPG functions is reported in Fig. 5.11.

CS has profound effects on the synthesis, activity, and turnover of key structural components of the ECM in connective tissues, such as type-II collagen and hyaluronic acid. For example, the synthesis of such macromolecules is fostered by TGF-β1 and HAS-2 growth factors, respectively, the expression of which is enhanced by CS binding to integrins (Bishnoi et al. 2016). Loss of CS from cartilage

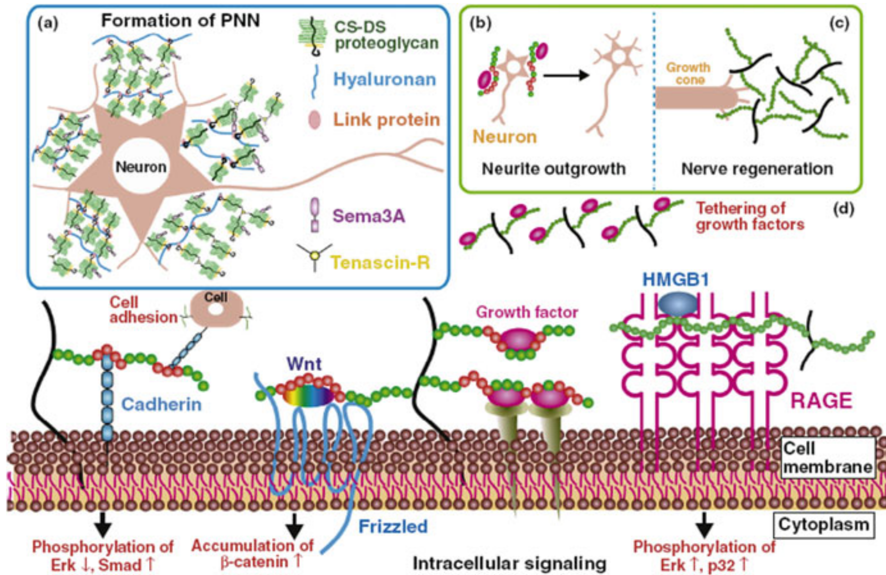


**Fig. 5.11** Various functions of CS/DSPGs: (**a**) formation of perineuronal nets (PNN), (**b**) neurite outgrowth activity, (**c**) inhibition of nerve cone growth after spinal cord injury, and (**d**) regulation of signal transduction, osteogenesis, and tumor metastasis through interaction of cell surface CS/DSPGs with growth factors and signaling proteins. (reprinted with permission from Mizumoto et al. 2015; ©2015 Elsevier)

tissues leads to osteochondral angiogenesis, one of the major cause of osteoarthritis (Bara et al. 2012), a very common disease of the aged population for which the administration of exogenous CS represents indeed a first-line therapy. Therapeutic efficiency of CS for osteoarthritis treatment is related not only to structural roles but also to an anti-inflammatory effect. The latter is due to the inhibition of signal transduction pathways, activated by damage-derived fragments of the ECM components in chondrocytes, synoviocytes, osteocytes, and osteoblasts and leading to a diminished translocation of pro-inflammatory transcription factors. In particular, CS oligosaccharides engage chondrocyte membrane receptors such as CD44 and ICAM1, to release IRAK-M, an IRAK inhibitor, and MPK-1, a MAPK dephosphorylating agent, both causing a reduction of nuclear translocation of NF-κB and therefore alleviating the inflammatory reaction. Furthermore, CS diminishes the proteolytic cleavage of kininogen to bradykinin, which induces the desensitization and internalization of B2R (du Souich 2014).

CS and DS are involved in hepatocellular proliferation and differentiation, by interaction with hepatocyte growth factors (HGF and HB-EGF). In particular, it seems that highly sulfated variants of CS and DS are involved in physiological processes (Lyon et al. 1998; Li et al. 2007), whereas in rat models with fibrous lesions due to liver cirrhosis, a decrease of the sulfation degree and an increase in CS

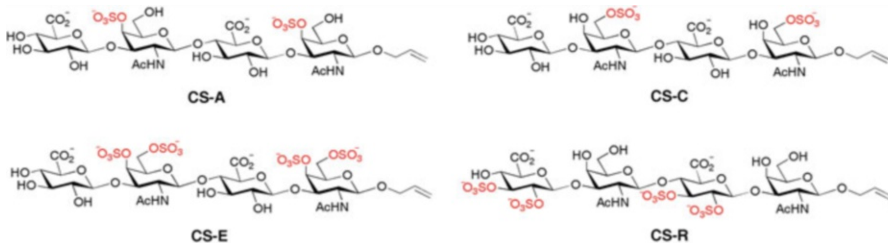


**Fig. 5.12** Chemical structure of CS-E, CS-C, CS-A, and CS-R tetrasaccharides assayed for their interaction with protein growth factors (Gama et al. 2006)

to DS ratio have been detected (Koshiishi et al. 1999). Furthermore, a deficiency in xylosyltransferase 2 (one of the enzymes initiating HS and CS/DS biosynthesis: see Paragraph 5.2.1) results in significant abnormalities of liver structure such as fibrosis, biliary tract hyperplasia, and cysts (Condac et al. 2007).

Like HSPGs, CSPGs play a significant role in CNS development (Silver and Silver 2014; Miller and Hsieh-Wilson 2015; Hayes et al. 2018). They are essential for FGF-2-mediated proliferation and maintenance of neural stem cells (Sirko et al. 2010) and interact with different growth factors (Rogers et al. 2011). The interaction with proteins is also determined by specific sulfation motifs in the CS chain. To define the sequence of sulfate groups and study the structure-function relationship of CSs, chemically synthesized CS tetrasaccharides (CS-A, CS-C, CS-E, and CS-R: Fig. 5.12; see also Fig. 5.4 for nomenclature of CS subunits) were assayed for their interaction with protein growth factors (Gama et al. 2006). A selective binding of midkine and neurotrophin BDNF to the CS-E tetrasaccharide was found, while none of the tetrasaccharides showed appreciable interaction with fibroblast growth factor FGF-1. Although CSPGs are known to repel extending axons, some sulfation variants of CS have been found in growth permissive regions of the CNS. Therefore, the authors investigated the effect of the sulfation patterns on the neurite outgrowth of hippocampal, dopaminergic, and dorsal root ganglion neuron cultures. Only the tetrasulfated CS-E stimulated the neurite outgrowth of the different cell types. Altogether, the results suggest that CS sulfation motifs can function as molecular recognition elements for growth factors and facilitate activation of the corresponding signaling pathways.

The fact that CS chains can stimulate or inhibit neuronal outgrowth depending on the sulfation sequence has been utilized to fabricate neuron-guiding substrates (Swarup et al. 2013). In particular, four major CS sulfation variants (CS-A, CS-C, CS-D, and CS-E) and DS found in mammalian brain were printed in stripes on preactivated poly-L-lysine-coated substrates. Hippocampal neurons were then cultured on printed CS stripes. Neurites preferred growing over CS-A, CS-E, and DS lanes and avoided penetrating into the CS-C containing lanes. The binary combination containing alternate lanes of neurite repelling (CS-C) and attracting (CS-A, CS-E, and DS) polysaccharide chains led to a significant alignment of neurites on the attracting lanes (Fig. 5.13).

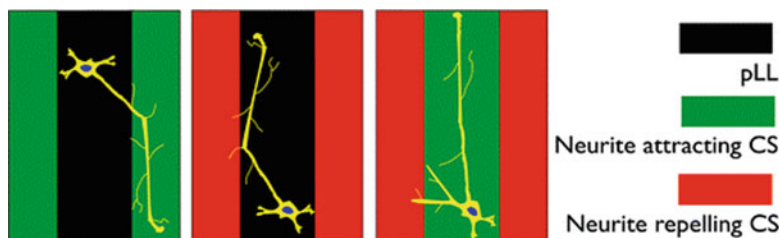


**Fig. 5.13** Fluorescent images of effect on neurite growth of poly-L-lysine (pLL)-coated glass surface printed with CSs having different sulfation patterns. (reprinted with permission from Swarup et al. 2013; ©2013 American Chemical Society)

CSPGs are involved in the control of neural plasticity, the ability of the brain to rewire the neural circuits in response to new experiences. In the mammalian CNS, there is a postnatal limited time window, which is called the critical period, during which neuroplasticity is at a high level. When the critical period ends, reticular structures, known as perineuronal nets (PNNs), deposit around the cell bodies and dendrites of neurons helping to stabilize the newly established synapses but restricting plasticity. PPNs contain components of the ECM with high concentration of CSPGs, which play an important role in maintaining stability and restricting plasticity (Bartus et al. 2012; Miyata and Kitagawa 2017). As an illustrative experiment to show that CS can suppress neuronal plasticity, it was reported that the injection of chondroitinase ABC, which catalyzes the eliminative degradation of CS, into the adult visual cortex of rats led to a reactivation of ocular dominance plasticity, even after the end of the critical period (Pizzorusso et al. 2002). In line with this study, Gogolla et al. (2009) showed that CSPGs of PNNs could be responsible for the resistance of fear memories to erasure by extinction in adult rats. In contrast to adult animals, extinction in young rats leads to erasure of fear memories. When adult mice were injected in the amygdala with chondroitinase ABC, the subsequently acquired fear memory was susceptible to erasure (Gogolla et al. 2009). Therefore, degradation of CSPGs in adult rats reenabled erasure of fear memories by extinction that is typical in young rodents.

The influence of specific sulfation patterns of CS on cortical plasticity was shown in transgenic mice that overexpress human C6ST-1 enzyme, which catalyzes the sulfation of CS chains at position 6 (Miyata et al. 2012). The authors found that the increase in GalNAc 4- vs. 6-*O*-sulfonation of CSPGs of the brain ECM leads to the termination of the critical period for ocular dominance plasticity in the mouse visual cortex. Consequently, they observed that the mice with upregulated 6-*O*-sulfonation had reduced PNN formation, and they retained juvenile-like plasticity as adults. The results suggest that the temporal shift of the 4S/6S ratio may be developmentally programmed to regulate the critical period for cortical plasticity.

CS plays a central role in traumatic injury of the brain and spinal cord, which is a major health problem worldwide and is associated with long-term physical difficulties. After injury in the CNS, there is a reaction which results in recruitment of glial cells and formation of a scar surrounding the injury site. Although these responses

could be beneficial since isolating the injury can minimize the damage, some ECM components produced by these glial cells can inhibit regeneration and thus retard functional recovery. It is well known that CSPGs released by reactive astrocytes are major impediments to axonal regeneration and the inhibitory activity depends upon CS sulfation pattern (Smith et al. 2015). In addition to increased expression of CSPG by astrocytes, there are also changes in the sulfation pattern of the CS chains (George and Geller 2018). Expression of 4- and 6-sulfotransferases, which modify the GalNAc unit, is upregulated. Although the exact mechanisms by which these CSPGs affect axonal regeneration are not entirely known yet, one possibility is that they interact with transmembrane receptors on the neuron cell surface. It was reported that a transmembrane protein tyrosine phosphatase, PTPσ, is a receptor for CS chains that mediates the inhibitory effect of CSPGs in the glial scar (Shen et al. 2009). The identification of this receptor provided a new target for therapeutic approaches. Thus, administration of a peptide that binds to PTPσ was reported to restore axon regeneration and facilitate functional recovery after spinal cord injury (Lang et al. 2014). A widely used approach tested in many injury models that can neutralize the inhibitory effect of CSPGs is treatment with chondroitinase ABC, which degrades the CS chains (Bradbury and Carter 2011).

Interestingly, CS and HS have been shown to have antagonistic effects in some biological functions. Thus, while CSPG restricts neuronal plasticity by stabilizing the existing synaptic connections, HSPG syndecan-2 was shown to induce dendritic spine formation in hippocampal neurons (Ethell et al. 2001). The opposite functions were described in neuronal polarization, in which CS stabilizes and the HS destabilizes the growth of axons (Nishimura et al. 2010). In another example, CS interacts with transmembrane receptors RPTPσ during inhibition of axon growth after injury, as discussed above, while HSPGs binding to this receptor promotes axonal growth (Aricescu et al. 2002). An interesting antagonistic activity was found for TβRIII transmembrane proteoglycan that exists with HS and/or CS chains linked to the protein core (Jenkins et al. 2016). This proteoglycan regulates Wnt signaling in cells with roles in development and cancer. HS chains result in inhibition of Wnt signaling, whereas CS chains promote the signaling cascade. The authors suggested that TβRIII proteoglycan state (HS and/or CS) may be regulated by the expression of the glycosyltransferases which initiate the synthesis of HS or CS chains. The final balance of HS and CS determines the ligand responses.

As illustrated in the above examples focused on the nervous system, the variety of processes in which HS and CS are involved depend largely on the distribution of sulfate groups along the sugar chain. This sulfation pattern is determined in a dynamic way by the enzymatic synthesis and modification of HS and CS. Evidence accumulated in the last two decades indicates that during development and damage repair, the nervous system makes use of spatial and temporal expression of sulfotransferases and sulfatases, which modify the sulfation profile of HS and CS, to regulate a number of biological processes. Considering the high molecular diversity that can potentially exist in HS and CS chains, it is not surprising that a "sulfation code" hypothesis has been proposed, whereby HS and CS encode functional information in a manner similar to those of nucleic acids and proteins (Bülow and Hobert 2004; Holt and Dickson 2005).

As well as HS, CS and DS have been also found to play pivotal roles in the biology of tumors. Tumor progression has been associated with several cases with accumulation and structural modification of CS/DSPGs (Wegrowski and Maquart 2004). For example, by comparing the structural characteristics of versican and decorin in human colon adenocarcinoma vs. normal tissues, a noticeable increase of CS vs. DS subunit content and a profound change of the sulfation pattern – from about 75% of iA subunits (see Paragraph 5.3.3.1 and Fig. 5.9) to approx. 65% of C subunits – were observed, together with a slight decrease in the length of the polysaccharide chain (Theocharis 2002). Overexpression and altered sulfation patterns of CS chains with respect to tissue from healthy organs were similarly reported for various phenotypes of breast, prostate, testicular, lung, gastric, and pancreatic malignant cancers (Theocharis et al. 2000, 2003; Li et al. 2008; Svensson et al. 2011). Structural alterations of CS chains in carcinoma cells result in various activities with respect to binding with growth factors, selectins, CD44 receptor, and metalloproteinases, all involved in tumor formation, progression, spreading, and metastasis (Afratis et al. 2012). In some cases, the specificity of CS sulfation patterns leading to enhanced binding has been revealed. For example, an enhanced level of A subunits specifically favors the complexation of CS with metalloproteinases in melanoma cells, leading to activation of the metastatic cascade (Iida et al. 2007), or affects integrin signaling pathways governing tumor cell motility (Clausen et al. 2016). In several cases CS-E subunits have been detected to give a specific interaction with growth factors involved in tumor growth and spreading. For example, expression of CS-E in ovarian adenocarcinoma ECM mediates VEGF binding, which promotes signaling pathways related to tumor progression, whereas in the physiological case, such kind of CS subunit is absent (Ten Dam et al. 2007). Analogously, in CD44-expressing pancreatic tumor cells, partially depolymerized CS-E chains specifically enhance CD44 receptor cleavage, a process involved in tumor cell motility (Sugahara et al. 2008). The overexpression and structural modification of CS and DS in tumors are currently under investigation also for exploiting them in targeting malignant cells or tissues. In particular, liposome or nanostructured delivery systems of anticancer drugs or genes have been formulated for the selective release to CS-overexpressing tumor cells (Lee et al. 2002). Antibodies against specific CS sequences blocking their action in tumor progression have been reported too (Afratis et al. 2012). Furthermore, chemical and/or enzymatic synthesis of CS oligosaccharides with tailored, well-defined structures has been exploited to inhibit interaction of CS chains overexpressed in tumor cells with their receptors (Mizumoto et al. 2012; Poh et al. 2015).

As already mentioned for HS, CS located on cell surfaces can be exploited by some microorganisms for adhering to and then entering animal cells (Yamada and Sugahara 2008). In particular, Lyme disease spirochete, HSV-1, and malarial parasite are known to interact with cell surface CS (Banfield et al. 1995; Alkhalil et al. 2000; Mårdberg et al. 2002). Concerning malaria, it has been demonstrated that CS sulfation pattern found on the placental syncytium is strictly related to the high susceptibility of pregnant women to *Plasmodium falciparum*, even despite previous immunity. Indeed, placental CS is different from CS present in other organ tissues, with the higher content of blocks of continuous CS-A disaccharide subunits in the

former allowing a stronger interaction with the unique protein VAR2CSA present on *P. falciparum* infected erythrocyte membranes (Sugiura et al. 2016).

The flexibility related to the presence of IdoA, a monosaccharide featuring several conformations such as $^{1}C_{4}$ and $^{4}C_{1}$ chairs, and $^{2}S_{O}$ skew-boat, whereas GlcA essentially displays only a $^{4}C_{1}$ chair conformation (Ferro et al. 1990), is usually invoked for explaining some special biological functions associated with DS or hybrid CS/DS chains, as discussed here below.

Collagen 3D structure formation is a really complex phenomenon entailing the engagement of a large number of collagen ligands, including several PGs (Kadler et al. 2008); the core protein of decorin CS/DSPG seems to be implicated in interaction with collagen through multiple binding domains (Schonherr et al. 1995), whereas the single GAG chain of decorin was shown to play an important role in collagen fibril formation at the early stages of fibrillogenesis (Rühland et al. 2007). In addition, DS can mediate the linkage of collagen with other macromolecules, for example, tenascin-X, an ECM protein important for the properties of connective tissues (Elefteriou et al. 2001). The specific nature of the GAG structures can influence the diameter of collagen fibers; for example, in tendons the highest diameters are observed in tissues more enriched with DS, whereas decreasing values are detected in tissues with higher amounts of CS and HA (Ryan et al. 2015).

There are several pieces of evidence about possible interactions of DS sequences with growth factors, events with relevant biological implications. For example, relatively short DS sequences can bind with high affinity to hepatocyte growth factor/scatter factor (HGF/SF) (Deakin et al. 2009), secreted by mesenchymal cells and implicated in the regulation of cell growth, motility, and morphogenesis. Endocan, a CS/DSPG specifically secreted by endothelial cells, was found to interact with HGF/SF, thereby stimulating mitogenic activity (Bechard et al. 2001). DS can potentiate the response of fibroblast growth factor type 2 (FGF-2) on skeletal muscle satellite cell proliferation and migration (Villena and Brandan 2004). GAG chains of syndecan-4 and glypican-4 PGs are directly involved in the activation of FGF in early stages of embryo development through initial formation of a GP-FGF complex and subsequent degradation of the proteoglycan core protein performed by a protease overexpressed at this stage, with consequent release of an active DS-FGF complex that stimulates long-range FGF signaling (Hou et al. 2007).

DS also plays a role by interacting with several mediators of coagulation, as typically ascribed to heparin structures. For example, decorin binds to von Willebrand factor, a protein implicated in platelet adhesion in wounded sites, through its single CS/DS chain. The affinity in this case is less influenced by IdoA content but, rather, by sulfation degree (Guidetti et al. 2004). DS chains, even if not especially long, can bind with high affinity to HCII, accelerating the inhibitory action of this protein toward thrombin (Maimone and Tollefsen 1990; Sarilla et al. 2010). In particular, a DS hexasaccharide fragment composed of three iB subunits (see Fig. 5.6) has been recognized as responsible for an approximately 1000-fold increase of the rate of HCII-mediated inhibition of thrombin by providing a template to which both HCII and thrombin bind (Tollefsen 1992).

DS is able to bind to α-defensin, a small neutrophil-derived peptide, and this interaction results in neutralization of the bactericidal action of α-defensin. This is a protective mechanism developed by some pathogenic bacteria such as *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Streptococcus pyogenes*. This process is triggered by the secretion of extracellular proteinases whose hydrolytic action leads to core protein degradation of PGs and liberation of the corresponding GAG chains (Schmidtchen et al. 2001).

DS is involved in the development of esophageal squamous cell carcinoma, as demonstrated by the reduced migration and invasiveness recorded for tumor cells with downregulated DS epimerase 1 (DS-epi1). This downregulation reduces the IdoA content and, consequently, the activation of pERK-1/2 signaling mediated by growth factors that has a critical role for tumor invasion (Thelin et al. 2012).

Genetic disorders associated with defective expression of sulfatase or epimerase enzymes engaged in the biosynthesis of DS chains in PGs are known. A deficient activity of the sulfotransferase DS4-1 has implications, as previously discussed (Paragraph 5.2.3), in the content of iduronic acid residues in the GAG chain (Pacheco et al. 2009c), and the altered functionality of the PGs, thus obtained, is manifested by an irregular assembly of collagen fibrils leading to connective tissue disorders (Zhang et al. 2010). Altered expression of DS-epi2, relatively abundant in the brain in healthy individuals, is the cause of bipolar disorders (Goossens et al. 2003).

### 5.4.3 Keratan Sulfate

KSPGs are biomacromolecules with multifunctional biological activities, localized in the cornea, brain, and cartilages. Several papers report on the connection of sulfate distribution to activity (Saito et al. 2008; Liles et al. 2010; Nakayama et al. 2013). Relatively little is known about their functional properties in comparison with the other sulfated GAGs. In the cornea membrane, KS short chains are mainly localized in keratocan, lumican, mimecan, and fibromodulin, KSPGs that are included in class II small leucine-rich proteoglycans (Iozzo and Schaefer 2015). KS chains have been also found interspersed within the CS-rich region of the aggrecan PG in many tissues, but their function has not been elucidated yet. Instead, KS chains located in the aggrecan G1 domain are reported to inhibit immune responses to this domain. Indeed, patients with rheumatoid arthritis showed enhanced immune reactivity toward the G1 domain only when KS chains were removed (Leroux et al. 1996; Guerassimov et al. 1998).

KSPGs display a significant role in regulating corneal transparency, also by controlling collagen fibril architecture (Ho et al. 2014). In addition, corneal KS binds with high affinity to FGF-2 and sonic hedgehog (Weyers et al. 2013), indicating that KSPGs regulate growth factor activity and morphogen gradient formation. One pathology involving KS in the cornea is keratoconus, a disorder of the eye leading to a progressive thinning of the cornea. People suffering from this pathology display

double and blurry vision, high myopia (nearsightedness), astigmatism, and light sensitivity (Funderburgh et al. 1989; Edrington et al. 1995; Espandar and Meyer 2010). It has been suggested that the changed KS antigenicity found in the cornea extracts from surgical patients of keratoplasty can be due to a decrease in the number of KS chains per proteoglycan molecule, a decreased or altered KS sulfation pattern, or reduction of KS chain length (Funderburgh et al. 1989).

Brain tissues are the second richest source of KS in the human body after the cornea. Highly sulfated KS-III chains are displayed by numerous large KSPGs in the brain, among which the SV-2 plays significant neuronal and synaptic regulatory roles (Scranton et al. 1993). The negative charges on KS chains of SV2 distributed around a synaptic vesicle interact with $Ca^{2+}$ ions and with neurotransmitters such as dopamine, thus forming a smart gel proteoglycan delivery complex (Caterson and Melrose 2018). It has been found that the interruption of SV2 functionality is connected to epilepsy (Wan et al. 2010).

Variation in the sulfation of KS chains has been revealed to be a marker for the presence of tumor cells. In podocalyxin, a cell surface mucin-like KSPG marker of human embryonic and induced pluripotent stem cells, the low sulfate content of KS chains has been detected by monoclonal antibody R-10G (Kawabe et al. 2013). In contrast, antibodies such as 5D4 or MZ14 (Caterson et al. 1983) revealed that tumor cells produce highly sulfated KS. In addition, podocalyxin has been found overexpressed in a variety of cancers. In particular, in high-grade serous ovarian carcinomas, it decreases adhesion of β-integrins at the cell surface either by altering their availability or their stability, contributing to the initial transperitoneal diffusion of metastasis (Cipollone et al. 2012).

Another report about the involvement of KS chains in diseases describes modified KS glycans in Alzheimer pathology (Lindahl et al. 1996; Zhang et al. 2017). The sulfotransferase GlcNAc6ST1 was found to be upregulated in the brains of transgenic mouse models (J20 and Tg2576) and of patients with Alzheimer disease (Zhang et al. 2017). KS chains have also been found to play key roles in the early phase of amyotrophic lateral sclerosis (ALS) (Hirano et al. 2013), a motor neuron-degenerative disease that leads to progressive muscle weakness and complete paralysis within 1 to 5 years after disease onset. Finally, it is worth noting that abundance of cell-associated KS in the endometrial lining varies markedly during the menstrual cycle, reaching a peak at the time at which embryo implantation occurs (Graham et al. 1994).

## 5.5 Outlook and Future Perspectives

Sulfated GAGs are the carbohydrate portion of PGS, a family of complex biomacromolecules ubiquitously found in the ECM and on cell surfaces and playing critical roles in a plethora of physiological and pathological events. In this chapter the chemical structure and biosynthesis of the four main sulfated GAG types are

firstly described, and then the most commonly employed isolation and purification methods as well as the spectroscopic and spectrometric techniques for their structural analysis are discussed, with a focus on the most recent advancements in the field. Lastly, an overview of the most important biological functions of the different sulfated GAGs is reported, with special attention to the molecular features – sulfation pattern above all – that have been revealed to play significant roles in the discussed biological events.

In spite of a myriad studies on sulfated GAG chemistry and biochemistry, especially in the last 25 years, structure-biological function relationships have been elucidated in detail up to now only in few cases. This is mainly due to the complexity of sulfated GAGs, showing structural heterogeneity that makes their profiling in cells, tissues, and biological fluids as well as the precise understanding of their interaction with proteins and other biomolecules very challenging (Ricard-Blum and Lisacek 2017).

We foresee that in the next years, many efforts in the field will be devoted to fill these gaps and in particular to unveil the details of the roles played by GAG sulfation code in as more biological events as possible.

Essential prerequisite for solving these issues are at least (Mizumoto et al. 2015):

(i) The development of methods and technical solutions for the streamlined sequence determination of sulfated GAG chain more complex than bikunin and decorin – the two simplest CSPGs for which the full structure could be sequenced (Ly et al. 2011; Yu et al. 2017)
(ii) An access to (semi)-synthetic sulfated GAG oligo- and polysaccharides (as well as analogs thereof: Lane et al. 2017; Gao and Edgar 2019) with a very well-defined structure through chemical and/or chemo-enzymatic procedures, which would be easier, cheaper, and more efficient than the procedures described up to now (Bedini and Parrilli 2012; Dulaney and Huang 2012; DeAngelis et al. 2013; Liu and Linhardt 2014; Mende et al. 2016)
(iii) The improvement of the biochemical efforts toward a more precise and complete comprehension of the regulatory aspects governing sulfated GAG biosynthesis
(iv) The implementation of computational tools for mining sulfated GAG-protein interactions and fully exploring the topology of ECM and surface interaction networks involving sulfated GAGs

Another urgent gap concerning sulfated GAGs to be filled in the next years is the development of multi-analytical methods for the rapid and efficient screening of purity and titer of sulfated GAGs employed as ingredients of drugs and nutraceuticals, due to the lack of robust data on this issue.

**Acknowledgment** Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) is acknowledged for funding to EB (FFABR 2017, "Fondo per il finanziamento delle attività base di ricerca"). AFM thanks the support of the Spanish Ministerio de Economía y Competitividad (Grant MAT2015-65184-C2-2-R).

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