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REVIEW ARTICLE

Control of tissue homeostasis by the extracellular matrix: Synthetic heparan sulfate as a promising therapeutic for periodontal health and bone regeneration

P. A. Miguez[1] | E. Bash[1] | M. L. Musskopf[1] | S. A. Tuin[2] | A. Rivera-Concepcion[2] | I. L. C. Chapple[3] | J. Liu[4]

[1]Division of Comprehensive Oral Health - Periodontology, Adams School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[2]Oral and Craniofacial Health Sciences, Adams School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[3]Periodontal Research Group, School of Dentistry, Institute of Clinical Sciences, College of Medical and Dental Sciences, Birmingham's NIHR BRC in Inflammation Research, University of Birmingham and Birmingham Community Health Foundation Trust, Birmingham, UK

[4]Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina, USA

Correspondence
P. A. Miguez, Division of Comprehensive Oral Health–Periodontology, Adams School of Dentistry, University of North Carolina at Chapel Hill, CB# 7455, Koury Oral Health Sciences, 385 S. Columbia St. Chapel Hill, NC 27599-7455, USA.
Email: miguezp@unc.edu

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1 | INTRODUCTION

This article aims to review key extracellular matrix components in the periodontium that have been found to be actively involved in immunity, tissue homeostasis, and cell signaling besides their well-known role as scaffolds to promote cell growth and migration. More recently, these extracellular matrix components have been investigated as therapeutic candidates to promote connective tissue homeostasis and regeneration. The organic environment in the periodontal tissues is primarily composed of collagen type I followed by noncollagenous proteins. A large component of the noncollagenous milieu are proteins rich in carbohydrate/sugar moieties called proteoglycans. These proteoglycans carry carbohydrates named glycosaminoglycans that can be of different lengths, disaccharide combinations and levels of sulfation, ultimately generating a series of diverse molecular signatures that act as codes to unlock specific functions. The application of glycosaminoglycan-based therapeutics represents a promising candidate as a next-generation biomaterial in bone and periodontal tissue homeostasis. The discovery of (a) specific glycosaminoglycan code(s) that is/are key in inflammation control, bone homeostasis and regeneration may lead to a novel line of cutting-edge regenerative therapeutics.

2 | PROTEOGLYCANS AND GLYCOSAMINOGLYCANS IN THE PERIODONTIUM MICROENVIRONMENT

The most abundant noncollagenous extracellular matrix proteins are proteoglycans. This class of glycosylated proteins is composed of an amino acid sequence that is post-translationally modified.[1] Post-translational modification happens to the core of a protein following its assembly and can be, for example, glycanation, glycosylation, enzymatic cross-linking or cleavage of core protein, all of which can ultimately dictate the protein function.[2] One of these post-translational modifications, the addition of a carbohydrate covalently bound to a core protein, leads to generation of proteoglycans (a polypeptide core with short N-linked oligosaccharides and large

O-linked disaccharides). These disaccharides are, in summary, carbohydrates with a repeating and specific sequence called glycosaminoglycans. Glycosaminoglycans are classified according to their composition into six types: heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid or hyaluronan[3,4] (Figure 1). The diversity of the glycosaminoglycan chains confers a variety of functions upon proteoglycans including collagen alignment, mineralization, cell signaling, mechanical property modulation, immune regulation, etc.[5] (Table S1). The disaccharide units of glycosaminoglycans consist of an *N*-acetyl–hexosamine (e.g., galactosamine or glucosamine), a hexose or hexuronic acid (e.g., galactose or glucuronic acid or iduronic acid, where either or both may be sulfated). The combination of the sulfate group and the carboxylate groups gives them a very high-density negative charge. Dermatan sulfate is distinguished from chondroitin sulfate by the presence of iduronic acid. The first steps of the synthesis of glycosaminoglycan chains of chondroitin sulfate/dermatan sulfate and heparan sulfate share a common pathway involving xylosyl transferases 1 and 2, β4-galactosyltransferase, β3-galactosyltransferase and β3-glucuronosyltransferases. Chondroitin sulfate/dermatan sulfate linkage region biosynthesis and chain extension require two additional enzymes, β4-galactose *N*-acetyl transferase, and chondroitin synthases 1, 2 and 3-like, which has both β3 and β4 glucuronosyltransferases activities required to form the repeating disaccharide unit. Lastly, several enzymes are involved in the modification of the chondroitin sulfate/dermatan sulfate glycosaminoglycan repeating unit (five enzymes) that adds sulfate to glucuronic acid/iduronic acid (2S) or *N*-acetyl galactosamine (4S and/or 6S).[6] Thus, their synthesis represents a highly complex event, and by default, difficult to replicate. Proteoglycans and their glycosaminoglycans are vastly and strategically distributed in mineralized and nonmineralized connective tissues.[7-10] Many of the proteoglycans within the periodontium (cementum, periodontal ligament, and alveolar bone), are key members of each specific cell environment and play a role in physiological turnover, cell differentiation, mineralization control, mechanical strength, and inflammation.[11-14] The small leucine-rich proteoglycan family are abundant proteoglycans within the periodontium.[15-18] Small leucine-rich proteoglycans have been classified into five subfamilies, a topic extensively reviewed in Iozzo et al.[5] These proteoglycans present a cluster of leucine repeats in their core that is known to promote protein–protein interactions.[19] They have been extensively studied in the context of connective tissue development and more recently in regeneration due to their ability to bind growth factors of the transforming growth factor-β family, such as bone morphogenetic proteins.[20] An example of small leucine-rich proteoglycans commonly found in connective tissues, decorin and biglycan, are depicted in Figure 2.

Although proteoglycans have been widely studied, the specific involvement of their glycosaminoglycan chains, either associated or not associated with the core (i.e., released free in tissue by

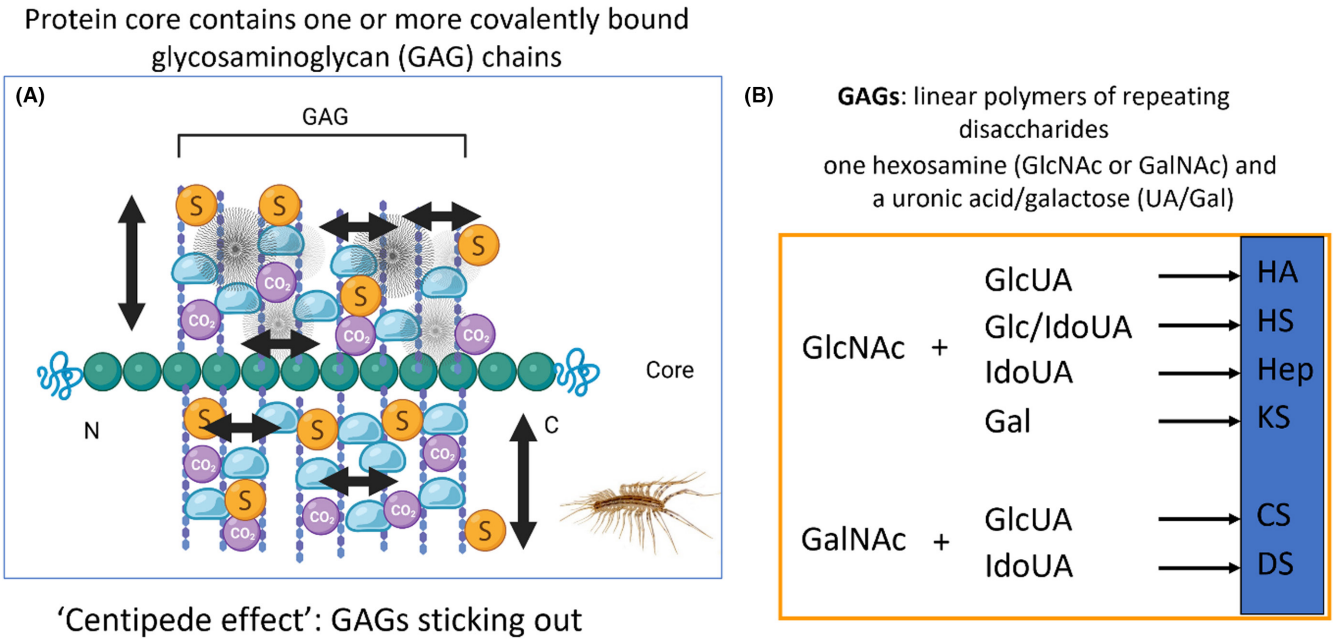

FIGURE 1 Proteoglycans and their glycosaminoglycans. (A) One or more glycosaminoglycans are attached to a protein core and can provide spacing (black arrows), mechanical support, influence water retention (blue droplet), tissue shape, mineralization, and signaling in connective tissues. Some proteoglycans have hundreds of glycosaminoglycans creating a centipede-like structure (i.e., aggrecan). (B) Glycosaminoglycans are linear polymers of repeating disaccharides that contain one hexosamine *N*-acetyl glucosamine or *N*-acetyl galactosamine and a uronic acid/galactose. Different combinations lead to six types of glycosaminoglycans. Partly created with Biorender. C, C-terminal; CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetyl glucosamine; GlcUA, glucuronic acid; HA, hyaluronic acid; Hep, heparin; HS, heparan sulfate; IdoUa, iduronic acid; KS, keratan sulfate; *N*, *N*-terminal.

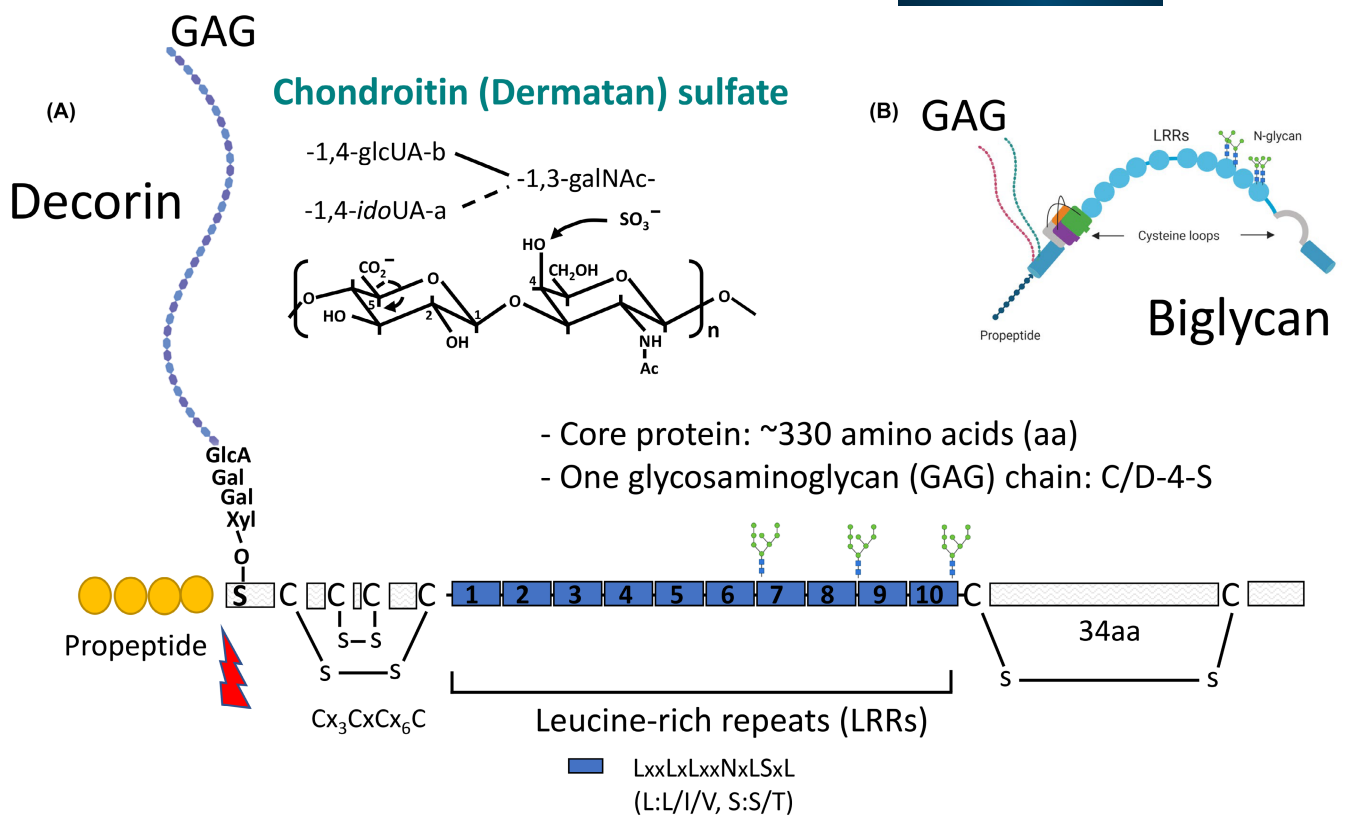

FIGURE 2 Small leucine-rich proteoglycans. (A) Decorin is a type of small leucine-rich proteoglycan highly abundant in connective tissues of the periodontium. It has one glycosaminoglycan of chondroitin sulfate or dermatan sulfate origin attached to the *N*-terminal. The leucine-rich repeats favor protein–protein interactions. Its *N*-terminal propeptide can be cleaved by bone morphogenic protein 1 (lightning shape). (B) Decorin, like biglycan, has a tertiary structure characterized by a C-shape and often the glycosaminoglycan(s) is larger than the core protein itself. C/D, chondroitin/dermatan; Gal, galactose; GalNAc, *N*-acetyl galactosamine; GlcUA, glucuronic acid; idoUA, iduronic acid; Xyl, xylose.

enzymes) in signaling processes has only recently gained traction. Glycosaminoglycans are emerging as critical components in many physiological processes, yet there is still a lack of understanding of their structure–function relationship.

A major challenge in studying glycosaminoglycan-protein interactions is the difficulty in acquiring an adequate and structurally homogeneous pool of glycosaminoglycans for research due to the extensive number and complexity of glycosides that are extracted from tissues.[21] Generating glycosaminoglycans in vitro through overexpression of glycosaminoglycan-synthesizing enzymes is also a challenge due to the involvement of multiple enzymes in the synthesis of glycosaminoglycans. Therefore, there is a need to develop new methods and strategies to produce glycosaminoglycans in larger quantities, with higher purity levels and intentional and predictable homogeny.[22] This will enable more detailed studies of the structural-functional relationship of glycosaminoglycans and their interactions with proteins.[23] The ability to generate synthetic glycosaminoglycans of heparan sulfate and chondroitin sulfate nature has given a new understanding of the role of these glycosaminoglycans in important events such as inflammation and tissue homeostasis.[24-27] This is a critical step in development of new agents and provides a novel avenue of therapeutics in the field of inflammatory diseases and bone ailments, which has the potential to make a paradigm shift in periodontal biology.

2.1 | Proteoglycans and glycosaminoglycans in periodontal ligament

The most abundant extracellular proteoglycans in periodontal ligament are reported to be members of the small leucine-rich family (which are considered small proteoglycans), such as lumican (with keratan sulfate glycosaminoglycans, thought to provide the exact alignment of collagen and aid in tissue repair), asporin (which does not have a glycosaminoglycan attached, is rich in aspartic acid and is thought to inhibit mineralization), biglycan (known for its contribution to the regulation of transforming growth factor-β/bone morphogenic protein signaling) and decorin (primarily known for its "decorating" ability of collagen and the regulation of its organization and mineralization)[28-31] (Figure 3). Decorin and biglycan are "sister" proteoglycans of the small leucine-rich class with very similar amino acid sequences, although decorin has one glycosaminoglycan and biglycan can have two. They both bind to collagen (decorin having the strongest affinity) and their glycosaminoglycans are primarily of chondroitin sulfate/dermatan sulfate nature. These small leucine-rich proteoglycans and their respective glycosaminoglycans have been implicated in attracting water, modulating mineralization, participating in collagen matrix organization and regulating cell differentiation and signaling.[32-34]

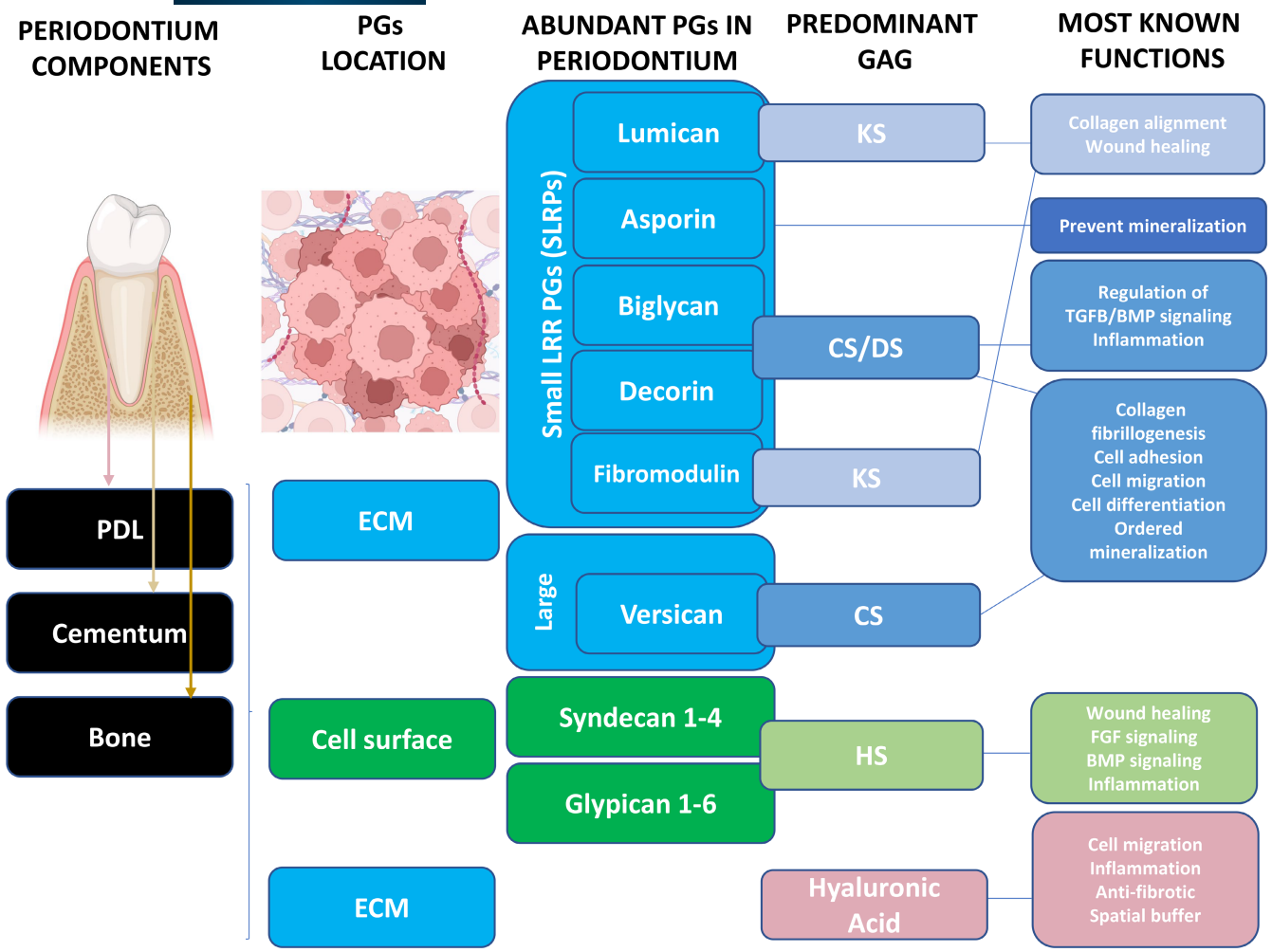

FIGURE 3 Classification of main proteoglycans and glycosaminoglycans found in periodontium based on their location and associated functions. CS, chondroitin sulfate; DS, dermatan sulfate; ECM, extracellular matrix; GAG, glycosaminoglycan; HS, heparan sulfate; KS, keratan sulfate; LRR, leucine-rich repeat; PDL, periodontal ligament; PGs, proteoglycan; SLRPS, small leucine-rich proteoglycans; TGFB: transforming growth factor beta; FGF, fibroblast growth factor; BMP: bone morphogenetic protein. Teeth and cells schematics created with Biorender.

Of the cell surface proteoglycans in periodontal ligament, syndecans and glypicans are examples commonly found around teeth and both present with heparan sulfate-type glycosaminoglycans.[35,36] Syndecans have been implicated in wound healing and modulation of inflammation,[37] and glypicans control cell growth and angiogenesis through various signaling pathways leading to Smads, β-catenin and AP-1 activation downstream of receptors such as bone morphogenetic protein, frizzled, low density lipoprotein receptor-related protein and fibroblast growth factor receptors.[38-40] Classification of the proteoglycans based on their location and associated glycosaminoglycans, is displayed in Figure 3. Proteoglycans and glycosaminoglycans that have been implicated in bone morphogenetic protein function, bind to bone morphogenetic proteins via the negative charge of proteoglycans conferred by glycosaminoglycans. They also bind the leucine-rich repeat to the *N*-terminal region containing basic amino acids and known to attract other proteins such as bone morphogenetic proteins.[41-43] Their critical role in transforming growth factor-β and bone morphogenetic protein signaling has not been as widely explored in periodontal ligament homeostasis as in bone.[44-46]

Particularly for heparan sulfate, it has been demonstrated that the degree of sulfation of heparan sulfate can impact the strength of the electrostatic interactions between heparan sulfate proteoglycans and bone morphogenetic proteins.[47] However, the binding of bone morphogenetic proteins to heparan sulfate proteoglycans does not entirely depend on sulfated glycosaminoglycans.[48] The significance of regulating bone morphogenetic protein biding through sulfation is of interest in development of heparan sulfate-based regenerative materials.

2.2 | Proteoglycans and glycosaminoglycans in the mineralized tissues of the periodontium

2.2.1 | Cementum

Large proteoglycans within cementum such as versican (with chondroitin sulfate-type of glycosaminoglycan), retain hydrostatic pressure and significantly influence the shape of connective tissues

given their bulkiness (i.e., aggrecan in cartilage, has several chondroitin sulfate-type of glycosaminoglycan, has a bottle-brush/centipede shape, it is over 2500 kDa, thus attracting large amounts of water, Figure 1),[28,49,50,51] while lower molecular weight extracellular proteoglycans (such as small leucine-rich proteoglycans) can maintain and fine tune the immediate cell-matrix interactions such as cementoblasts and cementocytes. Around cementum cells, biglycan is found with greater abundance, although versican and syndecan are also highly expressed.[52,53] Syndecans and glypicans, the heparan sulfate proteoglycans, play crucial role in cell signaling during tooth movement and inflammatory insult.[35]

The distribution of glycosaminoglycans in cementum was thoroughly characterized in the late 80's and early 90's and proved them to be mostly composed of chondroitin sulfate, dermatan sulfate and heparan sulfate with some free hyaluronan.[54-56] Hyaluronan is highly abundant in periodontium, is nonsulfated and is the most studied glycosaminoglycan in the context of periodontal homeostasis.[57-59] Its role in cell migration, inflammation, anti-fibrosis and as a spatial regulator is well recognized.[60] However, the role of hyaluronan size in the immune response needs to be further elucidated as low and high molecular weight hyaluronan can have opposite effects on inflammation.[61]

It has been pointed out that some small leucine-rich proteoglycans (such as biglycan and decorin), bind to collagen fibrils at very specific sites that are critical for hydroxyapatite deposition[62,63] preventing mineralization at those sites but ordering mineral deposition where their glycosaminoglycans extend to.[64,65] At the periodontal ligament-cementum and dentin-cementum interfaces, biglycan, fibromodulin and their glycosaminoglycans, connect with collagen and this protective proteoglycan-glycosaminoglycan layer is thought to maintain tooth structure integrity during orthodontics as well as to control osteoclastogenic activity to reach dentin during tooth exfoliation.[63,66] As teeth are subjected to loading, these glycosaminoglycans provide cushioning, protection via electrostatic interactions, steric hindrance, water retention, lubrication and control mineralization and resorption of these sites during tooth movement.[67] In a similar manner, the cementoid secreted by progenitor cells contains higher ratios of organic to inorganic matter due to, primarily, the presence of glycosaminoglycans. During mineralization, proteoglycans abundance diminishes in mineralized tissues such as dentin and bone.[68] This finding implies that proteoglycans and glycosaminoglycans are important for induction of mineralized tissue formation and, therefore, exploring this function in tissue regeneration is highly relevant to advance regenerative therapies.

2.2.2 | Bone

For some tissues such as bone, information regarding absolute amounts of extracellular matrix components has not been appropriately recorded. Estimates have been generated for several connective tissues in health and disease demonstrating that there can be variations in sulfation of glycosaminoglycans and collagen content. The predominant proteoglycans in bone have been reported to be small leucine-rich and heparan sulfate proteoglycans.[69-72] The predominant glycosaminoglycans in human alveolar bone have been described to be chondroitin sulfate (4S), although hyaluronan, dermatan sulfate and heparan sulfate are also attached to proteins, cells or free within tissue.[73-77] Interestingly, with age, glycosaminoglycans have been demonstrated to shorten and proteoglycans can be found completely deglycanated (so called nonproteoglycan form) or partially degraded (small leucine-rich proteoglycan fragmentation).[15,72]

Glycosaminoglycan composition has also been shown to change in ectopic bone, inflammatory diseases such as diabetes, rheumatoid arthritis and lupus.[33,78] The expression of proteoglycans and glycosaminoglycans on cell surfaces forming a glycocalyx dictates cell interactions with chemokines, receptors and other molecules in the endosteal and mineralized areas of bone.[33,79] Thus, changes with aging and with disease can have considerable impact on cell signaling and health.

Another important aspect of proteoglycans and glycosaminoglycans in bone, is the fact that they are critical mediators of mechanical properties.[11,80] Altered proteoglycan and glycosaminoglycan content can lead to brittle bone.[32] The common signaling pathways found across connective tissues that are modulated by proteoglycans and glycosaminoglycans are Wnt, Smads, mitogen-activated protein kinase, etc. all resulting from modulation of bone morphogenetic protein, fibroblast growth factor, platelet-derived growth factor, insulin growth factor, vascular endothelial growth factor and transforming growth factor signaling.[81,82] Many receptors have been found to interact with proteoglycans and glycosaminoglycans and the complexity of the interactions are illustrated in Figure 4.

Mice lacking proteoglycans and/or glycosaminoglycans provide clues to the function of such a variety of proteoglycans and glycosaminoglycans in bone formation and maintenance at both structural and signaling levels.[5,83,84,85,86,87] Decorin deficient mice are primarily characterized by a phenotype of skin fragility due to abnormal collagen fibrillogenesis and assembly.[88] In mice there are problems in dentin mineralization and periodontal homeostasis due to, in part, collagen fragility[89-91] as well as effects on cell adhesion, migration and differentiation. More recently, there have been several reports on the effect of decorin on innate immunity, angiogenesis and tumor progression.[92-96] Biglycan ablation has a more pronounced effect in bone leading to reduced bone mass with age.[83,84] Osteoblast numbers are decreased, and reduced bone morphogenetic protein signaling as well as osteoclast function is reported. Biglycan was also found to promote angiogenesis and lack of biglycan hinders vascular endothelial growth factor receptor 2 activation leading to delayed fracture healing.[97]

Overall, of all the six types of glycosaminoglycans, hyaluronan and heparan sulfate have been the most studied in the context of inflammation and, in turn, there is growing interest in these glycosaminoglycans in bone homeostasis. Heparan sulfate is well known for interactions with growth factors and cytokines, control of bone morphogenetic protein signaling, heparanase expression (an enzyme that cleaves heparan sulfate and is abundant in response to stress

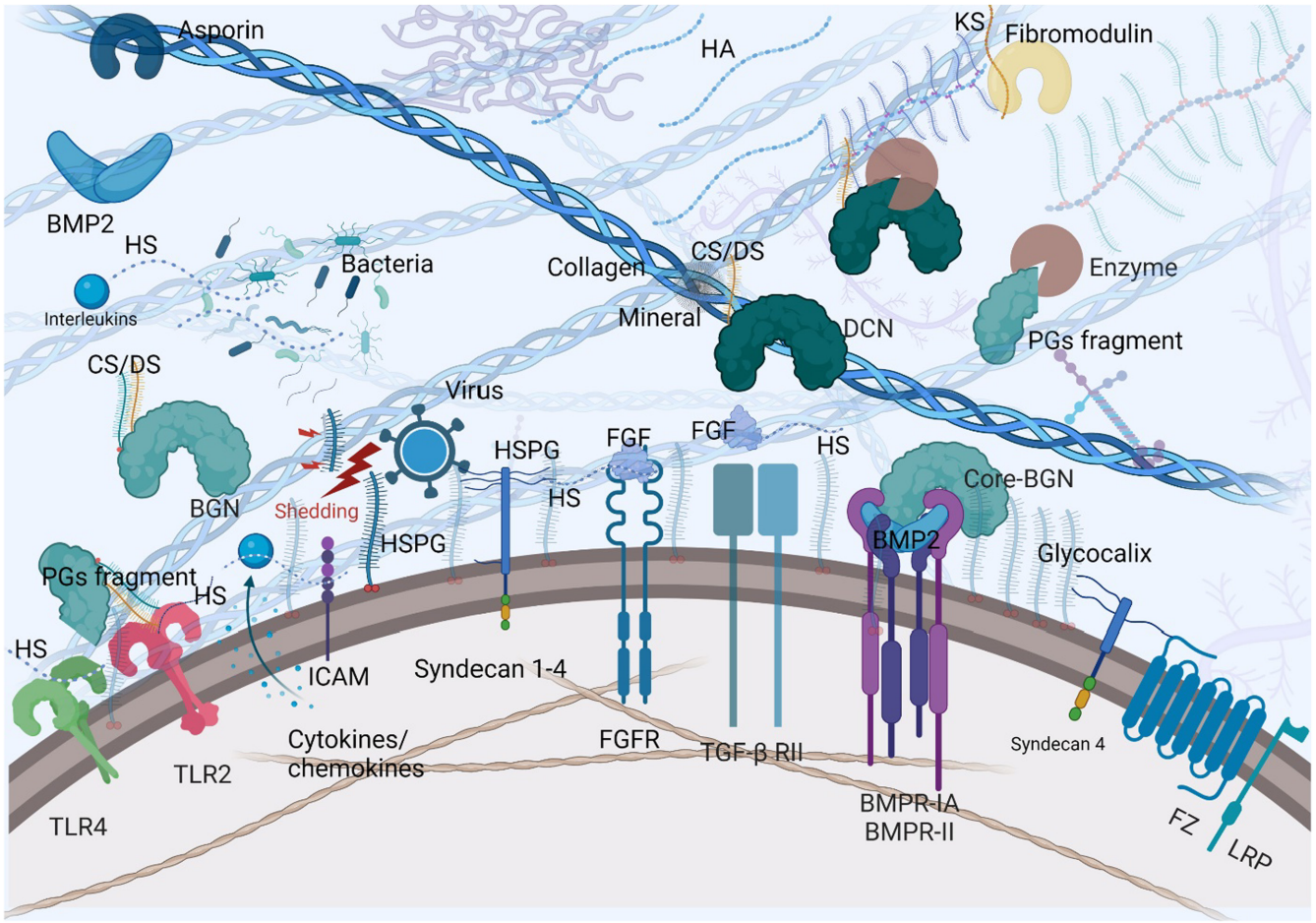

FIGURE 4 Various proteoglycans and glycosaminoglycans around progenitor cells in periodontal ligament and bone are in extracellular space or on the cell surface or peri-surface. They bind with various classes of protein (enzymes, growth factors, receptors, adhesion molecules, chemokines, pathogens). The nature of the interactions is thought to be determined by the length, sulfation pattern and type of disaccharides. The glycocalyx is composed of glycosaminoglycans surrounding cells and is of key importance in ligand/receptor interactions, virus/bacteria interactions, and its shedding (via heparanase) leads to binding of glycosaminoglycans and proteoglycans with other soluble molecules, mediating the inflammatory response and microenvironment of cells. Created with Biorender. BGN, biglycan; BMP2, bone morphogenetic protein 2; BMPR, bone morphogenetic protein receptor; CS, chondroitin sulfate; DCN, decorin; DS, dermatan sulfate; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FZ, frizzled; HA, hyaluronic acid; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; KS, keratan sulfate; LRP, low density lipoprotein receptor-related protein; PGs, proteoglycans; TGF-β, transforming growth factor beta; TLR, toll-like receptor.

such as sterile inflammation or infection),[98] fibroblast growth factor/bone morphogenetic protein interaction and noggin/gremlin (bone morphogenetic protein antagonists) function as heparan sulfate is ubiquitously expressed at cell surfaces and in extracellular matrix. Heparan sulfate has been proposed as a master regulator of stem cell fate due to its ability to modulate protein gradients and signal transduction.[99] Lack of heparan sulfate production causes excessive bone morphogenetic protein signaling and can provoke ectopic chondrogenesis and osteochondroma formation.[100,101]

Of the many cytokines of the transforming growth factor-β superfamily, around a third bind heparin and heparan sulfate. Growing evidence reveals that antagonists to these growth factors are also heparin-binding proteins.[102] Thus, it is likely that the structure of heparan sulfate plays a critical role in the fate of the molecule towards an anabolic or catabolic pathway. Since heparan sulfate, via variations in sulfation, can be constructed for a specific function (rather than using just size as dictator of function in the case of hyaluronan), this molecule is an attractive and pliable candidate for therapeutic development.

3 | PROTEOGLYCANS AND GLYCO SAMINOGLYCANS AS MEDIATORS OF INFLAMMATION

The past 15 years of research has shown that small leucine-rich proteoglycans can act as modulators of inflammation via interaction with innate immune receptors.[103,104] Fascinating evidence has revealed small leucine-rich proteoglycans to promote a switch between pro-and anti-inflammatory signaling pathways by choosing

which receptor to interact with. Pathogen- and danger-associated molecular patterns trigger a series of receptors that orchestrate inflammation such as toll-like receptors, and the receptor for advanced glycation end products. Small leucine-rich proteoglycans can orchestrate both pathogen-mediated and sterile inflammation during innate and adaptive immune response[105-107] (Figure 4).

A paradigm shift in understanding the role of proteoglycans in inflammation arose when the small leucine-rich proteoglycan biglycan was found to have a pro-inflammatory function by binding to toll-like receptors-2 and -4.[106] Biglycan knock out mice were subjected to lethal doses of bacterial lipopolysaccharide to induce sepsis and mutant mice presented with less systemic inflammation and had lower mortality rates. Macrophages secrete biglycan, which behaves as danger-associated molecular pattern triggering the innate immune toll-like receptors-2 and -4[106] and the purinergic P2X receptors.[108] Other evidence of biglycan as a pro-inflammatory molecule was found in toll-like receptors-2/4 activation in ischemic acute renal injury.[103,109] Its detection as a soluble molecule has raised the idea of detection of biglycan as a biomarker of renal stress.[110] The dermatan sulfate and chondroitin sulfate type of glycosaminoglycans have also been described to influence inflammation depending upon their length and by modulating nuclear factor kappa-light-chain-enhancer of activated B cells activation.[105,111] Our group[112,113] found that the core of biglycan was more prone to mediate osteogenic function as compared to glycosaminoglycan-rich biglycan. This is likely because the chondroitin sulfate/dermatan sulfate type of glycosaminoglycan of such small leucine-rich proteoglycan may favor inflammatory-receptor interactions rather than bone morphogenetic protein receptor(s) activation.

Decorin, similarly structured to biglycan, with either a dermatan sulfate or chondroitin sulfate single glycosaminoglycan, has various binding partners that are beyond the extracellular matrix component collagen. Some of the interactions involve glycosaminoglycan chains.[114] However, decorin can also serve as a ligand to toll-like receptor-2 and -4 and, similarly to biglycan, only when associated with a glycosaminoglycan it can trigger signaling through these receptors.[115] Certain inflammatory conditions exhibit overexpression of decorin corroborating its proposed role as a pro-inflammatory mediator, dependent upon its structure.[116,117]

Lumican, with its keratan sulfate glycosaminoglycan, has also been shown to mediate pathogen-induced inflammation. One proposed mechanism is that it promotes the binding of lipopolysaccharide-CD14 to toll-like receptor-4 leading to phagocytosis and subsequent stimulation of type I interferon production. Thus, lumican promotes further secretion of pro-inflammatory cytokines and mice lacking lumican exhibit a diminished inflammatory response.[118,119]

Another small leucine-rich proteoglycan, fibromodulin, is believed to play a role in the complement system in rheumatoid arthritis and osteoarthritis, which show evidence of fibromodulin activation of complement via C1q and C3b binding. Subsequently, following activation of the complement system, toll-like receptor activation and influence on adaptive immunity is reported.[104] Its role in wound healing is now accepted via modulation of angiogenesis and extracellular matrix organization.[120,121] Of the cell bound proteoglycans, heparan sulfate proteoglycans glypicans (heparan sulfate only glycosaminoglycans) and syndecans (heparan sulfate and chondroitin sulfate glycosaminoglycans), have heterogeneous glycosaminoglycan composition and, therefore, exhibit large differences in charge and flexibility at the cell surface.[122,123]

The different types of syndecans are characterized by differences in glycosaminoglycan position.[124] Syndecan-1 knockout mice have a hyper-inflammatory phenotype.[125] After myocardial infarction, inflammatory cell recruitment is increased in these mice and syndecan-1 deficiency accelerated the deposition of granulation tissue in the infarcted heart.[126] Human umbilical vein endothelial cells, when exposed to bacteria lipopolysaccharide, show increase in syndecan-4 and decrease in both −1 and −2 mRNA levels. Without syndecan-4, these cells express angiogenic growth factors and in vivo, the knockout mice upon lipopolysaccharide injection have a reduced immune response.[127,128]

Syndecan plays an important role in the migration of leukocytes via its heparan sulfate chains and this process has been described in several studies and is summarized in a recent review in Gopal.[129] Interestingly, this proteoglycan can undergo morphological changes, which acts as a trigger for the migration process. It has been reported to act on the actin cytoskeleton, via a feedback loop and to affect the release of matrix metalloproteinases, which are responsible for degrading the extracellular matrix allowing for leukocyte migration.[104]

Shedding of syndecan-1 (with heparan sulfate and chondroitin sulfate glycosaminoglycans) occurs by the action of heparanase as well as of matrix metalloproteinases (that are increased due to heparanase).[130] The exposure of receptors due to shedding of heparan sulfate (the main component of glycocalyx) that are players in the activation of the inflammatory response is a significant consequence of glycocalyx degradation.[131,132] A systematic review evaluated if glycocalyx components change in a variety of diseases and conditions. It was found that surgery and trauma can lead to glycocalyx degradation.[133] Thus, this process happens in both acute and chronic inflammation and is of high relevance to disease progression and tissue healing.

Regarding free heparan sulfate and heparin, research on inflammation has also explored them as anti-inflammatory molecules partly due to incidental findings in heparin-based research.[134] Heparin is a widely used anticoagulant, but it has also been found to have excellent anti-inflammatory properties in both animal models and human clinical trials as well as anti-apoptosis and anti-cancer properties as noted in a systematic review.[135]

Heparins may protect endothelial cells from damage through their effects on histone methylation (caused by severe inflammation) and through regulation of mitogen-activated protein kinase and nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathways.[136-138] Heparin binds with inflammatory cytokines, inhibits neutrophil chemotaxis by modulation of the formation of neutrophil extracellular traps, has a direct effect on leukocyte migration

and neutralizes the complement factor C5a.[139-141] Commercially available low molecular weight heparins have been reported to limit exaggerated formation of neutrophil extracellular traps, which can result in the chronicity of periodontitis.[142,143]

A number of studies have also demonstrated the effectiveness of heparin in treating, for example, inflammation associated with bronchial asthma.[144] Similarly, ulcerative colitis patients treated with heparin experienced a significant reduction in their symptoms.[145] Finally, heparin was also found to be effective in the treatment of burns, helping to reduce pain and inflammation.[146]

The most recent evidence of the contribution of heparin in controlling inflammation was with SARS-CoV-2.[147] While it was initially given to sick individuals with the purpose of preventing blood clots, its role as an anti-inflammatory is now more broadly accepted. This function likely played a key role in reducing the level of the 'cytokine storm' reported in several patients given the characteristic binding of the virus to heparan sulfate, reducing viral penetration of cells, decreasing glycocalyx perturbation by the virus and inhibiting heparanase.[140]

A few studies of heparin have demonstrated its effect is unpredictable, likely because it is extracted from tissues and marketed for anticoagulant purposes with little regulation.[148] Due to the international source of heparin (potential contaminants) and the heterogeneity of the molecule itself, variability of its interactions is extensive and likely leads to the reported unpredictable effects. Heparin's anti-inflammatory effect is dependent on glucosamine 6–0 sulfation, thus, other sulfation patterns did not promote the resolution of inflammation.[149] Thus, we believe that finding and producing a specific heparan sulfate/heparin structure is of key importance in its potential implementation as an effective anti-inflammatory and commercially available agent.

Compared to unfractionated (tissue-extracted, unmodified) heparin, the modified 2-*O*,3-*O*-desulfated heparin can block L- and P-selectins.[149] A synthetic heparin mimetic developed to mimic the anti-inflammatory effects of heparin, but without any anticoagulant effects was found to reduce airway inflammation by inhibiting human neutrophil elastase. This molecule has been shown to be a highly effective therapeutic agent for a variety of inflammatory conditions.[150]

Small leucine-rich proteoglycans such as biglycan, fibromodulin, lumican are being explored as therapeutics.[151-153] Recombinant protein application as therapeutics in orthopedics and dentistry has led to breakthroughs in regeneration strategies (e.g., bone morphogenetic proteins and platelet-derived growth factor), although concerns with cost, lack of important post-translational modifications, and evidence of induction of serious side effects are on the rise.[154,155] Carbohydrates could be alternatives to these proteins, and of all glycosaminoglycans, heparan sulfate is the most studied as a modulator of inflammation in the context of infection and most recently in sterile inflammatory processes. Similarly, hyaluronan has been investigated in immunity, although hyaluronan degradation in situ (due to bacterial lyase) can lead to changes in its function.[156] Due to newly developed technology, the ability to control size, degradation (sulfation can prevent cleavage) and sulfation pattern of heparan sulfate is now tangible, and this glycosaminoglycan offers the best opportunity as a therapeutic to predictably and cost-effectively control inflammation and other functions.

4 | HEPARAN SULFATE, HEPARIN AND HEPARAN SULFATE PROTEOGLYCANS IN PHYSIOLOGY

As described in previous sections, heparan sulfate and heparin are known for their ability to bind proteins. While some proteins bind heparan sulfate with high affinity, others bind because of its promiscuity, thus, greater understanding of structure–function in heparan sulfate is needed.

Heparin is synthetized in mast cells and basophils in the lungs, liver and intestines.[157,158] When these cells degranulate and degrade, heparin is secreted.[157] Heparin is typically purified from porcine and bovine sources and is used in clinic as an anticoagulant because it binds to antithrombin and accelerates the inactivation of the procoagulation enzymes thrombin and factor Xa. It has also been demonstrated to modulate inflammation, oxidation and vasodilation while exercising its anticoagulant function.[159] Compared to heparan sulfate, heparin has a much lower degree of sulfation leading to a decrease in the range of heparin binding sites, and it is also more susceptible to enzyme cleavage.[47,160]

In a recent study, it was found that heparan sulfate has three clusters of interactions with nearly 75% of all input proteins grouped with complement factors and chemokines. Besides the complement and chemokine cluster, a second cluster, as assessed by a systems approach, revealed interaction with growth factors and growth factor receptors, including members of the hepatocyte growth factor, platelet-derived growth factor, fibroblast growth factor, family of receptor tyrosine kinases and bone morphogenetic proteins. The third cluster was composed of major extracellular matrix components, which can act as scaffolds for other proteins involved in cell support and organization.[161]

Indeed, this cluster study supports the role of heparan sulfate and heparan sulfate proteoglycans in bone physiological functions such as weight bearing, endochondral biology,[162] fibroblast growth factor regulation of osteogenesis,[163] in skeletal development and growth[100] and their recently described role in osteoclastogenesis.[164] A knock in murine model of osteoprotegerin, a known binder of receptor activator of nuclear factor kappa-B ligand, which triggers osteoclast differentiation, demonstrated that heparan sulfate on the cell surface of osteoblasts (as it is not able to engage with osteoprotegerin) rather promotes receptor activator of nuclear factor kappa-B activation. Thus, heparan sulfate plays a key role in bone homeostasis.[165]

Besides its role in bone metabolism, another relevant physiological function in the context of the periodontium is the role of heparan sulfate in wound healing.[166] These roles in wound healing include: re-epithelialization, modulation of

coagulation, anti-inflammatory effect, regulation of inflammatory cell infiltration, angiogenesis, and cell proliferation and migration.[160] Syndecan null mice show defective re-epithelization of epidermis with thinner underlying connective tissue.[166] Briefly, its role in angiogenesis has been extensively reported via binding and regulation of vascular endothelial growth factor, fibroblast growth factor, transforming growth factor and platelet-derived growth factor.[167] In contrast, overexpression of syndecan-1 can switch heparan sulfate mediated fibroblast growth factor-7 signaling leading to its inhibition, which affects granulation tissue formation suggesting that the dose of heparan sulfate may also influence its function.[166] Heparan sulfate can also modulate wound healing by its interaction with toll-like receptor-4, leading to release of pro-inflammatory cytokines and influencing maturation of dendritic cells.[146,168] Indeed, heparan sulfate/heparin, are found to be increased in wound fluid after injury.[169]

5 | HEPARAN SULFATE AND HEPARAN SULFATE PROTEOGLYCANS IN DISEASE

5.1 | The glycocalyx

A large cohort of the associated diseases refer to glycocalyx degradation studies and some have found the shedding of heparan sulfate to be of diagnostic value as a biomarker for diseases.[170] Several pathological conditions have detectable circulating levels of heparan sulfate and heparan sulfate proteoglycans such as cardiovascular disease, cancer, trauma, sepsis, kidney disease, diabetes, etc.[170]

The degradation of glycocalyx occurs via matrix metalloproteinases and heparanase secreted by endothelial cells faced with an inflammatory trigger. Upon heparanase encounter, cleavage of the proteoglycans and heparan sulfate of syndecans and glypicans occurs. Matrix metalloproteinases 2, 7 and 9 have been reported to cleave these heparan sulfate proteoglycans mostly at sites of low sulfation or no sulfation, releasing small heparan sulfate fragments of 4–7 kDa.[171,172]

Evidence suggests that a damaged glycocalyx, found in aging, may initiate endothelial dysfunction leading to cardiovascular disease.[173] Accordingly, several studies have shown the association between circulating heparan sulfate proteoglycans levels, mainly syndecans-1 and -4 with myocardial infarction.[170] Several reviews on the heparan sulfate and glycocalyx axis are available for more in-depth knowledge.[174]

5.2 | Cancer

Elevated levels of syndecans have been shown to increase significantly in the plasma of patients with certain cancers such as multiple myeloma and further, have been correlated with a poor survival and response to treatment.[175,176] Glypicans have also been associated with liver carcinoma. The role of heparan sulfate proteoglycans in cancer has been found to be of diagnostic value, but has also been reported as tumor-suppressive, depending on the heparan sulfate proteoglycans and the cancer cell type.[177]

5.3 | Bone pathology

The development of osteochondromas in children and adults due to heparan sulfate deficiency has been extensively studied and it has been found that these tumors, rather than originate from bone marrow, are derived from progenitor cells in the perichondrium. With the loss of heparan sulfate or interference with heparan sulfate function, these cells are subjected to excessive bone morphogenetic protein signaling converting them to chondrocytes.[100] Heparan sulfate regulates bone morphogenetic function by storing it at the surface of cells or in the extracellular matrix, controlling its function. Similarly, lack of heparan sulfate at the cell surface limits noggin function.[178]

5.4 | Viral infections

In the context of infections, mutations in the heparan sulfate proteoglycans can lead to increase susceptibility to infectious diseases.[179] Several viral pathogens use heparan sulfate proteoglycans as receptors to facilitate entry in cells.[180,181] In viral infections, most pathogens do not synthetize heparan sulfate or heparin but have evolved heparan sulfate-binding or degrading capacity to exploit the glycocalyx and facilitate key host-pathogen interactions. Binding to heparan sulfate was reported for several coronaviruses.[181-184] Initially, heparan sulfate was found to help SARS-CoV-2 virus in cell attachment and virus entry.[185,186] Later, heparan sulfate binding to SARS-CoV-2 was found to be altered by modifying the heparan sulfate composition of the host cells.[187] Extensive reviews in heparan sulfate and viral infections are available.[180,188]

5.5 | Periodontitis

As a chronic inflammatory disease, various factors influence the etiology of periodontitis. From the perspective of the host response, multiple players are involved and the majority of research has focused on cells, their receptors and secreted inflammatory molecules.[189-192] In the early 1980's, several glycosaminoglycans free of proteoglycans were detected in gingival crevicular fluid of inflamed gingiva,[193] thus, being one of the first reports on the potential role of extracellular matrix molecules in the disease. It was initially thought that the changes in composition of the glycosaminoglycans from a healthy to a diseased periodontium was a reflection of inflammation and consequently mechanical loading of the inflamed tissue that had changed due to loss of attachment. At the time, extracellular matrix was thought to primarily serve to spatially distribute and promote support for cells.[194] However,

today, the role of glycosaminoglycans in promoting, sustaining, and controlling inflammation is undoubted although much more on mechanisms needs to be unveiled.

Loss of heparan sulfate proteoglycans on the surface of cells from patients with an inflamed periodontium was confirmed in subepithelial and subendothelial basement membranes and in infiltrating leukocytes.[195] When fibroblasts collected from healthy versus diseased gingiva were cultured, the fibroblasts from inflamed tissue secreted less heparan sulfate and more dermatan sulfate, a phenotype that persisted after several cell culture passages, demonstrating longevity of the phenotype.[196]

In different cells of the periodontium, periodontal ligament, gingival fibroblasts and osteoblasts, syndecan-2 exhibited a significant difference in expression. The presence of specific cell surface proteoglycans on periodontal cells implies distinctive functions.[36] Indeed, in the presence of periodontal inflammation, cell surface proteoglycan expression by lymphocytes is different to that in health.[14] *P. gingivalis* lipopolysaccharide influences the expression and processing of decorin and biglycan in extracellular matrix, altering alveolar bone-cell activity and osteoblast phenotype development.[197]

Overall, the role of the microenvironment in the pathogenesis of periodontitis is poorly characterized, despite the numerous reports from experimental studies about the critical involvement of these molecules in modulating various aspects of the inflammatory response, such as the formation of inflammatory mediator gradients, extracellular matrix remodeling in the resolution of inflammation and report of proteoglycan versus nonproteoglycan forms (enzymatically deglycanated) of these proteins having different interactions (i.e., with growth factor receptors vs. inflammatory receptors).[72,106,112] Syndecans and their heparan sulfate glycosaminoglycans are likely to regulate periodontal health through the various mechanisms involved in its biosynthesis and modification of their heparan sulfate glycosaminoglycans by enzymes such as exostosins, sulfotransferases, and heparanase. A recent study clearly indicated that some syndecans display different expression profiles in healthy and diseased periodontal tissues. Expression profiles of heparan sulfate proteoglycans and modifying enzymes also correlate with the presence of inflammatory fluid in healthy and diseased periodontal tissue.[198]

In a model based on data from immunofluorescence staining of human gingival samples, and reconstruction of a subset of heparan sulfate glycosaminoglycan-related proteins from the STRING protein interaction online database, investigators reported the role of heparan sulfate glycosaminoglycans on inflammation and alveolar bone loss. Further, it was found that increased expression of heparan sulfate glycosaminoglycan could stabilize the gingival inflammatory infiltrate, alveolar bone resorption and osteoprotegerin. This study suggests the use of exogenously added heparan sulfate glycosaminoglycans to control inflammation and periodontitis rather than just associating heparan sulfate with disease.[198,199]

Indeed, other inflammatory conditions such as acetaminophen-induced liver toxicity, can be significantly attenuated with the addition of exogenous HS glycosaminoglycan as we describe below. The role of soluble heparan sulfate glycosaminoglycans in gingivitis and periodontitis, is likely to depend on the fine structure of the carbohydrate.

6 | BIOSYNTHESIS OF HEPARAN SULFATE

The biosynthesis of heparan sulfate is a post-translational process that starts from a core protein and involves a cascade of enzymes. The heparan sulfate biosynthetic pathway encompasses the linkage region tetrasaccharide, heparan sulfate polysaccharide backbone synthesis, and modification steps (Figure 5). The synthesis of linkage tetrasaccharide is initiated from the core protein of heparan sulfate proteoglycans. First, xylosyltransferase transfers a xylose unit to a serine residue of the core protein. Two galactose units are then added to the xylose sequentially by galactosyltransferase-I and galactosyltransferase-II to form a trisaccharide unit of galactose-galactose-xylose-serine. A glucuronosyltransferase adds a glucuronic acid unit to the trisaccharide to lead to the linkage region tetrasaccharide, glucuronic acid-galactose-galactose-xylose-serine. This tetrasaccharide serves as a primer for the biosynthesis of heparan sulfate polysaccharide and chondroitin sulfate polysaccharides, depending on the subsequent enzymatic modification steps. Here, our focus is on the biosynthesis of heparan sulfate.

A complex of exostosin-1 and exostosin-2, from the exostosin family of proteins, catalyze the polymerization of heparan sulfate to yield the heparan sulfate proteoglycan backbone[200,201] (Figure 5). The backbone is then subjected to a series of enzymatic modifications. *N*-deacetylase/*N*-sulfotransferase transforms an *N*-acetyl glucosamine to an *N*-sulfo-glucosamine. The C5-epimerase converts a glucuronic acid to an iduronic acid. 2-O-sulfotransferase transfers a sulfo group to the 2-OH position of an iduronic acid unit to form iduronic acid 2S. The 6-*O*-sulfation and 3-*O*-sulfation of glucosamine unit is carried out by 6-O-sulfotransferase and 3-O-sulfotransferase, respectively. Heparan sulfate sulfotransferases are present in multiple isoforms except for 2-O-sulfotransferase. The isoforms are expressed at different levels and display different substrate specificities.[202] For example, *N*-deacetylase/*N*-sulfotransferase is present in four different isoforms, 6-O-sulfotransferase is present in three, and 3-O-sulfotransferase is present in seven isoforms.[203] The biosynthesis of heparan sulfate polysaccharides is not driven by a template. Consequently, the heparan sulfate isolated from a tissue is a complex mixture, consisting of different lengths in sugar chains and sulfation levels. Although the mechanism for the cellular control for the biosynthesis of heparan sulfate is not fully understood, it is generally accepted that the expression levels of individual heparan sulfate biosynthetic enzymes play essential roles for regulating the structures of heparan sulfate. Other factors contribute to the biosynthesis of heparan sulfate, that is, the expression of core proteins and the level of the sulfo donor that is used by sulfotransferases or sugar nucleotides used by exostosin 1/2 for the polysaccharide backbone synthesis.

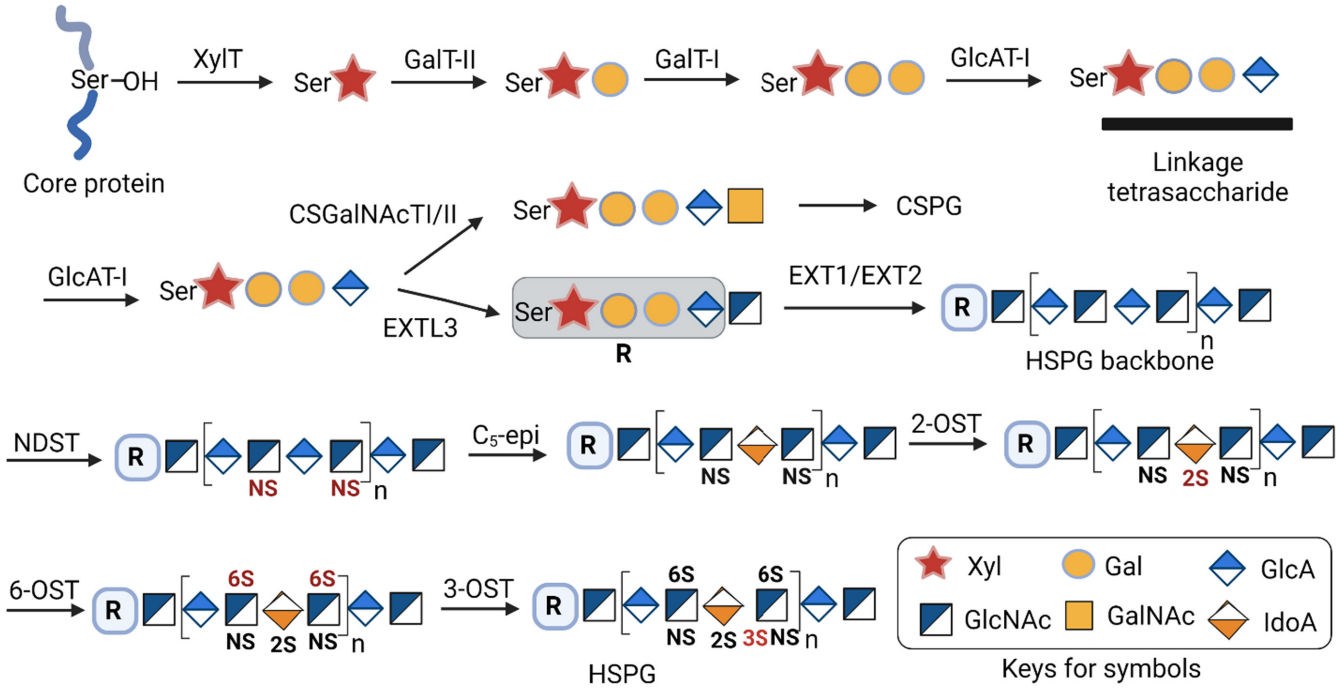

FIGURE 5 Biosynthesis of heparan sulfate. The biosynthesis of heparan sulfate is started with addition of a xylose followed by adding two galactose units and one glucuronic acid to yield the linkage region tetrasaccharide. EXTL3 enzyme adds one additional *N*-acetyl glucosamine to direct the synthesis towards heparan sulfate. Alternatively, chondroitin sulfate *N*-acetyl galactose I/II adds *N*-acetyl galactosamine to proceed the synthesis of chondroitin sulfate. Detailed explanations for each enzymatic reaction step are presented in the text. C5-epi, C5 epimerase; CSGalNAcT, chondroitin sulfate galactose *N*-acetyl transferase; CSPG, chondroitin sulfate proteoglycan; EXT, exostosin; Gal, galactosamine; GalNAc, *N*-acetyl galactosamine; Glc, glucuronic acid; GlcAT, acetyl glucosamine transferase; GlcNAc, *N*-acetyl glucosamine; HSPG, heparan sulfate proteoglycan; IdoA, iduronic acid; NS, nonsulfated; Ser, serine; ST, sulfotransferase; Xyl, xylose.

7 | CHEMOENZYMATIC SYNTHESIS OF HEPARAN SULFATE OLIGOSACCHARIDES

The functions of heparan sulfate are primarily dictated by sulfation patterns and the size of the sulfated saccharide domains.[204] A centerpiece in heparan sulfate-related research is to investigate the contribution of sulfation patterns in heparan sulfate to the biological functions. For this purpose, access to those structurally homogeneous or chemically pure heparan sulfate molecules is essential. Chemoenzymatic method offers a flexible and cost-effective approach for the synthesis of heparan sulfate oligosaccharides.[205] The method utilizes recombinant heparan sulfate biosynthetic enzymes. Unlike cellular synthesis, chemoenzymatic synthesis emphasizes controlling the completion at each enzymatic modification step to achieve the synthesis of structurally pure oligosaccharides after multi-step enzymatic reactions.

An example for the synthesis of a heparan sulfate hexasaccharide is provided in Figure 6. The synthesis is started from a commercially available *p*-nitrophenyl glucuronide. The monosaccharide is elongated to a hexasaccharide using the *p*-nitrophenyl glucuronide (heparosan synthase 2 from *Pasteurella multicide*), a glycosyltransferase that can elongate the monosaccharide to a hexasaccharide backbone. In contrast to exostosin 1/2, the mammalian heparan sulfate polymerase, recombinant *p*-nitrophenyl glucuronide enzyme is easy to obtain, facilitating the scale-up synthesis. The glucuronide uses two uridine diphosphate *N*-acetylglucosamine or uridine diphosphate glucuronic acid molecules for the synthesis of heparosan, a capsular polysaccharide from *Pasteurella multicide* that has the same structure as the heparan sulfate backbone.

The chemoenzymatic synthetic method uses a piece of technology to position a *N*-sulfated glucosamine unit. An unnatural sugar nucleotide donor, diphosphate *N*-trifluoroacetyl glucosamine is introduced to replace uridine diphosphate *N*-acetylglucosamine (Figure 6). The purpose of incorporating a *N*-trifluoroacetyl glucosamine unit is that it that can be readily converted into a glucosamine for the subsequent *N*-sulfation reaction by *N*-sulfotransferase.[206] This technique allows for precise controlling of the position of a *N*-sulfated glucosamine to progress the subsequent modifications. It is known that *N*-sulfation is essential to regulate the sites of action of C_5-epimerase, 2-O-sulfotransferase, and 3-O-sulfotransferase.[207] The chemoenzymatic method is now emerging as a highly effective approach to synthesize a wide range of structurally homogeneous oligosaccharides.[208,209] Access to these oligosaccharides has opened new opportunities for dissecting the biological functions of heparan sulfate and developing heparan sulfate-based therapeutics.

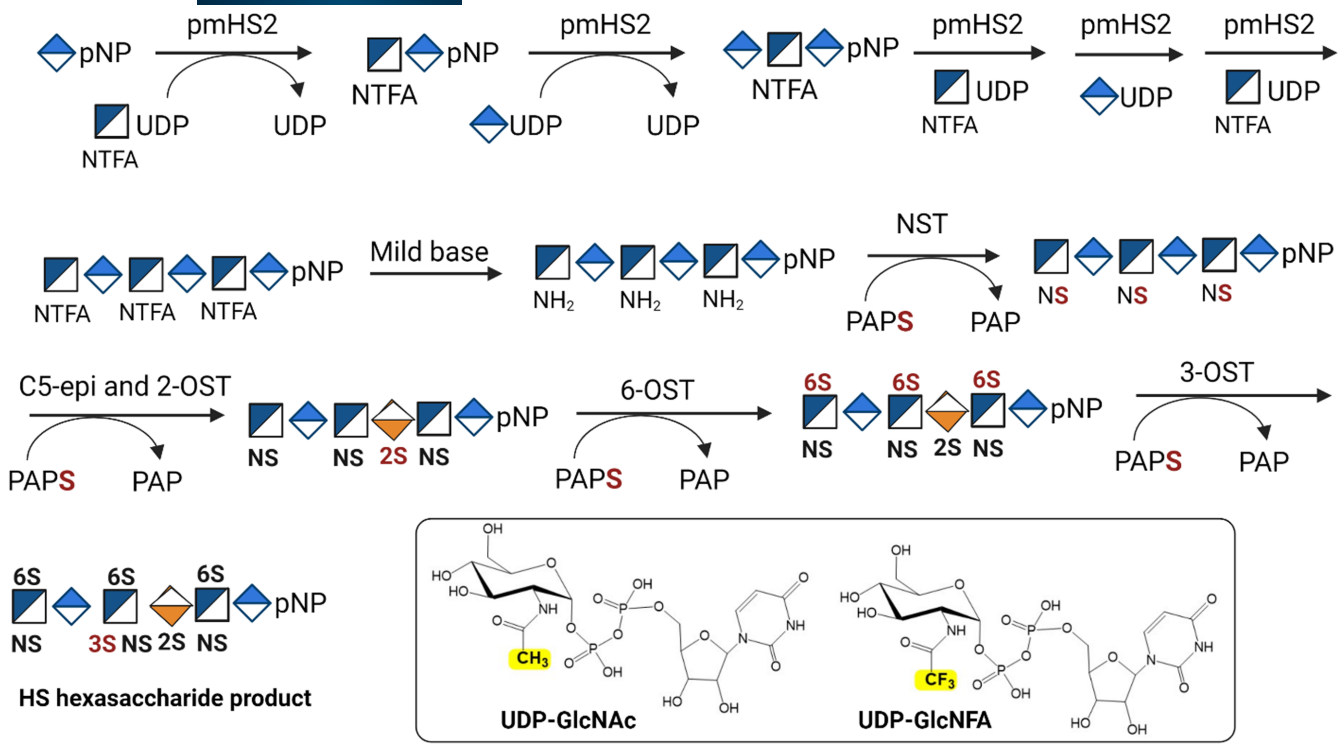

FIGURE 6 Chemoenzymatic synthesis of heparan sulfate hexasaccharide (i.e., 6-mer). The synthesis is initiated from a monosaccharide *p*-nitrophenyl glucuronide. An unnatural sugar nucleotide, diphosphate *N*-trifluoroacetyl glucosamine, is used in the synthesis. The structures of diphosphate *N*-acetyl glucosamine and diphosphate *N*-trifluoroacetyl glucosamine are shown in the box. The difference between two donors are highlighted in yellow. 3′-phosphoadenosine 5′-phosphosulfate is a sulfo donor used by sulfotransferases. EXTL3, exostosin-like 3; GlcA-Pnp, *p*-nitrophenyl glucuronide; GlcAT, glucuronyl transferase; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PmHS2, heparosan synthase 2 from *Pasteurella multicide*; UDP-GlcNFTA, diphosphate *N*-trifluoroacetyl glucosamine; Udp-GlcNAc, diphosphate *N*-acetyl glucosamine; UDP-GlcNFTA, diphosphate *N*-trifluoroacetyl glucosamine.

8 | POTENTIAL THERAPEUTIC ROLES OF HEPARAN SULFATE

8.1 | Anticoagulation

Heparin is a widely used anticoagulant drug in hospitals. There are several forms of heparin drugs in the marketplace, including unfractionated heparin, low molecular weight heparin and fondaparinux. Unfractionated heparin is isolated from porcine intestine and is a highly complex mixture of polysaccharides with different sizes (MW_{avg} ~ 16 000 Da). The low molecular weight heparin is a partially depolymerized unfractionated heparin with the average molecular weight (MW_{avg} 4500 to 6500 Da). Fondaparinux is a synthetic pentasaccharide, which is a pure chemical with a molecular weight of 1508 Da. With heparin, its interaction with antithrombin requires a specific pentasaccharide that contains a rare 3-O-sulfated glucosamine installed by 3-O-sulfotransferase. When the 3-O-sulfation is lacking, the binding affinity to antithrombin is reduced substantially, resulting in the loss of anticoagulant activity. Fondaparinux is a synthetic pentasaccharide that encompasses the pharmacophore of the drug heparin to display the anticoagulant activity. Knowing the structural requirements for the anticoagulant activity will also allow us to engineer the heparan sulfate oligosaccharides without the anticoagulant activity for different therapeutic purposes.

There are concerns over the relevance and safety of animal-sourced heparin.[210] Both the unfractionated form and its derivatives low molecular weight heparins are from animal source that go through a long and poorly regulated supply chain. Batches of contaminated heparin entered the world market, leading to 256 deaths in the US in 2008.[211] A reliable and cost-effective method to make synthetic heparin that can replace animal-sourced heparin is being actively pursued. Although fondaparinux is a synthetic, it does not cover all the pharmacological properties of heparin drugs because the size of carbohydrate chain is too short.[208] Synthesis of a dodecasacchride (12-mer) with similar pharmacological properties to low molecular weight heparin has been reported.[209] The chemoenzymatic synthetic approach plays a crucial role in the effort for developing a synthetic heparin to substitute animal-sourced heparin.

8.2 | Acetaminophen-induced acute liver injury

Heparan sulfate also displays anti-inflammatory properties by inhibiting the activity of pro-inflammatory proteins, which offers therapeutic application beyond anticoagulation. This has been demonstrated in a recent example of treating liver damage caused by acetaminophen overdose. Acetaminophen is an active ingredient in Tylenol®, an over-the-counter pain medication. Although it

is generally safe, acetaminophen overdose is the leading cause of acute liver failure in the US and Europe.[212] Acetaminophen-induced acute liver failure involves the axis of high mobility group box and the receptor for advanced glycation end products.[213] A synthetic heparan sulfate octadecasaccharide (18-mer) has been shown to protect against acute liver failure caused by acetaminophen overdose.[214] The 18-mer does not display anticoagulant activity. The 18-mer decreased high mobility group box 1-mediated neutrophil infiltration in an air pouch model. Treatment with both 18-mer and high mobility group box 1 neutralizing antibody offered very similar protection after acetaminophen overdose, suggesting that 18-mer and the antibody engaged high mobility group box 1 target. Interestingly, the anticoagulant 18-mer did not display the hepatoprotection in the acetaminophen overdose animal model. The chemical structure of anticoagulant 18-mer is different from the 18-mer. Further studies demonstrated that the anticoagulant activity from the anticoagulant 18-mer impaired the liver regeneration process by disrupting the generation for fibrin. These findings demonstrate the need for structurally homogeneous heparan sulfate oligosaccharides to probe the functions of heparan sulfate in disease models.

8.3 | Periodontitis

Periodontitis is a major chronic disease that causes alveolar bone and tooth loss and affects one out of every two American adults.[215,216] Currently, the therapy for periodontitis consists of biofilm disturbance and reduction with mechanical debridement of plaque and calculus around teeth, with or without concomitant systemic antibiotics.[217] Local antibiotics delivered to periodontal pockets have also been used; however, there is evidence that they are not always effective and do not prevent the re-emergence of periodontitis.[218,219] There is a line of evidence supporting the idea that periodontitis patients and healthy people have a similar biofilm with variations in bacterial ratio's causing deregulation/dysbiosis.[220] Thus, aiming for bacterial eradication via debridement and antibiotic treatment is not always the best approach as a microbial balance is necessary combined with inflammatory control.[220] Some studies support to primarily target the host inflammatory response to the intra-oral biofilm, alongside the reduction of biofilm accumulation (debridement, not eradication of bacteria with antibiotics). Indeed, about 30% of individuals do not respond well to any type of antibacterial periodontal therapy, including surgical therapy, and continue presenting with bone loss despite a minimal amount of pathogenic flora.[221] Part of this population is thought to have a hyperinflammatory response.[222]

The European Federation of Periodontology has recognized that part of the population affected does not respond to treatment, due to the therapeutic focus remaining primarily upon biofilm management (antibiotics, antiseptics) rather than modulation of inflammation, which is the primary reason for periodontal tissue damage.[223] The currently available antibiotics and other therapeutics such as systemic sub-antimicrobial dose doxycycline and antiseptic mouthwashes provide limited benefit.[224] Alendronates, nonsteroidal anti-inflammatory drugs, statins, probiotics are all examples of off-label nonantimicrobial adjuncts with no significant evidence for improving the outcomes of periodontal therapy, thus, there is currently no effective alternative to antibiotics.[224,225] A heparan sulfate of 18-mer size that specifically decreases inflammation and prevents acetaminophen-induced acute liver failure[25] has recently been investigated by our group and has shown promising results in protecting against murine liver disease and death due to inflammation. In evaluating similar-size synthetic heparan sulfate and unfractionated heparin, both heparin and the sulfated 18-mer were effective in preventing osteoclast differentiation in vitro, thus presenting potential therapeutic value in preventing bone loss. In an inflammatory bone challenge experiment in mice, heparin and the 18-mer, when locally injected into the gingiva offered protection of alveolar bone upon ligature insult (Figure 7), providing strong impetus to further investigate how this molecule affects periodontal tissue homeostasis, and the best way to explore it as a periodontal therapeutic agent. While heparin also showed protective effects, its characteristic heterogeneity, since it is tissue-extracted heparin, remains a concern. The variability in bone effects has been demonstrated in several studies that use heparins and low molecular weight heparins for long term treatment (i.e., for patients at risk of embolism such as post myocardial infarction patients, deep vein thrombosis, and pregnant patients). While some of these patients present with osteopenia, others do not display any specific bone phenotype.[226-228] Thus, as a therapeutic molecule in periodontitis, tissue-extracted heparin may present variable effects.

While these are proof-of-concept studies and more research needs to be undertaken to fully characterize the effect of the sulfated 18-mer in management of periodontitis, the data is very encouraging. To our knowledge, we are the first group to report that a specific heparan sulfate glycosaminoglycan code is beneficial in preventing bone loss in a murine model of periodontitis. The sulfated 18-mer could be a cost-effective therapeutic to promote alveolar bone homeostasis. Further mechanistic characterization of its effect at the level of inflammatory and bone-cells function is underway (likely involving high mobility group box 1 as described above, among other factors). In addition, dose-dependent studies including susceptible subjects such as with a hyperinflammatory phenotype are being investigated.

Other studies have attempted to investigate heparan sulfate/heparin in periodontitis management. A low molecular weight heparin derived from depolymerized tissue-extracted heparin (Clexane®) was subcutaneously injected for 60 days as a treatment for ligature-induced periodontitis in rats, although researchers did not see significant differences in bone levels.[229] Others have looked at a biomimetic of heparan sulfate, a regenerating agent named ReGeneraTing, which is dextran-based with added sulfate groups. Coyac[230] applied the biomimetic intra-muscularly for 8-weeks post-disease induction in a bacteria-induced mouse model of periodontitis and reported decreased levels of inflammation and fewer osteoclast numbers in mice treated with ReGeneraTing.

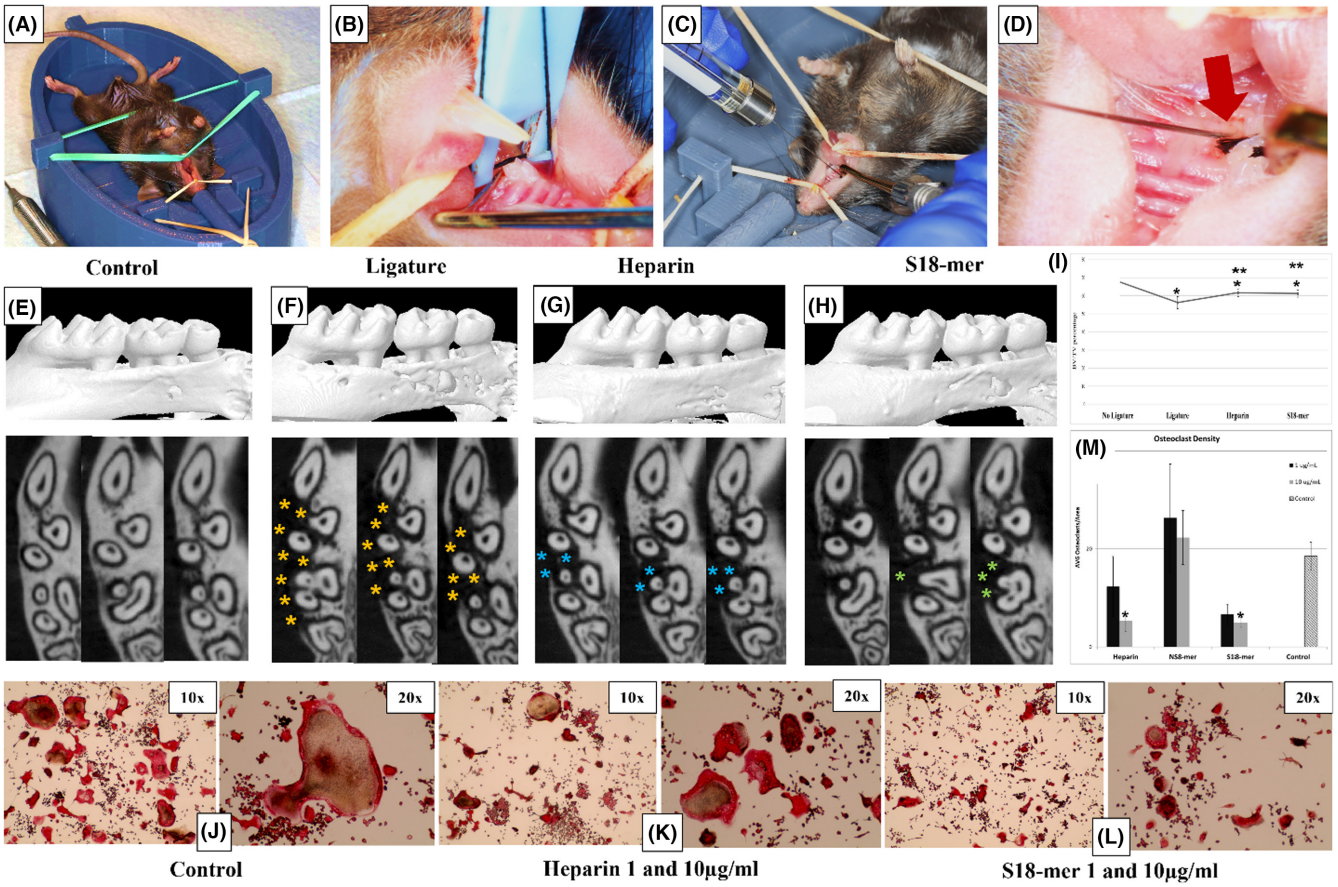

FIGURE 7 (A, B) Placement of silk ligature strip in mice to induce bone loss as a model of periodontitis. (C, D) Injection of vehicle, heparin or S18-mer in a 2 µL volume on the palatal tissue between mouse molars M1 and M2 was done daily during the course of the experiment (5 days) ($n = 7$ male C57BL/6 mice/group). (E–H) At 5 days, mice were sacrificed, and maxillae harvested for microcomputed tomography and quantification of bone loss performed by volumetric analyses. 3D (view of representative maxillae) and 2D (view of 3 representative maxillae) show clear differences in bone loss (represented by colored asterisks) in ligated mice as demonstrated in %BV/TV (I). Statistical difference is shown between no-ligature and all groups (*) and ligature and treated groups heparin and S18-mer (**) ($p < 0.05$). (J–L) Raw cells, a pre-osteoclast cell line, were cultured for 4 days in the presence of osteoclast differentiation media. Cells were treated with the oligosaccharides daily for 5 days and stained for tatrate-resistant acid phosphatase. (M) Quantification of osteoclasts/mm^3 area showed that compared to control (last gray bar), S18-mer showed the largest decrease in osteoclast numbers/differentiation at two different concentrations (1 and 10 µg), which was statistically significant ($p < 0.05$). Heparin significantly diminished osteoclast numbers at only one concentration (10 µg) ($n = 3$ for all groups). Another synthetic heparan sulfate of 8-mer was used as a proof-of-concept that the size and sulfation of the synthetic saccharide are important, showing no apparent effect on osteoclasts. Kruskal-Wallis with Dunn post hoc tests were performed to determine the differences among groups. *$p < 0.05$ compared to negative control group.

Practice guidelines for the management of various stages of periodontitis have been published recently.[225,231] Besides biofilm control, plaque removal, oral hygiene instructions, and management of other risk factors (such as metabolic diseases and smoking), there is a recommendation to provide adjunctive therapy, which may include host-modulation and antimicrobial agents in the treatment of periodontitis. These may be included concomitantly or after initial therapy. Lastly, therapy may include periodontal surgery (where regeneration may be attempted) and supportive periodontal maintenance at regular intervals. We envision that in the future, heparan sulfate could to be used as an adjunctive therapy and host-modulating agent. Another potential application for heparan sulfate is in the treatment of residual pockets through regenerative therapy. In regeneration, it is recommended that membranes or enamel matrix derivative with or without biomaterials be applied to infrabony pockets. This is based on a rigorous evidence-based assessment that identified most promising biomaterials.[225] Regenerative therapy leads to improved clinical attachment levels, including in molars with class II furcation involvement. Ultimately, heparan sulfate as a periodontal therapeutic, will need to go through careful, powerful, and with low risk of bias, randomized clinical trials in order to be recommended as a periodontal supportive and regenerative therapy in the future.

8.4 | Tissue engineering

Besides the management of inflammation, another area that has gained traction for heparan sulfate is in the tissue engineering of various connective tissues. Heparan sulfate-based biomaterials have

generated interest because molecules of the transforming growth factor-β superfamily bind to heparin and heparan sulfate.[102] Thus, heparan sulfate can facilitate controlled release of growth factors such as bone morphogenetic protein 2, fibroblast growth factor, platelet-derived growth factor, among others.[46] Heparin has been found to bind to the *N*-terminal of bone morphogenetic protein although further characterization of this feature is needed, thus changes in heparan sulfate structure may alter the binding pattern.[232]

Heparan sulfate proteoglycans can promote the formation of a complex between bone morphogenetic protein 2 receptors I and II sustaining the activity. Heparan sulfate proteoglycans can also bind noggin, a bone morphogenetic protein antagonist, helping to block the binding epitopes of bone morphogenetic protein receptors.[233,234] Heparan sulfate as a soluble molecule, can enhance bone morphogenetic protein signaling in cell culture and sustain Smad 1/5/8 phosphorylation.[43,235] Interestingly, when heparan sulfate is coupled with bone morphogenetic protein 2 in vitro, noggin is not able to antagonize it, which is a promising way to deliver bone morphogenetic protein, potentially sustaining signaling and preventing canceling of its function. Heparan sulfate or heparin bound to growth factors can also protect them from enzymatic degradation.[46]

There is growing interest in biomaterials development harboring glycosaminoglycans for the purpose of improving cell migration, decreasing inflammation, improving extracellular matrix organization and biomaterials that are capable of controlled and persistent release of bioactive molecules. A few studies have explored bone healing with adjunctive heparan sulfate and report improvements in the regenerative process due to improved stability, cell reception and flexibility in delivery of biomolecules in fractures, including nonunion or delayed union.[236,237]

The mechanism by which heparan sulfate and heparin interact with growth factors is primarily through ionic interactions and carboxylate groups.[44] Various polymers, nanoparticles and composite materials have been modified with heparin or low molecular weight heparin via chemical cross-linking, encapsulation or just pooled together.[238,239] Some of the reported biomaterial backbones utilized with heparan sulfate are collagen, other glycosaminoglycans, chitosan, etc.[239-241]

Besides the delivery of growth factors, the addition of a heparan sulfate molecule provides the scaffold with the ability to concomitantly be (potentially) anti-inflammatory and anti-scarring.[242,243] It is tempting to assume that the release of heparan sulfate could also potentially reduce viral and bacterial binding to cell receptors in the event of infection, thus, further controlling an excessive inflammatory response.

However, part of the literature has emphasized that heparin, when used long-term, can lead to osteopenia and even osteoporosis, potentially promoting poor bone regeneration.[227,244] Low molecular weight treatment did not show any obvious effects on fracture risk but heparin increased the osteoclast surface.[226,228,245] Low molecular weight heparin has several advantages such as increased half-life and bioavailability compared to full length heparin. Thus, added to the low concern for osteoporosis with long-term use of low molecular weight heparin, such risk is perhaps understated.

Other classes of heparins have been developed for tissue engineering such as biomimetics in an attempt to deliver a more consistently generated molecule with low degree of complexity during manufacturing (i.e., size, chemical modifications, etc.). Lallam et al.[246,247] reported a heparan sulfate biomimetic to use in the regeneration of the periodontium of hamsters. The biomimetic carries carboxymethyl and sulfate groups on a carbohydrate backbone and has shown promise in the resolution of inflammation and promotion of healing in muscle, skin wounds and ulcers of the cornea.[248,249] This compound was delivered systemically and promoted cell proliferation and changed the organization of healing tissues in periodontium. This is the beginning of a new era of glycobiology, where defined carbohydrate structures can predictably act as adjuncts in multiple clinical scenarios. Given the new tools available to generate such therapeutics, a clear safety profile and the ability to manufacture it at low cost, human translational studies should soon be underway.[45]

9 | CONCLUSIONS

The availability of synthetic heparan sulfate oligosaccharides will accelerate the understanding of the role of heparan sulfate in health and disease and contribute to a new era in biologics as therapeutics. Of interest, periodontitis and periodontal regeneration are promising targets for a myriad of defined heparan sulfate molecules with potential anti-inflammatory and anticlastogenic effects. Efforts are underway to also characterize their function in osteogenesis. In summary, recognizing the functions of these molecules in periodontal homeostasis may represent a paradigm shift in periodontics and regenerative dentistry.

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CONFLICT OF INTEREST STATEMENT

JL is founder and chief scientific officer of Glycan Therapeutics. Other authors declare no competing interest.

DATA AVAILABILITY STATEMENT

Any raw data supporting reported results can be requested by contacting the corresponding author.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the University of North Carolina at Chapel Hill, IACUC#16-162.

ORCID

P. A. Miguez https://orcid.org/0000-0003-1255-3887
I. L. C. Chapple https://orcid.org/0000-0003-2697-7082

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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