Sweet but Challenging: Tackling the Complexity of GAGs with Engineered Tailor-Made Biomaterials

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((Glycosaminoglycans (GAGs) play a crucial role in tissue homeostasis by regulating the activity and diffusion of bioactive molecules. Incorporating GAGs into biomaterials has emerged as a widely adopted strategy in medical applications, owing to their biocompatibility and ability to control the release of bioactive molecules. Nevertheless, immobilized GAGs on biomaterials can elicit distinct cellular responses compared to their soluble forms, underscoring the need to understand the

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interactions between GAG and bioactive molecules within engineered functional biomaterials. By controlling critical parameters such as GAG type, density, and sulfation, it becomes possible to precisely delineate GAG functions within a biomaterial context and to better mimic specific tissue properties, enabling tailored design of GAG-based biomaterials for specific medical applications. However, this requires access to pure and well-characterized GAG compounds, which remains challenging. This review focuses on different strategies for producing well-defined GAGs and explores high-throughput approaches employed to investigate GAG–growth factor interactions and to quantify cellular responses on GAG-based biomaterials. These automated methods hold considerable promise for improving our understanding of the diverse functions of GAGs.

In perspectives, we encourage the scientific community to adopt a rational approach in designing GAG-based biomaterials, taking into account the *in vivo* properties of the targeted tissue for medical applications.))

1. Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides that are major components of the extracellular matrix. Through electrostatic interaction depending on sulfation patterns along their chain, GAGs can bind a myriad of proteins, including growth factors (GF), cytokines, and chemokines. Consequently, GAGs can regulate the bioactivity of proteins and their delivery to the surrounding cells, thereby exerting key regulatory roles in many biological processes.[1–4] It is well-known that GAG content and fine structural composition vary across tissues and are developmentally regulated.[5–9]

For these reasons, the use of GAGs in biomaterials for delivery of GFs in tissue engineering is becoming a common approach.[10–17]. Over the last decades, a large variety of GAG-based biomaterials has been developed including scaffolds, coatings, microparticles, hydrogels, cryogels, coacervates or liposomes.[17,18] As previously reviewed, the primary applications of GAG-based biomaterials relate to bone, cartilage or tendon regeneration, and wound

healing.[19,20] Depending on the targeted application, the selection of the biomaterial type must be adapted to the desired properties, including dimensionality (2D/3D material), mechanical and chemical properties, as well as implantation or injection strategies. The fabrication of GAG-based biomaterials can be achieved through various functionalization approaches to control their shape, viscoelastic properties, cell adhesion properties, degradation properties, and the spatiotemporal release of bioactive molecules such as GFs. The use of GAG-based biomaterials for regenerative medicine application in combination (or not) with GFs has been exhaustively reviewed.[17,20,21] Hachim and co-workers provided a detailed examination of optimal combinations between GFs and GAGs, along with a comprehensive overview of all delivery systems for each type of GF and GAG.[17] Strategies for the fabrication of GAG-based hydrogels have been reviewed by Freudenberg and co-workers,[22] and those for GAG-based cryogels by Wartenberg and co-workers.[23] It is worth noting that several biomaterials featuring identical GAG types have been used for engineering different tissues and exhibited distinct properties. Generally, such property variabilities result from the combination of GAGs and GFs used, which may elicit specific functions related to the targeted tissue, such as vessel or neurite growth, bone formation or anti-inflammatory effects.[17]

Cytotoxicity and the ability to deliver GFs are the most frequently studied parameters of GAG-based biomaterials.[24–27] However, the molecular interactions between GAGs and GFs as well as their consequences on cellular responses remain poorly understood. In particular, the biological properties of the GF may depend upon its delivery mode, either in solution as conventionally studied,[28–32] or when physically bound to the GAG-based biomaterial. Indeed, the presentation of GFs in a bound form may better mimic the natural conditions, as in the extracellular matrix (ECM) or in the pericellular coat.

A significant challenge for the generation of GAG-based biomaterials is the structural variability of GAGs, which depends on the source, and the requirement for sufficient quantities of well-defined GAGs. Therefore, when designing biomimetic materials for medical applications or to studying the

effects of GAGs on cellular responses, it is crucial to consider the structural discrepancies between commercially available GAGs and those found *in vivo*. Novel methods for producing controlled oligosaccharides in sufficient quantities are therefore needed and are currently under development.

Here, we will focus on immobilized GAGs, which exhibit a greater degree of physiological relevance compared to their soluble counterparts. Improving our understanding of the molecular interactions between GAGs and proteins or GFs is a prerequisite for engineering biomaterials with more advanced functionalities. In this manuscript we highlight several aspects pivotal for the design of GAG-based biomaterials and their investigation at high content (**Figure 1**). In particular, the type, density and sulfation of GAGs are highly variable parameters *in vivo* that depends on the tissue, pathology or age, and should be considered in the design of biomaterials. To address this, two major issues must be considered: first, the need of methodologies for the synthesis/preparation of well-defined GAG structures, including highly pure, structurally defined oligosaccharides in substantial quantities; second, the implementation of high-throughput techniques to analyze the interactions between GAGs and other biomolecules such as GFs, and to evaluate their impact on cellular responses *in vitro.* Automated strategies for biomaterial fabrication and cell behavior analysis have recently emerged.[33,34] We anticipate that novel high-content methodologies for the study of GAG–GF interactions and the role of GAGs mediated presentation of GFs on cellular responses will emerge and will be further developed.

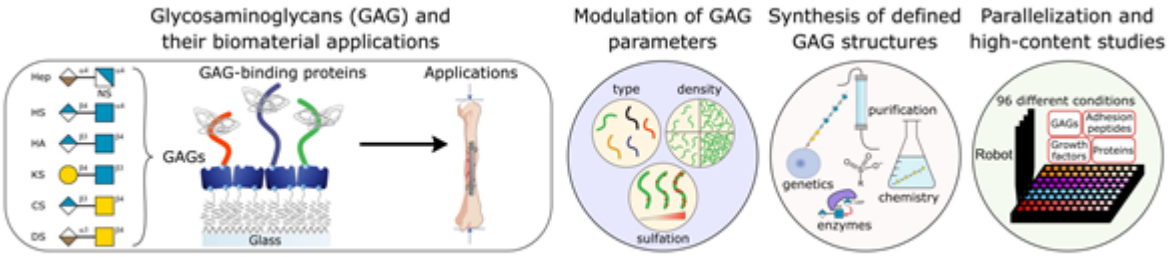


Figure 1. ((Schematic representation of the main topics of the review organized in four chapters to describe GAGs and their use in GAG-based biomaterials, the important parameters that can be tuned

when designing a GAG-based biomaterial, the techniques to obtain pure oligosaccharides with defined sulfation, and finally the high-throughput methods available to compare different properties of GAGs for molecular and cellular studies.))

2. GAGs: structure, biological functions and biomaterial applications

2.1. The GAG family

GAGs constitute a family of complex polysaccharides, encompassing Heparin (Hep), Heparan Sulfate (HS), Chondroitin Sulfate (CS), Dermatan Sulfate (DS), Keratan Sulfate (KS) and Hyaluronic Acid (HA). GAG chains are constituted by the repetition of a disaccharide unit, composed of an amino sugar (either glucosamine or galactosamine) and a hexuronic acid (glucuronic or iduronic acid). The nature of the saccharide constituents defines each GAG type and has been previously reviewed.[17] With the exception of HA, these chains are covalently bound to a protein core, forming a proteoglycan (PG) in a well-defined manner. Depending on their protein core, PGs can carry between one to more than a hundred GAG chains of one or different GAG types.[35], and can be found in various locations, including the ECM, cell surfaces, in the circulation or even in intracellular vesicles.[36] GAGs are synthesized directly in the Golgi, starting with the addition of a tetrasaccharide linker to a serine residue (alternatively, threonine or asparagine for KS) of the protein core. Next, they are further elongated and modified by many biosynthesis enzymes.[9,37–41] The most crucial modifications involve the transfer of sulfate groups to specific positions of the disaccharide units, which are catalyzed by various sulfotransferase enzymes. By controlling precisely the positions of sulfate groups along the GAG chain, these sulfation enzymes generate a wide array of sulfated saccharide sequences, resulting in tremendous structural diversity. These various combinations of highly negatively charged sulfate groups mediate interactions with a multitude of proteins. A recent extensive literature review unveiled the existence of more than 3400 distinct GAG-binding proteins.[42] Through these interactions, GAGs regulate protein activity, structural conformation, interaction with cell receptors and spatial localization.[43,44] Consequently, GAGs govern a large number of biological functions in the organism.[8] Mutations of the genes associated to GAG biosynthesis often lead to phenotypical

defects.[9] This suggests that GAG biosynthesis is highly regulated with evidence indicating the existence of compensatory effects between different GAGs. Notably, alterations of both HS length or sulfation in chondrocytes have been correlated with a strong increase in CS content.[45,46] Furthermore, GAGs play pivotal roles in ECM architecture, cohesion and hydration, conferring unique mechanical properties of tissues, such as skin, cartilage, synovial fluids or vitreous humor.[47]

2.2. Tissue prevalence, biological function of GAGs and their biomaterial applications

Altogether, the remarkable physical and biological functions of GAGs point them excellent candidates for biomaterial applications, particularly in the field of tissue regeneration **(Table 1)**.[17–20,22,23,48–50]

Structurally, GAGs are categorized according to the nature of their saccharide backbone and sulfation patterns. HA, Hep, HS and KS belong to the glucosaminoglycan sub-family, characterized by a glucosamine-containing disaccharide unit while CS and DS belong to the galactosaminoglycans family since their disaccharide units contain a galactosamine. HA is the structurally simplest GAG, as it is not sulfated and never linked to a PG but synthesized at the cell surface.

As reported in **Table 1**, except HS which is produced ubiquitously in the body at the cell-surface of virtually all cell-types, and is present in the ECM of all tissues, the other GAGs are tissue-specific. In particular, Hep has been first extracted from the liver, but is also present in the intracellular granules of mast cells, lung arteries, skin and in the bipotential glial progenitor cells. CS is found attached to aggrecan in cartilage.[41,51]. KS is predominantly found in the cornea, central and peripheral nervous systems, cartilage and bone. DS is also widely distributed, notably in skin, blood vessels, heart valves, cornea, tendons and lung. HA is abundantly found in soft tissues such as synovial fluid, articular cartilage, skin, vitreous humor, ECM of loose connective tissues and in the umbilical cord.

The functions of GAGs are really vast and summarized in **Table 1**. Several GAGs (HA, CS, DS and KS) share the common role of ECM formation, hydration, preservation and resilience to compression. HA

also regulates cell-adhesion and motility through interactions with its cell-surface receptors CD44 and RHAMM.[52,53] Many studies have reported that HA biological functions vary depending on its molecular weight.[54], high molecular weight HA being generally associated with tissue homeostasis and exhibits anti-inflammatory, anti-proliferative, anti-angiogenic and anti-metastatic activities. Conversely, low molecular weight HA is often considered as an alarm signal related to inflammation, angiogenesis and metastasis.[54] In cartilage, CS is mostly found attached to aggrecan, and confers its elasticity and anti-inflammatory properties to the tissue.[41,51] In the nervous system, CS chains serve as cues for guiding neural development and regeneration.[19,55] CS, particularly CS-E, plays also roles in angiogenesis.[56]. DS is involved in various biological functions including wound repair, ECM assembly with collagen fibers, inflammation, anti-coagulation, neural guidance and development, cell proliferation, invasion and metastasis.[6,57]. KS contributes to neural guidance, development and regeneration, notably playing a role in promoting neuron-glial cell interactions, myelination and axonal repair.[58]

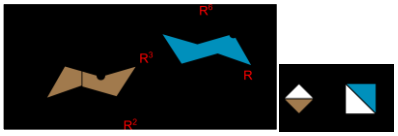
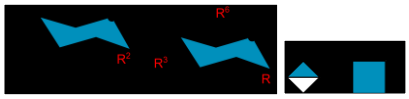
Hep, beyond its anticoagulant properties, can also inhibit cell proliferation, inflammation and tumor metastasis.[59] HS participates in wide range of functions, including cell differentiation, tissue morphogenesis, cell interactions and proliferation, and interaction with GFs, cytokines and cell adhesion molecules.[2,4,60–67]

Each GAG can exert various biological functions, which are often dictated by their interactions with specific proteins, as described elsewhere.[68]

Hep and HA are the most advanced GAGs for biomaterial applications. Indeed, due its large binding properties, many Hep-based biomaterials have been developed for biomedical applications, particularly in tissue engineering. Medical devices incorporating Hep for its anticoagulant properties have been successfully validated in clinical trials and are now commercially available.[69] HA is prevalent in a wide range of cosmetics and is in clinical trials for wound repair using different biomaterials like HA-based sponges,[70] or HA-based dermal substitute membranes.[71,72] HA-based scaffolds have also been tested in clinics for cartilage repair.[73–80] .

Various CS-based biomaterials have been developed for a broad range of applications. Notably, a membrane made of bovine tendon collagen and CS-C successfully completed clinical trials and is now

commercially available for skin repair of burn victims.[81,82] For cartilage repair, CS has already been used in clinical trials and is commercially available, though not as a biomaterial but as an intra-articular injectable solution.[83] Alongside, CS-based biomaterials such as hydrogels are currently being developed to repair damaged cartilage. [77–80,84,85]. Due to its structural diversity and of its numerous non-specific interactions with HS, there are no HS-based biomaterial under clinical trial, maybe due to its structural diversity or to the fact that it was rather considered as a secondary product during heparin purification. However, *in vitro* and *in vivo* studies have investigated HS-based biomaterials as listed in **Table 1**. Application of DS in biomaterials is progressing slowly compared to the other GAGs and **Table 1** illustrates emerging developments, Surprisingly, to our knowledge, no KS-based biomaterial has been developed.

	Disaccharide unit	Tissue prevalence	Biological functions	Biomaterial applications
Hep		Intracellular granules of mast cells [86] Lung arteries Liver and skin Bipotential glial progenitor cells	Anti-coagulation Anti-inflammation Anti-proliferation Anti-metastatic	Anti-coagulation [69] Anti-inflammation [87–89] – IL-4[87,88], IL-13[89] Bone repair [90–97] – BMP2 [90–97] Wound healing [98,99] – FGF2[98], HB-EGF [99] Cartilage repair [92,100,101] – BMP2[92],TGF-β3[100] Vascular reconstruction [49,102–117] – SDF-1α[103,104], VEGF[102,108–114], FGF2[115,116], Shh+IL-10[117] Tendon reconstruction [118–120] – PDGF-BB[118–120] Nerve growth [121–123] – GDNF[121], NGF [121–123] Cancer detection and apoptosis [124,125]
HS		Basement membranes Cell surface of all cell types ECM of all tissues	Cell differentiation and proliferation Cell-cell interactions Tissue morphogenesis and organ function Interactions with GFs, cytokines and cell adhesion molecules Inflammation	Bone repair [126] – BMP2 [126] Cartilage repair [127–129] – BMP2[127–129] Vascular reconstruction [110,130–133] – VEGF[110,130], FGF2[132,133] Wound healing [130] – VEGF[130] Stem cell therapy enhancement [10]
HA		Synovial fluid Articular cartilage	ECM assembly [134–136] Resilience to compression	Wound healing [70,137–139] – HB-EGF [139] Cartilage repair [73–80] Bone repair [12,92,140–144] – BMP2[12,92,140,141], SV[143], FGF2 [144]

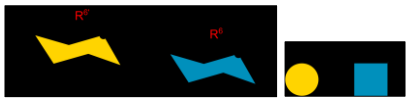
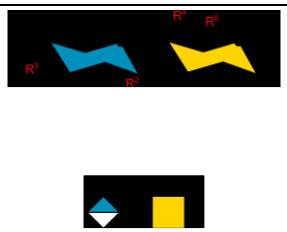
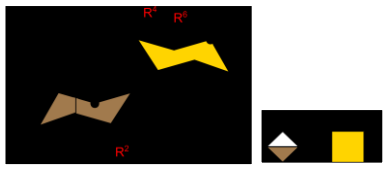
		Skin Vitreous humor ECM of loose connective tissues Umbilical cord	Molecular weight dependent: ▪ pro/anti-cancerous[54] ▪ pro/anti-inflammatory[54] ▪ pro/anti-angiogenic[54] ▪ pro/anti-proliferation[54]	Vascular reconstruction [145–147] Anti-inflammation [148,149] – IL-10[148] Vocal fold repair [150–152] Muscle regeneration [153,154] Lung tissue model [155] – FGF7+FGF10[155] Tendon / ligament repair [156] Nerve growth [157] Adipose tissue engineering [158]
KS		Cornea Bone Cartilage Central and peripheral nervous system	Corneal ECM assembly for light passage and hydration Resilience to compression Guidance of neural growth and regeneration	
CS		Cartilage Bone Brain Heart valves	Elasticity and anti-inflammatory properties Guidance of neural growth and regeneration Angiogenesis (CS-E)	Wound healing [82,98,159,160] – FGF2[98] Cartilage repair [77–80,84,85] Bone repair [126,161–164] – BMP2 [126,161,162], BMP4[163], PDGF-BB[164] Nerve growth [165,166] – NGF[165,166] Tendon regeneration [167,168] – IGF-1/GDF-5 [168] Angiogenesis [131]
DS		Skin Blood vessels Heart valves Cornea Tendons Lung	Wound repair ECM assembly with collagen Inflammation Anticoagulation Neural guidance and development Cell proliferation, invasion, and metastasis	Reduction of biofilm deposition [169] Vascular reconstruction [170,171] Bone repair [97,172] – BMP2 [97] Wound healing [173]

Table 1. ((Presentation of the structure, location, biological functions and biomaterial applications of the different GAGs. In the disaccharide unit column are presented both the chemical structure and the representation with the Symbol Nomenclature for Glycans (SNFG). R^2, R^3, R^4, R^6, $R^{6'}$ and R indicate H or SO_3^- groups. Biomaterial applications in green indicate the biomaterials that have already been used in clinical trials for the mentioned application. We specified in light brown which bioactive molecules, such as GFs, have been used in the biomaterials. BMP2/4: Bone Morphogenetic Protein 2/4; FGF2: Fibroblast Growth Factor 2; GDNF: Glial cell line-derived neurotrophic factor; HB-EGF: Heparin-Binding EGF-like Growth Factor; IGF-1: insulin-like growth factor-1; IL-4/-10/-13: Interleukin-4/-10/-13; NGF: Nerve Growth Factor; PDGF-BB: Platelet Derived Growth Factor –BB; SDF-1α: Stromal

cell-Derived Factor 1-Alpha; Shh: Sonic Hedgehog; SV: Simvastatin; TGF-β3: Transforming Growth Factor Beta-3; VEGF: Vascular Endothelial Growth Factor))

3. GAGs in vivo: Importance of tuning GAG density, type and sulfation

In tissues, the type of GAG, the density, the length and sulfation are precisely modulated. These parameters directly affect the interactions with bioactive molecules and consequently modifies cellular responses. Examples of the variation of these parameters in tissues are shown in **Figure 2.** Panel A illustrates differences in the distribution of GAG types, as exemplified with distinct localizations of HS and CS in mice eye sections during embryonic development. Panel B depicts variations in concentration of sulfated GAGs, as in mice growth plate exhibiting well-defined areas with high (dark blue) or low (white) concentrations. Panel C highlights differences of GAG sulfation in tissues. This panel shows glycan fragments detected by MALDI-FT-ICR mass spectrometry in patients with gastric cancer. Some of the glycans detected can be attributed to GAG structures. It is possible to identify a separation between regions containing sulfated or non-sulfated glycans.

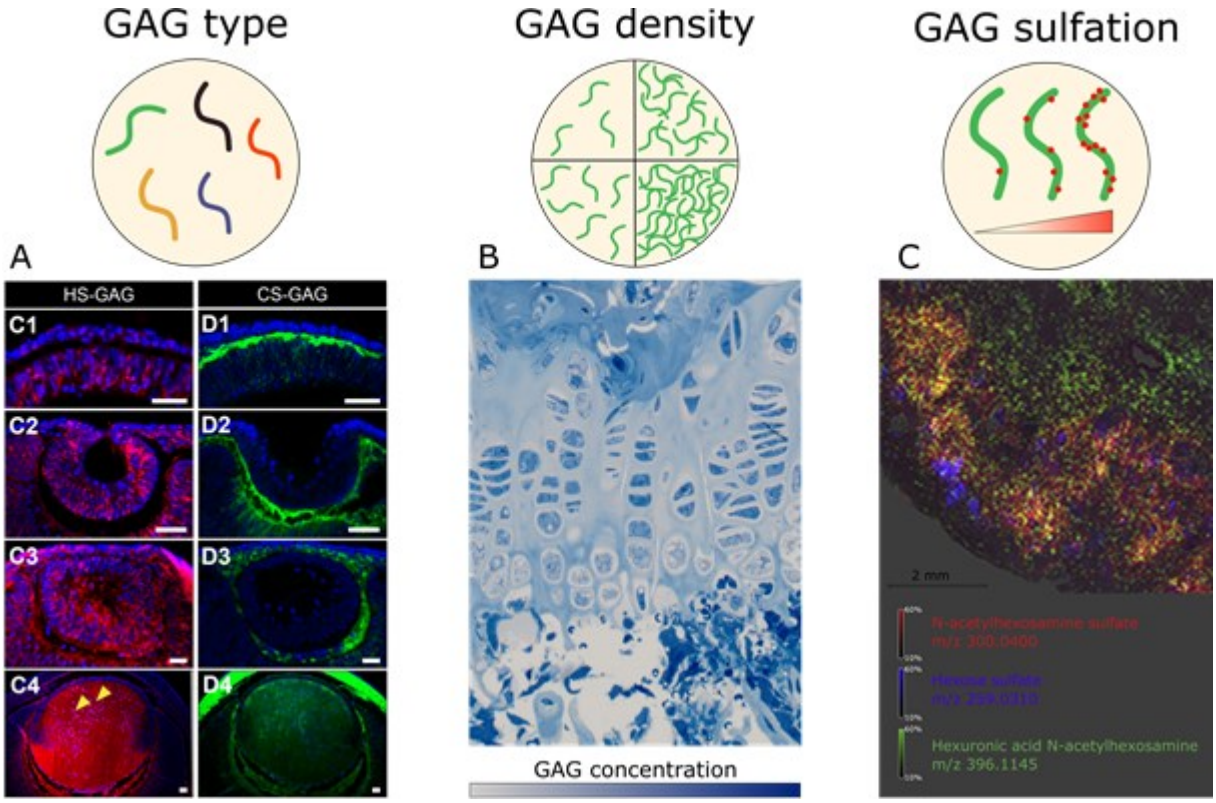


Figure 2. ((Schematic representation of different GAG parameters that naturally vary in animal tissues or cell cultures. A) Immunocytochemical staining of HS and CS in mice eye sections at embryonic days 9.5, 10.5, 11.5 and 14.5. Scale bars are 25 µm. Adapted with permission under terms of the CC-BY license.[174] Copyright 2023, Wishart *et* Lovicu., published by MDPI. B) Toluidine Blue staining representing the density variation of sulfated GAGs in growth plate of wild type mice. Adapted with permission.[175] Copyright 2017, Elsevier. C) Ion map of N-acetylhexosamine sulfate, hexose sulfate, and hexuronic acid N-acetylhexosamine glycan fragments detected by MALDI-FT-ICR mass spectrometry in whole tissue sections from a gastric cancer patient shows a separation between areas with sulfated glycans (red and blue) and without non-sulfated area (green). Adapted with permission under terms of the CC-BY license.[176] Copyright 2017, Kunzke *et al.*, published by Oncotarget))

3.1 Importance of GAG type and density *in vivo*

As seen in **Table 1**, GAG prevalence varies across tissues and fluids. In animal tissues, the density and distribution of GAGs is varying depending on tissue type but also throughout lifetime.[177,178] The spatiotemporal distribution of GAGs within tissues is a highly dynamic process, tightly regulated during development, homeostasis, and in response to various physiological and pathological stimuli. It relies on the fine balance between GAG biosynthesis and metabolism, exerting significant influence on tissue-specific mechanical properties, cell signaling, and overall tissue functions. As such, GAGs are intricately involved in tissue patterning, organogenesis and formation of morphogen gradients (hedgehogs, TGF-β, Wnt), which are particularly important in early development.[61,179–182]. Sulfated GAGs distribution also plays a crucial role in wound healing, where they orchestrate cell migration, proliferation, and differentiation. Additionally, production of HA at the wound site enables formations of a provisional matrix supporting tissue repair.[183] In contrast, alterations in the spatiotemporal distribution of GAGs have been frequently associated with various pathological conditions. For example, in osteoarthritis, an imbalance in the synthesis and degradation of CS leads to the degradation of cartilage ECM, resulting in joint degeneration.[184] Similarly, altered HS biosynthesis and/or catabolism have been associated with tumor growth, angiogenesis, and metastasis.[185–187]

3.2. Importance of sulfation in GAG-proteins interactions

GAGs binds to a multitude of proteins, including growth factors, cytokines, and chemokines.[43,44,60] Upon binding, GAGs can trigger different mechanisms regulating protein activity, such as protection from degradation, induction of conformational changes activating the protein function, or formation of complexes with cellular receptors. Protein/GAG interactions are mainly, though not exclusively, of electrostatic nature, involving negatively charged carboxyl and sulfate groups of the polysaccharides and clusters of basic amino acids at the surface of the proteins. Consequently, the binding properties of GAGs are tightly linked to their sulfate density and distribution.[44,188] The degree of GAGs sulfation varies among GAGs.

Hep is the most sulfated among GAGs, with approximately 1.6 to 3.0 sulfates per disaccharide unit.[189,190]. HS is less sulfated than Hep (between 0.4 and 2.0 sulfates/disaccharide) and features a distinctive molecular organization, in which non or low sulfated and modified (NAc domains) regions alternate with highly sulfated domains (NS domains).[38] CS and DS galactosaminoglycans can be variably sulfated, with different sulfation combinations classified by letters as specific units (A, B, C, D, E, etc…, see **Figure 3**). [191,192]. CS and DS can be distinguished by the nature of their disaccharide unit hexuronic acid, which is glucuronic acid for CS, and iduronic acid for DS.[9,190] Of note, DS was first been referred as CS-B, before the classification changed. Finally, KS structure is atypical (a galactose residue replaces the hexuronic acid in the disaccharide unit), for an overall sulfation degree is comprised between 0.9 and 1.8 sulfates/disaccharide.[190]

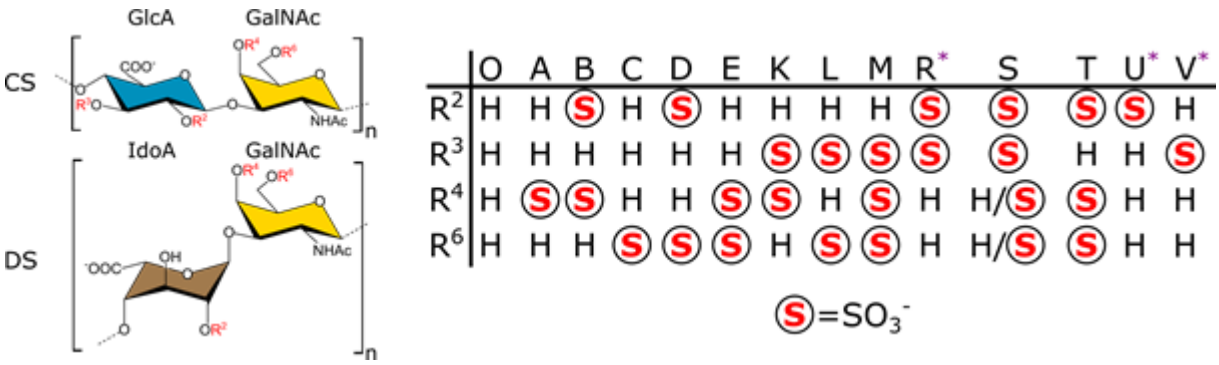


Figure 3. ((Sulfate combinations in CS/DS chains described with the O-,A-,B-,C-,D-,E- main units and the more rare K-,[193,194] L-,[193] M-,[193,195] R-,[196] S-,[197] T-,[198] U-,[199] and V-[199] units. The star symbol * indicates a unit that was synthesized but not identified nor extracted from natural source.))

However and despite tremendous interest, the vast structural heterogeneity of GAGs and the lack of dedicated tools have constituted severe limitations in the structural and functional characterization of GAGs and GAG/protein interactions.[200] The most documented example illustrating the impact of specific GAG motifs on proteins is the role of Hep, which activates Antithrombin III (AT III) by inducing a conformational change of the protein.[201] Significant efforts have been dedicated to identifying the precise structure within Hep responsible for mediating this process, and established that AT III bio-

activation is highly specific to Hep pentasaccharide sequences featuring a central 3-O sulfated glucosamine.[202–204] Compounds exhibiting this sulfation motif are therefore of particular interest for their anticoagulation properties.

Interactions of HS with members of the fibroblast growth factor (FGF) family, notably FGF1 and FGF2, have also been extensively studied. In 1991, two independent research groups demonstrated that cell-surface HS is a co-receptor of FGF2 and is required for the growth factor biological activity.[1,205] Subsequent studies highlighted distinct structural features for the binding to these growth factors. The interaction between FGFs and HS requires saccharide motifs of similar size (5-6 sugar units) and saccharide content (involving N-sulfated and 2-O-sulfated IdoA residues).[206,207] Interestingly, 6-O-sulfates were found essential for binding to FGF1, but not to FGF2.[208,209] FGF2 activity required presence of 6-O-sulfates and longer saccharide fragments (10-12 sugar units).[210] These observations therefore highlighted different structural requirements associated to FGF1 versus FGF2-induced biological responses, providing the first evidence of an uncoupling between HS binding properties and ligand-promoting activities. Structural imaging of the FGF/FGFR/HS complex structure by X-ray crystallography showed that 6-O-sulfates contributed to the stabilization of the ternary complex by establishing contacts with the FGFR.[211] Overall, a wealth of studies also demonstrated the importance of sulfation degree and specific sulfation motifs governing protein/GAG interactions in various biological contexts. These include regulation of the bioactivity of many signaling proteins, including cytokines and chemokines, members of the FGF family [212], hepatocyte growth factor (HGF), VEGF, IGFs and cell membrane receptors and coreceptors.[8,201]

In a study by Gama *et al*., CS-A, C, E and R (an unnatural GAG, which exhibits the same degree of sulfation as CS-E, but at different positions) tetrasaccharides were compared for their binding affinity to brain-derived neurotrophic factor (BDNF), midkine and FGF1.[196] Authors showed that CS-E exhibited the highest affinity with BDNF and midkine, while midkine and BDNF did not bind to CS-R and FGF1 did not bind to any CS. This demonstrates that the binding is not just related to non-specific charge interactions, but requires specific sulfation patterns. These results therefore support the concept of a 'sulfation code' wherein GAGs encode functional information in a sequence-specific manner, akin to DNA, RNA and proteins.

4. Modulation of GAGs parameters for biomaterials design

Given the inherent variability in GAG type, density and sulfation profiles, a rational design of biomaterials in relation to the specific *in vivo* attributes of the targeted tissue is critical for biomedical applications. Thus, biomaterial scientists are considering these parameters when engineering GAG-based biomaterials (**Figure 4**).

4.1 Modulation of GAG type in biomaterials

Incorporating GAGs into biomaterials can directly influence stem cell fate directly by activating some specific cell response, or indirectly by modulating the binding and activity of GFs. Given the distinct structural and functional properties of GAGs, along with their interactions with specific partners at varying affinities, it is expected that each GAG may differently modulate cellular responses.

When considering a biomaterial application, the choice of which GAG to incorporate should be made judiciously to elicit the desired effects (**Table 1**). Surprisingly, only a limited number of studies have addressed the effect of different GAGs on specific cellular processes. Dewey *et al.* investigated the effect of various GAGs incorporated into collagen scaffolds on osteogenic differentiation.[213] They found that ALP level and BMP2 gene expression, two markers of bone formation, were enhanced on Hep and CS-C scaffolds compared to CS-A scaffolds. Interestingly, CS-C notably increased the expression of SOX9, a marker of chondrogenic differentiation, while Hep markedly enhanced the expression of Osterix, a bone marker. These results therefore indicate that different GAGs may promote the formation of distinct tissues. In another study, Dewey *et al.* assessed the impact of different GAG types incorporated into collagen scaffolds on endothelial tube formation. They showed that CS-C induced the most robust endothelial tube formation compared to Hep and CS-A, which was correlated with higher VEGF expression.[214] (**Figure 4A**). All GAGs exhibited anti-inflammatory activities, but CS-C scaffolds lead to the most pronounced reduction in IL6 levels, as well as the strongest transition of macrophages from M1 to M2 phenotype. In the same study, Hep showed the

best potential to inhibit osteoclastogenesis. In another study, chitosan membranes grafted with various GAGs (HS, Hep, DS, CS-A, CS-C and HA) were used to investigate the effects on hMSCs adhesion and of osteogenic, adipogenic and chondrogenic differentiation induced by TGF-β.[215] Results showed that hMSCs adhered more effectively on HS and HA compared to other GAGs. Furthermore, adipogenic differentiation was predominantly enhanced by HA-chitosan membranes, albeit only at the lowest GAG density. The addition of TGF-β to the different GAG-modified chitosan membranes revealed that Hep and HA primarily induced chondrogenic differentiation of MSCs, compared to CS-A. With the aim of developing a biomaterial for tendon tissue engineering, Hortensius *et al.* engineered collagen-GAG scaffolds *via* freeze drying with either HA, CS or Hep.[168] They analyzed the cell proliferation and the expression of phenotypic markers in tenocytes cultured in these scaffolds containing IGF-1 growth factor. The authors observed an increase of COL1A2 proliferation marker associated with a decrease in tenascin-C tendon phenotypic marker with Hep, in contrast to HA-scaffolds. Due to the limited amount of studies which compared the role of GAGs on cellular responses, it is, so far, complicated to drive conclusions and to recommend a specific GAG for a specific biomaterial application.

4.2 Modulation of GAG density in biomaterials

As previously shown, GAG density is an important parameter for GAG function *in vivo*. Thus, for designing biomaterials, it is crucial to consider the GAG density of the targeted tissue, since it may impact cells behavior and biological responses, ultimately impacting the tissue regeneration properties of the biomaterial. The control of the GAG density in a biomaterial is complicated since it depends on the way of immobilization of the GAG. In a study by Wang and Yang, methacrylated HS and CS at different densities were incorporated in hydrogels of different stiffness to assess effects on the induction of hMSC chondrogenic differentiation.[216] **(Figure 4B**) They observed that chondrogenesis was enhanced with CS compared to HS, particularly in softer hydrogels. The highest neocartilage deposition of collagen types I and II and sulfated GAGs was achieved using intermediate CS concentrations, thus highlighting the importance of precisely regulating GAG density in tissues to induce specific and optimized cellular responses. In another study, collagen-GAG scaffolds were

generated *via* freeze-drying and osteoblasts were cultured on them for seven days.[217] Both collagen and GAG concentrations were adjusted to produce different scaffolds, on which cell number and metabolic activity were analyzed. Results showed that the highest metabolic activity and cell number were attained with the highest GAG concentration scaffolds. Another interesting study used PG-mimetic graft copolymers with tunable GAG density.[218] Both CS- and Hep-containing graft copolymers successfully delivered FGF-2 to cells, with the Hep low-density copolymer outperforming higher-density counterparts. Such biomaterials could be of great interest for future investigations into the role of GAG density directly on PGs' core protein. Nevertheless, further investigations on GAG density in biomaterials are greatly needed for achieving a more accurate replication of tissue structure and functions.

4.3 Modulation of GAG sulfation in biomaterials

Given the importance of GAG sulfation *in vivo* (see part 3), the control of sulfation degree and patterns of GAGs in biomaterials is of paramount importance for defining their biological properties (**Figure 4C**). Cellular studies using CS tetrasaccharide coatings showed that CS-E enhanced neurite outgrowth of different types of neurons including dopaminergic, DRG and hippocampal neurons.[196] This is explained by the ability of CS-E sulfation sequence to recruit specific growth factors to the cell surface, thereby activating downstream signaling pathways. In another study Tellier *et al.* coated micro particles with either Hep, N- or N,6-O-desulfated Hep or fully desulfated Hep to assess how Hep sulfation pattern regulates BMP2-induced ALP expression in C2C12 cells (**Figure 4C**).[219] The microparticles coated with the most sulfated compounds (Hep and N-desulfated Hep), combined with BMP2, elicited enhanced ALP expression compared to soluble BMP2. Conversely, microparticles carrying N,6-O-desulfated Hep and fully desulfated Hep failed to promote ALP expression with BMP2. This suggests that N-sulfation may not be crucial for efficient Hep-mediated BMP2 bioactivity. However, it is worth noting that another study did not support these findings, showing that N-sulfation of HS plays a significant role in both the binding and bioactivity of BMP2.[220] In this latter study, oligosaccharides of different sizes were used to define the minimum HS oligosaccharide length

required for BMP2 binding. SPR competitive binding experiments were then performed to assess the ability of selectively desulfated Hep to compete with surface-bound Hep for binding to BMP2. Results showed that N-sulfation is critical for BMP2 binding, followed by 6-O and 2-O sulfations. C2C12 cells were exposed to BMP2 that had been previously bound to soluble oligosaccharides, and BMP2 bioactivity was assessed by measuring osteogenic gene transcription, ALP activity at 5 days, and mineralization at 12 days. Results further supported the importance of N-sulfation. It is worth noting that in this study, the oligosaccharides were presented in solution for *in vitro* assays, and on a polycaprolactone tube combined with a collagen sponge for *in vivo* assays. Since GAGs are normally presented *via* the protein core of PGs, these different presentation modes of the GAG oligosaccharides may influence their interactions with BMP2. Likewise, in the study of Tellier *et al.*, the Hep was neither in the ideal biomimetic conformation, since it was not grafted by its reducing end but was interacting with the microparticles via the multiple thiolation of Hep derivatives. The same group developed a cell coating with layers of biotin and avidin functionalized directly on MSC aggregates, onto which biotinylated Hep were attached, to bind TGF-β1 or FGF2 growth factors.[221] In this study, the authors observed that FGF2 loaded on highly sulfated Hep triggered greater proliferation compared to FGF2 loaded on desulfated Hep. With TGF-β1, they observed a significant increase of chondrogenic differentiation, with strong upregulation of Collagen II and Collagen X gene expression. Interestingly, this effect was more pronounced with the low-sulfated Hep than with the high-sulfated one, providing further evidence of the crucial role of sulfation. Another study demonstrated that porcine aortic endothelial cell proliferation was more effectively stimulated by collagen hydrogels containing sulfated-HA compared to HA hydrogels, independently of the presence of $VEGF_{165}$.[222] Atallah *et al.* developed injectable hydrogels displaying Hep chains with variable sulfation patterns and observed the proliferation and migration of hMSCs.[223] Depending on the Hep sulfation pattern, distinct gradient distributions of platelet-derived growth factor-BB (PDGF-BB) were established, due to different interactions dynamics with the GF. They subsequently observed a slight increase in MSC proliferation and migration for the Hep hydrogels compared to the control, and a significant increase in proliferation and migration for the hydrogels with N,6-O-desulfated Hep that correlated well with the time dependent difference in the PDGF-BB gradient formed in the various hydrogels. In a study of Feng *et al.*, the chondrogenic differentiation of hMSCs encapsulated in methacrylated HA-hydrogels was investigated based on the sulfation degree of chemically modified HA.[224] After 14 and 28 days of

culture in chondrogenic medium with TGF-β1, the expression of aggrecan and ColII chondrogenic genes was increased in correlation with HA sulfation degree. A similar study compared the chondrogenic differentiation of MSCs encapsulated in PEG-based hydrogels containing CS or its non-sulfated form chondroitin. Surprisingly, they obtained opposite results in relation to GAG sulfation. MSCs in chondroitin hydrogels exhibited significantly greater gene expression of collagen II and aggrecan compared to CS hydrogels after 21 days of culture in chondrogenic medium containing TGF-β1. They even observed a greater early expression of SOX9 on day 7, suggesting that non-sulfated chondroitin materials may promote chondrogenic differentiation more efficiently than CS-materials.[225] Therefore, the choice of GAG sulfation degree or pattern for biomaterial applications should be carefully considered, depending on the specific tissue targeted for engineering applications. The sulfation pattern of GAGs has been consistently studied for a significant number of growth factors. The development of novel methods for synthetizing defined sulfated GAGs and oligosaccharide structures has made these advances possible.

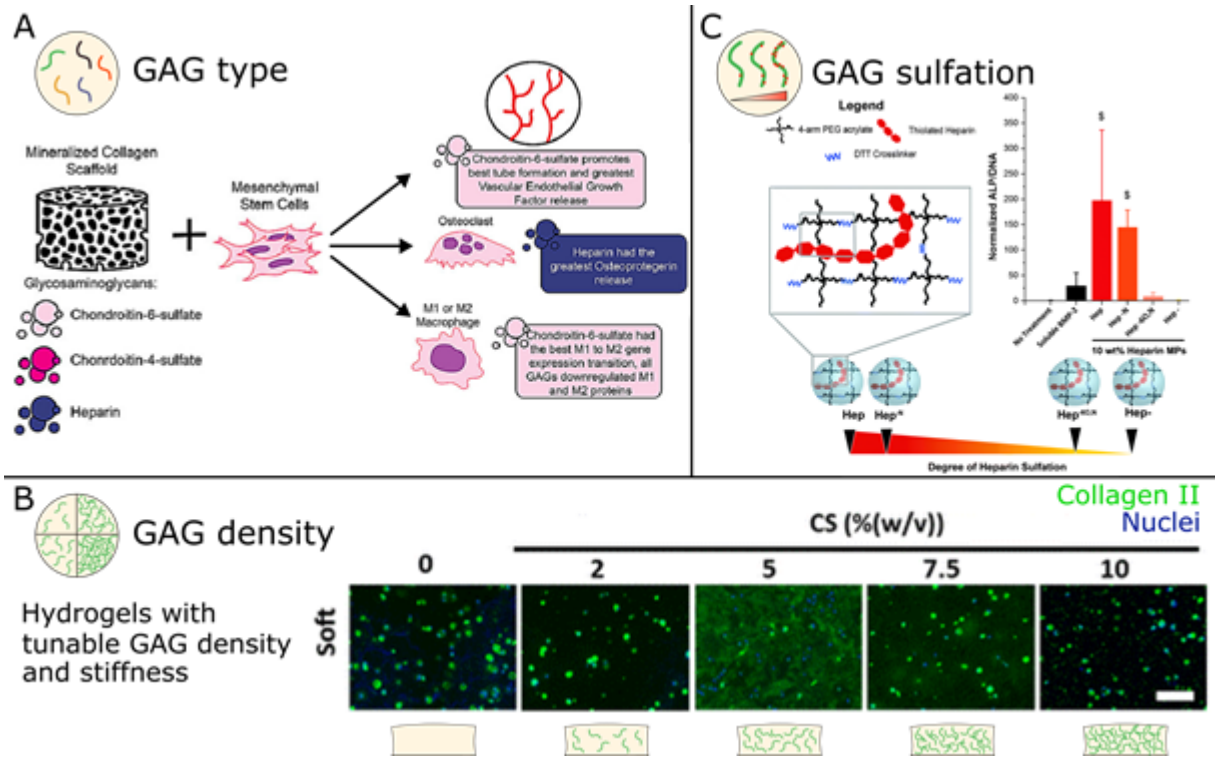


Figure 4. ((Various materials modulating specific GAG parameters to optimize their use for biomedical applications. A) Modulation of GAG type in collagen-GAG scaffolds induces distinct differentiation pathways. Adapted with permission.[214] Copyright 2021, Elsevier. B) Modulation of GAG density in hydrogels induces a stronger chondrogenic response for intermediate concentrations of CS, with a more important deposition of collagen II. Scale bar is 200 µm. Adapted under terms of the CC-BY license.[216] Copyright 2017, Wang et Yang, published by Springer Nature. C) Modulation of GAG sulfation degree/pattern in microparticles with BMP2 induce sulfation-dependent alkaline phosphatase (ALP) expression. Adapted with permission.[219] Copyright 2015, The Royal Society of Chemistry))

5. Synthesis of defined sulfated GAGs and oligosaccharides

A major challenge for studying GAGs and their mechanisms lies in the preparation of pure and well-characterized GAG materials, with defined length or sulfation patterns. To address this, various strategies can be adopted (**Figure 5**). We have listed and assessed these strategies (**Table 2**) based on several key parameters. These include the simplicity and efficiency of their implementation, the degree of purity achieved and the control of the obtained GAG structures in terms of length, sequence and sulfation. We have also evaluated them on a "versatility" criterion, by assessing their potential for translation in other research applications, such as cell-based assays, complete proteoglycan synthesis, or *in vivo* assays necessitating substantial quantities of material.

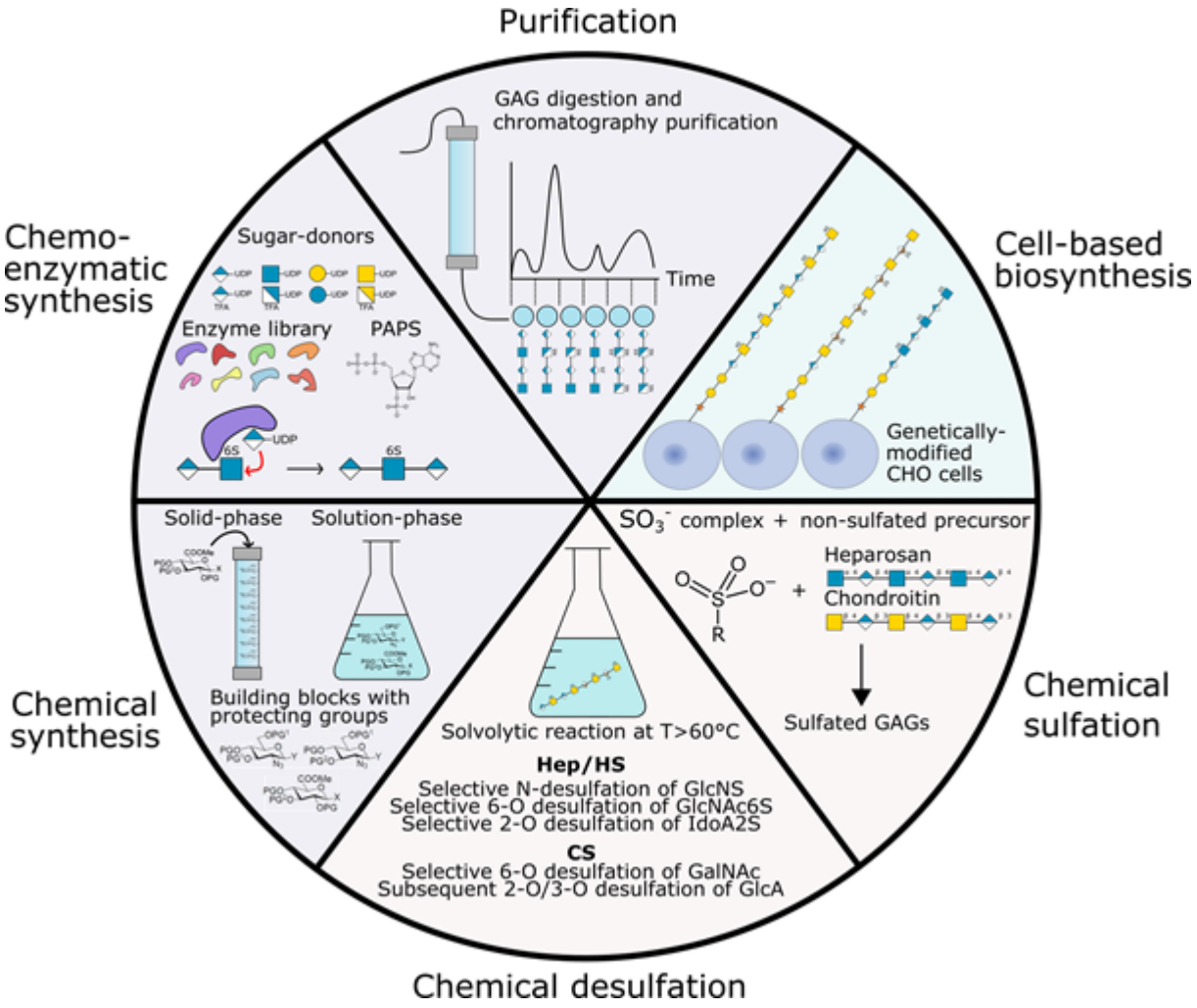


Figure 5. ((Schematic representation of the various methods for the preparation of GAG compounds with defined length, type of sulfation and sulfation pattern. The two beige-background approaches are commonly used chemical methods for modifying GAG sulfation, using natural sources of polysaccharide. Chemical desulfation consists in using regioselective solvent-based reactions to selectively remove sulfate groups at specific positions. Conversely, chemical sulfation uses SO_3^- complexes to add sulfate groups at specific locations on the GAG disaccharide unit. An alternative strategy is the recently reported cell-based biosynthesis approach (shown with a blue background), which involves manipulating CHO cells through knock-in or knock-out of various GAG-biosynthesis genes, to produce cell-surface GAGs with tailored sulfation patterns. The three light purple-background methods are particularly adapted for the synthesis of GAG oligosaccharides. The

"purification" approach consists in using a natural source of GAGs. GAG chains are depolymerized with digestion enzymes, and the generated oligosaccharides are purified according to size or charge using chromatography techniques. The chemo-enzymatic synthesis approach is based on the use of recombinant polymerase enzymes with sugar donors to generate oligosaccharide chains or sulfotransferases with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) sulfate donor to introduce sulfate groups at specific positions of the disaccharide motif. Alternatively, full chemical synthesis methods are use combinations of monosaccharide or disaccharide building blocks to generate structurally defined oligosaccharides.))

5.1. Specific desulfation

Methods for specific desulfation of sulfated carbohydrates can use different chemical strategies, including acid-catalyzed desulfation, solvolytic desulfation, alkali-catalyzed desulfation, and desulfation mediated by silylating reagents.[86,226–228]

For Hep and HS, chemical treatments have been developed to achieve specific 2-O, 6-O, or N-desulfation and are now commonly used.[223,229–234] N-desulfation is typically carried out through solvolytic desulfation with dimethyl sulfoxide (DMSO) containing methanol, without depolymerization of HS chains and minimal O-desulfation.[235] Specific 2-O desulfation is generally achieved using sodium hydroxide treatment.[231] Although this treatment does not alter other sulfation motifs, it leads to chain depolymerization, resulting in a ~25% reduction of Hep chain molecular weight.[231] The 6-O specific desulfation is generally performed using N-methyl-N-(trimethylsilyl)-trifluoro acetamide (MTSTFA) silylating reagent, enabling control of the desulfation degree through temperature and reaction time.[236,237] Complete 6-O desulfation of Hep results in a loss of ~20% of 2-O sulfate groups, without any other structural alteration or depolymerization.[238] In contrast, the specific removal of 3-O sulfate groups cannot be achieved without affecting other sulfation sites. Lyophilization of Hep under extreme alkaline conditions induces selective loss of 2-O and 3-O sulfates, while leaving intact 6-O and N-sulfation.[239] These strategies are suitable for examining the overall importance of one type of sulfation, but do not allow the study of precise sulfation sequences. Another strategy for the

selective desulfation of HS involves digestion of HS or Hep by sulfatase enzymes.[240] Human Sulf1 and Sulf2 (HSulf1 and HSulf2) are the only known extracellular post-synthetic HS modifying enzyme, with a substrate specificity for 6-O sulfation motifs. More precisely, HSulf2 essentially targets HexA(2S)-GlcNS(6S) trisulfated disaccharides, which are then converted into HexA(2S)-GlcNS disaccharides. HSulf2 has been shown to reduce dramatically the FGF1/FGF2-induced proliferation of FGFR1-IIIc-transfected BaF32 cells.[241] This result supports further the previously reported requirement of HS 6-O sulfation for promoting the bioactivity of these growth factors.[210,242] In addition, the authors showed that HSulf2 differentially regulated FGF1 and FGF2, thereby highlighting the involvement of specific 6-O sulfation pattern in these mechanisms.[241] However, this enzymatic desulfation approach is limited by the lack of sulfatases with alternative substrate specificities. The identification of new enzymes may pave the way for the development of novel strategies for preparation of GAGs compounds.

Chondroitin, the non-sulfated version of CS can be obtained from the reaction of CS with acidic methanol,[225,243] or in DMSO with a small amount of methanol.[244] These desulfation methods can also be applied to other GAGs, such as DS or KS.[232] For CS, regioselective methods for 4-O and 6-O desulfation have been established.[245] The 6-O desulfated CS can be obtained with MTSTFA agent without any depolymerization. A DMSO/methanol-based method was recently reported as an efficient regioselective method for the preparation of 4-O desulfated CS, albeit with some depolymerization. Surprisingly, the conditions used are very similar to the method of Nagasawa *et al*. for the preparation of chondroitin *via* global 4-O and 6-O desulfation of CS,[244] suggesting that minor protocol modifications may affect the reaction.

5.2. Specific sulfation

An alternative strategy for studying sulfation of GAGs involves the chemical sulfation of non-sulfated precursors such as HA, heparosan (non-sulfated HS) or chondroitin (non-sulfated CS).

For CS, the regioselective sulfation at the C-6 hydroxyl of the GalNAc with limited sulfation at the C-2 hydroxyl of the GlcA can be achieved using SO_3^--pyridine complex in dimethylformamide (DMF) at 0 °C.[246,247]

For HS, chemically sulfated chains can be obtained from the sulfation of heparosan. Effective O-sulfation requires prior N-sulfation of the heparosan chain. First, heparosan must undergo N-deacetylation with NaOH, followed by N-sulfation in Na_2CO_3 at 40-50 °C with SO_3^--pyridine complex or SO_3^--trimethylamine complex. The resulting N-sulfated HS can then be regioselectively O-sulfated at the C-6 hydroxyl of the GlcNS, with residual sulfation at the C-2 or C-3 hydroxyls of the GlcA.[226] This approach has been used in other studies for sulfation of HA with similar regioselectivity of the C-6 hydroxyl of the GlcNAc, followed by sulfation at the C-4 of the GlcNAc and at the C-2 and C-3 of the GlcA.[224,248,249]

5.3. Cell-based biosynthesis

Due to the complexity of GAG structures, recent research has turned towards producing GAGs using genetically modified cells to avoid complex chemistry or chemo-enzymatic steps. Dr. Jeffrey Esko's pioneering work in the 1980s in genetically modifying CHO-K1 cells laid the foundation for the establishment of a comprehensive cell library with specific knock-in (KI) and knock-out (KO) of GAG biosynthesis enzymes. Such a library referred to as the GAGOme, has been recently developed by Chen *et al.* and comprises various cell lines with distinct CS/DS and HS biosynthetic capabilities.[250] In total, 28 different genes of biosynthesis enzymes have been targeted to generate cell-lines displaying unique GAG structural features. However, while the recovery of GAGs from the cell lysates is feasible, the purity of the samples obtained remains an issue and could be affected by the presence undesired components. Additionally, GAG-biosynthesis enzymes may not catalyze structural modifications on all available substrates, yielding intra-variation in GAG chains with enzyme-processed and -unprocessed domains. Nevertheless, this genetic approach is highly versatile (Table 2) and holds huge potential as it allows for direct cell-based assays, or to synthesize entire PGs and xyloside-primed GAG chains, which can subsequently be used in microarray applications (see Section 6).

5.4. Preparation of well-defined GAG oligosaccharides

5.4.1. Purification

While natural GAGs from extractions offer a wide range of structural diversity and physiological relevance, they also exhibit significant structural heterogeneity. Alternatively, libraries of oligosaccharides with defined size and charges can be generated using a combination of different depolymerization and purification strategies. For this, GAG chains are first depolymerized into smaller fragment through enzymatic digestion. Different enzymes can be used, yielding different fragment structures. For example, heparinase III enzyme primarily cleaves HS/Hep in the low-sulfated NAc regions, releasing highly sulfated fragments from the NS domains. GAG samples are then purified by size-exclusion chromatography to separate them by their polymerization degree (dp2, dp4…). The generated oligosaccharides can be further separated according to charge by various chromatography techniques, such as anion-exchange chromatography or reverse-phase ion pair liquid chromatography, to isolate fractions with distinct sulfation patterns (for reviews, see [251–253]). However, due to the inherent charge heterogeneity of related oligosaccharides, it is likely to collect fractions comprising co-eluting compounds. To improve separation resolution, columns modified with cetyltrimethylammonium salts (CTA-SAX) were used in combination with volatile ammonium bicarbonate salt and enabled distinction of Hep hexasaccharides isomeric structures, which could not be achieved using conventional methods.[254] Another advantage of the strategy is the straightforward removal of the volatile ammonium salt through evaporation rather than by dialysis, reducing considerably sample loss and enabling direct coupling to mass spectrometry analysis. Polyacrylamide Gel Electrophoresis (PAGE) has also demonstrated interesting separating properties, achieving resolution of oligosaccharide species that could not be differentiated with conventional chromatography methods. Consequently, oligosaccharide co-eluting species obtained by strong-anion exchange high-performance liquid chromatography could be further resolved by PAGE, yielding pure compounds.[255] The preparation of oligosaccharide libraries using these purification strategies requires both time and expertise, but is more straightforward and accessible compared to oligosaccharide synthesis approaches (Table 2). However, access to specific oligosaccharide structures

is limited to those present in naturally occurring GAGs, by the resolution limits of the available separation techniques, especially for closely related structures and large oligosaccharides. Furthermore, the quantities of oligosaccharide that can be produced remain limited by the scale-up capacities of preparative chromatographies.[256] Another general constraint is that selective depolymerization enzymes are available for HS but not for CS. Consequently, libraries of CS oligosaccharides with defined structures still remains very challenging to produce.

5.4.2. Chemo-enzymatic synthesis

Chemo-enzymatic synthesis combines chemical reactions with the use of natural GAG-biosynthesis enzymes for oligosaccharide chain elongation and sulfate transfer. Thanks to the unique properties of these enzymes, this approach circumvents the technical challenges of chemical synthesis (see next section) such as regio- and stereo-selectivity, and avoids the need for numerous steps of group protection and de-protection for the transfer of sulfate groups at specific positions.

The activity and functional specificities of a large number of GAG biosynthesis enzymes are well characterized and have facilitated their use for the production of defined oligosaccharides. Elongation enzymes catalyze the transfer of monosaccharide compounds with the adequate glycosidic linkage. These enzymes rely on the presence of uridine diphosphate (UDP) sugar donors such as UDP-GlcNAc, UDP-GlcA, UDP-Glc, UDP-Gal or UDP-GalNAc. Sulfation enzymes transfer sulfate groups at specific position using 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as sulfate donor. The order of execution of successive reactions must be carefully designed to be compatible with the enzyme substrate specificities and to improve reaction yields. Various reviews provide more in-depth information about chemo-enzymatic synthesis and the different aspects of the approach.[257–260] While highly effective, the chemo-enzymatic strategy is complex and requires strong expertise in the production of recombinant biosynthesis enzymes, their roles and substrate specificities. Furthermore, the preparation of defined oligosaccharides requires the availability a wide range of enzymes, sugars and sulfate donors, representing a considerable initial investment. Although complex and time-consuming, this method yields oligosaccharides with exceptional purity and controlled structure. Quantities produced with this approach are suitable for *in vivo* assays, therefore accounting for its

versatility potential in Table 2. The enzymatic approach may also be used for sulfation of large GAG polysaccharide chains starting from non-sulfated heparosan or chondroitin, or to increase specific sulfation types in natural GAG samples.[261,262]

5.4.3. Chemical synthesis

The chemical synthesis of GAG oligosaccharides is based on the use of monosaccharides or disaccharides building blocks with protection groups in well-defined positions. Chain elongation is achieved by assembling these building blocks while controlling the stereochemistry of the glycosidic bonds formation and sulfation of specific positions is achieved following de-protection of the targeted groups. The introduction of hexuronic acid moieties is however an additional challenge due to their low reactivity. Chemical synthesis can be carried-out either in solution-phase or on-resin (automated solid-phase synthesis) to facilitate the removal of side products after each reaction. More details of the different strategies of chemical GAG oligosaccharide synthesis have been reviewed elsewhere.[263–265] The control of the various steps of the process is highly challenging and requires strong expertise in chemistry to properly design the successive reactions. Only a few research groups worldwide have the equipment and skills to achieve the chemical synthesis of large oligosaccharide libraries. However, the purity and the structural control of the chemically synthesized oligosaccharides are excellent.[266] Similar to chemo-enzymatic synthesis, quantities obtained are substantial and therefore compatible with *in vivo* assays.

5.4.4. Commercial GAG-oligosaccharide libraries

Thanks to the development of the above-mentioned techniques, a wide range of GAG compounds and oligosaccharides with defined length and sulfation pattern are readily accessible through commercial sources (Biosynth, Creative Biolabs, Glycan Therapeutics, Iduron…). Since most of these commercially available compounds are produced using chemical, chemo-enzymatic and to a lesser extent purification approaches, commercial libraries facilitate access to defined oligosaccharide structures with high purity. Although the structural diversity is limited to the available compounds, the repertoire

of structures is expanding rapidly and some companies offer custom synthesis service, which may provide a solution to address specific structural requirements. Furthermore, the cost of oligosaccharide compounds is relatively low compared to the time and effort required for the preparation of oligosaccharide libraries.

	Simplicity	Time saving	Purity	Control of GAG structure	Versatility
Chemical desulfation	++	++	-	-	-
Chemical sulfation	++	++	-	-	-
Cell-based biosynthesis	-	-	-	+	++
Chemical synthesis	-	-	+++	+++	+
Chemo-enzymatic synthesis	-	-	+++	+++	+
Purification	+/-	+/-	+	++	+/-
Commercial libraries	+++	+++	+++	+	-

Table 2. ((Comparison of the different methods for the production of defined GAG compounds based on various criteria: simplicity to establish the technique in a laboratory, time saving characteristics, purity of the produced materials and control over the structural features of the GAG materials produced, in terms of sulfation and length. The methods are also compared upon a "versatility" criterion that represents their potential applicability in various experimental contexts, such as cell-based assays, whole PG synthesis or *in vivo* assays. The two methods in light brown font correspond to straightforward chemical approaches typically used for sulfation modification of large GAG chains. The cell-based biosynthesis method, as the only genetics-based method, is shown with a blue font. The three methods in purple are particularly adapted for the synthesis of GAG-oligosaccharides. Commercial libraries, in black font, have emerged from the development of these other methods.))

6. Characterization and quantification of GAGs: from molecular to cellular interactions with GAG-based biomaterials using high content tools

As discussed earlier, the extensive functional repertoire of GAGs are intricately linked to their structure and sulfation patterns. This underscores the necessity for precise structural information on naturally occurring GAGs, for tailoring GAG structures in biomaterials to achieve specific biological properties. However, because of their natural diversity and complexity, quantifying and characterizing sulfated GAGs from tissues remains a formidable challenge, greatly impeding the development of new biomaterial applications.

As described above, various biomaterials have been developed to study different parameters of GAG presentation and structure, but only few studies combine molecular information with cellular responses. Both approaches are time consuming and complicated to achieve within the same experiment. Compared to genetics and proteomics, the field of glycobiology needs new tools and innovative biomaterials to accelerate and enhance the throughput for both molecular and cellular studies.

In this section, we review the state-of-the-art techniques used for the identification and characterization of GAGs, their sulfation pattern, and GAG-protein interactions, including their binding dynamics. We then present the recent developments using automation to study GAG-growth factor interactions and to quantify cellular responses to GAG-based biomaterials

6.1 Quantification of GAGs in tissues.

Quantification of GAGs in tissues can be complex. Determining GAG concentrations in body fluids such as urine plasma and serum is relatively straightforward and can be performed with ultra-high-performance liquid chromatography (UHPLC), coupled with mass spectrometry or fluorescent derivatization detection techniques.[175,267,268] In soft and solid tissues, however, assessing GAG

content is more complex, and only few quantitative values of tissue-specific GAG density are available in the literature, especially for human tissues. Various colorimetric assays such as Azure A, toluidine blue and 1,9-dimethyl-methylene blue (DMMB) enable the quantitative measurement of sulfated GAG content. It is however worth noting that these assays may be biased by other non-sulfated poly-anions such as DNA, RNA or HA, as well as variations in sulfation degree between GAG samples and standards.[269,270] Alternatively, the chemical carbazole assay allows for the recognition of all GAGs except KS, without interference from GAG sulfation.[270] All these assays require pre-digestion of tissue samples and generally a purification step to reduce possible artifacts of the assays caused by chloride ions, or other poly-anions. In clinics, several relatively non-invasive techniques based on MRI or computed tomography (CT) have been developed for the estimation of GAG concentration in cartilage.[271–274]

For structural analysis, specific monoclonal GAG-antibodies can be employed on explant tissues to qualitatively evaluate GAG composition, spatial distribution and sulfation. However the heterogeneity of GAGs and the unclear epitope recognition specificities of anti-GAG antibodies limit their use for quantitative measurements.[55,275,276] The current gold standard for naturally occurring GAG structural studies is the disaccharide composition analysis, which has been successfully applied to GAGs purified from tissues, requiring only limited material quantities. This is achieved by exhaustive enzymatic GAG chain depolymerization, and analysis of the generated GAG disaccharides using various standard separation techniques (SAX-HPLC, RPIP-HPLC, HILIC, or capillary electrophoresis…) coupled to MS or fluorescent derivatization approaches.[178,253,277–283] However, compositional analysis only provides partial structural information. Recent developments in analytical approaches are now offering news solutions to address this issue, enabling detailed structural characterization, up to GAG chain sequencing (for review, see Pérez *et al.*, 2023[200]). However, to date, there have been very few reported applications on tissue samples using these emerging technologies.

6.2 Immobilization of GAGs on a support

Advanced screening applications, such as microarrays (see part 6.4) biosensors and biomaterials, require immobilization of GAGs on a support, which can be a 2D surface or a 3D scaffold. For this, site-specific conjugation should be preferred over the physio-adsorption of GAGs on a support, since this strategy preserves GAG native structure and therefore its bioactivity and ability to interact with proteins.[284] As reviewed by Köwitsch *et al.* and Gemma *et al.*, GAGs feature multiple reactive groups that can be used for functionalization, such as the saccharide reducing non-reducing ends.[285,286] For biomaterials applications, functionalization through their reducing end is the most effective and straightforward strategy to achieve end-on (single point) attachment of GAGs. This method of immobilization is the most recommended, as it mimics the natural orientation of GAGs on proteoglycans. In the past, hydrazone ligation was a popular method for conjugating the reducing end of GAGs.[287–289] However, it was found to be inefficient for long GAG chains and unstable in aqueous solution.[290] Functionalization of GAG reducing end through oxime ligation is now favored, as it shows both improves yields and stability.[291] Interestingly, a range of oxiamine functionalizing agents are commercially available, including oxiamine biotin linkers.

In contrast, functionalizing the non-reducing end of GAGs remains challenging with only a few methods available.[286,292]

6.3 Techniques to characterize molecular interactions

Numerous techniques are available for characterizing molecular interactions. While specific interactions of GAGs with proteins, such as AT III and FGF2, have been demonstrated and extensively studied, the majority of GAG-binding proteins have only been identified by simple screening without a deeper investigation of the interaction dynamics.

Techniques, such as ELISA[293,294], fluorescence binding assays or fluorescence polarization assays [295–297], have been frequently used for studying GAG/protein interactions. However, techniques based on optical biosensors are currently considered most effective.[298] Surface Plasmon Resonance (SPR) is the reference for measuring kinetics constants, including the association rate constant (k_a or k_{on}), the dissociation rate constant (k_d or k_{off}) and the dissociation equilibrium constant ($K_D = \frac{k_d}{k_a}$). BioLayer Interferometry (BLI) was developed more recently and provides higher throughput capacity. SPR is a flow-operated technique, while BLI operates with analytes in solution in a 96-well plate, agitated at high speed to circumvent mass-transport limitations. SPR has been widely used for the analysis of GAG/protein interactions, revealing complex binding mechanisms, such as positive cooperative interaction of chemokine RANTES to Hep.[299] In contrast, there are still relatively few reported studies of GAG/protein interactions using the more recent BLI technique.[300] It should be noted that these techniques are not inherently specific to GAGs and their application to GAG-protein interaction studies may necessitate additional precautions. Because of GAG sequence heterogeneity and possible display of multiple protein binding sites on the same polysaccharide chain, GAG-protein interactions rarely correspond to simple interaction models. However, fitting models adapted to this type of interactions are not currently available, and the use of complex fitting models is generally not recommended. Consequently, simple 1:1 interaction models are typically used, which urges for careful consideration of the kinetic parameters obtained.

Quartz-Crystal Microbalance with Dissipation monitoring (QCM-D) technique enables characterization of molecular interactions at surfaces *in situ*[301–304]. However, due to mass transport limitations, QCM-D cannot be generalized for measuring k_a and k_d rates. QCM-D can be used to study the cross-linking of GAG chains upon binding to different proteins,[305] the conformational change of molecules,[306] and the effect of GAG density on molecular interactions.[307]

To identify the GAGs binding sites on proteins, a technique based on GAG-coated beads has been developed. GAGs are activated with EDC/NHS to form covalent complexes with the protein, which are subsequently proteolyzed. The fragments bound to the GAGs are then analyzed by N-terminal sequencing performed directly on the beads.[308]

Several other techniques provide additional information about GAG/protein molecular interactions. The stoichiometry of GAG-protein complexes can be determined using laser light scattering, while Ion Mobility Mass Spectrometry (IMMS) can be used to study complex shape. To further investigate the structure of GAG-protein complexes and potential conformational changes, techniques such as X-ray crystallography, NMR, and cryo-EM are the gold standard but are highly challenging. In the literature, only a few structures of GAG-protein complexes have been determined, due to the difficulty of obtaining pure GAG species in large quantities, and propensity of the polysaccharide to induce protein aggregation at high concentrations. In this respect, recent developments in cryo-EM may provide new insights into the structure of GAG-protein complexes.[309]

6.4 Parallelization of GAG-protein molecular interaction assays

The last decades have been marked by the development of microarrays to study molecular interactions. Microarrays are particularly adapted for screening interactions between GAGs and growth factors. They can be fabricated on various substrates including glass, silicon, or plastic. A large number of test sites can be generated by spotting ligands on the microarray surface at specific positions. Chips can include up to several thousand spots. The most common spotting methods are pin-based fluid transfer systems and piezo-based inkjet dispenser systems.[310–312] Although originally developed in the 1990s for nucleic acid research, microarrays have been rapidly adapted to the study of other molecules, such as peptides and polysaccharides. The first carbohydrate microarrays were reported in 2002,[313–316] and the first GAG-oligosaccharide microarray in 2006.[317] GAG compounds are generally spotted on the substrate in an oriented manner by chemical conjugation. The most standard immobilization procedure involves using an NHS-coated substrate to covalently attach amine-tagged oligosaccharides,[317–322], though other chemistries including non-covalent attachments have also been used.[323–325] The protein or growth factor of interest is then incubated on top of the GAG array, and the interaction can be monitored through fluorescence readout using a microarray scanner, SPR imaging, or mass spectrometry (MS) (**Figure 6**). The fluorescent detection approach generally requires the use of antibodies or fluorophore/tag (His tag or biotin)-labeled protein, which may in some cases interfere with the protein/GAG interaction. An alternative strategy involves using

label-free proteins and assessing binding by SPR imaging. Notably, the Biacore Flexchip SPR imaging instrument enables measurement of protein binding to biotinylated GAGs spotted onto neutravidin-coated gold chips, or to pyrrole–oligosaccharide electrocopolymerized on gold chips.[326–330] MS is another label-free detection method that can be used to analyze various GAGs simultaneously. Interestingly, MS has also been used in combination with glycan arrays to study the functional specificities of glycosyltransferase enzymes for the assembly of oligosaccharides.[331,332]

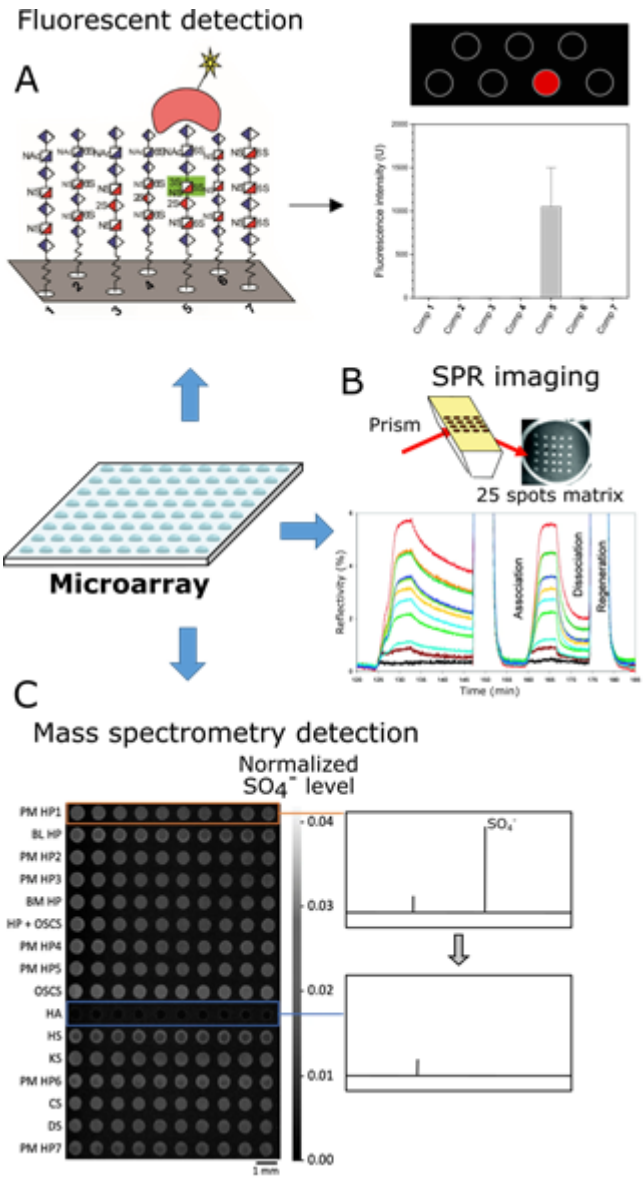


Figure 6. ((Different high-throughput detection methods adapted for microarrays. A) Microarrays can be used for fluorescent detection with a fluorescent protein probe or specific antibodies. Adapted with permission.[333] Copyright 2018, Elsevier. B) SPR imaging has also been coupled to microarrays, enabling measurement of binding curves and potential determination of kinetics parameters. Adapted with permission.[330]. Copyright 2008, American Chemical Society. C) Microarrays coupled with mass spectrometry detection enable analysis of varisous GAG samples, notably for assessing their sulfation degree. Adapted under terms of the CC-BY license.[334] Copyright 2021, Hook *et al*., published by Springer Nature))

One of the major advantage of microarrays is the moderate amount of protein required for performing incubation with coverslips or incubation chambers, and screening simultaneously the binding to numerous GAG compounds.[319,320] Only a few picomoles of proteins are needed to screen GAG-protein interactions. Furthermore, recent robotic arrayers require only a femtomolar quantities of GAG for each spot.[264]. Applications of high-throughput capacity microarrays for GAGs include the study of pathogen or protein interaction networks. For instance, comprehensive investigations have been conducted on the interaction networks of Leishmania pathogens and of endostatin with host ECM components, including GAGs. This led to the discovery of new endostatin GAG binding partners, namely CS and DS.[327,328] Another notable application of GAG microarrays is the study of biomolecule interactions with various GAG compounds, varying in type, length, and sulfation, to determine the structural features required for binding. In this context, microarrays have been used to analyze the binding of different proteins, including stromal cell-derived factor-1α (SDF-1α), interferon-γ (IFN-γ), with different GAGs.[330] SDF-1α exhibited strong binding to Hep, while binding weakly to CS and DS. IFN-γ bound to all GAGs, with a preference for Hep, followed by DS and then CS.

Limited access to compounds with defined structures have hindered the development of microarrays featuring sulfation-defined GAG oligosaccharides, but progress in GAG-oligosaccharide synthesis is steadily expanding the repertoire of available compounds. In 2006, the first GAG-oligosaccharide microarray featured only 5 different compounds screened for FGF1 binding.[317] This number rapidly

increased to 12, used for screening the binding of FGF1, FGF2 and FGF4.[320] In 2014, Nonaka and coworkers used 14 synthetic oligosaccharides and identified a non-naturally occurring monosaccharide, 2,4-O-di-sulfated iduronic acid, as a potential inhibitor of CCL20–HS interaction.[335] In 2017, Yang *et al.* used 14 oligosaccharides from their 21 compounds library in a microarray to probe the binding of 3-O-sulfotransferase isoform 1 and AT III.[321] During the same year, Zong *et al.* screened the binding of FGF-2 and chemokines CCL2, CCL5, CCL7, CCL13, CXCL8, and CXCL10 to 47 synthetized tetrasaccharides.[322] More recently in 2021, Chopra *et al.* characterized the binding of 11 proteins (AT III, HC-II, FGF2,7,9, BMP-2, FGFR-1, RAGE, Stab-2, Nrp-1, HSV-1 gD) to 27 rare 3-O-sulfated hexasaccharides.[318] The largest microarray featuring 95 HS structures (94 oligos + Hep) was used by Horton *et al.* to screen the binding to ATIII, FGF2, IL2 and platelet factor 4.[319] Remarkably, they determined the binding affinities (K_D constants) of AT III with 7 compounds and of FGF2 with 29 compounds.

In another context, microarrays were used to study the crosslinking of lectins to mucin glycans spotted at various surface densities.[336] The authors found that lectins such as SBA, WFL and VVA exhibited valency-dependent binding, while the HPA lectin showed strong avidities regardless of the glycan ligand density. However, the range of densities tested was restricted, due to detection limits of the experimental setup.

Finally, it is worth noting that this technique requires a highly specialized equipment, potentially impeding its widespread adoption in research laboratories.

High-throughput alternatives, including microsphere arrays,[337] or fluorescent polarization technique,[338] have also been used and reviewed.[264] Currently, both SPR and BLI systems are undergoing technological developments towards increasing their high-throughput capabilities. Such advances could revolutionize the field, by enabling analysis of a large number of interactions in a single run, while providing quantitative kinetic information (k_{on}, k_{off}, K_D). These may lead to the generation of large volumes of data, which will require standardization and new conventions for efficient processing. For instance, the extended Lawrence code for GAGs may be a useful tool for coding and representing disaccharide units and their sulfation.[8]

6.5 Cellular studies at high throughput using GAG-based biomaterials

Given the multitude of parameters (eg. GAG type, density, and sulfation), automated methods have started to emerge for studies on GAG-based biomaterials. High-throughput readouts are also needed to investigate how GAGs impact cell signaling (**Figure 7**).

A GAG microarray was used as a platform for a cellular study investigating the binding of chicken hepatocytes to different GAGs.[339] This approach identified GlcNAc residues as specific receptors for hepatocyte cell adhesion, independently of the linkage or orientation, while hepatocytes did not bind to galactose or N-acetylgalactosamine residues. As reviewed by Puvirajesinghe and Turnbull in 2016, microarrays can also be used to study cellular responses to GAGs.[340] For instance, a microarray slide functionalized with different Hep oligosaccharides was incubated in a cell culture dish with HS-deficient 3T3 cells, and the response of these cells to FGF2 was assessed by immunofluorescence staining of total and phosphorylated ERK1/2 markers.[341] Results showed the pERK1/2 signal increased with the size of the Hep oligosaccharide printed on the microarray. A 18-mer Hep yielded the strongest pERK1/2 signal compared to 12-mer Hep (**Figure 7A**). The 2-mer Hep did not elicit significant signal compared to the negative control. In another study, a cell-based microarray platform was developed to investigate GAG-induced FGF-FGFR signaling.[342] For all the FGFs studied, highly sulfated GAGs, especially those containing IdoA like Hep, 2-O desulfated Hep and DS, were the most effective in inducing FGF-mediated cell proliferation.

More recently, the automation of the fabrication of self-assembled materials in the form of streptavidin-based materials has been developed.[34] Such materials deposited in 96 wells cell culture microplates can be used for high-content studies of cellular responses. Notably, cells can be cultured on GAG-based biomaterials deposited directly at the bottom of the microplate, and can be stimulated by growth factors adsorbed onto the GAGs (**Figure 7B**). These materials being built with a basal layer of streptavidin, any type of biotinylated compounds, including GAGs and adhesion peptides, can be subsequently adsorbed. As a proof of concept, these biomaterials were co-functionalized with HS and an adhesion peptide (RGD). Several proteins from the BMP family were adsorbed on HS at increasing concentrations and their effect on cell differentiation to bone was assessed by following phosphorylated SMAD1/5/9 signaling, using a high content microscope.[343] C2C12 cell response to

increasing doses of either soluble of adsorbed BMPs on HS allowed determination of EC50 values. C2C12 cells were found to be more responsive to HS-bound BMP2 compared to other HS-bound BMPs. Such biomaterials may be used in the future to study the cellular response to different growth factors, by modulating the experimental conditions: GF presented via different GAGs, modulation of GAG density, response to GAG oligosaccharides with distinct sulfation patterns.

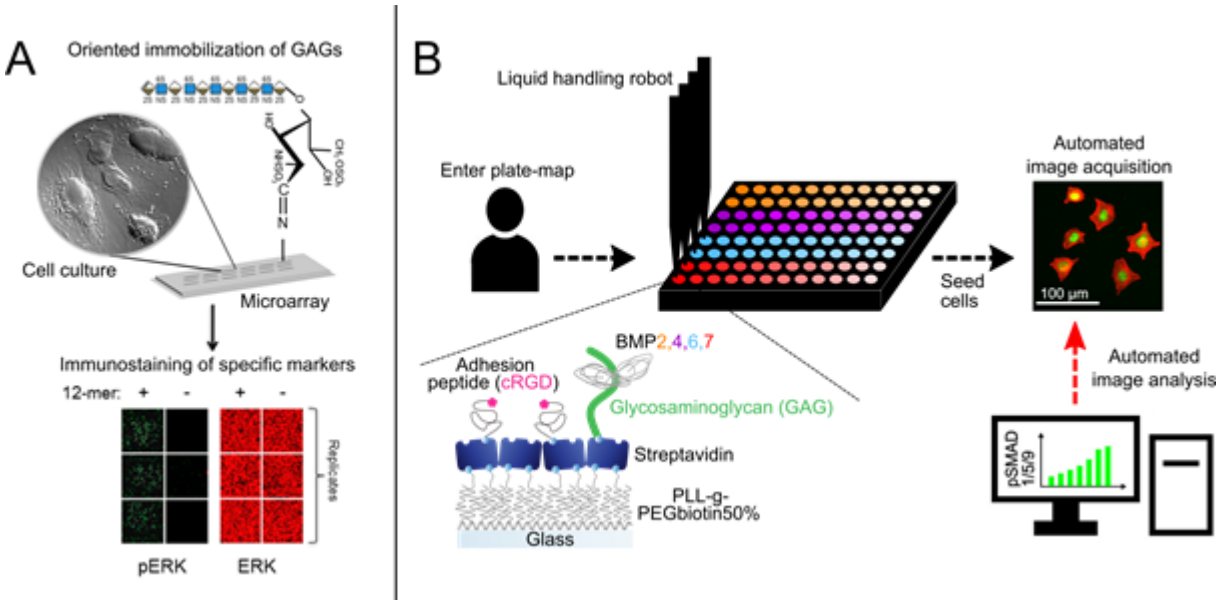


Figure 7. ((High-throughput strategies for studying the in vitro role of GAGs on cells. A) Immobilization of Hep oligosaccharides on a microarray as a support for cell culture. The levels of ERK and pERK were assessed by immunofluorescence after FGF2 stimulation. Adapted under terms of the CC-BY license. [340] Copyright 2016, Puvirajesinghe and Turnbull, published by MDPI. B) Fabrication of GAG-based biomimetic platforms in 96-well plate with liquid-handling robot to study the signaling pathway induced by GAG-bound BMPs. The nuclear pSMAD1/5/9 intensity was measured by immunofluorescence staining with a high-content microscope. A dose-response curve of nuclear pSMAD1/5/9 was established for all BMPs bound to HS. Adapted with permission.[34] Copyright 2022, American Chemical Society))

7. Conclusions and perspectives

Thanks to their vast biological functions, GAGs are promising candidates for biomedical applications. The design of GAG-based biomaterials is a growing field, with applications in tissue engineering, regenerative medicine and drug delivery. In this review, we point out the relevance of modulating parameters such as GAGs type, density, and sulfation pattern to engineer tailor-made biomaterials eliciting the desired cellular response and tissue regeneration properties. Given the considerable variability of these parameters amongst tissues and developmental stages, we also encourage the scientific community to correlate these parameters to the *in vivo* conditions of the targeted tissue engineering application, whenever possible. Developments of new strategies for producing structurally defined GAGs have significantly progressed during the past years. However, it is worth noting that efforts have mostly focused on HS and Hep, while other GAGs such as KS have been neglected. Technical limitations pose challenges in various area of glycobiology, including GAG sample analysis and tissue analysis. However, the emergence of innovative techniques, such as molecular nanopore sequencing and MALDI-FT-ICR mass spectrometry imaging holds the potential to unveil new exciting opportunities in these domains.

Regarding molecular interactions, the development of microarrays has drastically enhanced our ability to identify GAG structures specifically interacting with defined GFs. The development of technologies providing kinetics information (such as SPR or BLI) at high-throughput could revolutionize our practices and understanding of GAGs. Recent addition of automation in the design of GAG-based biomaterials and in the measurement of GAG-GFs or GAG-cell interactions opens new opportunities for conducting parallel tests on different GAGs and experimental conditions.

Altogether, recent developments in the field open new perspectives. First, the use GAG-based biomaterials presenting GFs bound to the biomimetic matrix may unveil novel cellular processes, in comparison to the soluble delivery of GAGs. Here, in-depth studies of GAG-proteins molecular interactions will be complemented by the studies of the effect of GAG-GFs at the cellular level in a biomaterial context. Second, there is a growing need for wider access to methods for producing purified oligosaccharides, within the scientific community. In addition, these methods must be optimized to yield sufficient quantities of material. Third, the complexity and diversity of GAGs, as well as potential modulation of these parameters, will necessitate testing a wide range of conditions within an experiment. However, new developments, including the use of well-defined GAGs, automated

fabrication of biomaterials, automated quantification of molecular and cellular interactions, will significantly facilitate the systematic study of GAG-GF-mediated cellular processes under similar experimental conditions. Therefore, these developments should provide novel insights into the impact of GAG structural features, such as GAG type, density and sulfation degree, in a given biological function.

Therefore, high content tools will enable to study the complexity and diversity of GAGs, and to gain knowledge on molecular mechanisms, which is currently limited to specific GFs, like AT III or FGF2. We expect that novel methods will be developed in the future to improve the automation of GAG immobilization, the high-throughput quantification of molecular interactions and the high-content study of cellular readouts.

Regarding their effect on cellular response, only a limited number of high content biomaterials and systems have been developed for such studies. Currently, most systems are using high content microarrays or versatile biomaterials that are compatible with high-throughput immunofluorescence analysis of nuclear translocation of markers. Additionally, the development of high content readouts will be required for analyzing other non-nuclear signaling markers, such as western blot and qPCR equivalent techniques.

Given the large volume of data that will be collected in the future, standardization and conventions will be necessary, as well as the assistance of bioinformaticians for analyzing batches of data.

In summary, GAG-based biomaterials is a rapidly growing field. We foresee the development of GAG-based biomaterials incorporating well-controlled oligosaccharides and GFs for specific medical applications in the future.

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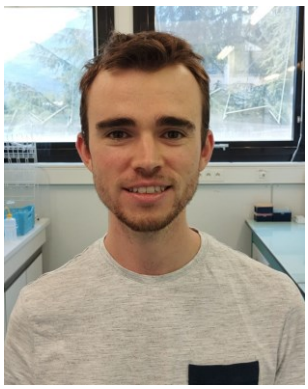

Jean Le Pennec is a PhD candidate in Biomaterials Science applied to Biology at the University Grenoble Alpes, France. He received in 2019 an engineering degree with specialization in Biomedical Engineering at the Grenoble Institute of Technology as well as a Master degree in Nanosciences and Nanotechnologies at the University Grenoble Alpes. He subsequently began his doctoral research at the University Grenoble Alpes under the supervision of Dr. Elisa Migliorini and Dr. Romain Vivès. His current research focuses on exploring the role of GAGs in the regulation of Bone Morphogenetic Protein 2 signaling using biomaterials.

Catherine Picart is research director, head of the Biology and Biotechnology for Health Joint Research Unit (INSERM/CEA/UGA). She also leads the "Biomimetism and Regenerative Medicine (BRM)" research team (EMR BRM 5000, CNRS/CEA/UGA). She was senior member of the Institut Universitaire de France (IUF) (2016-2021). Her research is focused on tissue engineering, biomaterials, medical devices, drug delivery, molecular and cellular biophysics. She received the CNRS Silver medal in 2016 and the Emilia Valori Prize from the French Academy of Science in 2019. Over the past 10 years, she was the PI of 4 ERC grants.

Romain Vivès is a CNRS Research director at the Institute of Structural Biology, Grenoble, France. After completing a PhD at the University of Manchester (UK) on the role of heparan sulfate (HS) in the

modulation of the response to bFGF, and a post-doctoral fellowship on the interaction of HS with HIV envelope glycoprotein gp120 (university of Grenoble-Alpes, France), he obtained a tenured position at CNRS in 2002. His research interests are the structural analysis of Glycosaminoglycan (GAG) structure and characterization of GAG/protein interactions, the biosynthesis and post-synthesis mechanisms regulating GAG structure and function and amongst them, the study of SULF extracellular endosulfatases. Romain Vivès is presently coordinator of the GLyco@Alps network (21 research laboratories in interdisciplinary glycosciences).

Elisa Migliorini, CNRS researcher since 2017, is working at BRM team at CEA-Grenoble (France) since 2020. Actually PI of national and international grants in 2015 she obtained the Marie-Sklodowska-Curie postdoctoral fellow working in Max-Planck Institute, Stuttgart, Germany after 2.5 years of post-doctorate between Grenoble and San Sebastian, Spain. Since the PhD (obtained in 2012 at the University of Trieste, Italy) she focused her studies at the interface between cells and substrates. She now aims to design biomaterials with controlled chemical functionalization and mechanical properties able to mimic selected aspects of the extracellular matrix presentation of growth factors and glycosaminoglycans.

Table of contents entry

((Scheme of the main review concepts: The field of biomaterials presenting glycosaminoglycans (GAGs) is an emerging field. Some of these biomaterials are applied for regenerative medicine but in vivo GAGs are highly diverse. Here, we review the methods employed to understand the complexity of GAGs. We focus on high-throughput methods to compare multiple parameters in the same experiment.))

Jean Le Pennec, Catherine Picart, Romain R. Vivès*, Elisa Migliorini**

Sweet but challenging: tackling the complexity of GAGs with engineered tailor-made biomaterials