Engineered Sulfated Polysaccharides for Biomedical Applications

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Sulfated polysaccharides are ubiquitous in living systems and have central roles in biological functions such as organism development, cell proliferation and differentiation, cellular communication, tissue homeostasis, and host defense. Engineered sulfated polysaccharides (ESPs) are structural derivatives not found in nature but generated through chemical and enzymatic modification of natural polysaccharides, as well as chemically synthesized oligo- and polysaccharides. ESPs exhibit novel and augmented biological properties compared with their unmodified counterparts, mainly through facilitating interactions with other macromolecules. These interactions are closely linked to their sulfation patterns and backbone structures, providing a means to fine-tune biological properties and characterize structural–functional relationships by employing well-characterized polysaccharides and strategies for regioselective modification. The following review provides a comprehensive overview of the synthesis and characterization of ESPs and of their biological properties. Through the pioneering research presented here, key emerging application areas for ESPs, which can lead to novel breakthroughs in biomedical research and clinical treatments, are highlighted.

1. Introduction

Natural sulfated polysaccharides are found in a large variety of living organisms and have widely diverse biological roles, depending on their molecular structure and interaction with other biomolecules. In animals, sulfated polysaccharides are referred to as glycosaminoglycans (GAGs), and in vertebrates the sulfated GAGs are comprised of chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate, heparan sulfate (HS), and heparin. When the GAGs are conjugated to a core protein, they are designated proteoglycans, and are ubiquitous in the extracellular environment of nearly all tissues. Heparin is a notable exception, as it is severed from its protein core and released into the circulatory system upon degranulation of mast cells. The remaining GAG, hyaluronan (HA), is nonsulfated and not conjugated to a core protein. The vertebrate proteoglycans and their associated GAGs are integral parts of the extracellular matrix (ECM) of tissues and are involved in intercellular signal transduction, reproduction and development, immunological responses, blood homeostasis and coagulation reactions, cell motility, and more.[1]

Sulfated GAGs and proteoglycans are also prevalent in invertebrates. Even in the most primitive invertebrates, marine sponges, sulfated polysaccharides are believed to facilitate cell-cell interactions as part of self-recognition and tissue formation.[2] Structural variants of heparin, CS, and HS have been identified,[3] as well as sulfated galactans and fucans with or without branching[4] in other marine invertebrates such as bivalves, echinoderms, and ascidians. Although these GAGs are studied less than their vertebrate counterparts, their biological functions in tissue development and maintenance are presumably related.[5,6] The only other known source of sulfated fucans is the cell wall and gelatinous outer layer of brown algae, whereas red algae and green algae have other sulfated polysaccharides, namely carrageenan and ulvan. There are several hypothesized biological roles of fucoids in brown algae, including osmoregulation, metabolism of reactive oxygen, and developmental biology.[7] Fucoidan, carrageenan, and ulvan have also structural properties for the maintenance of the algal cell walls.[8,9] Sulfated polysaccharides have also been isolated from capsules and biofilms of marine cyanobacteria, microalgae, and archaea, which show a vast structural diversity with presumbale roles in protection of cells from desiccation and other environmental stress.[10–12] Sulfated polysaccharides have been found in only a few species of plants, all of which are found in saline environments, again indicating a marine origin of these polysaccharides and a potential role in salt tolerance.[13,14]
GAGs have vital roles in the development and maintenance of tissues facilitated by interaction with soluble and membrane-associated proteins, which have inspired their use in the development of therapeutics and biomaterials. However, relatively few commercial applications exist today within the biomedical sector. The most prominent example is heparin, which has been the most widely used anticoagulant drug in the world since its discovery in the early 1900s.\[15\] GAGs are also important constituents of commercial biomaterials for cell cultivation and tissue engineering based on native ECM, and preclinical and clinical studies have evaluated heparin and other GAGs as antitumor and antiinflammatory agents.\[16\] The anionic nature of the sulfated polysaccharides further allows formation of electrostatic complexes toward dynamic sequestration of bioactive compounds and assembly of biomaterial structures such as hydrogels, films, or fibers for drug and cell delivery vehicles. For some of these applications, native GAGs may exhibit limitations with respect to production cost, batch standardization, immunogenicity, degradability, or other aspects. Engineering of polysaccharides can overcome some of these challenges and simultaneously unveil novel biomedical applications of sulfated polysaccharides (Figure 1).

Engineered sulfated polysaccharides (ESPs) are structural derivatives not found in nature, and often possess novel or enhanced properties compared with their natural sulfated counterparts. ESPs are made by functionalization of nonsulfated polysaccharides in order to facilitate novel biological interactions but can also result from structural tailoring of sulfated polysaccharides, e.g., by regioselective sulfation, desulfation, and backbone modification to enhance or tune inherent properties. Additional engineering can be employed to allow structuring of sulfated polysaccharides, for instance through ionic cross-linking, coacervation, or modifications for covalent cross-linking. As ESPs display unique structural features, they may exhibit altered pharmacokinetic properties compared with natural sulfated polysaccharides, such as in vivo degradability, revealing new possibilities for their use in biomaterials, drug delivery vehicles, and more. Several ESPs can be produced in large quantities at a low cost from renewable and/or underutilized raw materials or from microbial production, as a more environmentally and economically sustainable alternative to, e.g., isolation of GAGs from animal tissues.

Over the last two decades, a wide variety of ESPs have been synthesized and functionally characterized, uncovering

![Figure 1](https://www.afm-journal.de/graphics/afm-2021/afm-2021-f1.png)

Figure 1. Biomedical applications of engineered sulfated polysaccharides.
several biological interactions and potential biomedical applications. A selection of the most common ESPs referred to in literature and the present review is shown in Table 1. These polysaccharides have well-characterized structures, and some have regular repeating units contributing to the elucidation of structure–function relationships. However, as described

Table 1. Common polysaccharides (PS) used for sulfation with possible sulfation sites highlighted. Polysaccharides are here shown in the Haworth projection and the figure on bottom row indicates numbering of carbons on the pyranose ring to easily visualize sulfate positions as referred to throughout the review (3-O. 2-N, etc.).

| PS                        | Structure and sulfation sites                                                                 | Commercial sources | Notable properties                                      |
|---------------------------|------------------------------------------------------------------------------------------------|--------------------|---------------------------------------------------------|
| Alginate                  | ![Alginate structure](image) \(\beta-(1,4)-\text{ManA} \ \alpha-(1,4)-\text{GulA}\)          | Brown algae        | Cross-linked by divalent cations                         |
| Carboxymethyl dextran     | ![Carboxymethyl dextran structure](image) \(\alpha-(1,6)-\text{Glc} \ \alpha-(1,3)-\text{Glc} \ \alpha-(1,6)-\text{Glc-CMC}\) | Fermentation       | Used in multiple preclinical and clinical studies       |
| Cellulose                 | ![Cellulose structure](image) \(\beta-(1,4)-\text{Glc} \ \beta-(1,4)-\text{Glc}\)             | Plants and wood    | Can be sulfated in crystalline and fibrous forms         |
| Cellulose                 | ![Cellulose structure](image) \(\beta-(1,4)-\text{Glc} \ \beta-(1,4)-\text{Glc}\)             | Fermentation       | Can be sulfated in crystalline and fibrous forms         |
| Chitosan                  | ![Chitosan structure](image) \(\beta-(1,4)-\text{GlcNAc} \ \beta-(1,4)-\text{GlcN}\)         | Crustaceans        | Positive charged amino group                            |
| Chitosan                  | ![Chitosan structure](image) \(\beta-(1,4)-\text{GlcNAc} \ \beta-(1,4)-\text{GlcN}\)         | Fungi              | Positive charged amino group                            |
| Hyaluronan                | ![Hyaluronan structure](image) \(\beta-(1,3)-\text{GlcA} \ \beta-(1,4)-\text{GlcNAc}\)        | Animal tissues     | Regioselective sulfation well established                |
| Hyaluronan                | ![Hyaluronan structure](image) \(\beta-(1,3)-\text{GlcA} \ \beta-(1,4)-\text{GlcNAc}\)        | Fermentation       | Regioselective sulfation well established                |
| K5                        | ![K5 structure](image) \(\beta-(1,4)-\text{GlcA} \ \beta-(1,4)-\text{GlcNAc}\)              | Fermentation       | Structurally analogous to heparin and heparan sulfate   |

ManA = Mannuronic acid
GulA= Gularonic acid
GlcA = Glucuronic acid
Glc-CMC = Carboxymethylated glucose
GlcNAc = N-Acetylglucosamine
GlcN = Glucosamine
in the review (Section 3.4), several studies have produced ESPs from more complex plant polysaccharides showing large variations in monosaccharide composition and branching patterns, which may facilitate novel and specific biological interactions. Early work on ESPs was largely focused on their anticoagulating properties toward developing novel heparin analogs with tailored efficacy and pharmacokinetic properties. More recent research has also demonstrated the use of ESPs in tissue engineering scaffolds, serving as reservoirs and/ or coreceptors of signaling molecules and promoting tissue development, similar to the native GAGs. Innovative cross-linking strategies and composite designs now allow for the generation of complex biomaterials with tailored structural properties, which may solve central medical challenges in degenerative disease, congenital disorders, and regenerative medicine. Moreover, the antiviral properties of sulfated polysaccharides were recognized over 50 years ago and have, amid a global Covid-19 pandemic, again become highly relevant in the search for novel prophylactic and transmittance-reducing therapeutics.\[17\]

The rapidly increasing volume of research on ESPs hence warrants an overview of their chemical, physical, and biological properties, along with their potential future utilization as therapeutics and biomaterials. Because a wide variety of polysaccharides and innovative strategies for regioselective modification has been studied, the authors of the present review wish to particularly highlight structure–function relationships in central biomedical application areas.

2. Generation, Characterization, and Physical Properties of ESPs

2.1. Chemical Sulfation

Sulfation of polysaccharides as described in literature does not follow a universally standardized protocol and has been performed with a range of sulfate donors, solvents, temperatures, times, and molar ratios of reagents. In its simplest form, chemical sulfation involves a sulfate donor (commonly an acid) in a polar aprotic solvent and is performed in a one-step reaction. Controlling reaction conditions can allow for reproducible tuning of the DS (DS) and minimizing depolymerization and other side reactions. Importantly, different polysaccharides vary in their solubility and reactivity and may require specialized protocols for chemical modification; however, their structural nuances can also be exploited to introduce specific modification patterns. Enzymatic sulfation has been reported for GAG, namely, heparin and CS derivatives, and can be a significant future tool for generating highly specific sulfated moieties.\[18,19\] In biosynthesis of native GAGs, sulfotransferase (SULT) enzymes catalyze the transfer of a sulfo group from a donor molecule to an alcohol or amine group. Employing SULTs in large-scale synthesis of ESPs is, however, still not feasible, due to low commercial availability of enzymes and suitable substrates and has only been demonstrated at small scale.\[20–22\]

2.1.1. Sulfating Reagents

The first reported methods for nonspecific sulfation of polysaccharides employed sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) or derivatives such as chlorosulfonic acid (ClSO\textsubscript{3}H) or sulfamic acid (H\textsubscript{3}NSO\textsubscript{3}), which are still commonly used due to the relative simplicity of the reactions, high scalability, and low cost of reagents. One of the first examples of chemically sulfated polysaccharides came from degradation and defibrillation of cellulose (Cel) fibers using H\textsubscript{2}SO\textsubscript{4}, resulting in the formation of cellulose sulfate (sCel) esters in the process.\[23\] Several studies employ a pyridine salt intermediate (Figure 2), which can catalyze the sulfation reaction and potentially reduce degradation and other side reactions from heating with strong acids alone.\[24,25\] Certain sulfation procedures also start with forming a tetrabutylammonium salt of the polysaccharides to improve solubility in organic

Figure 2. Proposed reaction mechanism of sulfation of a glucose (Glc)-based polysaccharide with chlorosulfonic acid and a pyridine salt intermediate.\[23\]
solvents. These procedures target primary hydroxyl (OH) groups, whereas selective N-sulfation has been demonstrated primarily on N-acetyl-glucosamine-containing polysaccharides such as chitosan, chitin, HA, and heparin/HS derivatives using trimethylamine/triethylamine-sulfur trioxide. Generation of sulfur trioxide (SO$_3$) complexes with amides or amines in polar aprotic solvents can for many polysaccharides achieve a high DS under relatively mild conditions. As an example, for sulfation of HA with various methods, the SO$_3$/DMF complex has been reported to give the highest DS whereas SO$_3$/pyridine may provide a greater control over reaction kinetics and permit milder sulfating conditions at low to intermediate DS.

### 2.1.2. Tuning of Sulfation Degree

Several studies reviewed here have demonstrated the possibility of tuning the DS by varying reagent concentrations, reaction times, and/or reaction temperature. The highest obtainable DS and kinetics of the reaction additionally depends on the polysaccharide structure (i.e., available sulfation sites), site reactivity, and steric hindrance from other bulky functional groups, and on the solubility of the polysaccharide in the selected solvent. For instance, 2-O and 3-O sulfation of alginate confers steric hindrance and discourages dissubstituted (DS = 2) monosaccharides, while polysaccharides with an available hydroxyl group on carbon 6 (C6) can obtain a higher DS (e.g., HA, gellan gum, and glucose-based polysaccharides (glucans)). Studies on homogeneous sequences of alginate have additionally shown that sulfation of polyglucuronic acid (poly-G) with CISO$_3$H in formamide gives a higher DS than polymanuronic (poly-M) acid at high CISO$_3$H concentrations, when in fact the alternating poly-MG sequence demonstrates faster reaction kinetics, presumably due to higher solubility in the reaction mixture. Interestingly, full substitution has been reported for specific polysaccharides, for instance, for sulfated derivatives of amylose, cellulose, curdlan, and galactan with DS = 3 sulfates/monosaccharide. For cellulose, predissolution in ionic liquids provides a homogeneous system for sulfation and increases the obtained DS while reducing selectivity.

### 2.1.3. Side Reactions

As acidic conditions hydrolyze the glycosidic bonds and result in partial depolymerization, reaction parameters should additionally be optimized to minimize the reduction in molecular weight (MW) while tuning the DS. The use of acid scavengers such as 2-methyl-2-butene has been demonstrated to reduce depolymerization and cleavage of other acid-labile bonds, while allowing for tunable sulfation of carboxymethylated dextran. Chopin et al. carried out sulfation of a bacterial exopolysaccharide (GY785) using SO$_3$/pyridine in the ionic hydrophilic liquid 1-butyl-3-methylimidazolium chloride (BMImCl), demonstrating minimal depolymerization. As pointed out by the authors, a challenge remains in homogeneous solubilization of the polysaccharides, as the formation of aggregates during the reaction can lead to heterogeneous sulfation patterns. Similar ionic liquids have also been used for sulfation of Cel to improve solubility and provide a more homogeneous substitution, compared with H$_2$SO$_4$ and SO$_3$ complexes.

### 2.2. Regioselective Modification

Chemical sulfation reactions are not inherently regioselective, thus resulting in a random substitution pattern along the polysaccharide chain. However, structural patterns in the polysaccharide backbone, such as glycosidic bond location, rotational freedom, and the presence of other functional groups, can result in varying exposure and reactivity of the sulfation sites. Variations in the sulfation pattern of polysaccharides can result in different orientation and accessibility of the negative charges toward interactions with proteins. Thus, regioselective sulfation can be applied to study structure–function relationships and to potentially engineer binding epitopes with a high degree of specificity.

#### 2.2.1. Selective Sulfation

Sulfation studies of HA have indicated that carbon 6 (C6) of GlcNAc residues are heavily favored in the sulfation reaction and are completely substituted at DS > 1 per disaccharide. Similarly, C6 of polysaccharides such as HA, cellulose, and curdlan is generally more accessible than that of other hydroxyl groups and is therefore the predominant site for sulfation and modification with other bulky functional groups. However, the resulting modification patterns presumably also depend on the reaction conditions, as one study observed no 6-O substitution when sulfating a 1→3-linked fungal cell wall polysaccharide (Man–Gal–Glu) with sulfuric acid in n-propanol. For certain polysaccharides, the increased reactivity of C6 can be exploited to introduce protective groups such as acetyl, triphenylmethyl, dimethoxytrityl, or a benzoyl ester limiting sulfation to other hydroxyl groups. Primary hydroxyl groups are less distinct in reactivity and can display only moderate differences in sulfation selectivity. For dextran, a slight preference for 2-O and 3-O sulfation over 4-O has been reported. For sulfation of alginate, a minor preference for 2-O-sulfation over 3-O has been indicated for both mannuronic acid (M) and guluronic acid (G; GulA) moieties. To the authors’ knowledge, no successful attempts at regioselective sulfation of Alg has been described. However, chemical acetylation has been performed on Alg hydrogels, favoring M monomers not participating in ionic cross-links. This strategy can potentially be applied for sulfation to generate M-sulfated Alg with improved cross-linking properties, or to introduce protective groups on M followed by gel dissolution and selective sulfation of G. Specific conformational properties in polysaccharides can also allow regiospecific sulfation. For instance, β1→3 xylans exhibit a triple helical conformation resulting in sulfation predominantly at the outward-facing C4.

All GAGs and other polysaccharides such as chitosan contain monosaccharides N-acetylated at C2, namely, N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc). Here, the acetyl group acts as a protecting group, limiting sulfation
to C3 and C6, whereas the amine can be deacetylated to create zwitterionic polysaccharides.\[^{[46]}\] Moreover, the amine group can be specifically sulfated as described above. As an example, exclusive 2-N-sulfation has been achieved for HA with a relatively low substitution degree of 16% of the available amino groups, although the researchers behind the study were able to combine this with complete 6-O-sulfation for a second derivative.\[^{[47]}\] For chitin, regioselectivity in sulfation had also been demonstrated through temperature control, where sulfation performed at room temperature yielded only 6-O-sulfated chitin and increasing the temperature in the range of 45–75 °C additionally resulted in 3-O-sulfation with a tunable DS.\[^{[48]}\] Chitosan can further be sulfated in the gel state, resulting in selective N-sulfation.\[^{[49]}\] A selection of regioselective sulfation procedures is listed below in Table 2.

### 2.2.2. Desulfation

Sulfated polysaccharides may also be subjected to desulfating reactions (Figure 3) for simplifying structural characterization of the polysaccharide backbone or for generating specific sulfation patterns, resulting in altered physical and biological properties. While the structure and mechanism of action of some marine polysaccharide sulfatases have been characterized,\[^{[62]}\] these enzymes are to the authors' knowledge not commercially available. A common chemical approach is the use of methanol in a polar aprotic solvent, at various temperatures and mixing ratios to tune reaction kinetics.\[^{[63]}\] Chemical solvolytic desulfation is generally nonspecific and complete but can demonstrate regioselectivity similar to sulfation based on reactivity of the substitution sites and tuning reaction conditions. For instance, de-N-sulfation was many years ago shown to occur faster than de-O-sulfation in heparin,\[^{[64]}\] and selective 6-O-desulfation has been achieved in GAGs and sulfated galactans by means of a silylating agent.\[^{[65]}\] Regioselective sulfation of HA has also been demonstrated by first generating a high-DS (DS = 1.5) derivative with SO$_3$/DMF, followed by conversion into their respective pyridinium or tributylammonium salts. Next, a reaction with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) resulted in a 50% decrease in DS and selective 4-O and 6-O desulfation of GlcNAc residues.\[^{[30]}\] This strategy has similarly been applied for regioselective desulfuration of carrageenan from red algae and CS.\[^{[65,66]}\] Enzymatic desulfation may be employed to generate more specific sulfation patterns but is not well-established, presumably due to the lack of commercial enzymes. For a more extensive insight into selective sulfation and desulfuration of polysaccharides, the reader is referred to the recent review by Bedini et al.\[^{[55]}\]

### 2.2.3. Oversulfation

Chemical sulfation of native sulfated polysaccharides permits synthesis of oversulfated derivatives with altered physical and biological properties. An extensively studied example in nature is CS, which has several different subtypes with different DS and the same polysaccharide backbone. For instance, increasing the DS can result in potent anticoagulating properties,\[^{[68]}\] and different sulfation patterns in native CS can exhibit opposite effects in neural plasticity and regeneration.\[^{[69]}\] As an example of ESPs generated through oversulfation, k-carrageenan oligosaccharides with a repeating structure of 4-O-sulfated galactose and 3,6-anhydrogalactose were prepared from red algae, followed by sulfation with SO$_3$ in DMF. The authors observed complete 6-O sulfation on galactose and partial 2-O sulfation on galactose and anhydrogalactose with a 3–4-fold increase in the overall DS. The increased DS resulted in a 30-fold increase in anticoagulant activity compared with the native compound.\[^{[70]}\] By oversulfating a fucoidan consisting mainly of 1–3-linked 2-O-sulfated fucose, Qiu et al. demonstrated near full substitution using chlorosulfonic acid to target the only nonsubstituted hydroxyl group at C4.\[^{[71]}\] Another study employed SO$_3$-trimethylamine for oversulfation of DS, resulting in increased platelet aggregation when added to human plasma compared with native DS.\[^{[72]}\] Oversulfation has also been described for several microbial exopolysaccharides, resulting in increased potency in anticoagulation, antioxidation, and cell proliferation.\[^{[73]}\] Many of these polysaccharides, however, have complex monosaccharide sequences and patterns, complicating structural characterization and sulfation patterns and will not be covered extensively in the present review.

### 2.3. Polysaccharide Backbone Modification

The monosaccharide structure and sequence influence the physical and biological properties of sulfated polysaccharides, affecting the maximum DS allowed, orientation of functional groups, as well as macromolecular properties in solution and in cross-linked or aggregated systems. This can be exploited using natural structural derivatives of sulfated polysaccharides, whereas for certain polysaccharides, the monosaccharide sequence can be engineered prior to chemical sulfation, to tailor their properties.

#### 2.3.1. Enzymatic Epimerization

For specific polysaccharides, postsynthesis sequence modification is performed in nature by enzymatic epimerization of monosaccharides. Glucuronyl C5-epimerases (GLCE) perform in vivo conversion of glucuronic acid (GlcA) to iduronic acid (IdoA) in heparin, HS, and dermatan sulfate, and have been isolated for epimerization of heparin analogues, e.g., based on Escherichia coli K5 polysaccharide, also referred to as heparosan.\[^{[74]}\] The enzyme catalyzes a reversible reaction where GlcA and IdoA exist in an equilibrium in solution, and where optimization of the reaction condition allows conversion rates of up to 60%, which is reflected in heparin biosynthesis. IdoA has a crucial role in the binding of protein epitopes on GAGs through its conformational flexibility,\[^{[75]}\] and thus is important in engineering heparin-like polysaccharides.

Alginate has a well-established toolbox consisting of multiple microbial mannanuronan-C5-epimerases (AlgG) catalyzing the conversion of mannuronic acid (ManA) to guluronic acid (GulA). Alginate-producing bacteria in the Pseudomonas and Azotobacter genera secrete epimerases in the periplasmic space as a part of biofilms and capsular cyst formation. Seven such epimerases were initially
Table 2. Common sulfating reagents for regioselective sulfation of various polysaccharides (PS). The degree of sulfation (DS) corresponds to number of sulfate groups per monosaccharide.

| PS          | Sulfating reagents                      | Structure and sulfation sites | DS         | Ref.  |
|-------------|----------------------------------------|-------------------------------|------------|-------|
| Alginate    | HClSO₃/Formamide                        | 2-O together with 3-O Sulfation | 0.1–0.2    | [31,50–52] |
| Cellulose   | HClSO₃/DMF                              | 2-O, 3-O, and 6-O Sulfation | 0.2–2.6    | (C₆ > C₂ > C₃) [53,54] |
| Chitosan    | CH₃COOH, NaHCO₃/MeOH; HClSO₃/DMF        | 3,6-O Sulfation               | 1.0–1.5    | (C₆ > C₃) [55,56] |
|            | SO₃/Me₃N                                | N-Sulfation                   | 0.4–0.9    | [27,55] |
| Dextran     | Piperidine N-sulfonic acid/DMSO         | 2-O, 3-O, and 6-O Sulfation | 1.0–1.9    | [57] |
| Hyaluronic  | SO₃–pyridine complex/DMF                | 2-O, 3-O, 4-O, and 6-O Sulfation | 1.0        | [58–60] |
|            | BzCl/pyridine; SO₃–DMF complex/DMF     | 2-O, 3-O, and 6-O Sulfation | 1.5        | [30] |
identified and cloned from *A. vinelandii* (AlgE1-7), which demonstrate different modes of action and have furthermore resulted in multiple engineered, derivative enzymes. Through chemical sulfation of homogeneous sequences of alginates generated by epimerization, studies have demonstrated pronounced differences in biological activities based on polysaccharide sequence. The AlgG and GLCE enzymes are highly specific for ManA and GlcA, whereas these monosaccharides can be introduced by oxidation of mannose and glucose, respectively. One study demonstrated enzymatic epimerization of the galactomannan guar gum with AlgE enzymes, using galactosidase enzymes followed by C6-selective TEMPO oxidation of mannose residues. Epimerization of alginates prior to sulfation has been shown to increase interaction strength with specific growth factors, presumably due to increased polysaccharide chain flexibility and/or more favorable charge orientation.

### 2.3.2. Modification of Polysaccharide Conformation

Oxidation of the polysaccharide backbone has previously been performed on cellulose, chitin, alginate, and other polysaccharides using periodate to introduce hexuronic ring opening. This introduces flexible junction zones in the polysaccharide chain, and the resulting dialdehydes serve as highly reactive targets for further reactions, such as conjugation of functional groups, and can increase biodegradability of polysaccharides. The increased chain flexibility of periodate-oxidized sAlg has, similar to above, been shown to increase interaction strength with growth factors, approaching the potency of heparin. One study indicated that excessive sulfation of polysaccharides can itself alter the conformation of the monosaccharides. In one study, a shift in proton coupling constants was observed in fully sulfated polysaccharides through $^1$H NMR data, where the authors proposed a conformational change from $^4C_1$ to $^1C_4$ in amylose and from $^1C_4$ to $^2S_0$ in cellulose, curdlan and galactan.

#### 2.4. Chemoenzymatic Synthesis

Chemical synthesis of oligosaccharides beyond eight saccharide units remains extremely challenging. Moreover, there is no unique molecular structure, due to the previously described dynamic sulfation patterns. Kuberan et al. reported an enzymatic approach starting from nonsulfated K5 polysaccharide heparosan that allows for the generation of homogenous heparan oligosaccharides of any size and structure with biological activity. The K5 nonsulfated polysaccharide used as starting material itself resembles the unmodified nascent HS chain (Figure 4). As the synthetic strategy starts from a unique oligosaccharide and only targets a single product, it lacks the ability to produce HS chains with different sizes and structures. Another chemoenzymatic approach toward the comprehensive synthesis of variously sized oligosaccharides.

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**Table 2. Continued.**

| PS | Sulfating reagents | Structure and sulfation sites | DS | Ref. |
|---|---|---|---|---|
| SO$_3^-$DMF complex/DMF (stoichiometric) | ![Structure](image1) | ![Structure](image2) | 0.2 | [47] |
| K5 (Heparosan) | SO$_3^-$pyridine complex/DMF | ![Structure](image3) | 0.5–2.2 | [61] |

![Figure 3](image4). General mechanism of chemical and enzymatic desulfation of galactose (Gal) in carrageenan by nucleophilic (Nu) attack. 

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has been reported by Liu et al.\textsuperscript{83} In order to synthesize different sulfation patterns in a predictable way, the authors make use of disaccharide building blocks and a set of nine enzymes, namely, glycosyltransferases, a C5-epimerase, and SULTs.

2.5. Polysaccharide-Based Mimetics and Analogues

Natural GAGs such as heparin and HS can activate some of the signalling pathways in stem cell differentiation.\textsuperscript{83} Developmental studies have also shown that varying the sulfation pattern of embryonic stem cell (ESC)-associated HS is used to regulate cell fate.\textsuperscript{86} However, there are several drawbacks to working with natural GAGs in cellular engineering. As described in the previous section, de novo chemical synthesis of even short GAGs is very challenging and limits their availability. Second, isolated natural GAGs exhibit low chemical stability and are rapidly degraded under physiological conditions.\textsuperscript{87} Furthermore, the structures of natural GAGs are subject to several biosynthetic postmodifications that are highly specific to certain cell types; this may result in high structural heterogeneity such as varying sulfation patterns. Finally, natural GAGs are difficult to purify and can potentially cause immune reactions.\textsuperscript{88} These disadvantages have led to the development of corresponding mimetics and analogs that exhibit multivalent

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Chemoenzymatic synthesis of a heparan sulfate pentasaccharide. NDST = N-deacetylase and N-sulfotransferase, OST = O-sulfotransferase.\textsuperscript{84}}
\end{figure}
binding interactions with various biological targets. Herein, the specific chain configuration determines interactions with an array of possible receptors such as proteins and other multivalent saccharides (Figure 5). The monosaccharide sequence, charge density and the molecular weight of GAGs all play a crucial role in determining their biological activities. Thus, by providing control over structure and sulfation, their binding affinity can be tuned by adjusting the molecular weight dispersity and degree of sulfation. However, a comprehensive review of their properties and applications is beyond the scope of this review, and we will instead showcase polysaccharide-derived mimetics and analogs that possess a similar chemical nature to GAGs. In general, GAG mimetics with multivalent interaction characteristics can be prepared by copolymerizing sulfated mono- and oligomers (Figure 5A). In a study by Wang et al., a novel approach was proposed for the synthesis of GAG analogs, by first separating the sulfated saccharide building blocks into functional mono- and polymers, i.e., a glyco unit (2-methacrylamido glucopyranose) and sulfonated unit (4-vinyl-benzenesulfonate) (Figure 5B). Both units were copolymerized to yield various GAG analogs by reversible addition–fragmentation chain transfer (RAFT) radical polymerization. These synthetic polymers were then analyzed in the context of ESC proliferation and differentiation, showing similar bioactivity to natural GAGs, such as heparin.

2.6. Structural Characterization of Sulfated Polysaccharides

Provided that the structure of the polysaccharide backbone is known, structural characterization of ESPs mainly encompasses determination of the DS, study of substitution patterns and assessment of changes to the average molecular weight (i.e., chain length).

2.6.1. Analysis of Sulfation Degree

Multiple methods have been described for measuring the degree of sulfation (DS) of polysaccharides, which in the present review will be designated as the average number of sulfate groups per monosaccharide, unless otherwise stated. The traditional approach for quantification generally involves acid hydrolysis of the sulfate groups and formation of insoluble salts with benzidine or barium derivatives, which can further be quantified through colorimetric analyses, gravimetric analyses, or titration methods. Additionally, conductometric titration has been applied to assess changes in electrical conductivity in chitosan as a result of sulfation. However, more precise and faster DS estimations, as well as additional structural data, can be obtained using CHNS/O elemental analyzers or inductively coupled plasma mass spectrometry-based analysis of elemental sulfur. If the monosaccharide structure is known, including the presence of associated counterions and water, the degree of sulfation (average sulfate groups per monosaccharide) can be estimated with high accuracy through a mass balance equation. These methods, however, depend on atomization of the samples, and therefore other methods are required to assess sulfation patterns qualitatively and quantitatively.

2.6.2. Infrared Spectroscopy

Infrared (IR) and Raman spectroscopy can provide a qualitative assessment of sulfation, showing characteristic absorption
The sulfation reactions are generally acidic and performed at moderate to high temperatures, which can lead to hydrolysis of glycosidic bonds in the polysaccharides, requiring analysis of change in the average MW and polydispersity, i.e., the size distribution of the polysaccharide chains. Gel permeation and size-exclusion chromatography (SEC) are commonly employed to separate and accurately measure the MW of polysaccharides, using light scattering methodology. One important parameter for characterizing the MW of chemically sulfated polysaccharides is the refractive index increment (dn/dc), related to the density or specific volume of the polysaccharides. The dn/dc for polysaccharides typically varies from 0.10–0.18 mL g⁻¹, where ESPs generally exhibit lower dn/dc values than their unmodified counterparts, indicating a more extended conformation.[78,99,100]

2.7. Solution Behavior and Hydrolysis of ESPs

The behavior of ESPs in different solvents and in solid phase systems is largely dependent on the native polysaccharide properties, such as charge, pKa, molecular weight, and intra- and intermolecular forces. Sulfation generally enhances the solubility of polysaccharides in water and other polar solvents by increasing the molecular affinity to the solvent as well as promoting a more elongated conformation preventing inter- and intramolecular association. As the pKa of sulfate esters (≈2.0) is lower than the carboxylate groups in uronic acid-containing polysaccharides like heparin[101] and green algae-derived ulvan[102] sulfation can significantly improve solubility at low pH. This is further dependent on the DS, exemplified by the natively sulfated carrageenans where the highly sulfated λ-carrageenan is highly water soluble and the less sulfated κ-carrageenan promotes precipitation and hydrogel formation in the presence of potassium ions. At high DS, the resulting conformational change and increase in chain stiffness can stem from electrostatic repulsion and/or from the relatively bulky sulfate groups reducing rotation around the glycosidic bonds.

Hydrolysis of polysaccharides is commonly performed as a part of their structural characterization or to prepare oligosaccharides for specific applications. As elaborated in previous sections chemical sulfation promotes hydrolysis of the glycosidic bonds and partial depolymerization of the polysaccharides, primarily attributed to the acidic conditions and often high temperatures of the sulfation reactions. There have been few systematic studies on how chemical sulfation alters the stability of the polysaccharide backbone, whereas studies on native sulfated polysaccharides indicate a significant influence of sulfate position, polysaccharide conformation and hydrolysis conditions. Early studies have demonstrated lower rates of glycosidic bond hydrolysis in sulfated polysaccharides compared with their nonsulfated counterparts at high temperature and acidic conditions,[103] whereas the opposite has also been observed for other polysaccharides and hydrolysis conditions. For example, a
3. Biological Properties and Biomedical Applications of ESPs

3.1. Anticoagulant Properties

Heparin was the first anticoagulant used in clinical medicine and is today the most widely used anticoagulant to treat and prevent blood clots by direct injection as well as coating of surgical devices. Heparin exerts its main anticoagulant activity by binding and activating antithrombin III (AT), which subsequently inhibits factor FXa and thrombin at crucial steps in the coagulation cascade. Importantly, the heparin-AT interaction remains one of the few well-documented examples of highly specific interactions between sulfated polysaccharides and proteins, represented by a minimally varying pentasaccharide sequence found in around 30% of the polysaccharide chains in unfractionated heparin.[25] Heparin additionally potentiates the thrombin inhibitor heparin cofactor II (HCII). Conversely to AT, HCII binds less specifically to heparin and can form complexes with a variety of sulfated polysaccharides but inhibits thrombin at a slower rate and does not inhibit Factor Xa.[31] Despite the high efficacy of heparin as an anticoagulant and antithrombotic drug, there have been and still are complications related to its animal origins, such as disease leading to scarcity of animal materials and risks of contamination. In the future, utilizing semisynthetic heparin analogs may provide a more sustainable supply and potentially tunable pharmacological properties. Thus, a large research effort has been directed toward chemoenzymatic engineering of heparin, toward generating molecular weight-specific fractions with varying pharmacokinetic and pharmacodynamic properties, synthetic AT-binding analogs, as well as non-anticoagulating heparins toward other biomaterial applications. The use of such derivatives can also contribute to alleviating adverse effects such as thrombocytopenia, osteoporosis, and hemorrhagic complications.[122] Besides GAGs, numerous studies have assessed the anticoagulant and antithrombotic properties of naturally occurring sulfated polysaccharides, many of which are isolated from macroalgae and marine invertebrates. The anticoagulant activity of sulfated polysaccharides has previously been reviewed by Mestechkina and Shcherbukhkin, who discussed the influence of polysaccharide structure on their anticoagulant activity.[113] Here, we show additional examples from more recent research. The negative charge of the sulfated polysaccharides mediates electrostatic interactions with multiple protein factors in the coagulation cascade, whereas other structural factors such as glycosidic bond orientation, monosaccharide structure, sulfate positions, and the presence of other functional groups influence the interactions and may confer a degree of specificity.

There are two major pathways triggering the blood coagulation cascade, referred to as the extrinsic tissue factor (TF) pathway and the intrinsic contact pathway (Figure 6). The TF pathway primarily triggers coagulation during normal hemostasis, whereas the contact pathway is usually triggered by blood contact with artificial surfaces.[114] The two pathways eventually converge where the final steps of coagulation are referred to as the common pathway. Conventional and standardized methods for assessing anticoagulant properties include measuring the activated partial thromboplastin time (APTT), the prothrombin time (PT), and the thrombin time (TT). The APTT test utilizes an artificial surface and thus emulates the contact pathway, and additionally involves all clotting factors of the common pathway. PT measures the integrity of the TF pathways as well as factors common to both main pathways, and the TT assesses the activity of fibrinogen in the final phase of clotting in the common pathway. In addition, the antifactor Xa assay measures AT-catalyzed inhibition of coagulation factor Xa at the first step of the common pathway.

3.1.1. Effects of Sulfation Degree on Anticoagulating Properties

The importance of the sulfate groups is evident, as sulfated polysaccharides are commonly observed to prolong coagulation times compared to their nonsulfated controls, and the anticoagulant properties of natural sulfated polysaccharides can be enhanced through oversulfation.[115–117] For instance, oversulfation of fucans derived from brown algae resulted in an increased APTT compared with the native sulfated fucan as well as heparin.[118] Generally, the anticoagulating action is observed to increase with the DS of the polysaccharides.[119] Moreover, sulfated polysaccharides generally exert a more potent anticoagulant effect compared to other polyanions, e.g., phosphorylated polysaccharides, presumably due to their more acidic nature.[120] The first publication known to the authors employing sAlg demonstrated prolonged APTT, TT, and PT of sAlg (DS = 0.4–0.9) compared with unmodified
alginate, where the increased coagulation time was directly correlated with the DS of the samples.\(^{[121]}\) The anticoagulant properties of sulfated carboxymethyl starch (sCMS) have also been studied, where an increase in APTT was observed here, similarly as for sAlg, as a function of DS. Whereas the study did not compare sCMS with heparin at equivalent concentrations, heparin showed an approximately threefold increase in the APTT compared with sCMS, and no apparent effects on the PT and TT were observed from either sAlg or sCMS.\(^{[26,50]}\) For several chemically sulfated polysaccharides studied, the increase in APTT has been found to be equivalent to heparin only at high dose ranges, indicating less specific anticoagulant activity. As an example, the anticoagulant efficacy of microcrystalline cellulose sulfate (MCS) at DS = 1.7 has also been assessed. Here, MCS showed a greater prolongation of the APTT compared with heparin at equivalent and relatively high treatment concentrations. MCS was also found to increase the TT in a concentration-dependent manner, but with a lower efficacy compared with heparin. MCS was further shown to inhibit coagulation factors Xa and thrombin, and prolonged coagulation time in mice with a similar dose-dependence pattern as heparin, although with a slightly lower efficacy.\(^{[122]}\) In a similar study, sAlg with various DS (0–1.3) showed a DS-dependent prolonging of APTT and TT and a complete inhibition of coagulation at relatively high DS and dose concentrations.\(^{[96]}\)

### 3.1.2. Effects of Sulfation Patterns on Anticoagulant Properties

Studies of naturally sulfated polysaccharides have highlighted the importance of sulfation patterns, which can be reproduced in engineered analogs through employing regioselective sulfation and desulfation strategies as previously described in the present review. For example, specific CS-like polysaccharides from sea cucumbers have fucan branches with different sulfation patterns, and a study demonstrated a higher anticoagulant potency from 3,4-O-sulfated branches compared with 2,4-O-sulfated branches.\(^{[123]}\) As previously described, the C6 hydroxyl group generally displays higher reactivity and can for certain polysaccharides be specifically sulfated or desulfated to study its impact in biological interactions. Citrus pectin consisting primarily of 1→4-linked galacturonic acid (GalA) can be subjected to carboxy-reduction to form galactose (Gal), allowing sulfation at C6 in addition to C2 and C3. One study found that 6-O-sulfation resulted in a more potent prolongation of the APTT and AT-induced thrombin inhibition compared with 2-O and 3-O sulfated pectins, although inactivation of thrombin by HCII was not significantly affected by the different sulfation.\(^{[124]}\) For cellulose and other glucans, 6-O sulfation occurs at relatively mild sulfation conditions. It has been observed that 6-O sulfated cellulose (DS = 0.9) had no significant effect on the APTT, whereas increasing the DS to 1.7 with 2-O and 3-O sulfation resulted in a prolongation of the coagulation time equivalent to that of heparin.\(^{[138]}\) Chitosans with either 3-O or 6-O sulfation (DS = 1.0) have previously demonstrated little effect on prolonging coagulation, but chitosan with combined 3,6-O-sulfation (DS = 1.3–1.6) resulted in a tenfold increase in the APTT.\(^{[48]}\) To study structure–function relationships in anticoagulating actions, a selection of modified carrageenans was prepared through regioselective desulfation and oversulfation. The APTT was found to increase as a function of DS and sulfation of C6 was found to have a significant contribution to the anticoagulating activity. Interestingly, a procoagulant effect...
was observed for two derivatives of \( \tau \)-carrageenan with different DS and molecular weights, indicating that the polysaccharide backbone structure and position of the sulfate groups contributed to the observed effects.\[125\] The position of the glycosidic bond can determine the orientation of the sulfate groups, and the effect on anticoagulant properties has also been studied through complete sulfation (DS = 3.0) of homopolysaccharides with \( 1 \rightarrow 4 \) (xylan, amyllose, cellulose) or \( 1 \rightarrow 3 \) glycosidic linkages (curdlan, galactan) as well as 6-O-desulfated derivatives. Here, it was found that \( 1 \rightarrow 3 \) linked curdlan exhibited more potent antithrombin activity compared to the \( 1 \rightarrow 4 \) linked polysaccharides. 6-O desulfation resulted in a near 50% reduction in anticoagulant activity for the \( 1 \rightarrow 3 \) linked polysaccharides but had only minimal effects on the \( 1 \rightarrow 4 \) linked polysaccharides.\[32\]

### 3.1.3. Effects of Molecular Weight on Anticoagulant Action and Elimination Mechanisms

The influence of MW on the anticoagulant properties of sulfated polysaccharides has been assessed in multiple studies and varies depending on the polysaccharides and model systems used. One study compared highly substituted dextran sulfates (DS = 2.8) at 20 and 150 kDa with respect to coagulation time and platelet aggregation. Here, low-molecular weight derivatives exhibited a greater anticoagulation effect by a mechanism independent of antithrombin and factor Xa.\[126\] In a separate study, fractions of sulfated propylene glycol esters of alginate (PsAlg) were prepared with MWs ranging from 5 to 50 kDa at DS = 1.2. Here, the relatively high molecular weight fractions demonstrated prolongation of the coagulation time with comparable efficacies to low molecular weight heparin (LWMH, 4–6 kDa), whereas little effect was observed from the unfractionated and low molecular weight fractions. The authors proposed that PsAlg inhibited thrombin and to a lesser extent Xa in the presence of antithrombin III (AT III) and heparin cofactor II (HCII), although direct interactions of AT and HCII were not demonstrated.\[127\] The molecular weight can influence coagulation where interaction between a sulfated polysaccharide and a large protein requires a minimum chain length. Systematic elucidation of this relationship requires relatively pure fractions of short oligosaccharides, where minimal changes in biological activity are expected above a threshold in the MW. This can in part explain why little influence of MW is observed in studies employing a narrow range of relatively high-MW polysaccharides.\[8,56,128\] At high MWs there can also be multivalent binding to one or several proteins, or aggregation effects between polysaccharides influencing the results. Of note, Melo et al. demonstrated that highly sulfated galactans can bind antithrombin at MW between 15 and 45 kDa but requires a longer chain length to form a multimeric complex bridging AT and thrombin.\[129\] As studies of anticoagulant activity usually employ doses below the critical polymer concentrations, there are presumably few intra- and intermolecular entanglements, but aggregation can potentially occur in sChit or polymers with hydrophobic functional groups. For in vivo studies, the MW will additionally influence elimination mechanisms and thus prolong or shorten anticoagulating effects. This was observed in a study on MCS, where higher-MW samples showed increased coagulation times in vitro while the opposite was observed in vivo.\[130\]

Heparin has a relatively short half-life when injected intravenously, at \( \approx 60–90 \) min depending on the molecular weight fractions. Low-MW heparins are primarily eliminated through renal excretion, whereas unfractionated/high-MW heparins are taken up by endothelial cells and macrophages and are enzymatically degraded by heparinases and desulfatasers.\[131\] While this process is still not fully understood, the enzymes are presumed to be highly specific for heparin, indicating that other sulfated polysaccharides are eliminated by renal excretion alone or by other slower-acting mechanisms. Wang et al. subcutaneously injected mice with 0.3–1.5 mg kg\(^{-1}\) of MCS or heparin and withdrew intracardial blood over a period of 12 h, demonstrating that MCS persisted in the circulation up to four times as long as heparin.\[122\] If engineered heparin analogs bypass normal elimination and feedback regulation their potential prolonged activity can have adverse clinical effects. On the other hand, resistance to enzymatic depolymerization can be highly beneficial for more stable coating of biomaterials and surgical devices, e.g., through hydrogel coating, surface grafting or layer-by-layer assembly. In one study, sAlg were conjugated to a polyethersulfone surface via a dopamine linker, where coating of the surfaces suppressed platelet adhesion and activation and lowered the concentration of activated complement factor C3a compared to the noncoated controls. These inhibitory effects were also found to be correlated with an increasing DS of the sAlg.\[132\] In a different study PET surfaces were coated with anticoagulating sCel with varying DS, resulting in increased blood compatibility compared with noncoated materials.\[138\]

### 3.1.4. Mechanisms of Anticoagulating Properties in ESPs

ESP\s can prolong coagulation through nonspecific binding and sequestration of various factors in the coagulation cascade. Most of the published studies on anticoagulating effects of sulfated polysaccharides measure clotting time in assays such as APTT which include a multitude of factors and potential interaction targets. Presently, there is little experimental evidence showing specific binding and activation of antithrombin due to the high specificity of the interaction. A notable exception is the biosynthetic and chemoenzymatic engineering of “neo-heparin” based on the \( E. \ coli \) K5 polysaccharide sharing the same fundamental backbone structure as heparin and HS.\[133\] Yet, several of the reviewed studies on analogous sulfated polysaccharides have showed a dependence on having AT in the plasma for exerting their anticoagulant effects. Interactions with antithrombin have been investigated through various affinity assays. For instance, through studies employing surface plasmon resonance (SPR), sChits have been found to only interact with AT nonspecifically through monophasic binding, whereas heparin exhibits biphasic binding consistent with an induced conformational change in the protein.\[134\] Highly sulfated galactans have through affinity chromatography been shown to bind antithrombin, but at a different site than heparin and thus do not induce a conformational change. However, the anticoagulant activity of the galactans were dependent on the
AT concentration in the plasma, indicating a role of the polysaccharides in AT-mediated coagulation inhibition.\[129\] This is consistent with the fact that heparins that are devoid of the specific AT-binding pentasaccharide still display some anticoagulant activity. Conversely to AT, HCII binds less specifically to heparin and can form complexes with a variety of sulfated polysaccharides but inhibits thrombin at a slower rate and does not inhibit Factor Xa.\[111\] HCII-induced thrombin inhibition has been found to be significantly more potent for specific sulfated glycolipidmannans compared with heparin over a large dosing range, despite having a significantly lower DS (0.2).\[135\] Activation of HCII can thus have a significant role in the anticoagulant activity of the sulfated polysaccharides studied (Figure 6). Sulfated chitin,\[136\] galactans,\[137\] microcrystalline cellulose,\[122\] and other polysaccharides have been proposed to directly bind factor Xa, preventing its conversion of prothrombin to thrombin and can thus modulate a critical step in the common coagulation pathway. Factor Xa, IXa, and thrombin also exhibit heparin-binding domains\[138\] and can presumably directly associate with ESPs, although few of the reviewed studies have demonstrated this.

3.2. Antiviral Properties

Many viruses depend on interactions with sulfated GAGs for surface attachment and subsequent infection of mammalian host cells. This has been demonstrated in multiple studies, where the infection rate is reduced either through enzymatic removal of cell-surface GAGs or through addition of soluble GAGs, such as heparin, serving as an antagonist shielding the viruses against cell attachment.\[139,140\] Indeed, the antagonizing effects of sulfated polysaccharides has been exploited for the development of antiviral therapeutics preventing virus attachment and infections, such as carrageenan-containing nasal sprays (e.g., Betadine, Salinex).\[141\] More recently, heparin and fucoidan were demonstrated to effectively bind the spike protein of SARS-CoV-2, showing a great potential for use in inhaled prophylactic therapeutics.\[17\] Several mechanisms have been proposed for the observed antiviral properties in sulfated polysaccharides, including binding to cell surface receptors that act as viral receptors, polysaccharide cell entry and binding to viral enzymes and transcription factors, and enhancement of immunological responses (Figure 7).\[142\] For certain viruses, the interaction between GAGs and capsular proteins requires specific structural motifs in the sulfated polysaccharides which may aid the virus in targeting correct cell types and preventing cross-binding.\[143,144\] Thus, engineering of sulfated polysaccharides can potentially aid in targeting specific viruses and improve the efficacy in novel antiviral therapies. Increasing the specificity may also be required to prolong the bioavailability of ESP-based therapies, as heparin and its structural analogs can bind a multitude of plasma proteins resulting in reduced efficacy in vivo compared with in vitro.

Witvrouw and De Clercq have previously reviewed antiviral properties of various semisynthetic as well as natural sulfated polysaccharides and have proposed that their inhibiting properties generally increase with increasing DS and molecular weight.\[145\] The optimal molecular weight was further shown to vary between different viruses, indicating a level of specificity related to the size of the epitopes and potentially multimeric binding.\[145\] The authors further emphasized the importance of polysaccharide backbone structure, coinciding with multiple studies on the interaction of sulfated polysaccharides and

![Figure 7. Viral infection of cells and proposed antiviral mechanisms of ESPs. 1) Initial attachment of viral capsid proteins to cell surface proteoglycans and subsequent binding to adhesion and entry receptor proteins. 2) Receptor-mediated endocytosis of virus. 3) Viral transcription and reproduction. 4) ESP binding to viral capsid proteins to inhibit proteoglycan interaction. 5) ESP binding to adhesion and entry receptor to inhibit endocytosis of virus. 6) Endocytosis and release of ESPs to cytosol to inhibit viral transcription factors and reproductive processes.](image-url)
soluble proteins, as previously described in the present review. The dependence on DS in ESPs has been demonstrated for sulfated dextrans, where anti-HIV actions were observed at DS = 1.0 but not at DS = 0.7.[107] Furthermore, oversulfuration of naturally sulfated mannans and xylomannans has been shown to result in a near 20-fold reduction of cell adsorption by herpes simplex virus type 2 and dengue virus serotype 2. Here, abundant 2-O sulfation appeared to be a decisive factor for the increased activity, whereas elimination of the xylose side chains in the xylomannans resulted in a loss of antiviral activity.[146]

Heparin-like K5 polysaccharides with different DS were prepared and found to inhibit infection and syncytia formation of different strains of HIV-1, by preventing association between envelope glycoprotein gp120 and cell-surface syndecans. The K5 derivatives were also found to inhibit replication of virus isolates, where the derivative with the highest DS demonstrated the greatest efficacy.[147] More recent studies have demonstrated inhibition of respiratory syncytial virus (RSV) and human cytomegalovirus, preventing both attachment on initial exposure as well as cell-to-cell spread when added post-infection. Both studies showed that N-sulfation was not sufficient to exert antiviral properties and proposed O-sulfation to be a requirement for K5 binding to the virus.[148,149] For RSV, O-sulfated K5 polysaccharides interestingly exhibited greater antiviral potency than O,N-sulfated derivatives. One study characterized the effects of highly sulfated and monophosphorylated mannan oligosaccharides (PI-88), highly sulfated xylan (pentosan polysulfate – PPS) and heparin and demonstrated reduced infectivity of dengue virus and flaviviral encephalitis in mice. Whereas differences in DS and modification patterns were not discussed, heparin and PPS demonstrated the highest potency in vitro, proposed related to their higher molecular weight.[150] Binding of 3,6-O-sulfated chitosans to HPV16 capsid proteins of Human papilloma virus has been demonstrated, resulting in inhibition of adhesion and subsequent infection of the virus. The authors of the study further proposed that the sulfated chitosan could inhibit viruses indirectly by interfering with cell autophagy pathways in the host cells, preventing viral entry.[151] For a more comprehensive overview of the antiviral activities of polysaccharides, including sulfated derivatives, the reader is additionally referred to the recent review by Chen and Huang.[152]

3.3. Antimicrobial Properties

Only a few studies have demonstrated direct antimicrobial effects of engineered or natural sulfated polysaccharides, with microbial surface protein interactions and membrane disruption as proposed mechanisms. Certain poly- and oligosaccharides may also affect the minimum inhibitory concentration (MIC) of coadministered antibiotics,[153] although this has to our knowledge not been systematically studied for ESPs. As described above for viruses, ESPs can antagonize microbial carbohydrate-binding proteins, lectins, that normally bind cellular GAGs to initiate colonization and infection. Surface attachment and colonization by microorganisms can also be inhibited based on physical effects, as for instance the surfaces of Gram-negative bacteria are rich in negatively charged lipopolysaccharides, which may result in electrostatic repulsion from ESP-coated surfaces. ESPs may further bind medium components such as essential cations and peptides, preventing effective uptake by the microorganisms. Antimicrobial and antifouling properties have been reported for a few naturally sulfated polysaccharides, where many studies have focused on sulfated fucans and galactans derived from marine algae. Fucoidan exhibits growth inhibiting properties particularly toward gram positive bacteria with synergistic effects with antibiotics and has been proposed to target cell wall synthesis.[154] In a different study, researchers found that depolymerization of the fucoidan into oligosaccharides was required to observe significant antibacterial activity toward E. coli and Staphylococcus aureus, attributed to interaction with cationic membrane proteins and a resulting membrane-disrupting effect.[155] Sulfated galactans from green algae have also demonstrated growth inhibition of S. aureus at high treatment concentrations (50 mg mL⁻¹).[156] Microbial growth assays can be highly sensitive toward small changes in the growth medium, and there can be a challenge in obtaining pure samples of sulfated polysaccharides by extraction from biological tissues. As several studies report a high MIC, a correspondingly high presence of contaminants can cause inaccuracies in observed growth inhibiting or potentiating effects. ESPs can allow utilization of more pure and well-characterized fractions. Furthermore, specific noncharged polysaccharides may exhibit antimicrobial properties but little to no solubility in water, where sulfation can vastly improve solubility and allow their use in therapeutic formulations.

Of the ESPs, sChits have demonstrated antimicrobial properties, showing a twofold reduction of the MIC for E. coli and S. aureus compared to the unmodified control. Conversely, the same study showed an increase in the MIC for sChit for the yeasts Arthrinium sacchari and Botryts cinerea.[157] Recently, researchers sulfated a 1→6-linked fungal β-glucan (DS = 0.2) and reported growth inhibiting properties of the sulfated polysaccharides against selected Gram-negative bacteria and yeast compared with the nonsulfated control.[158] In a different study 1→3-linked β-glucans were sulfated (DS = 0.9), resulting in improved solubility and antimicrobial properties against multiple bacterial strains with an MIC in the range of 1–5 mg mL⁻¹. Sulfated dextrans (sDex) have been demonstrated to block proteoglycan-mediated cell attachment of both gram negative and positive bacteria within the genera Helicobacter,[159] Staphylococcus,[160] and Borrelia.[161] In these studies, only a single structural variant of sDex was studied in comparison with natural GAGs and the impact of DS was not explicitly discussed. In extensive studies by Thomas and Brooks, sDex showed potent inhibitory actions toward the attachment and subsequent infection of lung epithelia by Bacillus anthracis compared with unmodified dextrans.[162] Conversely, selected Gram-negative bacteria in the genera Burkholderia, Pseudomonas, and Legionella were inhibited by both the sulfated and the unmodified dextrans, indicating that sulfation is a negligible factor compared with monosaccharide sequence, or that the polysaccharides have different interaction targets.[163] Toxoplasma gondii is a unicellular parasite that can infect various types of human cells and can cause
severe disease particularly in immune-compromised patients. Cell entry is mediated by microbial adhesion proteins binding carbohydrate or protein epitopes on the cell surface. Dextran sulfates have been demonstrated to reduce infectivity of *T. gondii*, presumably by antagonizing lectins that normally bind sulfated polysaccharides in the ECM.[163] Inhibition of the malaria-causing parasite *Plasmodium falciparum* has been studied using a series of highly sulfated oligosaccharides (di- to hexasaccharides) varying in monosaccharide sequence and linkage types. Here, treatment with PI-88, a drug composed of sulfated mannose oligosaccharides, resulted in 50% inhibition of cell attachment compared with a nontreated control. *P. falciparum* utilizes chondroitin-4-sulfate as a receptor and addition of soluble CS resulted in 90% inhibition in the same study, indicating a high level of structural specificity.[164] Interestingly, Thomas and Brooks demonstrated that cell adhesion by gram-positive bacteria *Bacillus anthracis* is inhibited by sDex but not by unmodified dextrans.[162] sCels were developed in the early 2000s as a topical contraceptive and microbiocide, designated Usherell, as a preventative treatment to HIV infection.[165] However, the development was abruptly stopped when patients in a phase III trial showed increased rates of infection compared to the placebo. Preclinical studies of sCel showed antibacterial properties against several pathogenic bacteria associated with sexually transmitted infections, proposed to be caused by interactions with surface proteins required for cell division and pathogenicity.[163,166] These studies were however performed with relatively high dose concentrations (up to 10 mg mL⁻¹) indicating little specificity in the antimicrobial actions. Moreover, the sCels were not compared with a nonsulfated control, presumably due to solubility issues, to clearly demonstrate the effect of sulfation.

3.4. Immunomodulatory Properties

The immune system is activated by way of pathogen invasion, tissue injury, or exposure to foreign material surfaces, and plays a central role in host defense and tissue repair and regeneration. The two categories of immune responses are innate and adaptive immunity. Dendritic cells (DCs), natural killer (NK) cells, monocytes, and macrophages are the most important cells that are involved in innate immunity, while T lymphocytes and B lymphocytes are critical to adaptive immunity.[167,168] Macrophages are considered the key players of the innate immune system, and chronic inflammation results from persistent presence of inflammatory stimuli, with macrophages representing the driving force. However, macrophages can also be anti-inflammatory and are critical to the resolution of inflammation and tissue regeneration. These diverse functions are typically promoted by different macrophage subsets, originally referred to as M1 (proinflammatory) and M2 (anti-inflammatory) macrophages, which are modulated through crosstalk with other cells as well as by the presence of pro- or anti-inflammatory cytokines within the local environment.[166–172] Interactions between ESPs and these cytokines can contribute to the regulation of the immunological response, where ESPs potentially act as coreceptors or antagonists.

It has been shown that both natural polysaccharides and semisynthetic derivatives can regulate the function of the immune system through multiple pathways.[173] Of the different polysaccharide modifications, sulfation is one of the most examined with respect to immunomodulation effects, and many studies have shown that sulfation of polysaccharides can intensify the immunomodulatory properties of these polymers.[174,175] Moreover, it has been demonstrated that removal of sulfate groups on naturally sulfated polysaccharides leads to a markedly reduced immunomodulatory activity,[176–178] However, immunological processes are highly complex, and as evident in literature, sulfated polysaccharides may play a dual role, bringing about either immunostimulating or immunosuppressing effects (Figure 8).[179] A summary of key studies regarding the immunomodulatory roles of ESPs, concerning both immunostimulating and immunosuppressing effects is provided in Table 3. The structure of sulfated polysaccharides, such as molecular weight, degree of sulfation, sulfation pattern, as well as the model systems and experimental conditions used, are all critical factors in characterizing the immunomodulatory effects of ESPs.

3.4.1. Immunostimulation

Natural sulfated polysaccharides enhance activity of macrophages, e.g., through increased phagocytic activity,[186–190] and this has also been shown for ESPs such as sulfated starch and chitosan.[191–194] Furthermore, macrophages communicate with other immune cells and induce inflammation by secreting nitric oxide (NO), reactive oxygen species (ROS), and various inflammatory cytokines.[190] Multiple studies have shown that ESPs can induce expression and production of NO, ROS, and proinflammatory cytokines by macrophages, compared to their nonsulfated counterparts.[184,191–196] Yu et al. showed that a chemically sulfated polysaccharide (DS = 0.1) extracted from the plant *Cyclocarya paliurus* significantly increased NO, tumor necrosis factor alpha (TNF-α), and interleukins IL-1β and IL-6 secretion by macrophages, compared to the nonsulfated control.[194] Similar immunostimulating effects were observed in different studies on macrophages treated with sChit[184] or an ESP based on sulfation of a polysaccharide extracted from the fungi *Ganoderma atrum*. In an in vivo experiment, mice were treated with an intragastrically-administered ESP prepared from sulfation of another fungal polysaccharide from *Polyporus albicans* mycelia, which demonstrated that the stimulatory effect on NO and ROS production by macrophages was significantly enhanced in a dose- and DS-dependent manner.[195] In addition to macrophages, studies have found that sulfation of naturally nonsulfated polysaccharides enhances their immunostimulating effect on other cell types involved in innate immunity, including DCs[197] and NK cells.[196] Yao et al. showed that sulfation of a polysaccharide from *Echinacea purpurea* enhances its effect on phagocytosis as well as NO, IL-2, and IFN-γ production by DCs and also inhibits production of anti-inflammatory IL-4 and IL-10.[197] It was shown that sulfated K5 with a high degree of O-sulfation (DS = 0.3) enhances the apoptotic induction by NK cells.[196]

Surface receptors on macrophages known as pattern recognition receptors can recognize foreign ligands during...
initial phases of the immune response. Stimulation of macrophages by sulfated polysaccharides presumably occurs through their interaction with multiple pattern recognition receptors. The most notable example is the toll-like receptor (TLR)-4, present on macrophages, neutrophils, DCs, and several other cell types during inflammation. TLR-4 is the primary receptor for lipopolysaccharide (LPS) but can also be activated by several polysaccharides. Moreover, TLR-4 has been shown to play a key role in the immunomodulatory effects of both natural and ESP. According to one study, sChit treatment increases TLR-4 expression on RA W264.7 macrophages, and pretreatment of cells with TLR-4 inhibitor (TLF-4-IN-C34) partly decreases immunoactivating effects of the sChit. The authors further showed that the stimulation is regulated through NF-κB and AP-1 transcription factors involving p13K-Akt signaling. Similarly, treatment of DCs with an ESP from C. paliurus resulted in significant TLR-4 and -2 upregulation compared to the nonsulfated control, mediated by NF-κB and MAPK pathways. Blocking TLR-4 and -2 reduced this observed immunoactivating effect. Interestingly, a recent study showed that nonsulfated polysaccharides from C. paliurus downregulated TLR-4 expression in a mouse model of carbon tetrachloride (CCl4)-induced liver inflammation, resulting in decreased inflammatory response.

Regarding adaptive immunity, sulfated polysaccharides may enhance both cellular and humoral immunity by stimulating lymphocyte proliferation as well as cytokine and antibody secretion. Induction of lymphocyte proliferation by concanavalin A (ConA) or LPS has been used as a model for assessing stimulatory effects on the adaptive immunity, and it has been shown that ESPs alone or in combination with ConA or LPS can enhance lymphocyte proliferation significantly more than the nonsulfated polysaccharide control. ESPs from P. albicans mycelia significantly increased both ConA- and LPS-induced lymphocyte proliferation in a DS-dependent manner, where the highest DS (0.8) demonstrated the highest efficacy. When studying sulfated K5 with different degrees of sulfation and sulfation patterns, the strongest stimulatory effect on lymphocyte proliferation and cytokine secretion was observed for O-sulfated K5 with DS = 0.3. ESPs may further exert their immunoactivating effects through stimulating secretion of inflammatory cytokines such as TNF-α, IL-2, IL-4, IL-6, IFN-γ. ESPs from C. paliurus (DS = 0.1 and 0.5) significantly enhanced TNF-α, IL-2, and IL-4 secretion by T cells compared to the nonsulfated control. Interestingly, a higher DS favored more IL-2 secretion, which leads to Th1 type lymphocyte activation, while a lower DS favored more IL-4 production which leads to Th2 type cellular response. Based on their observed immunoactivating properties, ESPs have been evaluated as potential adjuvants for example in a Newcastle disease (ND) vaccine. A chemically sulfated polysaccharide from Astragalus plants showed improved adjuvant activity for the ND vaccine in a DS-dependent manner where the highest DS (1.5 compared to 1.2 and 1.3) had the highest efficacy. Guo et al. showed that sulfation of lentinan, licensed as potent immunoactivating drug in Japan, enhances its adjuvant activity and protection rate against ND virus through increased antibody production and lymphocyte proliferation with maximal effect at a medium DS (1.0) compared to low (0.7) and high (1.4) DS. In a similar experiment, polysaccharides derived from Lycium barbarum were sulfated to obtain ESPs with

Figure 8. ESPs show immunosuppressing and immunoactivating effects on lymphocytes, depending on the structural nature of the ESP and the model system employed. Left: ESPs exhibit immunosuppressing/anti-inflammatory properties through interference with the complement cascade (including assembly of the terminal complement complex (TCC)) and LPS-induced TLR4 activation. Inhibition of these pathways lead to reduced expression of pro-inflammatory cytokines. Right: ESPs activate TLR4 and promote expression of inflammatory cytokines and nitric oxide (NO).
different DS. The ESPs showed enhanced immunoactivating effect on lymphocyte proliferation and serum antibody levels compared to the nonsulfated control, and the ESP with an intermediate DS (1.5) was more effective than the one with a higher D (1.9).\textsuperscript{[210]}

It is evident that the ability of sulfated polysaccharides to stimulate and activate immune pathways strongly depends on the degree of sulfation. In studies applying ESPs with various DS, some show a positive correlation between increasing DS and the immunoactivating effects.\textsuperscript{[195,207,214]} While other studies have achieved the highest efficacy at an intermediate DS.\textsuperscript{[191,206,210,211]} As an example immunomodulatory effect of three ESPs derived from \textit{Ganoderma} fungal polysaccharides (DS = 0.6, 0.8, 3.4) were studied on macrophages from mice peritonea. The ESP with moderate DS (0.8) and a molecular weight of 4 kDa exhibited the highest efficacy in increasing the macrophage phagocytosis capacity and TNF-\textit{\(\alpha\)} production. As the DS was further increased to 3.4, the TNF-\textit{\(\alpha\)} secretion was dramatically decreased.\textsuperscript{[193]} Since the different DS in most of the studies is coupled with different MW, carbohydrate content and in some cases sulfation pattern, which all can affect the immunomodulation properties, it is not straightforward to draw a direct structure–function relationship.

Aside from the degree of sulfation, other structural features of polysaccharides, such as MW, monosaccharide composition and linkage type, can strongly affect immunoregulatory properties of the ESP. While both nonsulfated and sChit oligosaccharides with a degree of polymerization between 2 and 6 have shown immunosuppressing effects on LPS-induced RAW 264.7 macrophage cells,\textsuperscript{[215–217]} nonsulfated higher molecular weight chitosan (3 and 50 kDa) exhibited immunostimulatory activity.\textsuperscript{[218]} In a study to investigate the effect of sulfation on immunomodulatory properties of chitosan, \(\alpha\)-chitosan, and \(\beta\)-chitosan with different MWs were sulfated and their effect on NO production by RAW 264.7 macrophages was measured. It was found that while sulfation in general increased NO production, \(\beta\)-chitosan sulfate had a more stimulatory effect than \(\alpha\)-chitosan sulfate, presumably due to its higher solubility and reactivity, and among its different MWs (3, 5, and 15 kDa), the 5 kDa \(\beta\)-chitosan sulfate exhibited the highest stimulatory effect.\textsuperscript{[184]}

\begin{table}
\centering
\caption{Reported immunoactivating and immunosuppressing effects of selected ESPs in various model systems. The degree of sulfation (DS) is expressed as the average number of sulfate groups per monosaccharide, where reported, or as %wt sulfate in the polysaccharide sample.}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{ESP} & \textbf{DS} & \textbf{Model system} & \textbf{Effect of ESP} & \textbf{Ref.} \\
\hline
\textit{sChit} & 30\% & RAW 264.7 macrophages & Increased NO, TNF-\(\alpha\), IL-1\(\beta\), IL-6, and PGE2 secretion & [184] \\
\textit{sKS} & 0.1–0.5 & RAW 264.7 macrophages and mice spleen lymphocytes & Increased macrophage viability, TNF-\(\alpha\) and IL-1\(\beta\) secretion, spleen lymphocyte proliferation and IL-2, IFN-\(\gamma\), IgG1a, and IgG2b secretion & [196] \\
\textit{P. albicans} & 0.5 & Intragastrical administration to mice & Increased splenocyte proliferation & [195] \\
\textit{ESP} & 0.6 & & Increased NO and ROS production & [195] \\
\textit{ESP} & 0.8 & & & [195] \\
\textit{ESP} & 3.4 & & & [195] \\
\hline
\textit{Ganoderma} & 0.7 & Mice peritoneal macrophages & Increased TNF secretion and phagocytic activity & [197] \\
\textit{ESP} & 0.8 & & & [197] \\
\textit{C. paliurus} & 3.4 & & & [197] \\
\hline
\textit{C. paliurus} & 0.1 & RAW 264.7 macrophages & Increased NO, TNF-\(\alpha\), IL-1\(\beta\), and IL-6 secretion and phagocytic activities & [194] \\
\hline
\textit{C. paliurus} & 0.1 & Spleen lymphocytes & Increased TNF-\(\alpha\), IL-2, and IL-4 secretion & [211] \\
\textit{ESP} & 0.5 & Spleen lymphocytes & Increased spleen lymphocyte proliferation & [211] \\
\hline
\textit{sAlg} & 0.3 & Human whole blood & Decreased IL-1\(\beta\), TNF, IL-6, IL-8, MIP-1a secretion & [180] \\
\textit{sAlg} & 0.9 & Chondrocytes & Decreased IL-6, IL-8, COX-2, and ADAMTS-5 expression & [219] \\
\textit{sAlg} & 0.1–1.1 & Human chondrocytes THP-1 cell line (human monocyte) & Decreased IL-6 and CXCL8 gene expression in chondrocytes. Decreased expression of TNF-\(\alpha\) in M1-like macrophages & [220] \\
\textit{sHA} & 1.7 & Human macrophages & Decreased TNF-\(\alpha\), IL-12(p40), IL-6, and MCP-1 secretion & [221] \\
\textit{sHA} & 0.5 & Human blood monocytes & Decreased IL-1\(\beta\), IL-8, IL-12p40, TNF-\(\alpha\), and RANTES secretion and increased IL-10 and MCP-1 secretion & [222] \\
\textit{sK5} & N.a. & Human mononuclear cells & Decreased IL-1\(\beta\), IL-6, and TNF-\(\alpha\) secretion & [223] \\
\textit{S. cristaefolium} & 1\% & RAW 264.7 macrophages & Decreased NO production & [182] \\
\textit{ESP} & 9\% & & & [182] \\
\textit{C. paliurus} & 51\% & RAW 264.7 macrophages & Decreased NO production, phagocytic activity, and IL-6 and IL-1\(\beta\) secretion in vitro & [224] \\
\hline
\textit{Astragalus} & 1.4 & Caco2 cells & Decreased TNF-\(\beta\), IL-1, and IL-8 expression & [225] \\
\hline
\end{tabular}
\end{table}
The reviewed literature further shows that different polysaccharides with vastly different DSs and MWs can show similar levels of immunoactivation, indicating that other structural properties can have significant effects and/or large variations in the model systems and experimental conditions used. It should also be noted that most of the reviewed studies in this section are based on plant or fungal polysaccharides with highly complex and often incompletely characterized structures. It is thus difficult to elucidate clear relationships between monosaccharide sequences and the observed immunoactivating effects.

3.4.2. Immunosuppression

Inspired by the anti-inflammatory properties of naturally sulfated polysaccharides such as heparin,\textsuperscript{226–228} researchers have tried to develop ESPs from different polysaccharide sources and investigate their immunosuppressive and anti-inflammatory characteristics. Moreover, since use of heparin as an immunosuppressing agent is hindered due to its anticoagulating effects, development of other non-anti-coagulating heparin-like materials for immunomodulation is encouraged. Interestingly, and in contrast to the previous section, it has been shown that sulfation of polysaccharides can induce anti-inflammatory properties or enhance the already-existing immunosuppressive effects of the polysaccharide. It has been demonstrated that sulfation can suppress or dampen immunological processes mainly by blocking inflammatory signal transduction induced by pro-inflammatory cytokines and suppressing the activation of the complement cascade.\textsuperscript{180,229}

ESPs have been demonstrated to inhibit (LPS)-stimulated inflammation in immune cells. Preincubation of RAW264.7 macrophages with sulfated fungal polysaccharides from *Antrodia cinnamomea* before LPS stimulation has been shown to have significantly stronger inhibitory effect on TNF-\(\alpha\) and IL-6 production as compared to the nonsulfated polysaccharide.\textsuperscript{229} ESPs with different sulfate contents (0.8–50\%) based on a naturally sulfated polysaccharide from the brown alga, *Sargassum cristaefolium*, were produced via oversulfation and desulfation of the natural polysaccharide and were added to the LPS-containing culture media of macrophages. All the ESPs showed inhibitory effect on NO production via macroporphs in a sulfate content-dependent manner, where ESPs with an intermediate sulfate content (9.4\%) exhibited a greater inhibitory effect compared with low (0.8\%) and high (31.1\%) sulfate content.\textsuperscript{182} Wang et al. showed that preincubation of macrophage with ESPs from *C. paliurus* polysaccharide with DS=0.4 suppressed phagocytosis, NO production, and the release of IL-6 and IL-1\(\beta\) after LPS-stimulation. These effects were also observed for the nonsulfated polysaccharide, but with a much lower potency compared with the ESP. Moreover, the ESP improved the thymus and spleen index values, over, the ESP improved the thymus and spleen index values, and complement activity through the classical and alternative pathways.

This study indicates that the presence of the sulfate group as well as stereochemistry and sulfate position are key structural factors that determine the immunomodulatory properties of the ESPs. The exact mechanisms involved in inhibition of LPS-induced inflammation is not well elucidated in literature. It has recently been shown that suppression of the NF-\(\kappa\)B activation pathway through restoration of LPS-induced p38, ERK1/2, and JNK phosphorylation in a concentration-dependent manner,\textsuperscript{182} Furthermore, decreased expression of the TLR-4 receptor has been reported for an LPS-stimulated human epithelial cell line when preincubated with sulfated polysaccharide.\textsuperscript{225}

The complement system plays an important role in host immune defense against infection; however, its overactivation may result in various diseases such as systemic lupus erythematosus, rheumatoid arthritis, and acute respiratory distress syndrome.\textsuperscript{229} Heparin shows profound anticomplement activity, but its undesirable anticoagulating effect limits its application in the treatment of complement-associated diseases.\textsuperscript{230} Here, ESPs with tailored structures have been synthesized, which have potent anticomplement activities with few or no anticoagulating effects.\textsuperscript{232} Sulfation of a polysaccharide from the roots of *Saussurea costus* resulted in ESPs with five different DS values ranging from 0.4 to 2.2. All ESPs demonstrated potent anticomplement activity through the classical and alternative pathways in a dose-dependent manner, in contrast to the native polysaccharide, which did not exhibit such activity. The effect was positively correlated with the DS of the ESP and at high DS (>2) the effect was stronger than that of heparin. Also, all the ESPs had limited anticoagulating effects, proposed to be related to their low MW (3.6 kDa) and lack of specific antithrombin activity.\textsuperscript{233} sAlg have also shown anticomplement effects.\textsuperscript{31,219} Coincubation of sAlg with different monosaccharide sequences and complement-stimulated human plasma inhibited the formation of the terminal complement complex, where the most efficient alginate sequence was an epimerized, alternating MG structure. The increased efficacy of this repeating disaccharide sequence was proposed to be related to a higher relative chain flexibility compared with poly-M and poly-G.\textsuperscript{14} Chemical sulfation of a *Ganoderma lucidum*-derived polysaccharide conferred anticomplement properties, inhibiting both the classical and alternate pathways. The authors suggested two hypotheses for the potential mechanisms of the ESPs which need elaboration through further experiments; one was that the ESP inhibited the formation of C3- or C5-convertase, and the second was that the ESP bound to C3 or C5, protecting them from activation by proteolytic cleavage.\textsuperscript{234}

Integration of biomaterials into tissues is often disturbed by an adverse immune response to foreign surfaces, involving migration of monocytes to the implantation site and their
differentiation to inflammatory M1 macrophages. While high-molecular weight HA (HMW-HA, >1000 kDa) has previously demonstrated anti-inflammatory properties, low-molecular weight HA (LMW-HA, ≈250 kDa) exhibits immunoactivating and inflammatory activities. In one study, LMW-HA (28–50 kDa) was sulfated at low and high degrees of sulfation (DS = 0.6 and 1.6) and the effect on the differentiation of monocytes into macrophages was assessed in a sterile inflammation model. Where low-DS sulfated hyaluronic acid (sHA) still induced an inflammatory response, high-DS sHA stimulated a shift in the differentiation of monocytes from the M1 to the M2 phenotype, resulting further in reduced release of inflammatory cytokines (IL-1β, IL-8, IL-12(p40), and TNF-α), while increasing release of IL-10 and MCP-1 and cell surface expression of CD163. In a follow-up study, the authors investigated the impact of high-DS sHA on fully differentiated human inflammatory macrophages, resulting again in a reduced cytokine response of macrophages after inflammatory activation. The macrophages were found to internalize sHA by a mechanism involving CD44 and scavenger receptors, CD36 and Lox1. This resulted in increased gene expression of SOD2, SOD3, and GLRX3, which was additionally linked with reduced activation of the transcription factors NFκB, STAT1, and IRF5 (Figure 9). These results interestingly indicate that ESPs may have effects on intracellular signaling pathways involved in immunological responses, in addition to binding extracellular signaling molecules and receptors.

Microbeads and microspheres have attracted much attention for applications like cell-encapsulation or drug delivery but still face biocompatibility challenges, motivating assessment of immunosuppressing modification strategies. One example is Alg microspheres, where sAlg have been incorporated within the hydrogel network or used as a secondary coat on poly-L-lysine (PLL)-coated microspheres to assess anti-inflammatory properties in a human whole blood model. It was shown that microspheres containing sAlg suppressed inflammatory cytokine (IL-1β, TNF, IL-6, IL-8, MIP-1α) production and complement activation compared to unmodified microspheres and a saline control. Microspheres coated with sAlg showed a reduced cytokine response compared with PLL coating, where a low DS (0.3) was more effective than a high DS (0.8), but showed a simultaneously elevated complement response, presumably due to electrostatic binding and aggregation of complement factors on the microbead surface. Indeed, a large amount of complement factor C3 was found to adhere to the surface of the coated capsules, indicating a strong interaction with sAlg.

Immunosuppressing biomaterials are of further interest in inflammatory diseases such as osteoarthritis (OA). In a study to explore anti-inflammatory properties of sAlg for cartilage tissue engineering, primary human chondrocytes were encapsulated in sAlg hydrogels and subjected to inflammatory induction by IL-1β. Gene expression of proinflammatory markers IL-6, IL-8, COX-2 and aggrecanase were significantly lower in the sAlg gels compared with the nonsulfated control. Moreover, matrix metalloprotease-13 (MMP-13) expression was suppressed by sAlg under nonstimulated conditions but not following IL-1β stimulation. The results further indicate multiple roles of ESPs in immune regulation, where they interact with soluble signaling factors, as well as cell surface receptors and intracellular signaling pathways, as described in previous sections. In a follow-up study, it was shown that sAlg has DS-dependent anti-inflammatory and immunomodulatory properties. Supplementation of sAlg in the culture media resulted in reduced IL-1β-mediated expression of IL-6 and CXCL8 by chondrocytes and reduced...
expression of TNF-α and other inflammatory markers in M1-like macrophages. The potency of these effects was found to increase with the DS of the sAlg.[220]

3.5. Antioxidant Properties

Oxidative stress is believed to be a primary factor in various degenerative diseases, such as cancer, rheumatoid arthritis, atherosclerosis, and Alzheimer's disease, as well as in the normal process of aging.[236–238] Thus, research efforts have been made to characterize novel antioxidant agents for biomaterials, pharmaceuticals, and nutraceuticals, which can scavenge or prevent the production of ROS and protect cells from oxidative damage.[238] Polysaccharides have attracted a lot of interest in this field, being natural compounds and because of their ability to donate hydrogen atoms to free radicals.[239,240] The presence of sulfate groups could increase their ability to provide hydrogen atoms by activating the hydrogen atom of the anomeric carbon, promoting the antioxidant activity of polysaccharides.[191,241]

In vitro free radical systems, such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and hydroxyl radicals are commonly used to evaluate the antioxidant activities of polysaccharides. It has been shown that sulfation of Codonopsis pilosula polysaccharides at DS = 1.8 significantly improves its scavenging activity for all three radicals and that the performance of the sulfated polysaccharide was close to that of vitamin C.[242] Moreover, a different study showed that hydroxyethyl sChit could scavenge DPPH and carbon-centered radicals effectively, although it did not exhibit any scavenging activity against hydroxyl radicals.[245]

Multiple studies have found that the degree of sulfation has a positive correlation with antioxidant activities,[246–250] although the opposite trend has also been observed. Studies on chemically sulfated polysaccharides from C. paliurus with DS = 0.1–0.5 and different MWs showed that the polysaccharide with the lowest DS and MW exhibited the greatest antioxidant activities in hydroxyl, superoxide, DPPH radical, and b-carotene-inoleic acid assays.[251] In one study, regioselective sulfation of Chit was performed, followed by a comparison of the antioxidant effects of 2-N/3-O/6-O, 3-O/6-O, or 6-O-sulfated sChit. All sChit samples showed improved scavenging activity of superoxide radicals compared to nonsulfated control. Interestingly, the 6-O-sulfated sChit demonstrated similar scavenging activity to the higher sulfated samples, indicating that the sulfation site has a high importance for its antioxidant activity.[252]

Hydrogen peroxide (H2O2) is an efficient oxidative agent that can change cell permeability and cause cell membrane damage and is frequently used for cell oxidative damage models. In a study, two sulfated polysaccharides from C. paliurus with DS = 0.1 and 0.4 were found to reduce H2O2-induced oxidative stress compared to the nonsulfated polysaccharide. Specifically, the authors found that the low-DS ESPs maintained the viability of the H2O2-stimulated RAW264.7 cells and inhibited lipid oxidation more effectively than the high-DS samples. Meanwhile, the higher-DS ESPs increased superoxide dismutase (SOD)
activity in the cells, which is one of the representative antioxidant enzymes. A later study proposed that the underlying protective effect on RAW264.7 cells from H$_2$O$_2$-induced oxidative damage was through maintaining the cellular structure, improving the activity of antioxidant enzymes such as SOD and glutathione peroxidase (GSH-Px), and inhibiting caspase-3 activation and DNA fragmentation. Since there is a strong correlation between oxidative stress and diabetes, dietary supplementation with an antioxidant can potentially contribute to its treatment. Oral administration of a chemically sulfated polysaccharide isolated from the plant Achyranthes bidentata was found to significantly reduce blood glucose levels and malondialdehyde concentration, while increasing activities of GSH-Px and SOD in diabetic rats. The nonsulfated polysaccharide displayed much lower protective effects.

### 3.6. Therapeutic Delivery

#### 3.6.1. Growth Factors

Growth factors (GFs) play a pivotal role in cell proliferation and the development and homeostasis of tissues. However, direct injection of GFs in tissue repair are problematic because of their short biological half-lives in vivo, and low supraphysiologic concentrations. Many studies have therefore focused on developing suitable carriers and controlled delivery systems for optimal delivery of GFs. Physical entrapment or covalent binding of GFs in polymeric matrices are among the most studied methods, but lead to either too fast or slow release rates. Inspired by the controlled-release mechanism found in the native ECM, another class of delivery system called affinity-based systems has been proposed. Heparin is known to naturally bind and stabilize numerous protein therapeutics such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factors (TGF), bone morphogenic protein (BMP), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) families with medium- to high-affinity. Thus, many of the affinity-based systems include heparin or other sulfated GAGs in GF delivery systems by physical incorporation or chemical conjugation. However, aside from the adverse clinical effects of heparin, its heterogeneity makes it challenging to reproduce and to control its binding. Therefore, next-generation biomimetic affinity-based systems for GF delivery have been designed by incorporating ESPs, allowing for functionalization of hydrogel-forming polysaccharides and polysaccharides with regular repeating units, to increase in vivo stability of the delivery vehicles and avoid complications related to structural heterogeneity. Besides serving as physical carriers, ESPs can act as coreceptors and/or reservoirs for growth factors to facilitate signaling pathways and stabilize proteins against enzymatic hydrolysis. Table 4 includes a summary of studies focused on developing growth factor delivery carriers based on different ESPs.

Desulfation of chondroitin CS was shown to significantly increase the release of the positively charged model protein histone from CS hydrogels, confirming that sulfation plays a significant role in modulating protein interactions with GAGs.

| Table 4: Immobilization of growth factors in ESP-based materials. The degree of sulfation (DS) is expressed as the average number of sulfate groups per monosaccharide, where reported, or as %wt sulfur in the polysaccharide sample. N.a. = not available. |
|---|---|---|---|---|
| **ESP** | **DS** | **Material** | **Growth factor** | **Ref.** |
| sAlg 7.9% | Ionomically cross-linked microspheres | HGF | [265] |
| sAlg 7.9% | Ionomically cross-linked microspheres | bFGF | [31] |
| sAlg 7.9% | Ionomically cross-linked freeze-dried scaffold | VEGF, PDGF, TGFβ1 | [268] |
| sAlg 7.9% | Ionomically cross-linked microspheres | HGF, IGF-1 | [269] |
| sAlg 0.6 | Ionomically cross-linked hydrogel | FGF-2 | [270] |
| sAlg 0.4, 1.0, 1.4 | Immobilized on gold and polystyrene substrates | FGF-2 | [271] |
| sAlg 0.4, 1.0, 1.4 | Films | FGF-2 | [272] |
| sAlg 0.8, 3.4, 12.4% | Electrospun nanofibers with PVA | TGF-β1 | [273] |
| sAlg 0.2 | Bioink (Agarose, Pluronic F127) | BMP-2 | [274] |
| sCMC 1.0 | EDC/NHS cross-linked scaffolds with gelatin | TGF-β1 | [280] |
| sCMC 1.4 | HRP cross-linked hydrogel | TGF-β1 | [281] |
| sHA 0.5, 1.5 | Collagen I-based scaffold | TGF-β1 | [276] |
| sHA 1.6, 2.9% | Photocross-linked hydrogel | TGF-β1 | [109] |
| sHA 1.5 | Collagen I-based microgels | TGF-β1 | [277] |
| sHA 1.4 | Photo-cross-linked hydrogel | SDF-1α | [278] |
| sHA 0.6 | Collagen I-based scaffold | EGF | [279] |

In a separate study, a set of selectively desulfated heparin derivatives was utilized for sustained FGF-2 delivery from hydrogels formed by cross-linking amino end-functionalized four-arm star-polystyrene glycol (PEG) with activated carboxylic acid groups of heparins. While generally lower amounts of FGF-2 were immobilized with decreasing sulfate content, the absolute quantities of released GFs after 4 days were found to be dramatically elevated. Removal of only one sulfate residue (DS = 1.0) resulted in a threefold increase of the cumulative protein release. This effect was further enhanced by removing both the 6-O- and N-sulfate (DS = 0.6), resulting in a fivefold increase in FGF-2 release levels. Additional elimination of the 2-O-sulfate for (DS = 0.3) boosted the release efficiency by up to an 11-fold increase compared to that of standard heparin. Thus, the study provided a good example of how tuning the DS and sulfation pattern can contribute to control of growth factor affinity and subsequent release kinetics from ESP-based biomaterials.

Freeman et al. found that sAlg at DS = 1.0 has a high affinity to heparin-binding growth factors, as revealed by SPR analysis. No such interactions were observed with the unmodified Alg or for nonheparin-binding peptides with sAlg. In the same study, only bFGF showed stronger binding to heparin than to sAlg. This could be partly due to differences in DS of the two polymers and partly due to other interactions such as hydrogen bonding and van der Waals packing which may be reduced in Alg or its sulfated form. In a follow-up study, delivery of
three angiogenic factors (VEGF, PDGF-BB, and TGF-β1) from an Alg/sAlg hydrogel was demonstrated, exploiting the differential affinity of the GFs to sAlg to obtain a diffusion gradient. The release of all three growth factors from the sulfated scaffold over an 8-day period was significantly slower than their release from the nonsulfated scaffold over an 8-day period. Moreover, the kinetics of release from sAlg alginate scaffolds agreed with their measured binding affinity, which was VEGF < PDGF-BB ≈ TGF-β1. This mimicked the signal cascade acting in angiogenesis, namely, the initiation of the process by VEGF, followed by vessel stabilization by PDGF-BB, and finally, vessel remodeling induced by TGF-β1. These triple-growth factor-loaded sulfated scaffolds led to the formation of more stable and mature blood vessels three months after implantation in rats, compared to nonsulfated scaffolds.[268]

sAlg has furthermore been demonstrated to interact with HGF in multiple studies.[31,78,265,269] One study observed that the release rate of HGF from sAlg hydrogels was three times lower than for unmodified Alg hydrogels over an incubation period of one week.[265] Sequential delivery of HGF and insulin-like growth factor-1 (IGF-1) from sAlg has been achieved for promotion of myocardial repair. Despite similar affinity binding and even stronger binding of IGF-1 to sAlg, greater initial release rate was achieved for IGF-1, due to its smaller size and tenfold higher molar concentration in the sAlg hydrogels compared to HGF.[268] The release of FGF-2 from sAlg hydrogels has also been studied. Hydrogels containing sAlg with DS = 0.6 showed a significantly lower cumulative release of FGF-2 at all chosen time points compared to nonsulfated hydrogels, and almost 40% of initially loaded FGF-2 was retained in the scaffold after two weeks. In such affinity-based release systems, the release rate is controlled both through affinity interaction strength and through matrix pore size. The presence of sulfate groups results in increased swelling and permeability and therefore increased hydrogel pore size, an effect which could partially reduce the overall affinity of the material for GFs. This may be the reason for the observed similar release profile of FGF-2 from sAlg hydrogels with DSs ranging from 0.2 to 0.6.[270] In a different study, sAlg films were prepared, and FGF-2 interactions were assessed by QCM-D and immunostaining. Here, sAlg with DS = 1.0 showed a higher degree of interaction with FGF-2 compared with DS = 1.4, attributed to a higher relative chain flexibility.[271] This was not observed for interaction studies of sAlg in solution or in layer-by-layer films, where a similarly high DS resulted in the highest degree of FGF-2 binding.[272] Recently, release of growth factors from nanofibrous and printed structures based on sAlg has been investigated. It was shown that the release of absorbed TGF-β1 from the electrospun nanofibrous scaffold composed of sAlg and PVA is much slower compared to an Alg/PVA scaffold.[273] BMP-2 release from a 3D-printed Alg/sAlg bioink has also recently been studied for bone tissue engineering applications, and showed that increasing sAlg concentration in bioink solution resulted in slower release of the GF over a 10-day period.[274]

Hintze et al. found that sulfation of HA increases its interaction with BMP-4 and TGF-β1 in a DS-dependent manner.[93,275] Moreover, sHA exhibited higher binding strength to TGF-β1 than CS of the same degree of sulfation and similar MWs, which could be due to different molecular geometries in the polysaccharide backbone and different sulfation patterns.[275] TGF-β1 release from scaffolds composed of sHA and collagen I (Coll I) has also been studied, and slower GF release and more retention was observed in the presence of sHA in a DS-dependent manner.[276] Photocross-linked hydrogels composed of sHA and incorporated TGF-β1 have been developed for cartilage tissue engineering. The released amounts of TGF-β1 from the low- and high-sulfated hydrogels were respectively 40% and 50% lower than those of the nonsulfated hydrogels after 7 days of incubation at 37 °C.[109] In a recent study, Hintze et al. fabricated sHA/Coll I photocross-linked microgels by a microfluidic technique, incorporated them into bulk HA/Coll I hydrogels and performed a second cross-linking before measuring uptake and release of TGF-β1 over 15 days. As a control, the researchers mixed sHA directly into HA/Coll I bulk hydrogels, to investigate the effect of the microgel system on release kinetics. Both scaffolds demonstrated higher uptake and slower release of GFs compared to scaffolds without sHA. Moreover, scaffolds containing microgels bound more TGF-β1 after overnight incubation and had significantly slower releases compared to sulfated scaffolds without the microgels, and they retained more GF after 15 days of release (24% vs 15% of the initial amount). Here, a higher release rate of GFs from bulk hydrogels can be affected by diffusional release of sHA from the scaffold, as opposed to its retention in the cross-linked microgel structure.[277] In a different study, release of stromal cell-derived factor-1 (SDF-1) from HA/sHA photocross-linked hydrogels was studied, and it was found that the presence of 10% wt/wt sHA in the hydrogel significantly slowed SDF-1 release over a 12-day period compared to pure unmodified HA hydrogels.[278] In a follow-up study, the authors further reported enhanced binding and significantly slower release of EGF from photocross-linked sHA/Coll I scaffolds compared to nonsulfated ones.[279]

One study on sulfated carboxymethyl cellulose (sCMC) scaffolds showed high TGF-β1 affinity of the ESPs, sequestering 92% of initially loaded protein after overnight incubation and releasing only 25% of the loaded amount after 18 days with an initial burst release of only 6% on the first day.[280] In another, similar study, injectable hydrogels based on CMC and gelatin, using HRP enzyme for enzymatic cross-linking, were developed for TGF-β1 delivery. It was shown that sulfated hydrogels could sequester more than 90% of the total TGF-β1 loaded for about 4 weeks, compared to 70% sequestration for the nonsulfated control.[281] Using a library of dextran derivatives functionalized with carboxymethyl, benzylamide, and sulfate, one study demonstrated that interaction with platelet-derived growth factor BB homodimers (PDGF-BB) increased as a direct function of DS.[282] In a different study, similarly modified dextran demonstrated binding to TGF/β1 at DS > 0.5, where hydrophobic interactions with benzylamide were also shown to contribute to the binding affinity.[283]

Polysaccharide chain extension can influence protein interaction strength by altering charge orientation and density in sulfated polysaccharides. As previously described, iduronic acid in heparin exhibits conformational flexibility, presumably as a part of specific and cooperative binding to proteins, whereas periodate oxidized sAlg have demonstrated increased chain flexibility resulting in enhanced GF interaction.[78] For certain polysaccharides, these effects can be reduced by
altering the pH or salt concentrations, causing protonation or shielding of negative charges with counterions. Studies on displacement of HGF from the cell surface by sAlg demonstrated that increasing the DS from 0.5 to 1.0 resulted in a three- to fourfold increase in relative interaction strength for sulfated poly-M and poly-G sequences. Interestingly, an alternating poly-MG structure showed equally high interaction values at DS = 0.5 as at 1.0, indicating an additional influence of chain flexibility from increased rotation around the glycosidic bond.[131] The influence of saccharide chain length on protein interactions can be studied systematically by employing defined oligosaccharides, prepared through chemical or enzymatic depolymerization and preparative SEC prior to sulfation. This approach was used to identify the minimum chain length of sAlg displacing HGF and FGF-2 from HS, and to achieve interaction strengths equivalent to the polysaccharides of the same DS.[78] The same methodology was earlier employed to study the binding of chemically synthesized HS oligosaccharides to FGF-2 and VEGF.[284] For native or ESPs with complex and heterogeneous sulfation patterns such as HS, utilization of oligosaccharides and regioselective sulfation/desulfation strategies can additionally identify critical substitution patterns for biological activities, as demonstrated in functional studies on sulfated GAGs.[285]

3.6.2. Enzymes

ESPs have also been used for enzyme immobilization, either through direct complexation with the enzymes or as polyanions in polyelectrolyte complex (PEC) formulations as encapsulation devices.[286] Sulfated dextran (sDex) has been used in coatings to adsorb proteins contained in a crude extract of E. coli and has shown better performance compared to commercial carboxymethyl cellulose (CMC) and aspartate-modified dextrans, due to higher negative charge density of the sDex leading to an improved adsorption capacity. The sDex coating was also effective in immobilization of industrial enzymes including β-galactosidases and lipases at both pH values of 5 and 7, where the enzymes also maintained full activity.[287] Moreover, sDex complexes with poly(methyleniminophosphazene) hydrochloride have been shown to be effective in immobilization of insulin and its protection against proteolytic degradation.[288] sCel has also been used in PEC formulations for encapsulation of enzymes. Glucose oxidase was shown to be effectively loaded in a formulation consisting of a low-DS (0.2) sCel as polyanion and poly(dimethyldiallylammonium chloride) as polycation in ionic liquids. This formulation resulted in capsules with improved mechanical stability compared to water soluble sCel with higher degrees of sulfations (DS > 0.3).[289]

Lysozyme is a natural enzyme involved in the innate immune response, and its application as an anticancer drug is also under investigation.[298,299] It is further used as a positively charged model protein for testing therapeutic carriers of positively charged polypeptides such as growth factors. Regioselective synthesis of 6-O-sulfated chitosan (C6S), 2-N-6-O-sulfated chitosan (C26S), and 3,6-O-sulfated chitosan (C36S) was performed to investigate their direct interaction with lysozyme. It was found that all sChit derivatives exhibited lysozyme binding as opposed to nonsulfated chitosan, but with different strengths. Even though C6S possessed the lowest DS among the three sChits, it exhibited the highest binding activity with lysozyme, indicating that 6-O-sulfate groups may be responsible for the high affinity and presumed specific interaction of sChit with lysozyme.[292] Lysozyme release from photocross-linked scaffolds containing sHA has been investigated, demonstrating enhanced binding and retention and slower release of lysozyme over 8 days compared to nonsulfated HA. Here, there was no significant difference in the release profile for scaffolds containing sulfated HA with or without 6-O-sulfation.[293]

3.6.3. Small Molecules

In addition to enzymes and other polypeptides, ESPs have also been used in carriers for small molecule drugs. N-alkyl-O-sulfated chitosan (NOSC) containing alkyl groups as hydrophobic moieties and sulfate groups as hydrophilic moieties has an amphiphilic character and the ability to form micelles around 100–400 nm in size.[294,295] Such micelles have been evaluated as drug delivery carriers, e.g., for the anticancer Paclitaxel (PTX).[296,297] Here, in vivo studies in rats showed that the oral bioavailability of Paclitaxel loaded in NOSC micelles was increased sixfold in comparison to that of an orally dosed Taxol, the commercially available product, and had significantly less toxicity. In addition, it was shown that the effect of NOSC micelles on enhancing the absorption of PTX results from inhibition of P-gp, which functions as an ATP-dependent efflux pump to transport the chemotherapeutics out of the cells. Moreover, the mechanism of P-gp inhibition by NOSC was proved to be competitively impeding the binding of PTX with P-gp and reducing the fluidity of the cell membrane.[298,299] Murali et al. prepared sChit nanoparticles loaded with an antifungal, Amphotericin B (AmpB), to treat intracellular Candida glabrata infections. The prepared particles, with a diameter of ~300–310 nm, showed superior intracellular killing of C. glabrata in infected RAW264.7 macrophage cell lines compared to the drug alone and nonsulfated nanoparticles. The more potent antifungal activity of the AmpB-loaded sulfated nanoparticles could be attributed to the presence of sulfate groups, which aids phagocytosis of the particles by the RAW264.7 cells as opposed to uptake of nonsulfated nanoparticles through a general endocytic process and simple diffusion of AmpB alone.[300] sAlg has been used for ionic complex formation with small molecule cationic drugs as a potential delivery system. One study found that sAlg forms complexes with the cationic drug tetracycline hydrochloride (TCH), while no TCH–polymer complexes were observed using unmodified alginates.[301] In a different study, sulfated dextran-based nanocarriers were developed for targeted delivery of methotrexate (MTX) as an antiinflammatory drug to activated macrophages in inflamed cartilage tissue. Amphiphilic self-assembled nanocarriers with an sDex hydrophilic shell and a hydrophobic drug reservoir of 5β-cholanic acid were synthesized. The sDex sulfate shell improved targetability of the system to inflamed tissues.
through scavenger receptor class A, which is upregulated by the inflamed cells. The MTX-loaded nanoparticles exhibited significantly improved therapeutic efficacy against collagen-induced arthritis in mice compared to free MTX alone.\cite{302}

3.7. Regenerative Medicine

3.7.1. ESPs in Bio- and Nanomaterials

ESPs are of great interest in scaffold-based tissue engineering and other cell- and drug-encapsulation systems, due to their structural and functional analogy to the GAGs of the ECM. Their utilization in cross-linked hydrogels and other biomaterial types is therefore described in multiple recent studies. The most common strategies for ESP incorporation in biomaterials involve ionic cross-linking, exploiting inherent properties of the polysaccharides or through complexation with cationic polymers, or modification with reactive side groups forming covalent bonds. Alternatively, polysaccharides can be modified with hydrophobic side groups leading to coacervation. Cross-linking further allows formation of 3D hydrogel networks, fibers, layer-by-layer structures, particles/micelles, and other types of structures (Figure 11).

Alginites are commonly used due to their inherent ability to form hydrogels in the presence of divalent cations, which can be retained after sulfation. As the DS increases, the hydrogel stiffness decreases to the point where the sAlg are unable to form stable gels, as the bulky sulfate groups prevent sufficient intermolecular cross-linking.\cite{219,270,363} For sAlg cross-linked with calcium, a threshold has been demonstrated around DS $= 0.3–0.4$, where higher levels of sulfation result in a significant drop in hydrogel stiffness.\cite{219} This threshold will also depend on the type and molecular weight of alginate used, methods of gelation and rheological measurement, and choice of gelling ions. As alginate hydrogel stiffness is directly correlated with molecular weight, sulfation will also influence gel properties through partial depolymerization in the sulfation reaction. The sulfate groups associate with gelling ions and presumably form weak and transient cross-links, resulting in an overall decrease in stiffness and an increase in osmotic swelling.\cite{219} Interestingly, the combination of hydrogel beads with a low relative content of sAlg mixed with unmodified alginites (20/80 mixing ratio) has demonstrated less long-term swelling in saline compared to pure unmodified alginate beads. This is presumably due to increased electrostatic potential leading to retention of gelling ions in the hydrogel.\cite{219} Specific natural and engineered carrageenans form thermoreversible hydrogels in the presence of K$^+$.\cite{304} Sulfation of other polysaccharides with inherent gel-forming properties such as agarose and gellan is described only in a few studies,\cite{305–307} and the effects of sulfation on the gelation kinetics and resulting rheological properties have, to the authors’ knowledge, not been demonstrated. As gelation occurs by temperature-induced formation of helical fibers, sulfation may result in weaker fiber aggregation due to steric and electrostatic repulsion. It should be noted, however, that the sulfate groups in carrageenan directly contribute to K$^+$-mediated gelation and can similarly have a role in gelation of sulfated gellan and agarose.

For sulfated polysaccharides that do not have inherent cross-linking properties, the polysaccharides are frequently modified to allow covalent cross-linking. This can be performed by methacrylation and UV-induced cross-linking in the presence of a photoinitiator, which has been employed for sulfated hyaluronan (sHA).\cite{109} An alternative strategy employs modifying polysaccharides with peptides and forming isopeptide bonds catalyzed by transglutaminase.\cite{308–310} ESPs have also been incorporated in hydrogels without cross-linking, which can lead to gradual leakage by diffusion. Sulfated hyaluronan (sHA) has been incorporated in collagen hydrogels, conferring a high level of structural and physical analogy to the native ECM.\cite{276,311} It was, however, observed that more than half of the sHA was released from the hydrogels after 1 h incubation in PBS due to swelling of the scaffolds and a lack of intermolecular interactions.\cite{276} While the leached fraction was not compared to the retained sHA in the study, low-molecular weight and high-sulfated chains are assumed to diffuse faster out of hydrogels. Larger polysaccharides can potentially be retained longer in hydrogels through molecular entanglements and/or intramolecular interactions.

Besides hydrogels, nanofibrous scaffolds can be prepared through combination of sulfated polysaccharides with

\[ \text{Figure 11. Strategies for ionic and covalent cross-linking of ESPs and common biomaterial types incorporating ESPs.} \]
fiber-forming polymers. In one study, sAlg was combined with polyvinyl alcohol (PVA) and electrospun, followed by physical cross-linking by heating. Addition of sAlg or unmodified alginates both resulted in increased homogeneity in the fibers but a decrease in tensile strength compared to PVA alone.\[273\] Furthermore, coacervate hydrogels, fibers or nanoparticles can be prepared from ionic interactions between sulfated polysaccharides and cationic polymers. This interaction has previously been exploited to prepare complexes between chitosan and dextran sulfate toward drug delivery applications.\[312,313\] Chitosan and synthetic cationic polymers have additionally been used for layer-by-layer combination with sCel to prepare thin films or encapsulation complexes for cell cultivation and drug delivery applications.\[314–316\] Depending on the sulfation and deacetylation degrees of the polysaccharides, the resulting biomaterials can have a net positive or negative charge influencing interactions with encapsulated soluble compounds. For example, sAlg have been applied as a secondary coat on PLL-coated alginate microspheres.\[180\] Here, some destabilization of the PLL coating was observed for sAlg compared to coating with unmodified alginate, indicating that the sAlg competes with binding of the PLL to the inner Alg microsphere promoting detachment rather than layering in sandwich-like structures. Presumably, these interactions can be tuned by altering the DS and the monosaccharide sequence determining charge orientation. An alternative strategy for using ESPs in coats and films is end-point functionalization on the reducing end such as biotinylation and conjugation to streptavidin-coated surfaces.\[271\] The zwitterionic nature of sChit has additionally been exploited to form stable coatings on silver nanoparticles, as the amino groups serve as coordination sites for polymer–metal complexation and the negative charged sulfate groups provide repulsion to prevent aggregation.\[136\]

3.7.2. Tissue Engineering

ESP offer a wide range of advantages for use in tissue engineering in comparison to naturally sulfated polysaccharides. The most striking advantage is the ability to tune the degree of sulfation, which leads to a more faithful representation of the tissue of interest. Figure 12 shows the proposed modes of action of ESPs in tissue engineering scaffolds, as well as the applications discussed in the present review. Different native tissues have varying GAG contents that also change temporally, i.e., GAG content change in development plays an important role in directing several signaling pathways. Furthermore, some tissues, such as cartilage, have a stable and uniquely high GAG content, as cartilage is not fed by vasculature but rather relies on the synovial fluid for exchange of biomolecules as well as nutrition. The level of sulfation within tissues can also mean onset of pathological changes that can be mimicked with ESPs.\[317\] Directed sulfation can be coupled with tailored cross-linking mechanisms in order to tune the mechanics of the engineered tissues. This makes it possible to tune biochemical and mechanical properties in a controlled manner which enables studies of the effects of such coupled changes in tissues, whether they act synergistically or antagonistically, in the context of both regeneration and degeneration. Control over the molecular weight of polysaccharides is another advantage that ESPs offer, i.e., changing the molecular weight of the starting polymer prior to sulfation is known to affect the viscoelasticity of engineered networks which has been shown to guide several

![Figure 12. Modes of action of engineered sulfated polysaccharides in tissue engineering (TE) with corresponding applications. Illustrations in bottom panel created with BioRender.com.](https://www.afm-journal.de/)

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signaling mechanisms involving differentiation, migration and malignancy.[318,319] Lastly, as mentioned previously, most ESPs are presumably not recognized by catabolic enzymes in the body, which facilitates their use in long-lived and highly stable tissue-engineering matrices.[109] These advantages, combined with their ability to mimic the native GAGs in interacting with protein signal factors as reservoirs, coreceptors and/or antagonists, highlight ESPs as excellent candidates for use in tissue engineering. Here, we summarize their use for several tissue types, including cartilage, bone, heart, vascular, and neural tissues as well as wound healing processes.

**Cartilage:** Table 5 summarizes some of the key studies developing ESP-based scaffolds for cartilage tissue engineering as well as the type of growth factor used in these scaffolds. Articular cartilage is frequently damaged by trauma and degenerative joint diseases such as OA. Due to its very limited self-healing capacity, articular cartilage injuries remain one of the unresolved challenges in clinics. Currently available surgical options do not provide complete regeneration; therefore, emerging tissue engineering approaches aim to develop new strategies for restoring tissue function and replacing damaged tissue with functional healthy tissue.[320,321] Articular cartilage has a low cell density embedded within an elegantly organized ECM composed of collagen and proteoglycans.[312] Multiple cell-based approaches using chondrocytes or stem cells have been developed for cartilage tissue regeneration. Growth factors and other bioactive molecules, such as the TGF-β family, are also usually used to act as signals to regulate cell function and direct chondrogenic differentiation.[322] In most of the approaches, a supportive scaffold material—to guide cell differentiation and matrix synthesis and organization—is a crucial requirement. Moreover, the regulation of activity and availability of growth factors is largely mediated by matrix structure and biochemistry.[321] Natural cartilage is rich in sulfated GAGs, including CS, KS, and dermatan sulfate, resulting in a high fixed negative charge of the ECM. This negative charge gives the tissue its compressive strength and osmotic swelling properties by entrapping water, and it also plays an important

### Table 5. Growth factor-potentiating effects of ESPs in cartilage and bone engineering.

| ESP   | Scaffold/carrier | Growth factor | Model system | Effect of ESP                                                                 | Ref.                  |
|-------|------------------|---------------|--------------|-------------------------------------------------------------------------------|-----------------------|
| sAlg  | Ionically X-linked gel | FGF-2          | Chondrocytes | Increased cell proliferation and ECM production                               | [270]                 |
| sAlg  | Ionically X-linked gel | –             | Chondrocytes | Increased cell proliferation and phenotype maintenance                        | [303]                 |
| sAlg  | Covalently X-linked gel | TGF-β3         | Chondrocytes | Increased ECM gene expression                                                 | [309]                 |
| sAlg  | Nanocellulose bioink | –             | Chondrocytes | Improved chondrogenesis                                                       | [325]                 |
| sAlg  | Ionically X-linked gel | TGF-β1         | hMSCs        | Improved chondrogenesis                                                       | [329]                 |
| sChit | Dissolved in medium | –             | Chondroprogenitor cells | Improved chondrogenesis                                               | [326]                 |
| sCMC  | Gelatin nanofibres  | TGF-β3         | hMSCs        | Improved chondrogenesis                                                       | [330]                 |
| sCMC  | Gelatin nanofibres  | TGF-β3         | hMSCs        | Improved chondrogenesis                                                       | [331]                 |
| sCMC  | Gelatin gel       | TGF-β1         | hMSCs        | Improved chondrogenesis                                                       | [280]                 |
| sHA   | Photo-X-linked hydrogel | TGF-β1         | hMSCs, in vivo implantation | Improved chondrogenesis and inhibited hypertrophy | [109]                 |

**Bone**

| ESP   | Scaffold/carrier | Growth factor | Model system | Effect of ESP                                                                 | Ref.                  |
|-------|------------------|---------------|--------------|-------------------------------------------------------------------------------|-----------------------|
| sAlg  | Agarose, Pluronic F127 | BMP-2         | MC3T3-E1 osteoblasts | Improved osteogenesis                                                         | [274]                 |
| sChit | Dissolved in medium | BMP-2         | C2C12 cells | Improved ALP activity                                                         | [340]                 |
| sChit | Dissolved in medium | BMP-2         | C2C12 cells | Increased ALP activity                                                        | [341]                 |
| sChit | Nanoparticles in gelatin sponges | BMP-2 | HUVECs | Increased bone formation in mice                                              | [342]                 |
| sChit | Nanoparticles in photo-linked gelatin | BMP-2 | Implantation in rabbit bone defect | Improved angiogenesis and angiogenesis in vivo | [343]                 |
| sChit | Immobilized on PLGA | BMP-2         | C2C12 cells | Improved cell attachment                                                      | [344]                 |
| sChit | PLGA particles in gelatin/26SCS photo-X-linked gel | BMP-2 | HUVECs/MC3T3-E1 | Improved angiogenesis and osteogenesis in vivo | [347]                 |
| sHA   | Collagen I gel | –             | hMSCs        | Improved osteogenesis                                                         | [351]                 |
| sHA   | Collagen I gel | –             | hMSCs        | Improved osteogenesis                                                         | [350]                 |
| sHA   | Collagen I gel | –             | hMSCs        | Improved osteogenesis                                                         | [353]                 |
role in growth factor sequestration and bioavailability.\cite{320} To mimic native cartilage ECM and to provide a reservoir for controlled and prolonged growth factor delivery, ESPs have recently been considered as optimal biomaterials for cartilage tissue engineering scaffolds. The tunability of the degree of sulfation and sulfate pattern allows for tailoring of growth factor interaction, whereas the choice of polysaccharides and cross-linking strategies can provide the desired mechanical properties of the scaffolds.

It has been shown that ESPs have a positive effect on chondrogenic cells’ viability and proliferation as well as on their spreading and attachment within a polysaccharide-based scaffold.\cite{270,303} Zenobi-Wong and colleagues fabricated ionically cross-linked hydrogels based on sAlg for 3D culture of chondrocytes, resulting in a significant promotion of the proliferation and viability of encapsulated chondrocytes compared to nonsulfated hydrogels.\cite{303} In a follow-up study, the authors proposed that increased proliferation of encapsulated chondrocytes was due to higher retention of mitogenic growth factor FGF-2 in the sulfated hydrogel network and protection from proteolytic cleavage, while facilitating growth factor-receptor interaction to initiate downstream signaling events (\textbf{Figure 13}).\cite{270} Such FGF-2-mediated mitogenic activity has additionally been reported for sChit and sCel.\cite{324} Interestingly, it was also observed that sAlg induces chondrocytes’ spreading and attachment to the hydrogel matrix. This effect was speculated to be mediated via beta1 integrins and increased synthesis and secretion of collagens from the chondrocytes.\cite{303}

Moreover, significantly increased Col II and proteoglycan deposition and decreased Col I production have been reported for chondrocytes encapsulated in sAlg hydrogels, with a positive correlation to the DS.\cite{270} Recently, sAlg was modified with tyramine to facilitate tyrosinase-mediated cross-linking in situ and demonstrated strong adhesion to native cartilage tissue.\cite{309} Aggrecan and SOX9 gene expressions, as two important chondrogenic markers, were significantly upregulated in chondrocytes in sulfated hydrogels compared to those in the nonsulfated hydrogels. Moreover, sAlg hydrogels with encapsulated human chondrocytes demonstrated a high degree of in vivo stability and cartilage matrix deposition upon subcutaneous implantation into mice for 4 weeks.\cite{309} Due to the promising effects of sAlg on chondrogenic cells, it has been used in bioink formulation along with nanocellulose for cartilage bioprinting. Here, the sAlg maintained its positive effect on bovine chondrocyte spreading and proliferation in bioink formulation and showed good printing properties.\cite{325} Sulfation of other polysaccharides has also shown enhanced chondrogenesis and enhanced cartilaginous matrix production by chondrocytes. Lord et al. conjugated arginine to chitosan, allowing dissolution of the polysaccharide in water, followed by sulfation. After 7 days of cell culture, chitosan–arginine was found to promote an osteogenic phenotype in human fetal chondro-progenitor cells, whereas sChit–arginine promoted a chondrogenic phenotype in the same cells, as indicated by significant upregulation of Coll II, aggrecan, perlecan, and SOX9 genes.\cite{326} TGF-\(\beta\) signaling has been shown to be critical for cartilage morphogenesis and chondrogenic differentiation of stem cells.\cite{327} However, optimal GF dosage with sufficiently long and sustained exposure to stem cells are two important prerequisites for stem cell differentiation.\cite{328} The ability of ESPs to interact with growth factors and to prolong their retention in the scaffold network makes them ideal biomaterials for chondrogenic culture, mimicking the natural ECM mechanism for GF presentation. Re’em et al. showed that freeze-dried

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure13}
\caption{A) Schematic depiction of the mechanism of activation of FGF signaling in sulfated alginate (sAlg) hydrogels. sAlg exerts high affinity for FGFs, mediating growth factor–receptor interactions that leads to initiation of downstream signaling, promoting potent proliferation, cartilage matrix deposition, and suppression of dedifferentiation and catabolism. B) Fluorescence imaging of phalloidin–rhodamine (gray) and DAPI (blue) stained aggregates of chondrocytes in sAlg and alginate (Alg) hydrogels, Scale bar: 50 \(\mu\)m. C) Immunofluorescence imaging of Col2 (green) antibody staining of chondrocytes encapsulated in alginate and sAlg hydrogels after 42 d of culture. Rhodamine phalloidin (red) and DAPI (blue) were used as counterstains. Scale bar: 100 \(\mu\)m. Adapted with permission.\cite{270} Copyright 2020, Wiley-VCH.}
\end{figure}
scaffolds containing a mixture of Alg and sAlg caused attenuated TGF-β1 release and consequently improved chondrogenesis of entrapped human mesenchymal stem cells (hMSCs) both in vitro and in vivo compared with scaffolds lacking sAlg.\(^{[329]}\)

In a different study, photocross-linked injectable hydrogels were fabricated from sHA and their potential for cartilage tissue engineering was investigated. The study showed that sHA hydrogels promoted chondrogenesis of hMSCs in chondrogenic media while inhibiting their hypertrophy in hypertrophic culture, correlated with their DS. Since the hypertrophic culture of cells followed their chondrogenic culture in media containing TGF-β1, the positive effect of sHA in inhibiting hypertrophy is presumably due to its greater TGF-β1 binding and retention compared with unmodified HA. From the same study, in vivo tests showed more cartilage ECM production in sulfated cell-laden hydrogels after 28 days of subcutaneous implantation in mice. Furthermore, improved articular cartilage regeneration in an OA-induced rat upon sHA hydrogel injection compared to nonsulfated controls was reported.\(^{[109]}\) Waghmare et al. recently fabricated macroporous scaffolds based on sCMC and gelatin cross-linked using EDC-NHS chemistry. The scaffolds with either TGF-β1 loaded in the constructs or supplemented in the media both showed improved chondrogenic differentiation of seeded mesenchymal stem cells (MSCs) with similar efficiency, despite a twofold higher amount of TGF-β1 supplemented through media. This may be due to the homogenous and long-term presentation of TGF-β1 throughout the culture period by loaded scaffolds, whereas diffusion of media-supplemented TGF-β1 into the scaffold may be limited by high affinity binding to the sCMC in the outer layer of the scaffolds.\(^{[280]}\) Arinzeh and co-workers showed that adding sCel to an induction media containing TGF-β1 significantly enhanced chondrogenic differentiation of hMSCs.\(^{[330]}\) The authors of the study further fabricated hydrolytically stable electrospun nanofibers based on sCel and gelatin, where inclusion of sCel was found to increase TGF-β1 absorption to electrospun scaffolds. Moreover, hMSC chondrogenesis after 28 and 56 days in media containing TGF-β3 was significantly enhanced for the sulfated scaffold in a concentration-dependent manner. An optimum concentration of sCel in scaffold formulation was determined in this study, above which chondrogenesis was inhibited, indicating that increased bioavailability of GF for long duration may have an inhibitory effect on chondrogenesis.\(^{[310,311]}\)

To obtain a greater understanding of the effects of DS on chondrogenesis, one study investigated chondrogenic differentiation of MSCs encapsulated in scaffolds composed of poly(ethylene glycol)-diacrylate with either CS or highly desulfated CS. Interestingly, it was here found that desulfated CS upregulated gene expression of chondrogenic markers by MSCs compared to native CS, when cultured in a chondrogenic medium containing TGF-β1. This result seems contradictory, since it was shown in the same study that CS has higher sequestration of TGF-β1 and slower release of it compared to desulfated CS. Since this upregulation of chondrogenic markers did not happen in the absence of TGF-β1, this positive effect is probably due to more favorable interaction of biomaterial with GF. The authors argued that the high negative charge density of CS could potentially decrease TGF-β1 activity or inhibit transport within the hydrogel network and effectively inhibit intercellular communication.\(^{[346]}\)

**Bone:** Table 5 includes a summary of key studies focused on developing ESP-based scaffolds for bone tissue regeneration as well as the type of growth factor used in these scaffolds. Bone tissue has, in contrast to cartilage, a high density of resident cells and extensive vascularity, providing abundant nutrients and signaling factors that stimulate tissue repair and can promote healing of small defects.\(^{[332]}\) However, regeneration of large defects resulting from traumatic bone lesions, osteoporosis, and osteolytic bone metastases are still unresolved clinical challenges necessitating allografts, which can only be obtained in limited quantities. Hence, the regeneration of bone by emerging tissue engineering approaches has attracted significant attention of researchers.\(^{[333]}\) Here, the main objective has been to incite and promote the natural healing processes of bone that do not naturally occur in critical-sized defects. Growth factors, hormones and other stimulatory factors are normally used in bone tissue engineering to stimulate bone regeneration.\(^{[334]}\) However, BMP-2 has a very short half-life in vivo and the use of high doses can lead to adverse side effects such as bone resorption or an exaggerated ossification of the tissue.\(^{[336]}\) Therefore, protective carriers for controlled and sustained BMP-2 delivery in local bone defect areas are highly coveted in bone tissue engineering. Sulfated polysaccharides, due to their inherent affinity to growth factors, have been considered as an ideal and yet simple candidate for such carriers in bone tissue engineering. It has, for instance, been shown that sulfated polysaccharides such as heparin and heparin sulfate can stimulate the biological activities of BMP-2 in vitro and significantly increase its half-life in culture medium.\(^{[337]}\) However, using heparin for bone treatment has shown adverse effects, including delayed healing in short term and increased risk of osteoporosis in long term.\(^{[338,339]}\) This has encouraged exploration of heparin mimetics in carrier biomaterials that can facilitate sustained release of BMP-2 in bone defects, while displaying minimal adverse effects and optimized pharmacokinetic properties. Sulfation of polysaccharides has been found particularly effective in inducing osteogenesis. One study employed different chemical modifications including sulfation, carboxylation, and carboxymethylation on cellulose followed by investigation of their osteogenic potential on myoblastic C2C12 precursor cells. The researchers found that sulfation is the most effective modification with respect to enhancing osteogenic activity in cooperation with BMP-2, indicating a synergistic effect.\(^{[340]}\) sChit is one of the most studied ESPs for bone tissue engineering and its synergistic effect on BMP-2 bioactivity has been indicated in multiple studies.\(^{[340,341]}\) Liu and colleagues first synthesized 2-N-, 6-O-, and 2-N,6-O-sulfated chitosans, designated 2SCS, 6SCS, and 26SCS, to study their effect on osteogenesis and BMP-2 bioactivity.\(^{[342]}\) All sChit variants enhanced bioactivity of BMP-2 evaluated by alkaline phosphatase (ALP) activity in C2C12 cells, compared with heparin and unmodified chitosans. However, none of the ESPs were able to stimulate osteoblastic
differentiation in C2C12 cells in the absence of BMP-2. Moreover, 26SCS was the most potent BMP-2 enhancer, while 25SCS was the weakest, revealing the importance of sulfation in C6 position for the observed effects. The greater efficacy of 26SCS compared with heparin was proposed to be due to its higher DS and higher MW. Interestingly, BMP-2 receptor binding and osteogenesis were enhanced by a low dose of 26SCS, whereas increasing concentration of 26SCS resulted in lower efficacy. This concentration-dependent effect of sChit on promoting BMP-2 bioactivity has been indicated in other studies as well.

Furthermore, simultaneous administration of BMP-2 and 26SCS in vivo dose-dependently induced larger amounts of mineralized bone tissue compared with BMP-2 alone. In a follow-up study, 26SCS-based nanoparticles were fabricated as carriers for BMP-2 and were incorporated in gelatin sponges for controlled delivery and enhanced activity of BMP-2. The implant showed sustained release of BMP-2 over 21 days and the released BMP-2 significantly induced ALP activity compared with supplementation of BMP-2 in the culture media.

To obtain a more robust scaffold, the authors incorporated the BMP-2 loaded 26SCS nanoparticles in photocross-linked gelatin hydrogels. The scaffold enabled sustained release of BMP-2 over one week and resulted in improved bone formation in rabbit radius critical defect compared with BMP-2 loaded hydrogels without the nanoparticles.

In another approach, 26SCS was electrostatically self-assembled on the surface of aminoloyzed PLGA scaffolds. The immobilized 26SCS increased surface hydrophilicity, C2C12 cell attachment, and facilitated adsorption and sustained release of BMP-2. In a recent study, bioinks composed of Alg/sAlg were prepared and loaded with BMP-2. The bioinks demonstrated good printability and the inclusion of sAlg resulted in slower release of BMP-2 from printed constructs over 10 days of incubation. Moreover, printing of bioinks containing both BMP-2 and osteoblasts was successfully performed, resulting in osteogenic differentiation in sAlg-containing constructs after 7 days of culture, presumably due to a higher degree of GF retention in the hydrogel scaffolds.

Since angiogenesis is a critical factor for bone tissue regeneration, the effect of ESPs on angiogenesis and new blood vessel formation during bone regeneration has been assessed. Further studies on 26SCS nanoparticles demonstrated increased viability and VEGF secretion of encapsulated human umbilical cord vascular endothelial cells (HUVECs), as well as vascular tube formation in a concentration-dependent manner. The proangiogenic effect of gelatin implant incorporating BMP-2-loaded 26SCS nanoparticles resulted in substantial repair of a critical-sized bone defect in rabbits. The new vessel formation effect of the implant in vivo was proposed due to the stimulatory effect of released 26SCS on the proliferation and VEGF secretion of endothelia cells (ECs) in nearby blood vessels, leading to the migration of ECs out of the vessel and the formation of growing sprouts. In a recent study by the same group of researchers, expression patterns of angiogenic and osteogenic genes were analyzed, showing that VEGFR2 signaling, which is significantly upregulated in the presence of 26SCS, plays a key role in stimulating the angiogenic potential of MSCs.

Due to the synergistic effect of 26SCS on both angiogenesis and osteogenesis, a dual growth factor delivery system incorporating BMP2 and VEGF has been designed for tunable delivery of the two GFs for effective bone regeneration. BMP2 has been loaded in PLGA microparticles for a more sustained delivery over longer times and were loaded in photocross-linked gelatin scaffold containing VEGF and 26SCS. The system showed superior performance in bone regeneration compared to single GF delivery and compared to scaffold without 26SCS.

In contrast to the solid bone ECM that is rich in inorganic minerals, the bone marrow niche ECM surrounding MSCs with
Osteogenic potential has a high content of HS proteoglycans, presumably involved in regulating the osteogenic differentiation processes. Hempel et al. investigated the effect of sHA on osteogenic differentiation of hMSCs. The researchers fabricated scaffolds composed of Col I and either nonsulfated HA, sHA with varying DS (1.0–3.0) or CS. Compared with scaffolds containing CS or Col I alone, scaffolds with sHA-induced calcium phosphate deposition, tissue nonspecific alkaline phosphatase (TNAP) activity, and osteoblast-specific gene expression in hMSC. Interestingly, these effects were also observed in the absence of dexamethasone, which is an established osteogenic supplement. Later, the researchers showed that combined usage of sHA and dexamethasone had synergistic effects for osteogenic differentiation of hMSCs. Interestingly, the osteogenic effect of sHA on hMSC was independent of the DS, indicating the importance of the sulfation on C6 position, which was sulfated in all derivatives. This hypothesis was further confirmed by showing that CS with a similar DS as sHA, but primarily 4-O sulfation, lacked this pro-osteogenic effect.

To further characterize the underlying cellular mechanisms, sHA interaction with fibronectin, as a major interaction partner of GAGs in the ECM and a key player in the commitment of stem and progenitor cell populations to induce cardiomyocyte differentation,[358] has been investigated.[359] It has previously been shown that heparin-induced conformational changes of fibronectin within the ECM promote hMSC osteogenic differentiation.[359] Here, it was shown that while both sHA and heparin affected fibronectin conformation, only sHA increased fibronectin levels and led to thinner fibrils and later only sHA showed pro-osteogenic effect, pointing the probable involvement of fibronectin regulated osteogenic effects. Moreover, quantitative proteomic studies revealed an sHA-mediated upregulation of proteins associated with important cellular functions such as cell adhesion, cell signaling, matrix remodeling, and endocytosis of hMSCs.[354] In addition to MSCs, the effect of sHA on osteoblast and osteoclast function has been studied. Here it was found that treatment of osteoblasts with highly sulfated sHA suppressed their ability to support osteoclast differentiation and resorption.[355,356] Moreover, sulfation of HA and oversulfation of CS led to a significant inhibition of osteoclast differentiation of monocytes and RAW264.7 cells as well as osteoclast resorption, where the inhibitory effects increased with the DS of the ESPs.[357]

Harnessing the osteogenic capacity of sHA as a coating material for bone implants has been evaluated as a means to induce bone formation in vivo without any osteoconductive materials. Titanium-coated polymer ether ketone plates were coated with Col I supplemented with either nonsulfated HA, highly sulfated HA (DS = 3.0), CS (DS = 0.9) or highly sulfated CS (DS = 3.0) and inserted into critical-size bone defects in rat femora. The highest amounts of newly formed bone were observed for unmodified CS and highly sulfated HA, indicating that oversulfation increased the osteogenic effect of HA but reduced that of CS.[358] It has further been shown that thermopolymerized lactide-based scaffolds coated with Col I and highly sulfated HA accelerate bone defect regeneration in diabetic rats by promoting bone mineralization and decreasing the amount of nonmineralized bone matrix. The proposed underlying mechanisms were improved osteoblast function and enhanced binding of sHA to the Wnt inhibitor, sclerostin.[359,360] The Wnt signaling pathway is a critical mechanism of bone formation and bone remodeling, and sclerostin is a secreted glycoprotein acting as an endogenous inhibitor of Wnt signaling. Preincubation of sclerostin with sHA led to a sulfate- and dose-dependent loss of its bioactivity.[360]

Heart: Coronary heart disease is the leading cause of death worldwide and presents a major healthcare burden. Myocardial infarction (MI) stems from occlusion of coronary arteries and manifests as a series of events including an ischemic attack that induces cardiomyocyte death, a strong inflammatory response to necrosis, ECM remodeling and scar formation, hypertrophy in the myocardium, and left ventricular remodeling leading to aberrant contractility and congestive heart failure.[361,362] Tissue engineering approaches to promote cardiac tissue regeneration have gained wide interest in recent years due to the adverse effects and lack of long-term improvements from currently used clinical therapies for MI, such as pharmacological and surgical regimens.[362,363] Recently described tissue engineering approaches commonly involve the combination of biomaterials with cells and bioactive molecules with the aim of achieving myocardial regeneration.[363–365] Different stages of disease progression have been targeted, and treatment attempts have included inhibition of cardiomyocyte death, controlling post-MI inflammation and fibrotic ECM formation, promoting vascularization to suppress ischemia, and attracting stem and progenitor cell populations to induce cardiomyocyte regeneration.[366–372] The importance of paracrine factors (i.e., growth factors, chemokines, cytokines) and signaling in these stages of post-MI heart failure has been established by several studies.[362,366–372] Therefore, the use of ESPs as the biomaterial platform in cardiac tissue engineering has been pursued as a means of providing a reservoir for these paracrine factors and to facilitate their signaling mechanisms.[362] Among the bioactive molecules that are involved in myocardial regeneration and neovascularization are FGF,[373] IGF-1,[374] granulocyte colony-stimulating factor,[375] SDF-1,[376] VEGF,[377] and HGF.[378]

Using sulfated polysaccharides as a delivery vehicle can overcome several drawbacks of systemic administration of the factors alone, such as high dosing requirements, uncontrolled adverse effects, lack of local targeting, and fast elimination. A bioengineered cardiac patch from microporous Alg/sAlg scaffolds loaded with IGF-1, SDF-1, and VEGF and seeded with neonatal rat cardiomyocytes was found to induce vascularization when implanted into rat omentum.[379] These scaffolds, when re-implanted into infarcted hearts, demonstrated successful structural and electrical integration in the infarct site and improved cardiac function. Furthermore, these beneficial effects were observed even when the scaffolds did not contain any cells, but only the cocktail of growth factors.[378] Alg/sAlg scaffolds were shown to facilitate sustained release of HGF and provide cytoprotection in rat neonatal cardiomyocytes via an ERK1/2-mediated mechanism.[365] An injectable form of Alg/sAlg scaffold loaded with IGF-1 and HGF was used in an acute myocardial infarct model.[360] The scaffolds further allowed sequential delivery of the factors with a fast release of IGF-1, to provide a survival signal to prevent cardiomyocyte death, followed by a prolonged release profile for HGF to ensure vascularization and to control fibrotic remodeling. Injection of the scaffolds led to a reduction in cell death and fibrotic scar formation, promoted angiogenesis, and prevented infarct expansion.
in rats.\textsuperscript{269} Nanoparticles of sAlg with basic FGF, VEGF and HGF have been fabricated and shown to induce angiogenesis in a murine model of hindlimb ischemia as well as regeneration in a rat model for myocardial infarction.\textsuperscript{380} In a separate study, complexes of sAlg with VEGF were synthesized and shown to increase VEGF receptor 2 activation, induce endothelial sprouting and promote recovery of perfusion in a murine model for hindlimb ischemia.\textsuperscript{381} Another strategy for using sAlg in cardiovascular research has been to control the level of the main cholesterol transporter low-density lipoprotein (LDL), to prevent atherosclerotic plaque formation and cardiovascular disease progression. Polysulfone membranes with sAlg have been shown to successfully enable LDL apheresis.\textsuperscript{382}

ESPs have also been used in immunomodulatory approaches toward cardiac repair. miRNA-21, which is a microRNA upregulated by peritoneal macrophages post-phagocytosis of dead cells, as a means to resolve an inflammatory reaction, has been complicated with sHA into nanoparticles and delivered to infarct sites to control macrophage-mediated inflammation.\textsuperscript{383,384} miRNA-21-sHA nanoparticles were found to target cardiac macrophages at the infarct site, inducing a change in their phenotype from proinflammatory to regenerative, which ultimately resulted in angiogenesis and a reduction in cardiomyocyte death and fibrotic remodeling.\textsuperscript{385}

Low-molecular-weight sulfated dextran has been shown to act as a protector of the cardiac endothelium through inhibiting complement-mediated cytotoxic effects in hamster-to-rat and in pig-to-human xenotransplantation models.\textsuperscript{385,386} such protection has been revealed to prevent ischemia/reperfusion-induced organ damage in a porcine model.\textsuperscript{387} Similarly, sDex was found to decrease endothelial cell activation with immune cell infiltration, and attenuate ischemia/reperfusion-mediated acute graft injury in a rat model.\textsuperscript{388} sDex has also demonstrated protective effects on cardiomyocytes against free radical injury induced by hydrogen peroxide, hypoxic challenge or conditioned medium obtained from endothelial cells that were damaged by ischemia-reperfusion. The study further showed that sDex was superior to heparin in suppressing free radical injury caused by hydrogen peroxide or hypoxia-reoxygenation.\textsuperscript{389} These studies reveal the potential for incorporating these ESPs into cardiac tissue engineering applications, enabling control over the cardiomyocyte phenotype and providing protection against microenvironmental challenges such as free radicals, severe underoxygenation, and inflammation. An HA-based hydrogel scaffold incorporating sDex and matrix metalloproteinase (MMP) inhibitors was used for on-demand MMP-induced release when injected into pig hearts in an MMP-rich infarct microenvironment. This on-demand release suppressed the adverse MMP-mediated left ventricular remodeling in the heart.\textsuperscript{190} Enriching the cellular complexity of engineered tissues with recruitment of supporting cell types such as fibroblasts and endothelial cells is crucial in cardiac tissue engineering, to ensure the most faithful recapitulation of physiological conditions and mature phenotype in cardiomyocytes. In line with this, incorporation of native sulfated polysaccharides or ESPs such as sDex into engineered tissues was also shown to affect and modulate the phenotype and behavior of relevant cell types, including human vascular endothelial cells, fibroblasts, or smooth muscle cells.\textsuperscript{191}

**Neural Tissues:** As an integral part of proteoglycans, sulfated GAGs regulate many aspects of neuronal patterning and synaptogenesis. There is ample evidence that native GAGs such as HS\textsuperscript{392–396} and CS\textsuperscript{393,396–402} are involved in neurite outgrowth and central neural system (CNS) repair.\textsuperscript{406–411} For neuronal TE applications, HS/heparin and CS seem particularly promising. The interactions of HS/heparin chains with GFs (pleiotropin,\textsuperscript{412,413} various FGFs,\textsuperscript{413} midkine\textsuperscript{414–415},) axon guidance molecules (ephrins,\textsuperscript{416} netrin1,\textsuperscript{417} semaphorin 5A\textsuperscript{413–415,418}), axon guidance molecules (ephrins,\textsuperscript{416} netrin1,\textsuperscript{417} semaphorin 5A\textsuperscript{418},) morphogens (WNT\textsuperscript{419–421}) and chemokines (chemokine ligand 2\textsuperscript{422},) are both specific and structure dependent. In addition, CS chains can interact with many of the binding partners of HS/heparin, though with lower affinity.\textsuperscript{423} An overview of developmentally and physiologically relevant processes is provided in reviews elsewhere.\textsuperscript{393,422} These are both specific and structure dependent. In addition, CS chains can interact with many of the binding partners of HS/heparin, though with lower affinity.\textsuperscript{423} An overview of developmentally and physiologically relevant processes is provided in reviews elsewhere.\textsuperscript{393,424} Based on published literature, the specific sulfation pattern of GAGs is crucial in neuronal patterning. The role of sulfated GAGs in synaptic patterning and function has also been investigated, with many of the studies focusing on the neuromuscular junction. Other important hallmarks include functional redundancy not only for sulfated GAGs themselves, but also for the core proteins of proteoglycans.\textsuperscript{408,424} This versatility of sulfated GAGs has been exploited in the biomedical field and various tissue engineering applications for the purpose of CNS repair.

To treat spinal cord injuries, both neurite outgrowth and myelination of neurons is required. Much of current literature thus focuses on the engineering of scaffolds that offer advantageous characteristics to support reinnervation by glial cells. Astrocytes are the largest subgroup of CNS glial cells. Their main function is the support of CNS neurons, and thus they are involved in tasks such as synaptogenesis or secretion of neuroactive agents.\textsuperscript{425–430} The effect of GAG-mediated GF delivery on the transformation from normal into a stellate morphology, as well as on the activity of human astrocytes has also been demonstrated.\textsuperscript{430}

In one study addressing spinal cord injury, partially (DS = 0.8) or fully (DS = 2.3) sCel was mixed with gelatin to produce aligned fibrous hydrogel scaffolds using electrospinning. Native CS variants CS-A and CS-C were used as controls.\textsuperscript{431} Whereas NGF binding was higher in scaffolds containing either of the sCel variants, neurite outgrowth from dorsal root ganglion explants was highest in fully sulfated cellulose. Decreased neurite outgrowth was detected in scaffolds containing partially sulfated cellulose, followed by CS-C and CS-A, respectively. This indicated that the alignment of electrospun fibers supports neurite outgrowth, with a positive correlation between neurite length and the degree of sulfation.\textsuperscript{431} McCanney et al. reported another approach to tackling the lack of endogenous repair and glial scar formation by elevated astrocyte recruitment following a spinal cord injury.\textsuperscript{432} Here, a library of selectively 2-O- and/or
6-O-desulfated heparins, as well as N-acetylated heparins, was prepared. These low sulfated heparin mimetics were structurally similar to resident HS.[412,413]

To assess whether the heparin ESPs supported remyelination and neurite outgrowth required for treating spinal cord injuries, the authors created lesioned and demyelinated neural cell cocultures, followed by the addition of low- or highly sulfated heparins. Whereas the former enhanced neurite outgrowth, the latter had an attenuative effect. As low sulfated heparin had no effect on controls containing uninjured and myelinated cocultures, modulation or sequestration of inhibitory factors following injury were considered the primary functions of these ESPs. Furthermore, the authors postulated that the generated heparins not only modulated cell signaling by sequestration of ligands, but also acted as cofactors during ligand-receptor complex-formation.[432] This finding is in agreement with previous reports regarding the attenuative role played by native low-sulfated heparin moieties toward astrocytes in the CNS.[407,432] This finding is in agreement with previous reports regarding the attenuative role played by native low-sulfated heparin moieties toward astrocytes in the CNS.[407]

A recent study employed sAlg for controlled growth factor binding and delivery for engineering of neural tissues. Biotinylated sAlg (b-sAlg) with varying DS per disaccharide (0.0, 0.8, 2.0, 2.7) were deposited on streptavidin-modified gold substrates resulting in thin 2D films capable of binding FGF-2 (Figure 15A). As expected, the DS correlated positively with FGF-2 binding. A peak was observed at DS = 2.0, after which binding affinities again decreased due to sterically hindered accessibility. At similar DS, the binding affinity for b-sAlg was even higher than for heparin. Similarly, viability was highest at DS = 2.0 for both neural and glial cell lines, emphasizing the possible benefits of sAlg for cell culture applications. Even though average neurite length was comparable between b-heparin, b-sAlg0.0, b-sAlg0.8, and b-sAlg2.0, a significantly higher percentage of cells (75–90%) cultured on b-sAlg exhibited neurites compared to cells grown on b-heparin (10%). Besides controlled growth factor delivery, the grafting of b-sAlg may unlock novel biosensing applications.[271]

Yamada et al. made similar observations for astrocytes cultured in the presence of sHA and FGF-2. The authors showed that the effect of sHA was dependent on the level of sulfation and that the combination of sHA and FGF-2 had a stronger influence on stellation (outgrowth of lamellipodial processes) and gene expression levels of neurotrophic factors than sHA alone. Among the neurotrophic factors, FGF-2, midkine, NGF-1, and insulin-like growth factor -1 (IGF-1) were upregulated most in sHA/FGF-2 cocultures, compared to cultures containing only control medium. Relative gene expression for brain-derived neurotrophic factor (BDNF) was highest in pure sHA cultures, i.e., in the absence of exogenous FGF-2. FGF-2, midkine, and BDNF are directly involved in neurogenesis, neuroprotection, and the expansion of neural dendrites. The activation of astrocytes leads to survival of neurons and aids the regeneration of neural networks following an injury (Figure 15B).[430]

In yet another interesting approach, biotinylated sHA-derived tetrasaccharides with specific sulfation patterns were immobilized on sensor chips using biotin-streptavidin interactions. Strong binding affinity toward 6-O-sulfated HA was

**Figure 15.** A) Molecular assembly and binding mechanism of a biotinylated sulfated alginate (b-sAlg)-coated substrate. The gold sensor is part of the QCM-D quartz crystal microbalance setup to measure the amount of deposited material. (i) Assembly of biotinylated oligoethylene glycol subunits as self-assembled monolayer (Biotin SAM). (ii) Streptavidin bound to Biotin SAM provides binding sites for subsequent binding of b-sAlg via their end-on biotin moiety. (iv) FGF-2 bound to b-sAlg. (iv) Increasing sulfation yields higher FGF-2 binding.[271] B) Mechanism of the effect of hyaluronan sulfate on astrocyte activation and neurotrophic factor production.[430] Reproduced with permission.[271] Copyright 2020, Royal Society of Chemistry (Figure A). Illustration in Figure B partially created with BioRender.com.
observed for pleiotrophin and midkine. This binding affinity translated into a significant increase in total neurite length of hippocampal neuron cultures maintained on tetrasaccharide-coated PLL substrates. By contrast, no binding was observed for sHA monosulfated at 4-0 and disulfated at the 4-0 and 6-0 positions even at high treatment concentrations. BDNF exhibited overall weak binding affinities for 6-o-sulfated and 4-o and 6-o-disulfated, with no binding detected again for monosulfation at the 4-0 position. Correspondingly, neurite outgrowth on these low-affinity variants was negligible compared to PLL controls. The study thus showed that 6-O-sulfation is critical to effectively bind growth factors and stimulate neurite outgrowth in vitro.[434]

Tully et al. determined the minimal structural elements of CS polysaccharides required for modulation of neuronal growth in hippocampal neurons. Chemically synthesized 4-O-sulfated di- or tetrasaccharides were immobilized on poly-€-ornithine substrates. The study showed that only neurons cultured on 4-O-sulfated tetrasaccharides were able to rapidly form extended neurites, indicating that the sulfated tetrasaccharide is the minimal structural motif required for CS activity.[435]

3.7.3. Cutaneous Wound Healing

Chronic wounds are known as the silent epidemic, as they are slow to heal and affect a large proportion of the global population. It is estimated that within developed countries 1–2% of the total population will experience a chronic non- or slow-healing wound during their lifetime.[250] In contrast to acute skin wounds that generally heal within a few weeks, chronic wounds fail to proceed through a regulated reparative process. This results in an often poor anatomic and functional integrity of the injured site.[431] Tissue repair following injury involves a complex series of events mediated by the interactions between cells and signaling molecules within their 3D ECM. HS is an essential part of the cutaneous ECM, capable of storing, protecting, and regulating the availability of signaling molecules required during tissue repair. If this interaction is broken, e.g., through catabolic degradation of HS, cytokines are released and affect the availability of signaling molecules affecting cell migration, proliferation, and differentiation during the wound healing process in vitro.[436]

To induce the healing process and re-establish tissue homeostasis in cutaneous chronic wounds, engineered polysaccharides that more closely mimic the function of HS were introduced over a decade ago in the form of carboxylated and sulfated dextran agents designated “ReGeneraTing Agents” (RGTAs).[34,439] Already before that, the role of functionalized dextran RGTAs as a mimic of heparin in skin had been well established.[440,441] Since then, the effects of RGTAs in the treatment of skin-associated chronic wounds, ulcers, burns, and wounds stemming from traumatic or plastic surgery have been investigated in several clinical cases and studies. For a more comprehensive overview, the reader is referred to elsewhere.[437,442–448] The most prominent example of these ESPs is the already-mentioned OTR4120 (cf. Figure 17), which localizes to the injured tissue, where it binds to structural proteins present in the ECM, for instance collagen, fibronectin, and laminin.[439] In addition, the ESP supports the healing process by maintaining the bioavailability of HS-bound growth factors and polypeptides such as inflammatory mediators, chemokines, angiogenic factors, and morphogens. RGTAs are resistant toward glycans (glycan-hydrolyzing enzymes) and as such are not subject to repeated cycles of ECM destruction and reconstruction that otherwise characterize chronic wounds. Whereas the application of OTR4120 in mouse models had no effect on typical markers for keratinocyte proliferation or differentiation (CK10, CK14, CK16), the material was able to modulate collagen type I and III expression levels to resemble the normal dermal matrix.[438] In addition to its application in chronic wounds, OTR4120 has also been identified as a possible treatment for skin burns.[449] The positive effects of RGTAs in chronic wound healing have been highlighted in several animal models, including rats (burn wounds,[449] surgical excision wounds,[450,451] dermal ischemia ulcers,[452] diabetic wounds[453]) and mice (necrotic skin ulcers,[454] diabetic wounds[455] and mice (necrotic skin ulcers[439]). A broad range of cutaneous wound-related treatments have been investigated in numerous studies and clinical cases. For a broader overview, the reader is referred to Barriault et al.[442] The effectiveness of such RGTAs as OTR4120 in wound healing is proposed to be related to stimulation of neo-vascularization and the resolution of inflammatory processes. During wound closure, epidermal proliferation and migration are promoted so that re-epithelialization is greatly enhanced. Lastly, improved formation of granulation tissue and collagen maturation contribute to normal cutaneous wound repair and the restoration of healing processes often impaired in chronic wounds.[450] Rouet et al. studied another RGTAA variant designated RGTA1192, which was obtained from modification of dextran T40 (average MW 40 kDa) with carboxymethyl, sulfate and benzylamine groups. When compared with heparin, RGTA1192 did not exhibit any anticoagulant activity which would be detrimental to wound healing applications. Once applied to the wound site, RGTA1192 was found to replace degraded HS and constitute a niche for sequestration and protection of heparin binding GPs.[460]

Carboxymethyl benzylamide sulfonate dextrans (CMDBS; Figure 16) are another important group of functionalized dextran agents that exhibit stimulatory effects on wound healing in vivo, mainly via their GF storing capability which in turn induces cell migration, proliferation, and differentiation during the
wound healing response. Interaction of CMDBS with various heparin binding growth factors such as EGF, FGFs, PDGF, and TGF-β has been shown to potentiate their effects, presumably by assisting in receptor binding and by providing a localized reservoir. CMDBS has also demonstrated a protective effect against proteolytic degradation of some growth factors. Cutaneous wounds treated with CMDBS demonstrated improved wound healing kinetics and quality of the restored skin compared to a pure collagen-based wound dressing.

Some studies have specifically focused on growth factor-binding and -concentrating properties of ESPs. Van der Smissen et al. utilized HA and CS with either low or high degrees of sulfation in collagen type I-based substrates for the purpose of promoting wound healing responses. The study showed that initial adhesion and cell proliferation of dermal fibroblasts increased with the DS, although lower synthesis of collagen and HA was observed at higher degrees of sulfation. One explanation could be that incorporation of ESPs into the scaffold reduces overall stiffness, thus adversely impacting the matrix secreting phenotype of fibroblasts. Using a combination of collagen and HA with DS = 3.0 per disaccharide, the highest adhesion rate of fibroblasts was achieved. The study showed that combinations of collagen with highly sulfated HA (DS = 3.1) or CS-A (DS = 3.1) yielded the highest levels of cell adhesion. In comparison, both sHA and CS-A with low degrees of sulfation (DS = 1.0 and DS = 1.8, respectively) yielded comparably weaker adhesion when combined with collagen. Increased electrostatic interactions between the charged cell surface and sulfate groups and general hydrophilic properties of the ECM were seen as main contributing factors toward increased cell attachment. The trends observed for adhesion were again seen in proliferation rates, with high DS consistently outperforming variants with lower DS.

Tissue-degrading MMPs and their counterparts, tissue inhibitors of metalloproteinases (TIMPs), are two crucial players in the maintenance of tissue homeostasis. An imbalance between MMPs and TIMPs can lead to pathologic ECM degradation, eventually resulting in chronic wounds. Therefore, materials that reduce MMP and increase TIMP expression are important in shifting the TIMP/MMP balance to achieve healing phenotype. Some sulfated GAGs actively interfere with GAG/TIMP interactions, a process that can be tuned by changing GAG concentration, DS and chain length. Whereas their nonsulfated counterpart has little influence on MMP activity, sHA and CS can actively control ECM remodeling via the stabilization and accumulation of TIMP. These results are in line with previous reports demonstrating reduced MMP-1 synthesis of dermal fibroblasts cultured on substrates consisting of highly sulfated HA and collagen type I. Similarly, reduced MMP-2 activity was reported for MSCs cultured on highly sulfated HA-containing collagen type I-based substrates. MMP-2 is a critical player in the interaction between fibroblasts and keratinocytes during late stage wound healing and tissue remodeling processes. Other groups have reported promising results of artificial ECMs based on sulfated GAGs and collagen type I for wound healing applications. Other groups have reported promising results of artificial ECMs based on sulfated GAGs and collagen type I for wound healing applications where the use of ESPs can offer additional benefits by improving the mechanical properties of the substrates and allow fine-tuning of the biological effects.

3.7.4. Angiogenesis and Vascularization

Proangiogenic Effects: Several studies have implicated heparin and HS in angiogenesis, i.e., the development of new blood vessels from an existing vasculature. This process relies heavily on endothelial cell proliferation and is essential not only during wound healing, but also a requirement for the development and maintenance of all vascularized biological tissues. This section serves to give a broad but nonexhaustive overview of the different areas where ESPs have been used to support vascularization in vitro or angiogenesis in vivo. Similar to their natural analogs, ESPs are thought to potentiate angiogenic activity of FGF-2 and VEGF while at the same exhibiting low anticoagulant activity. This makes them interesting for applications that aim at supporting vascular wound healing and promoting growth of collateral blood vessels during ischemic repair. In the previously described study by Freeman et al., increased retention of heparin/HS binding proteins by sAlg was demonstrated. Despite an observed depolymerization accompanying the sulfation process, stable sAlg/Alg hydrogels were still formed after incorporation of up to 75% of the ESP relative to the unmodified alginate. In vivo, the sAlg scaffold showed enhanced angiogenic properties post-implantation compared with unmodified alginate. Interestingly, sAlg with bound FGF-2 increased the fraction of matured vessels, as assessed by the expression of smooth muscle actin (SMA).

The aforementioned dextran-based ESP CMDBS has demonstrated stimulatory effects on the proliferation and metabolism of endothelial cells and associated smooth muscle cells. This was partially explained by the potentiating effect of CMDBS on FGF-mediated cell proliferation, mainly by complex formation between the negatively charged chains of CMDBS and positive amino acid side chains of FGF. Similarly, the RGTA family member OTR4120 was found to enhance VEGF-mediated proliferation and migration of HUVECs in vitro, as well as angiogenesis in a chick chorioallantoic membrane (CAM) in vivo assay. Similarly, the RGTA family member OTR4120...
was found to enhance VEGF-mediated proliferation and migration of HUVECs in vitro, as well as angiogenesis in a chick chorioallantoic membrane (CAM) on ovo assay.\[34,44]\ Thus, the role of OTIR420 during wound healing is intimately related to its stimulatory effect on endothelial cell proliferation. Another study based on the CAM model assessed several linear and branched synthetic sulfated oligosaccharides for their potential to induce and later sustain a capillary network. Interestingly, optimal proangiogenic efficiency was only observed for chain lengths ranging from hexa- to deca-saccharides. Mechanistic data suggested that cellular responses toward extracellular sulfated oligosaccharides are initiated at the plasma membrane αvβ3 integrin and involve the activation of ERK1/2 (MAPK signaling pathway).\[466]

sChits have been used in several applications that aim at enhancing capillary tube formation either in vitro or in vivo. Herein, 2-N,6-O-sulfated chitosan (26SCS) has attracted the most attention, not only due to its rather broad binding affinity for several growth factors but also for its versatility within different fields (cf. Section 3.7.2, Bone). Han et al. specifically investigated the effect of different sulfation patterns on HUVEC viability and proliferation rates. Whereas also 6-O and 3-O,6-O-sulfated chitosan exhibited favorable biological properties and induced capillary-like tube formation, the effect was most pronounced for 2-N,6-O-sulfation. Furthermore, gene expression studies revealed higher transcript levels for angiogenic-related genes VEGF, von Willebrand factor and CD31 in the case of 2-N,6-O-sulfation.\[467]

**Antiangiogenic Effects:** VEGF has two known isoforms, i.e., proangiogenic VEGF$_{165}$ and antiangiogenic VEGF$_{165b}$. Some angiogenesis-related pathologies such as cancer or macular degeneration may require selective inhibition of VEGF$_{165}$, while leaving interactions with VEGF$_{165b}$ largely undisturbed.\[288\] Such distinct differences in binding profiles were observed in the case of 6-O sulfation of hyaluronic acid. Whereas varying the MW of HA had little influence on GF binding, the DS strongly impacted the affinities with which these isoforms were able to bind to sHA. The high affinity observed for VEGF$_{165}$ (K$_D \approx 1 \times 10^{-9}$ m) ultimately inhibited endothelial cell survival and proliferation by sequestering available VEGF$_{165}$.\[468\] This differential binding was explained by differences in the heparin-binding domain structures of both isoforms.\[469\] A recent study describes sChit as another potent inhibitor of angiogenesis in vivo, where an increase 6-O sulfation was shown to increase the inhibiting effect on angiogenesis.\[468\] This differential binding was explained by differences in the heparin-binding domain structures of both isoforms.\[469\] A recent study describes sChit as another potent inhibitor of angiogenesis in vivo, where an increase 6-O sulfation was shown to increase the inhibiting effect on angiogenesis. Assessing different sulfation sites, the authors concluded that the 6-O sulfation is the main factor affecting VEGF sequestration. Combined with the inhibiting effect on endothelial cell proliferation, 6-O-sulfated chitosan could thus serve as a potent inhibitor of the VEGF/VEGFR2 pathway.\[460\]

Whereas heparin has long been known to exhibit proangiogenic activity,\[312\] inhibiting effects have been described for the oral administration of heparin or heparin fragments.\[473\] Here, the accompanying release of non-anticoagulant heparin fragments in the serum in the presence of cortisol resulted in antiangiogenic effects. Similar to LMW heparin, sulfated K5 polysaccharides are able to bind FGF-2 and prevent the formation of HSPG/FGF-2/fibroblast growth factor receptor (FGFR) ternary complexes. FGF-2-mediated endothelial cell proliferation is effectively inhibited as a direct consequence of the prevention of GF-mediated attachment of FGFR-expressing cells to HSPG-bearing cells. While a potent FGF-2 antagonist activity was demonstrated in vitro, similar antiangiogenic properties were shown in vivo.\[474\] Inhibitory effects even stronger than those observed for heparin were described for sChit. These derivatives were found to block the VEGF/VEGFR signaling pathway, resulting in suppressed proliferation, migration, and tube formation of HUVECs. The authors observed a general trend toward stronger inhibitory effects at lower molecular weights. As an important advantage over native heparin, sChit did not show anticoagulant activity in vivo. 6-O sulfation was again identified as the main contributing factor affecting VEGF binding.\[470\] Antiangiogenic effects of chitosan and various derivatives were reported in several instances, in particular in regards to tumor-induced angiogenesis.\[475,476\] In one study, oppositely charged chitosan and dextran sulfate were allowed to self-assemble into nanoparticles and served as degradable delivery vehicles for an antiangiogenic hexapeptide. 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As an important advantage over native heparin, sChit did not show anticoagulant activity in vivo. 6-O sulfation was again identified as the main contributing factor affecting VEGF binding.\[470\] Antiangiogenic effects of chitosan and various derivatives were reported in several instances, in particular in regards to tumor-induced angiogenesis.\[475,476\] In one study, oppositely charged chitosan and dextran sulfate were allowed to self-assemble into nanoparticles and served as degradable delivery vehicles for an antiangiogenic hexapeptide.\[312\] An interesting approach toward generating a non-anticoagulant antiangiogenic heparin derivative (ST1514) was presented by Casu et al.\[477\] Selectively 2-O desulfated heparin was split between C2–C3 bonds of all nonsulfated uronic acid residues, effectively destroying the essential glucuronic acid of the antithrombin-binding site. Whereas ST1514 no longer exhibited anticoagulant activity, it gained FGF-2 antagonistic effects. Similar to LMW heparin, sulfated K5 polysaccharides are able to bind FGF-2 and prevent the formation of HSPG/FGF-2/fibroblast growth factor receptor (FGFR) ternary complexes. FGF-2-mediated endothelial cell proliferation is effectively inhibited as a direct consequence of the prevention of GF-mediated attachment of FGFR-expressing cells to HSPG-bearing cells. While a potent FGF-2 antagonist activity was demonstrated in vitro, similar antiangiogenic properties were shown in vivo.\[474\] Inhibitory effects even stronger than those observed for heparin were described for sChit. These derivatives were found to block the VEGF/VEGFR signaling pathway, resulting in suppressed proliferation, migration, and tube formation of HUVECs. The authors observed a general trend toward stronger inhibitory effects at lower molecular weights. 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and angiostatic activity. More recently, an LMW variant of the above glycol-split heparin derivative with negligible anticoagulant and strong antiangiogenic properties was reported.\textsuperscript{[478]} Finally, various sulfated oligosaccharide derivatives have been shown to inhibit angiogenesis, e.g., sulfated mannopentaose phosphate (PI-88)\textsuperscript{[479,480]} maltolactose and maltohexose sulfate,\textsuperscript{[481]} or the heparanase inhibitor oligomannurate sulfate.\textsuperscript{[414]} An interesting approach toward generating a non-anticoagulant antiangiogenic heparin derivative (ST1514) was presented by Casu et al.\textsuperscript{[477]} Selectively 2-O desulfated heparin was split between C2–C3 bonds of all nonsulfated uronic acid residues, effectively destroying the essential glucuronic acid of the antithrombin-binding site. Whereas ST1514 no longer exhibited anticoagulant activity, it gained FGF-2 antagonistic and angiostatic activity.

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3.7.5. Stem Cell Maintenance and Differentiation

ESC have for a long time been a valuable cell source for tissue engineering and regenerative medicine applications. In the past, most of the relevant research has focused on murine cell lines. With the advent of cell reprogramming techniques, induced pluripotent stem cells (iPSCs) have opened additional possibilities such as using stem cells derived from human somatic cells.\textsuperscript{[482–484]} Evidence suggests that GAGs play a crucial role in homeostasis and lineage commitment of pluripotent stem cells.\textsuperscript{[86,485–489]} Even though a multitude of signaling pathways involved in these processes have been identified, the mechanisms driving these interactions between GAGs and proteins are not clear. This is mostly due to the difficult purification of large quantities of natural GAGs with a homogeneous, defined sulfation pattern. For this reason, semi-synthetic strategies are essential to generate sufficient quantities for cell studies and tissue engineering applications. Indeed, previous biochemical studies have shown that specific sulfation patterns in GAGs are required for stem cell differentiation.\textsuperscript{[85,490–492]} A low content of sulfated GAGs within the stem cell niche not only minimizes growth factor sequestration and hence exposure, but also reduces GAG-mediated receptor binding. As a result, premature transition from self-renewal toward differentiation can be avoided. In general, differentiation is accompanied by an increase in the degree of sulfation, thus resulting in differential binding of growth factors.\textsuperscript{[85,485,493]} The following sections are focused on the effects of several ESPs on the behavior of various pluripotent and multipotent stem cells. Whereas a large part of the literature focuses on neural differentiation of ESCs or chondrogenic and osteogenic differentiation of MSCs, less is known about how sulfated polysaccharides influence iPSCs. A more thorough discussion of the role that sulfated GAGs play in the mediation of stem cell niches can be found in the review by Mikami and Kitagawa.\textsuperscript{[491]}

**Stem Cell Maintenance:** With respect to ESCs, HS accounts for the vast majority (80%) of expressed GAGs within the stem cell niche, with CS constituting the remaining 20%. Here, HS functions mainly as coreceptor and reservoir for various growth factors and morphogens (e.g., BMP, Wnt, FGF) involved in self-renewal\textsuperscript{[85,488–497]} Early studies have shown that sustained and elevated levels of FGF-2 support long-term maintenance of ESCs while suppressing BMP signaling-mediated differentiation.\textsuperscript{[495–498–500]} This implies that sequestration and sustained release of elevated doses of FGF-2 by HS and HS-like ESPs can contribute to stem cell renewal and differentiation.\textsuperscript{[486,488]} Besides the specific sulfation patterns, Pickford et al. demonstrated that length and structure of the polysaccharide backbone are important factors in stem cell maintenance and differentiation.\textsuperscript{[501]} In particular, under-sulfated HS appears to be characteristic of pluripotent cells.\textsuperscript{[502]}

Keeping human iPSCs in a pluripotent and undifferentiated state is dependent on FGF-2, which usually makes maintenance of these cells very costly. To mitigate these problems, Miura et al. developed a novel feeder-free and FGF-2-free culture system.\textsuperscript{[503]} Although the specific regulation of GAG-mediated signaling in human iPSCs has not been investigated so far, evidence presented by the authors suggests that shA maintains stemness by facilitating FGF-2 signaling. To investigate the effect of sulfation on stem cell maintenance, shA with different DS was added directly to cell cultures. At high DS (0.9), 90% of the cells remained undifferentiated under feeder-free and FGF-2-free conditions. At low DS (0.5) and for nonsulfated HA, less than 10% of the cells remained undifferentiated. Essential transcription factors for keeping iPSCs in an undifferentiated state (OCT4, SOX2, NANOG) were significantly increased in high-DS shHA. Moreover, embryoid bodies formed from cells previously cultured with highly sulfated HA showed increased expression levels of markers specific to the ectoderm (βIII-tubulin), endoderm (GATA6) and mesoderm (α-SMA). Taken together, these results clearly demonstrate that highly sulfated HA maintains both the undifferentiated state as well as pluripotency of iPSCs.\textsuperscript{[501]}

Mhanna et al. synthesized sAlg derivatives with different DS.\textsuperscript{[272]} To engineer a stem cell niche, thin films consisting of sAlg and collagen were alternately deposited in a layer-by-layer fashion on 2D substrates. This multilayered construct was then used to study the effects of sulfation density on FGF-2 affinity and its effect on the stemness of adipose derived stem cells (ADSCs). The study showed that FGF-2 binding improved at higher DS, such that the following trend could be observed for sAlg: sAlg\textsubscript{0.4} > sAlg\textsubscript{0.6} > sAlg\textsubscript{0.9} (per monosaccharide). Stemness, as defined by the expression of surface markers CD73, CD90, and CD105 as well as the ability to differentiate along the mesenchymal lineage was highest for colonies grown on sAlg\textsubscript{0.3} and decreased at lower levels of sulfation.\textsuperscript{[272,504]} In addition to their effect on stem cell maintenance, the sAlg-based hydrogels were found to promote proliferation and sustain viability of encapsulated stem cells.\textsuperscript{[505]}
GAG-derived mimetics and structural analogs have also been employed for the purpose of regulating stem cell maintenance and differentiation. In one attempt to mimic natural GAGs, Wang et al. employed a copolymerization strategy that yielded various GAG analogs by RAFT radical polymerization (cf. Section 2.5). These synthetic polysaccharides were analyzed in the context of ESC proliferation and differentiation, showing biological activities similar to native sulfated GAGs. Murine ESCs cultivated under feeder-free conditions and in the presence of the copolymer analogs exhibited up to 60% higher cell proliferation compared with heparin. The analogs further elicited threefold higher βIII-tubulin gene expression levels (qPCR) compared with heparin, hence proving their potential to induce neural differentiation.[93]

To maintain the undifferentiated state of iPSC-derived neural stem cells, a fully synthetic HS-based neural tissue construct was developed. Synthetic HS oligosaccharides were modified with triazide PEG linkers which could undergo 1,3-dipolar cycloadditions with cyclooctyne-modified 4-arm PEG derivatives to yield stable hydrogels. It was hypothesized that the placement of sulfated oligosaccharide units would lead to functional interactions with the HS-binding protein FGF-2. Compared to cultures on Geltrex (matrix substitute containing complex mixture of ECM proteins), stem cells encapsulated in the ESP hydrogel scaffold exhibited significantly higher proliferation and self-renwal. The study showed that the HS disaccharides protected FGF-2 from degradation, extending its otherwise short biological half-life. Furthermore, self-renewal markers SOX1 and astrocytic markers GFAP were significantly upregulated, indicating an increased potential of the sulfated HS oligosaccharides to induce cell differentiation.[906]

**Stem Cell Differentiation:** Whereas HS has important functions in stem cell maintenance, both HS and CS are also involved in ESC transition from a state of self-renewal to cell fate commitment into multiple lineages. Irrespective of the specific stem cell fate, an overall increase in HS content and DS has been observed during differentiation.[85, 486, 488, 494–497] Here, HS acts as a coreceptor mediating the interaction between FGFRs and FGFs. Following FGF binding, extracellular signal-regulated kinases 1 and 2 (ERK1/2) are phosphorylated and initiate downstream signaling events that eventually lead to neural differentiation of stem cells.[507] Of note, it has been reported that a structural transition from low to high sulfation and thus an overall change in sulfation pattern is characteristic for the development of mESC into neuroectodermal progenitor cells.[85, 485, 486, 488, 494–497] Here, HS acts as a coreceptor mediating the interaction between FGFRs and FGFs. Following FGF binding, extracellular signal-regulated kinases 1 and 2 (ERK1/2) are phosphorylated and initiate downstream signaling events that eventually lead to neural differentiation of stem cells.[907] Of note, it has been reported that a structural transition from low to high sulfation and thus an overall change in sulfation pattern is characteristic for the development of mESC into neuroectodermal progenitor cells.[485] However, this ability of promoting stem cell differentiation into the neural or other mesodermal lineages is lost following removal of 2-O and the majority of 6-O sulfate groups. Interestingly, rescue of the neural specification potential was possible by adding FGF-4 and soluble heparin to mESC cultures. These data showed that heparin can substitute for HS by binding to growth factors and modulating signaling pathways, and further implies that ESPs can assume a similar role.[486, 502]

Several studies have highlighted the use of ESPs for promoting differentiation in various stem cell types. For example, in one study the pro-osteogenic effect of sHA on human bone marrow-derived mesenchymal stem cell (hBMSC) cultures was demonstrated. Here, the authors treated hBMSCs with various sulfated and nonsulfated HA derivatives. sHA treatment led to increased fibronectin (FN) secretion and changes in FN fibril assembly. Analysis of TNAP activity, a marker for early osteogenic differentiation, was significantly increased for sHA treated cultures.[153] This result was in agreement with earlier studies that described the pro-osteogenic effect of sHA on hBMSCs within collagen-based matrices.[151, 155] As referred to in a previous section, osteogenic differentiation of hBMSCs into osteoblasts was also observed when culturing cells osteogenic medium conditioned with sHA and dexamethasone. The latter constitutes an important activator of several downstream pathways involved in osteoblast differentiation. Based on TNAP activity and deposition of calcium phosphate, a clear synergistic effect was observed for sHA and dexamethasone cocultures. Proteomic analysis highlighted several pathways involved in the osteogenic differentiation (EXCR4, ERK/MAPK, EIF2).[350] Several studies utilized sHA to promote the differentiation of mesenchymal stem cells. Feng et al. encapsulated hMSCs within sHA hydrogels to promote chondrogenic differentiation and found that hypertrophy was suppressed both in vitro and in vivo after implantation. The effect was attributed mainly to sustained release of exogenously added TGF-β.[109]

Di Giacomo et al. analyzed synthetic sulfated oligosaccharide mimetics of HS.[508] EP80031, a synthetic octosaccharide was analyzed with respect to its ability to mobilize hematopoietic stem cells (HSCs) in peripheral blood. Following intravenous injections into C57BL/6 mice, the authors observed a maximum fivefold increase in the mobilization of multipotent HSCs. It was reasoned that these cKit+/Lin−/Sca+ (KLS) cells could hence serve as an alternative to endogenous HS.[508]

Sulfated alginate has been shown to indirectly induce chondrogenic differentiation of hBMSCs by initial sequestration and subsequent release of TGF-β1 when used in hydrogel scaffolds. Differentiated hBMSCs demonstrated increased deposition of collagen type II and cartilage-specific ECM deposition.[129] A similar study addressed the effect of recombinantly produced TGF-β1 on ADSCs encapsulated in SA hydrogels. Again, it was found that sustained GF-release is the main contributing factor to chondrogenic differentiation.[509] In addition to bulk hydrogels, sAlg was also combined with PVA to form complex electrospun nanofibrous scaffolds.[510] Electrospun scaffolds were able to promote proliferation as well as neurogenesis of hBMSCs in vitro. The effect of sAlg on neuronal differentiation was assessed through MAP2 gene expression levels. Interestingly, increased MAP2 mRNA levels and neural-like morphology were observed even in the absence of external differentiation agents (β-carotene), thus highlighting the neuroinductive effect of sAlg.[510] Ding et al. demonstrated the ability of murine ESCs (mESCs) to differentiate into nerve cells using differentiation media supplemented with various sulfated chitosan derivatives.[512] In this study, chitosan was selectively
2-N, 6-O, and 3,6-O-sulfated and the authors also assessed the effect of varying the DS. While all sChit variants directed ESCs into neural differentiation, the efficiency of differentiation was not the same across the different modifications. Even though the DS values for 2-O- and 6-O-sulfated chitosan were similar (≈0.5), ESCs cultured in the presence of 6-O-sulfated chitosan expressed higher levels of late-stage neuron-specific marker βIII-tubulin. They also exhibited neuron-like morphologies with protruding axons and dendrites. Increasing the DS of 6-O-sulfated chitosan to 0.8 resulted in a 10-fold increase in βIII-tubulin expression compared to the variant with lower DS. Nevertheless, increasing the overall DS by introducing other sulfation sites did not promote neural differentiation to the same extent. In fact, the introduction of additional sulfation sites, such as at the 2-N site, may even decrease the neural differentiation potential. The results show that the sulfation pattern is a more important factor than DS for differentiation in this specific model, and they further imply that the process by which sChit promotes neural differentiation of ESC is similar to heparin/HS.\(^{511}\)

sCel has been investigated for use in chondrogenic differentiation of MSCs through incorporation into a fibrous gelatin scaffold using electrospinning.\(^ {512}\) The degree of sulfation (DS = 0.8) for sCel was tailored to more closely mimic naturally occurring 6-O-sulfated CS (C6S). Scaffolds containing sCel retained TGF-β more effectively than gelatin alone, although no significant difference between high (5.0 wt%) and low concentrations (0.1 wt%) of sCel was observed. By contrast, chondrogenic differentiation of MSCs—as measured by gene expression of ACAN, COL2A1, SOX9—was significantly increased for scaffolds containing high concentrations of sCel. A follow-up study using fully sulfated cellulose (DS = 3.0) showed diminished chondrogenic differentiation of MSCs seeded on scaffolds containing high concentrations of sCel. This was proposed to be partly caused by removal of growth factors from the surrounding medium due to the high charge density of the scaffold. Interestingly, control experiments using only cell pellet cultures showed that basal medium containing 1% sCel promotes chondrogenesis even in the absence of chondrogenesis-inducing factors.\(^ {513}\) sCel further exhibited increased binding affinity toward FGF-2. Zhang et al. found that sCel with intermediate to high 2-O sulfation and maximum 6-O sulfation showed superior growth factor binding compared to low or no sulfation.\(^ {51}\) Even though overall binding only reached ≈60% of that observed for heparin, sCel appears to be an interesting scaffolding material for stem cell differentiation due to its structural properties and potential for regioselective modification.\(^ {513}\)

Sulfated dextran derivatives serve as HS-analogues and have garnered wider recognition as main building block of RGTAs. Nevertheless, only a few reports have been published concerning the role of sulfated dextran derivatives in stem cell maintenance and differentiation. OTR\(_{4120}\) and OTR\(_{4131}\) (Figure 17A,B) are derivatives of dextran T40 and composed of ≈2500 glucosidic units linked by α-1→6 bonds.\(^ {14}\)

Whereas both compounds are sulfated (DS = 1.2), only OTR\(_{4131}\) has been acetylated (DAc = 0.2). Rat MSCs cultured with the HS-analogue OTR\(_{4120}\) exhibited a higher colony forming efficiency compared to heparin or OTR\(_{4131}\). In the absence of exogenous growth factor FGF-2, only OTR\(_{4131}\) showed significantly increased MSC proliferation and enhanced migration.\(^ {514}\) These findings agree with previous results suggesting that RGTAs can provide a beneficial matrix environment for the migration of osteoblastic progenitors.\(^ {515}\) However, both molecules exhibited similar potential concerning the regulation of stem cell differentiation. There were no marked differences in osteogenesis induction of MSCs, revealing that the dominant factor driving stem cell differentiation is sulfation rather than acetylation.\(^ {34}\) In one study, the depolymerization of high MW (1000 kDa) marine-derived polysaccharide GY785 yielded a slightly sulfated low MW (15 kDa) variant designated GY785 DR. GY785 was produced by the bacterium A. infernus and a more detailed characterization can be found in the study by Raguenes et al.\(^ {516}\) Subsequent sulfation yielded its oversulfated isoform GY785 DRS (20 kDa). Both ESPs displayed high structural stability and absent immunoactivity when compared to natural GAGs. Furthermore, GY785 DRS exhibited a 100-fold stronger binding affinity for TGF-β1 compared with GY785 DR. The higher binding affinity subsequently translated into stronger promotion of chondrogenesis of cultured ADSCs.\(^ {74}\) Another study showed that algal fucoidans (DS = 0.8–1.0) were more efficient in mobilizing hematopoietic stem cells compared with both RGTAs variants.\(^ {517}\) Demonstrating again that higher DS does not always correlate with higher potentials in the regulation of stem cells, as described above for regioselectively sChits.

As previously described, membrane-associated HS have central roles in modulating stem cell behavior, and recent research

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**Figure 17.** Structures of RGTAs A) OTR4120 and B) OTR4131.\(^ {443,446}\)
has described various strategies for integrating ESPs in the plasma membrane. Huang et al. produced various synthetic sulfated “neoproteoglycans” (neoPGs) with high affinity for FGF-2, which were further functionalized with a phospholipid tail and anchored into the plasma membrane of ESCs (Figure 18A). Consistent with previous literature, both 2-O-sulfation of the uronic acid and 6-O-sulfation of the glucosamine were required for FGF-2 binding. Interestingly, the highest binding affinities were observed for 2,6-O-disulfated units. The lipid-anchored chains remained on the cell surface for several hours and induced ERK1/2 signaling, leading further to neural differentiation of the ESCs, evident by the formation of nestin+ neural rosettes and a decrease in Oct4 expression.[507] The lab of Hsieh-Wilson reported a cell-surface modification based on dodecanone-containing liposomes as delivery vehicles. Sulfated CS chains were functionalized with an aminooxyacetic group that allowed stable integration of the GAG into liposomes (Figure 18B).[518] Liposomes were then fused with the target cell membrane. Upon cell-surface modification, embryonic PC12 progenitor cells upregulated NGF-mediated signaling and increased neurite outgrowth.[519] A similar strategy was employed to incorporate lipid-anchored biomimetics (copolymerized from synthetic GAG analogues) into ESC membranes (Figure 18C).[519] Upon cell-surface modification, embryonic PC12 progenitor cells upregulated NGF-mediated signaling and increased neurite outgrowth.[518] A similar strategy was employed to incorporate lipid-anchored biomimetics (copolymerized from synthetic GAG analogues) into ESC membranes (Figure 18C).[519] To prolong the display time of the ESPs, an anchoring strategy involving HaloTag proteins (HTPs) was devised (Figure 18D). HTPs are modified alkane dehalogenases that were linked covalently to chloroalkane-modified HS (HS-CL). As a result, the lifetime of membrane-anchored HS GAGs increased significantly (up to 1 week), thus allowing long-term presentation on membranes. Modified mESCs were able to exit the self-renewal stage early and commit to a neural lineage via activation of ERK/MAPK signaling pathways.[520]

Various other heparin and HS-mimicking structures have been developed over the years. However, due to their distant nature from sulfated polysaccharides, they mostly fall outside the scope of this review. For a more thorough treatment of GAG mimetics based on glycopolymer systems, the reader is referred to the comprehensive reviews by Wang et al.,[88] Miura et al.,[89] and Paluck et al.[92]

4. Conclusions and Outlook

The reviewed research presents many fascinating examples of how ESPs can mimic the biological roles of natural sulfated GAGs, while offering benefits over their natural counterparts that may facilitate their use in biomedical applications. The most significant difference between ESPs and natural sulfated polysaccharides is the possibility of regioselective modification and fine-tuning of the DS. Regioselective sulfation of ESPs allows for systematic exploration of structure–function aspects, such as identifying which sulfate positions are crucial for specific biological interactions and which additional positions that can enhance or diminish the effects of the interactions. It can further be used to increase the potency of a given biological effect, reduce cross-reactivity, or allow novel and highly specific interactions like the activation of antithrombin by heparin.

Most of the observed effects of ESPs on cellular processes and tissue development are attributed to their interactions with growth factors, cytokines, and chemokines. The differential binding of specifically modified ESPs can be exploited for sequential release of these signaling factors to mimic the natural progression of complex multistep biological processes, additionally avoiding nonphysiological burst release to achieve long-lasting effects and an improved safety profile compared with other biomaterials. An important aspect of introducing ESPs in implantable biomaterials is the possibility of using polysaccharides with a natural capability to form hydrogel networks or fibers, allowing generation of tissue-like structures. Here, the DS and pattern of ESPs are closely linked to the physical and structural properties of the materials, necessitating independent control of ESP and matrix properties to obtain robust biological data. As the ESPs can be based on nonmammalian polysaccharides and/or have specific sulfation patterns not found in nature, they can be protected from enzymatic depolymerization to maintain structural properties and prolong their biological effects, e.g., in drug delivery and tissue engineering applications.

Studies have revealed a high level of functional versatility driven by electrostatic interactions, presenting new opportunities in developing multifunctional materials and drugs. This versatility of ESPs also presents a challenge in the forms of cross-reactivity and of characterizing their effects in complex biological systems. This is well indicated in studies on their effects on coagulation and immunological processes, where ESPs have multiple interaction targets and can have contradictory effects depending on their structural properties or on the specific model system employed. As described in previous sections, ESP-protein interactions can be potentiating or synergistic, with the ESPs serving as coreceptors and/or reservoirs, or inhibiting, through sequestration of proteins from their receptors or antagonization of the binding sites. Functional redundancy is also observed in nature, for instance, with a single GAG binding multiple different growth factors, although GAGs can exhibit different structural motifs with similarly different binding affinities. The biological responses resulting from these interactions are presumably concerted events of multiple signaling pathways, where temporal and spatial control of chemokine and GAG expression are likely to play key roles.

Thus, some scrutiny must be applied for isolated events studied in vitro, particularly when using poorly characterized ESPs with complex and nonregular structures that may exhibit a low degree of specificity. Here, strategies for regioselective modification will be crucial to identify interaction targets and increase specificity. Generating ESPs with a high degree of non-specific activity may also have a use, for example, in novel antiviral/antimicrobial prophylactics as they can potentially target surface proteins of several different organisms, and they do not require systemic administration which can carry high risks of adverse effects. Regardless of the application, it is apparent that a more systematic approach utilizing well-defined structures in
Figure 18. Stem cell specification based on cell surface engineering strategies. A) The HS-mediated interaction between FGF-2 and FGFR in embryonic stem cells (ESCs) can be mimicked by the insertion of HS mimetics (neoPG) into the plasma membrane using various anchoring strategies.\cite{507} B) Sulfated CS chains ($n \approx 110$ disaccharide units) are coupled to lipid anchors and indirectly incorporated into the plasma membrane using liposomes.\cite{518} C) Synthetic GAG analogues are functionalized with phospholipid anchors and incorporated into cell membranes of ESCs to promote short-term differentiation into neurons.\cite{519} Stem cell specification based on cell surface engineering strategies. (A) The HS-mediated interaction between FGF-2 and FGFR in ESCs can be mimicked by the insertion of HS mimetics (neoPG) into the plasma membrane using various anchoring strategies.\cite{507} (B) Sulfated CS chains ($n \approx 110$ disaccharide units) are coupled to lipid anchors and indirectly incorporated into the plasma membrane using liposomes.\cite{518} C) Synthetic GAG analogues are functionalized with phospholipid anchors and incorporated into cell membranes of ESCs to promote short-term differentiation into neurons.\cite{519} D) HaloTag proteins (HTP) allow the long-term presentation of HS-derived chains on the cell surface of mESCs.\cite{520} Adapted with permission. Copyright 2020, American Chemical Society (Figure A–C), Wiley-VCH (Figure D).
multiple different model systems will provide more robust data on the efficacy and safety of ESPs. This will, in turn, provide a foundation for new clinical applications in which observed in vitro effects are translated to a complete biological system.

In recent years, we have seen a vast increase in the research output on ESPs, following a greater understanding of the biological roles of natural sulfated polysaccharides, their implications in pathologies, and how they can be exploited in medicine and medical technology. As new and highly advanced technology for structural characterization, material fabrication and biological characterization emerges for increasingly sophisticated model systems, we foresee an increase in novel ESP-based innovations and breakthroughs in clinical trials within the coming few years, and we strongly encourage the continued interdisciplinary participation of the research communities.

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Conflict of Interest

The authors declare no conflict of interest.

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[1] R. Raman, V. Sasishekar, R. Sasishekaran, Chem. Biol. 2005, 12, 267.
[2] D. R. Coombe, C. R. Parish, in Invertebrate Historecognition (Eds: R. K. Grosberg, D. Hedgcock, K. Nelson), Springer Science & Business Media, Boston, MA, USA 1988, pp. 31–54.
[3] C. F. Medeiros, A. Mendes, R. A. B. Castro, E. C. Bau, H. B. Nader, C. P. Dietrich, Biochim. Biophys. Acta 2000, 1475, 287.
[4] O. Bertue, B. Mullay, Glycobiology 2003, 13, 29R.
[5] P. A. S. Mourao, M. S. Pereira, Trends Cardiovasc. Med 1999, 9, 225.
[6] R. Harel, M. L. Tanzer, FASEB J. 1993, 7, 1115.
[7] A. V. Skriptsova, Russ. J. Mar. Biol. 2015, 41, 145.
[8] M. Lahaye, M. A. V. Axelos, Carbohydr. Polym. 1993, 22, 261.
[9] S. Mabeau, B. Kloareg, J. P. Joseleau, Phytochemistry 1990, 29, 2441.
[10] N. Sooanen, E. Da Silva, C. Gardarin, P. Michaud, C. Laroche, Biotechnol. 2016, 213, 231.
[11] G. Squillaci, R. Finamore, P. Diana, O. F. Restaino, C. Schiraldi, S. Arubucci, E. Ionata, F. La Cara, A. Morana, Appl. Microbiol. Biotechnol. 2016, 100, 613.
[12] L. Richert, S. Golubic, R. Le Guedes, J. Ratiskol, C. Payri, J. Guezenneec, Curr. Microbiol. 2005, 51, 379.
[13] R. S. Aquino, C. Grativol, P. A. S. Mourao, PLoS One 2011, 6.
[14] N. Dantas-Santos, D. L. Gomes, L. S. Costa, S. L. Cordeiro, M. S. S. Costa, E. S. Trindade, C. R. C. Franco, K. C. Scortecci, E. L. Leite, H. A. O. Rocha, Int. J. Mol. Sci. 2012, 13, 961.
[15] D. Wardrop, D. Keeling, Brit. J. Haematol. 2008, 141, 757.
[16] A. Kowitsch, G. Y. Zhou, T. Groth, J. Tissue Eng. Rejuven. Med. 2018, 12, e23.
[17] P. S. Kwon, H. Oh, S. J. Kwon, W. H. Jin, F. M. Zhang, K. Fraser, J. J. Hong, R. J. Linhardt, J. S. Dordick, Cell Discovery 2020, 6.
[18] Y. M. Xu, C. Cai, K. Chandarajoti, P. H. Hsieh, L. Y. Li, T. Q. Pham, E. M. Sparkenbaugh, J. Z. Sheng, N. S. Key, R. Pawlinski, E. N. Harris, R. J. Linhardt, J. Liu, Nat. Chem. Biol. 2014, 10, 248.
[19] N. Sugita, T. Shoimi, M. Chiba, T. Sato, H. Narimatsu, K. Kimata, H. Watanabe, J. Biol. Chem. 2012, 287, 43390.
[20] A. S. Saribas, A. Mobasseri, P. Pristatsky, X. Chen, R. Barthelson, D. Hakes, J. Wang, Glycobiology 2004, 14, 1217.
[21] A. Prechoux, C. Halimi, J. P. Simonne, H. Lortat-Jacob, C. Laguri, ACS Chem. Biol. 2015, 10, 1064.
[22] E. Tykesson, A. Hassinen, K. Zielinska, M. A. Thelin, G. Frati, U. Ellervik, G. Westergren-Thorsson, A. Malmstrom, S. Kellokumpu, M. Maccarana, J. Biol. Chem. 2018, 293, 13725.
[23] E. C. Sherrard, G. W. Blanco, Ind. Eng. Chem. 1923, 15, 611.
[24] R. A. Al-Horani, U. R. Desai, Tetrahedron 2010, 66, 2907.
[25] C. C. Calegari, V. A. Q. Santos, A. M. Barbosa-Dekker, C. Busso, R. F. H. Dekker, M. A. A. da Cunha, Food Technol. Biotechnol. 2019, 57, 490.
[26] L. Fan, Y. Gong, M. Cao, S. Gao, Y. Sun, L. Chen, H. Zheng, W. Xie, J. Appl. Polym. Sci. 2013, 127, 4856.
[27] K. R. Holme, A. S. Perlin, Carbohydr. Res. 1997, 302, 7.
[28] V. Crescenzi, M. Dentini, D. R. Gasco, S. Spadoni, G. Skjåk-Bræk, D. Capitanli, L. Mannina, S. Viel, Biomacromolecules 2004, 5, 537.
[29] K. Lidholt, M. Fjelstad, K. Jann, U. Lindahl, Glycoconjugate J. 1993, 10, 221.
[30] J. Becher, S. Möller, D. Weiss, J. Schiller, M. Schnabelrauch, Macromol. Symp. 2010, 296, 446.
[31] G. Arlov, F. L. Aachmann, A. Sundan, T. Espevik, G. Skjåk-Bræk, Biomacromolecules 2014, 15, 2744.
[32] A. Chairedegumjorn, H. Toyoda, E. R. Woo, K. B. Lee, Y. S. Kim, T. Toida, T. Imanari, Carbohydr. Res. 2002, 337, 925.
[33] Z. M. Wang, L. Li, K. J. Xiao, J. Y. Wu, Bioreour. Technol. 2009, 100, 1687.
[34] D. Papy-Garcia, V. Barbier-Chassafere, V. Rouet, M. E. Kerros, C. Klochendler, M. C. Tournaire, D. Barrault, J. P. Caruelle, E. Petit, Macromolecules 2005, 38, 4647.
[35] N. Chopin, C. Sinquin, J. Ratiskol, A. Zykwinska, P. Weiss, S. Cerantola, J. Le Bideau, S. Colliec-Jouault, Biomed. Res. Int. 2015.
[36] T. Liebert, J. Wotschadlo, M. Gerice, S. Kohler, P. Laudeley, T. Heinze, ACS Symp. Ser. 2009, 1017, 115.
[37] A. Magnani, S. Lamponi, R. Rappuoli, R. B. Barbutti, Polym. Int. 1998, 46, 225.
[38] M. Gerice, A. Doliska, J. Stana, T. Liebert, T. Heinze, K. Stana-Kleinschek, Macromol. Biosci. 2011, 11, 549.
[39] C. S. B. Telles, D. A. Sabry, J. Almeida-Lima, M. S. S. P. Costa, R. F. Melo-Silveira, E. S. Trindade, G. L. Sassaki, E. Wisbeck, S. A. Furlan, E. L. Leite, H. A. O. Rocha, Carbohydr. Polym. 2011, 85, 514.
[40] T. Groth, W. Wagenknecht, Biomaterials 2001, 22, 2719.
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