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Author(s):
Arlov, Øystein; Rütsche, Dominic; Asadi Korayem, Maryam; Öztürk, Ece; Zenobi-Wong, Marcy

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Engineered sulfated polysaccharides for biomedical applications

Øystein Arlov[1], Dominic Rütsche[2*], Maryam Asadi Korayem[2*], Ece Öztürk[3,4] Marcy Zenobi-Wong[2],

1. SINTEF Industry, Department of Biotechnology and Nanomedicine, Richard Birkelands vei 3B, 7034 Trondheim, Norway
2. ETH Zurich, Tissue Engineering + Biofabrication Laboratory, Otto-Stern-Weg 7, 8093 Zurich, Switzerland
3. Koç University, School of Medicine, Rumelifeneri Yolu, Sariyer, Istanbul, 34450, Turkey
4. Koç University Research Center for Translational Medicine (KUTTAM), Rumelifeneri Yolu, Sariyer, Istanbul 34450, Turkey

* D.R. and M.A.K. contributed equally to the manuscript.

Abstract

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, we highlight key emerging application areas for ESPs, which could lead to novel breakthroughs in biomedical research and clinical treatments.

Author biographies

Øystein Arlov

Øystein Arlov is a researcher at the Department of Biotechnology and Nanomedicine at SINTEF in Norway. His main research interest is within marine biomasses and the isolation, modification, and functional characterization of polysaccharides, including alginates, fucoidans, laminarins and chitosans. He holds a PhD in Biotechnology from the Norwegian University of Science and Technology (NTNU) where he worked on chemically sulfated alginates, focusing on structural tailoring and characterizing effects on macromolecule interactions and inflammatory processes.

Maryam Asadi Korayem

Maryam Asadikorayem obtained her BSc and MSc in Chemical Engineering with a Biomedical Engineering direction from Sharif University of Technology. She is now PhD student in the Tissue Engineering and Biofabrication laboratory at ETH Zürich, Switzerland under supervision of Prof. Marcy Zenobi-Wong. The main focus of her research is development of advanced biomaterials for cartilage tissue regeneration.

Dominic Rütsche

Dominic Rütsche obtained his BSc and MSc in Interdisciplinary Sciences with biochemical-physical direction from ETH Zürich, Switzerland. As a PhD student in the laboratory of Prof. Zenobi-Wong, his main research interests are the chemical modification of biopolymers and *de novo* design of bio-inductive matrices for tissue vascularization.

Ece Öztürk

Ece Öztürk is an Assistant Professor at Koç University School of Medicine and Koç University Research Center for Translational Medicine (KUTTAM). She obtained her PhD from ETH Zurich where she worked on developing biomimetic and functional materials based on sulfated biopolymers for cartilage regeneration under the supervision Prof. Zenobi-Wong. She then worked at Columbia University for her postdoctoral research where she conducted studies on lung tissue engineering and breast cancer metastasis modeling. Currently, her research direction involves building biomimetic lung tumor models, tumor microenvironment and lung cancer metastasis.

Marcy Zenobi-Wong

Marcy Zenobi-Wong is a Professor at ETH Zürich where she leads a multidisciplinary team focusing on bioprinting, two-photon polymerization, and advanced biomaterials for tissue regeneration. She is currently President of the Swiss Society for Biomaterials and Regenerative Medicine and General Secretary for the International Society of Biofabrication (ISBF). She serves on editorial boards for *Biofabrication* and *Advanced Healthcare Materials* and is a Fellow to the European Alliance for Medical & Biological Engineering and Science (EAMBES).

ToC text

The review provides a comprehensive overview of engineered sulfated polysaccharides (ESPs), including their synthesis, characterization, and biological properties. The functions of ESPs are closely linked to their sulfation pattern and backbone modification, and the review emphasizes study of structure-function relationships in emerging applications within biomedical research and clinical treatments.

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 (DS), 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 non-sulfated and not conjugated to a core protein. The vertebrate proteoglycans and their associated GAGs are integral parts of the extracellular matrix 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 fucoidans 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 presumable 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 anti-coagulant 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 extracellular matrix (ECM), and pre-clinical and clinical studies have evaluated heparin and other GAGs as anti-tumor and anti-inflammatory 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).

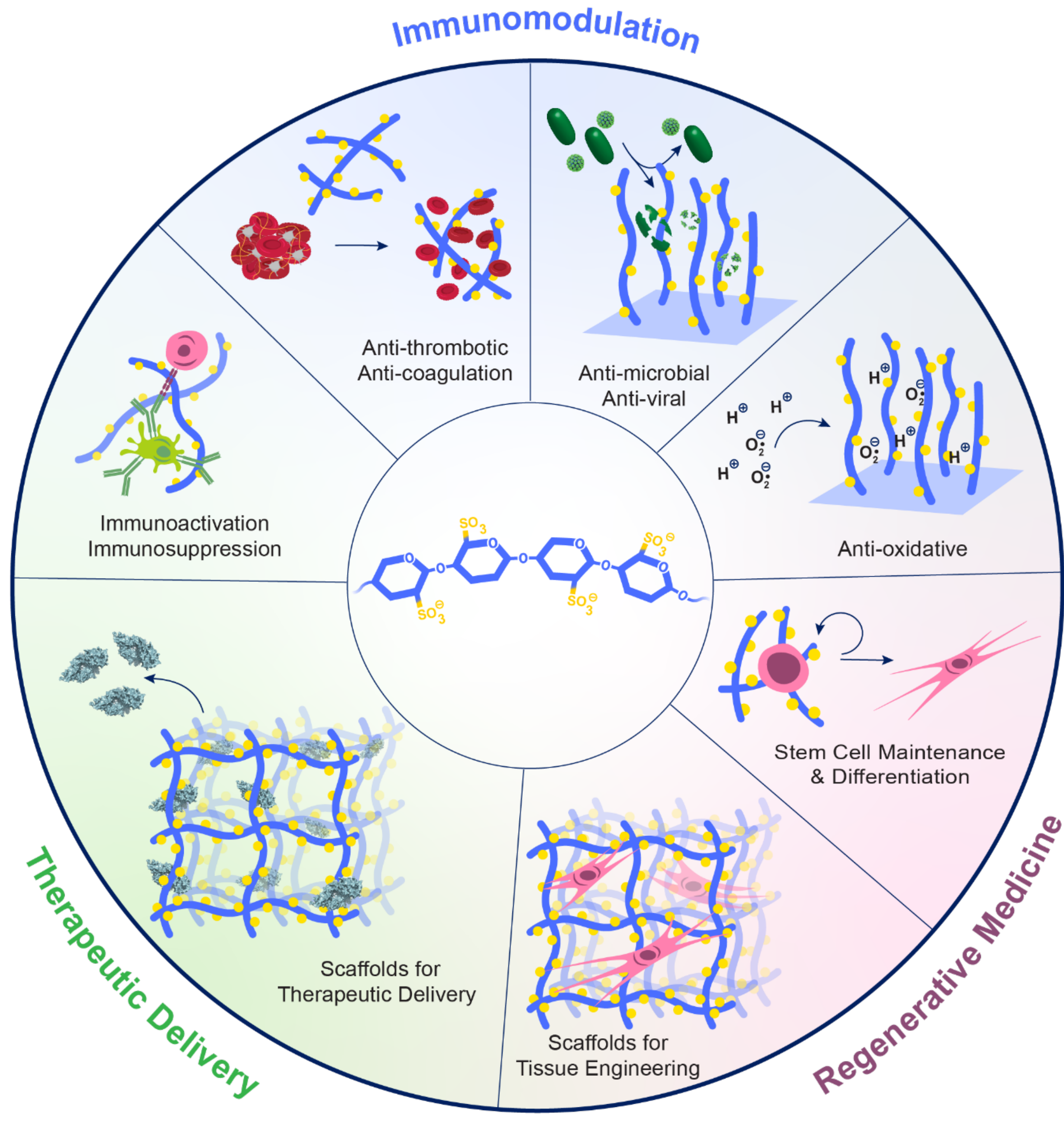

Figure 1: Biomedical applications of engineered sulfated polysaccharides

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 non-sulfated polysaccharides in order to facilitate novel biological interactions but can also result from structural tailoring of sulfated polysaccharides, e.g., by regioselective sulfation, de-sulfation, 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 engineered sulfated polysaccharides 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 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 later 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 engineered sulfated polysaccharides was largely focused on their anti-coagulating 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 co-receptors 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 anti-viral 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 engineered sulfated polysaccharides 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.

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	β-(1,4)-ManA α-(1,4)-GulA	Brown algae	Cross-linked by divalent cations
Carboxymethyl Dextran (RGTA)	α-(1,6)-Glc α-(1,6)-Glc-CMC	Fermentation	Used in multiple pre-clinical and clinical studies
Cellulose	β-(1,4)-Glc β-(1,4)-Glc	Plants and wood Fermentation Tunicates	Can be sulfated in crystalline and fibrous forms
Chitosan	β-(1,4)-GlcNAc β-(1,4)-GlcN	Crustaceans Fungi	Positive charged amino group
Hyaluronan	β-(1,3)-GlcA β-(1,4)-GlcNAc	Animal tissues Fermentation	Regioselective sulfation well established
K5	β-(1,4)-GlcA β-(1,4)-GlcNAc	Fermentation	Structurally analogous to heparin and heparan sulfate

ManA = Mannuronic acid
GulA = Guluronic acid
GlcA = Glucuronic acid

Glc = Glucose
Glc-CMC = Carboxymethylated glucose
GlcNAc = N-acetylglucosamine
GlcN = Glucosamine

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 sulfation degree (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 glycosaminoglycans, namely heparin and chondroitin sulfate 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 non-specific sulfation of polysaccharides employed sulfuric acid (H_2SO_4) or derivatives such as chlorosulfonic acid ($ClSO_3H$) or sulfamic acid (H_3NSO_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 fibers using H_2SO_4, resulting in the formation of cellulose sulfate 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 (TBA) salt of the polysaccharides to improve solubility in organic solvents.[26] These procedures target primary hydroxyl (OH) groups, whereas selective N-sulfation has been demonstrated primarily on N-acetyl-glucosamine-containing polysaccharides such as chitosan,[27] hyaluronan,[28] and heparin/heparan sulfate derivatives[29] 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 sulfation degree under relatively mild conditions. As an example, for sulfation of hyaluronan with various methods, the SO_3/DMF complex has been reported to give the highest sulfation degrees, whereas SO_3/pyridine may provide a greater control over reaction kinetics and permit milder sulfating conditions at low to intermediate sulfation degrees.[30]

Figure 2: Proposed reaction mechanism of sulfation of a glucose (Glc)-based polysaccharide with chlorosulfonic acid and a pyridine salt intermediate.[25]

2.1.2 Tuning of sulfation degree

Several studies reviewed here have demonstrated the possibility of tuning the sulfation degree by varying reagent concentrations, reaction times, and/or reaction temperature. The highest obtainable sulfation degree 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 disubstituted (DS=2) monosaccharides,[31] while polysaccharides with an available hydroxyl group on carbon 6 (C6) can obtain a higher sulfation degree (e.g., hyaluronan, gellan gum, and glucose-based polysaccharides (glucans)). Studies on homogeneous sequences of alginate have additionally shown that sulfation of polyguluronic acid (poly-G) with $ClSO_3H$ in formamide gives a higher sulfation degree than polymannuronic (poly-M) acid at high $ClSO_3H$ concentrations, when in fact the alternating poly-MG sequence demonstrates faster reaction kinetics, presumably due to higher solubility in the reaction mixture.[31] 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.[32] For cellulose, pre-dissolution in ionic liquids provides a homogeneous system for sulfation and increases the obtained sulfation degree while reducing selectivity.[33]

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.[34] Chopin and coworkers 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.[35] 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 cellulose to improve solubility and provide a more homogeneous substitution, compared with H_2SO_4 and SO_3 complexes.[36]

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.[37] Similarly, C6 of polysaccharides such as hyaluronan, 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.[38] 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.[39] For certain polysaccharides, the increased reactiveness of C6 can be exploited to introduce protective groups such as acetyl,[40] triphenylmethyl (TPM),[41] dimethoxytrityl (DMT),[42] or a benzoyl ester,[30] 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.[43] For sulfation of alginate, a minor preference for 2-*O*-sulfation over 2-*O* has been indicated for both mannuronic acid (M) and guluronic acid (G) moieties.[31] To the authors' knowledge, no successful attempts at regioselective sulfation of alginate has been described. However, chemical acetylation has been performed on alginate hydrogels, favoring M monomers not participating in ionic cross-links.[44] This strategy can potentially be applied for sulfation to generate M-sulfated alginates 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, β-1→3 xylans exhibit a triple helical conformation resulting in sulfation predominantly at the outward-facing C4.[45]

All glycosaminoglycans 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 hyaluronan 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 sulfation degree.[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.

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	$HClSO_3$/ Formamide	***2-O*** together with ***3-O* Sulfation**	0.1-0.2	[31, 50-52]
Cellulose	$HClSO_3$/ DMF	***6-O*** together with ***2,3-O* Sulfation**	0.2-2.6 ($C_6 > C_2 > C_3$)	[53, 54]
	$HClSO_3$/DMF	***2-O*, *3-O*,** and ***6-O* Sulfation**	0.5-3.0	[33]
Chitosan	$HClSO_3$/DMF	***3,6-O* Sulfation**	1.0-1.5 ($C_6 > C_3$)	
	SO_3/Me_3N	***N*-Sulfation**	0.4-0.9	
	$CuSO_4$, HCO_2H; SO_3-Pyridine complex/DMF	***6-O* Sulfation**	1.0	[55]
	Piperidine *N*-sulfonic acid/DMSO	***2-O, 3-O, 4-O,*** and ***6-O* Sulfation**	1.0-1.9	[57]

Hyaluronic acid	SO_3-pyridine complex/DMF	**6-*O* Sulfation**	1.0	[58-60]
	BzCl/pyridine; SO_3-DMF complex/DMF (excess)	**4-*O*** together with **2,3-*O* Sulfation**		[30]
	SO_3-DMF complex/DMF (stoichiometric)	***N*-Sulfation**		[47]
K5 (Heparosan)	SO_3-pyridine complex/DMF	**6-*O*** together with ***N*-sulfation, 3-*O* sulfation**	0.5-2.2	[61]

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 non-specific 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 hyaluronan 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 desulfation of carrageenan from red algae and chondroitin sulfate.[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 desulfation of polysaccharides, the reader is referred to the recent review by Bedini and coworkers.[55]

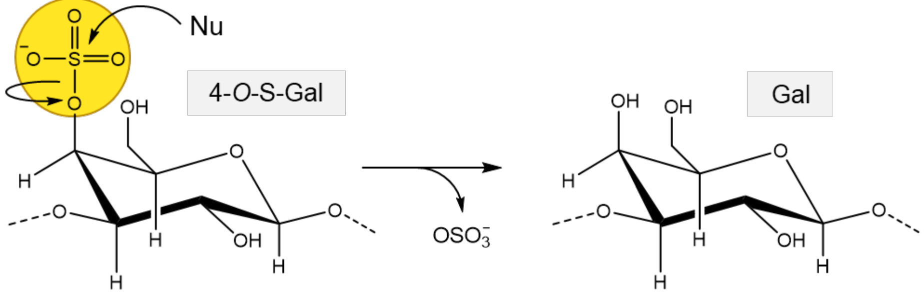

Figure 3: General mechanism of chemical and enzymatic desulfation of galactose (Gal) in carrageenan by nucleophilic (Nu) attack.[62, 67]

2.2.3 Over-sulfation

Chemical sulfation of native sulfated polysaccharides permits synthesis of over-sulfated derivatives with altered physical and biological properties. An extensively studied example in nature is chondroitin sulfate, which has several different subtypes with different sulfation degrees and the same polysaccharide backbone. For instance, increasing the sulfation degree can result in potent anti-coagulating 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 over-sulfation, κ-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 sulfation degree. The increased DS resulted in a 30-fold increase in anti-coagulant activity compared with the native compound.[70] By over-sulfating a fucoidan consisting mainly of 1→3-linked 2-*O*-sulfated fucose, Qiu and coworkers demonstrated near full substitution using chlorosulfonic acid to target the only non-substituted hydroxyl group at C4.[71] Another study employed SO_3-trimethylamine for over-sulfation of dermatan sulfate (DS), resulting in increased platelet aggregation when added to human plasma compared with native DS.[72] Over-sulfation has also been described for several microbial exopolysaccharides, resulting in increased potency in anti-coagulation, anti-oxidation 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 sulfation degree 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, post-synthesis 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, heparan sulfate, and dermatan sulfate, and have been isolated for epimerization of heparin analogues, e.g., based on *E. 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.

Alginates have a well-established toolbox consisting of multiple microbial mannuronan-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 identified and cloned from *A. vinelandii* (AlgE1-7),[76] which demonstrate different modes of action and have furthermore resulted in multiple engineered, derivative enzymes.[77] Through chemical sulfation of homogeneous sequences of alginates generated by epimerization, studies have demonstrated pronounced differences in biological activities based on polysaccharide sequence.[31] 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.[28] 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.[31, 78]

2.3.2 Modification of polysaccharide conformation

Oxidation of the polysaccharide backbone has previously been performed on cellulose,[79] chitin,[80] alginate,[81] 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.[81] The increased chain flexibility of periodate-oxidized and sulfated alginates has, similar to above, been shown to increase interaction strength with growth factors, approaching the potency of heparin.[78] 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.[32]

2.4 Chemoenzymatic Synthesis

Chemical synthesis of oligosaccharides beyond eight saccharide units remains extremely challenging.[82, 83] Moreover, there is no unique molecular structure, due to the previously described dynamic sulfation patterns. Kuberan and coworkers 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.[84] 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.[84] Another chemoenzymatic approach towards the comprehensive synthesis of variously-sized oligosaccharides has been reported by Liu and coworkers.[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 sulfotransferases.

Figure 4: Chemoenzymatic synthesis of a heparan sulfate pentasaccharide. NDST=N-Deacetylase And N-Sulfotransferase, OST=O-Sulfotransferase.[84]

2.5 Polysaccharide-based mimetics and analogues

Natural glycosaminoglycans (GAG) such as heparin and heparan sulfate can activate some of the signalling pathways in stem cell differentiation.[85] Developmental studies have also shown that varying the sulfation pattern of embryonic stem cell (ESC)-associated HS is used to regulate cell fate.[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.[87] Furthermore, the structures of natural GAGs are subject to several biosynthetic post-modifications 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.[88] These disadvantages have led to the development of corresponding mimetics and analogs that exhibit multivalent 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).[89, 90] The monosaccharide sequence, charge density and the molecular weight of GAGs all play a crucial role in determining their biological activities.[91] Thus, by providing control over structure and sulfation, their binding affinity can be tuned by adjusting the molecular weight dispersity and degree of sulfation.[92] 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).[89] In a study by Wang and coworkers, 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).[93] Both units were co-polymerized 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.[93]

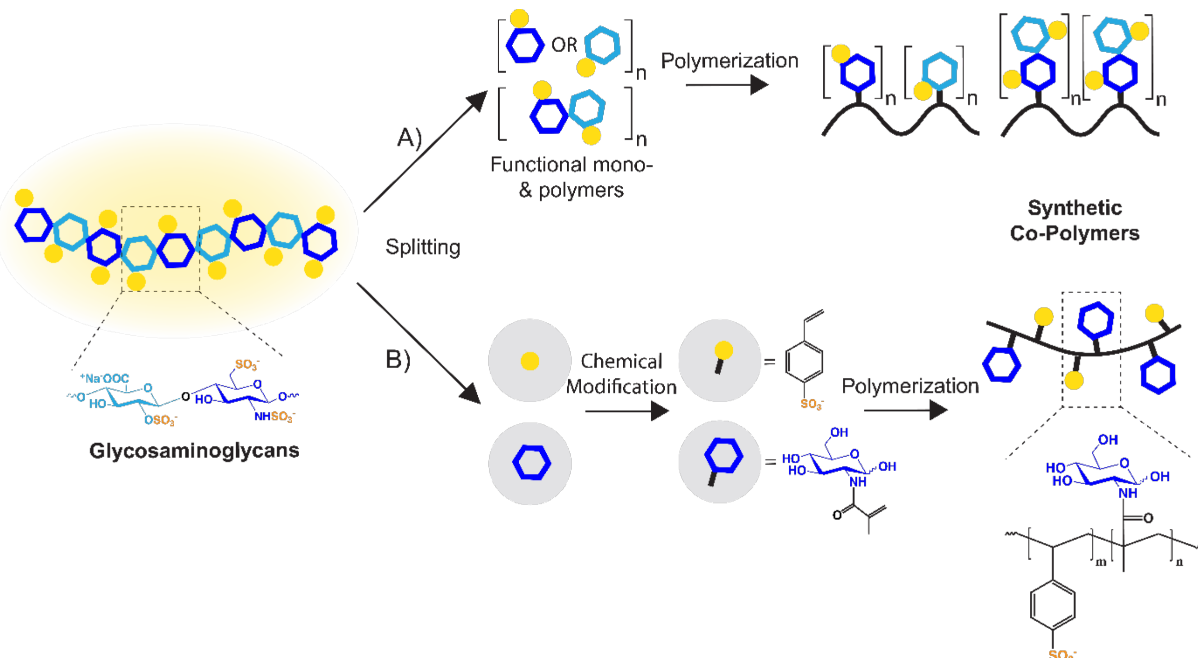

Figure 5: A) Preparation of GAG mimetics with multivalent properties.[89, 92] **B)** Derivatization of synthetic copolymers: using RAFT polymerization, the split and chemically modified subunits can be reassembled into linear synthetic co-polymers with properties similar to naturally sulfated glycosaminoglycans.[93]

2.6 Structural characterization of sulfated polysaccharides

Provided that the structure of the polysaccharide backbone is known, structural characterization of engineered sulfated polysaccharides mainly encompasses determination of the sulfation degree, 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.[94] 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 (ICP-MS)-based analysis of elemental sulfur.[31] If the monosaccharide structure is known, including the presence of associated counter-ions 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 peaks generally around 1300-1000 cm^{-1} and 900-800 cm^{-1} for the S=O and C-O-S bonds, respectively. Small variations in the wave numbers are primarily based on the polysaccharide backbone structure. IR spectroscopy is particularly useful due to the quick readout, small sample requirements, and the potential to analyze solid substrates. Raman spectroscopy has been specifically employed as a rapid method for precise quantification of the sulfation degree, demonstrated by correlating results with NMR and elemental analysis.[95]

2.6.3 Mass spectrometry

Mass spectrometry (MS)-based analyses are advantageous in their high sensitivity for quantification and low sample amount requirement. Multiple methods have been described for different polysaccharides and will not be covered in detail in the present review. Of note, methods based on matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) in tandem with various MS methods have shown great promise for rapid and reproducible structural elucidation of heterogeneous sulfated polysaccharides and have frequently been applied for characterization of GAGs. Intact polysaccharides cannot be detected by MS methods due to their low volatility and are enzymatically or chemically hydrolyzed to oligosaccharides followed by sample ionization and fragmentation. In MALDI-MS, the sulfate groups are commonly cleaved during ionization, which can complicate characterization of substitution patterns in heterogeneous mixtures. This can be overcome, e.g., through sequential permethylation, desulfation by methanolysis, and acetylation[96]/permethylation,[97] replacing the labile sulfate groups with more stable and distinguishable acetyl/ methyl groups. Subsequent fragment analysis can provide structural information on monosaccharide composition, linkages, branching patterns, and sulfate groups, as well as other functional groups.

2.6.4 Nuclear magnetic resonance spectroscopy

^{1}H and ^{13}C NMR can, alongside MS-based methods, provide structural data on sulfated polysaccharides, including substitution preferences. Clear advantages of NMR are the capability to analyze intact polysaccharides in solution or solid-state, simple identification of sample contaminants, and the fact that the analysis is non-destructive toward the samples. While there is no strict requirement for

derivatization, desulfation and/or substitution of the sulfate groups can be performed to obtain more accurate structural data. Sulfation influences the NMR signals of protons and carbons adjacent to the substitution site on the same monosaccharide, as well as on adjacent monosaccharides in the polysaccharide chain. This generally results in highly complex spectra with multiple overlapping signals, complicating exact quantitation of DS from NMR alone. The resolution of the spectra can be improved by various 2D-NMR techniques such as total correlated spectroscopy (TOCSY), homonuclear correlation spectroscopy (COSY), and heteronuclear single quantum coherence spectroscopy (HSQC).[31] Such methods have been described for distinguishing, i.e., "fingerprinting," GAGs and other polysaccharides in heterogeneous mixtures.[98]

2.6.5 Molecular weight measurement

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 molecular weight (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, 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 engineered sulfated polysaccharides 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 (~3.2),[102] sulfation can significantly improve solubility at low pH. This is further dependent on the sulfation degree, 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 sulfation degrees, 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 non-sulfated counterparts at high temperature and acidic conditions,[103] whereas the opposite has also been observed for other polysaccharides and hydrolysis conditions. For example, a lower depolymerization rate has been demonstrated for dextran sulfate compared to dextran at pH=1, and a higher relative hydrolysis rate at pH>1 attributed to accumulation of protons around the negatively charged polysaccharides at intermediate to low proton concentrations in solution.[104] Hydrolysis of the sulfate ester has also been

shown to occur faster for dextran sulfate compared to carrageenans and heparin, indicating a dependence on polymer structure and chain flexibility.[105] κ-carrageenan has shown a 5-6-fold higher rate of depolymerization compared with ι-carrageenan, where 2-*O* sulfation of the 3,6-anhydrogalactose moiety is proposed to reduce the reactivity of the glycosidic linkage and stabilize the polymer backbone.[106] Studies of fucans have indicated that desulfated fucose residues are more susceptible to acid hydrolysis than the sulfated moieties, attributed to steric and/or electrostatic effects.[107] Contrary to these results, addition of sulfate groups to bacterial cellulose has been shown to reduce the temperature requirement for thermal degradation in sulfuric acid.[108] With respect to biodegradability, sulfation can reduce the susceptibility of polysaccharides to enzymatic hydrolysis as the polysaccharide-modifying enzymes often demonstrate high specificity and the sulfate groups confer steric and electrostatic hindrance.[109] Sulfation of cellulose reduces the binding domains of cellulases, resulting in an inhibition of enzymatic hydrolysis. This has similarly been demonstrated for hyaluronan derivatives, where sulfation reduces depolymerization by hyaluronidase.[109] Rapid enzymatic degradation has been a limiting factor for the use of native sulfated GAGs in certain *in vivo* applications, encouraging the use of engineered sulfated derivatives.

3. Biological properties and biomedical applications of ESPs

3.1 Anti-coagulant properties

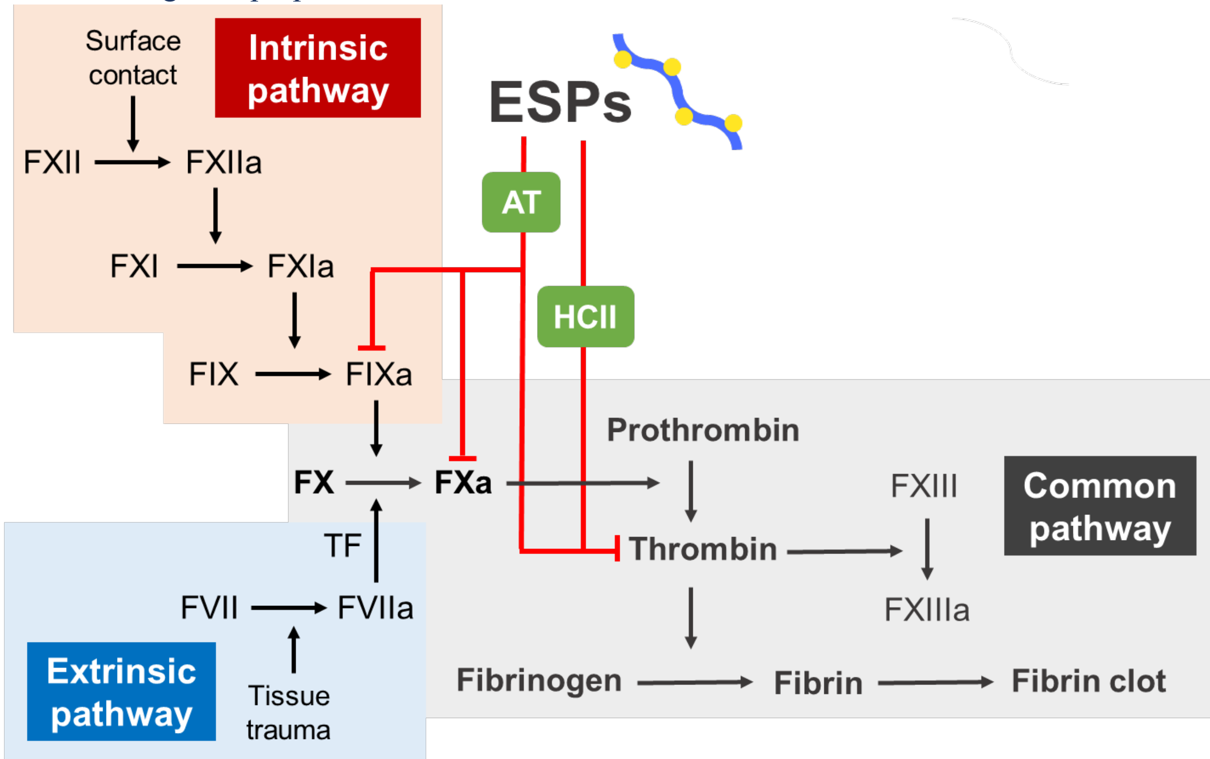

Figure 6: Main coagulation pathways and proposed mechanisms of engineered sulfated polysaccharides (ESPs) as anti-coagulating agents. The intrinsic pathway (mediated by contact with negatively charged surfaces) and the extrinsic pathway (mediated by exposure of tissue factor (TF) to the blood through trauma) converges in the common pathway ultimately leading to a fibrin clot.[110] ESPs with a high structural homology to heparin can activate antithrombin (AT), which in turn inhibits coagulation factors FIXa, FXa and thrombin. ESPs can additionally bind and activate the thrombin inhibitor heparin cofactor II (HCII) or exert anti-coagulant actions through binding and sequestering other coagulation factors (not shown).

Heparin was the first anti-coagulant used in clinical medicine and is today the most widely used anti-coagulant to treat and prevent blood clots by direct injection as well as coating of surgical devices. Heparin exerts its main anti-coagulant 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.[75] 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.[111] Despite the high efficacy of heparin as an anti-coagulant and anti-thrombotic 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 semi-synthetic 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-anti-coagulating heparins toward other biomaterial applications. The use of such derivatives can also contribute to alleviating adverse effects such as thrombocytopenia, osteoporosis and hemorrhagic complications.[112] Besides GAGs, numerous studies have assessed the anti-coagulant and anti-thrombotic properties of naturally occurring sulfated polysaccharides, many of which are isolated from macroalgae and marine invertebrates. The anti-coagulant activity of sulfated polysaccharides has previously been reviewed by Mestechkina and Shcherbukhin, who discussed the influence of polysaccharide structure on their anti-coagulant 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 trigged 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 anti-coagulant 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 anti-factor 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 anti-coagulating properties

The importance of the sulfate groups is evident, as sulfated polysaccharides are commonly observed to prolong coagulation times compared to their non-sulfated controls, and the anti-coagulant properties of natural sulfated polysaccharides can be enhanced through over-sulfation.[115-117]For instance, over-sulfation of fucans derived from brown algae resulted in an increased APTT compared with the native sulfated fucan as well as heparin.[118] Generally, the anti-coagulating action is observed to increase with the sulfation degree of the polysaccharides.[119] Moreover, sulfated polysaccharides generally exert a more potent anti-coagulant 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 sulfated alginates 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 sulfation

degree of the samples.[121] The anti-coagulant 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 sulfation degree. 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 anti-coagulant activity. As an example, the anti-coagulant 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, sulfated alginates with various sulfation degrees (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.[50]

3.1.2 Effects of sulfation patterns on anti-coagulant 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 chondroitin sulfate-like polysaccharides from sea cucumbers have fucan branches with different sulfation patterns, and a study demonstrated a higher anti-coagulant 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.[38] 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 10-fold increase in the APTT. [48] To study structure-function relationships in anti-coagulating actions, a selection of modified carrageenans was prepared through regioselective desulfation and over-sulfation. The APTT was found to increase as a function of sulfation degree and sulfation of C6 was found to have a significant contribution to the anti-coagulating activity. Interestingly, a procoagulant effect was observed for two derivatives of ι-carrageenan with different sulfation degrees 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 anti-coagulant properties has also been studied through complete sulfation (DS=3.0) of homopolysaccharides with 1→4 (xylan, amylose, cellulose) or 1→3 glycosidic linkages (curdlan, galactan) as well as 6-*O*-desulfated derivatives. Here, it was found that 1→3 linked curdlan exhibited more potent anti-thrombin activity compared to the 1→4 linked polysaccharides. 6-*O* desulfation resulted in a near 50% reduction in anti-coagulant activity for the 1→3 linked polysaccharides but had only minimal effects on the 1→4 linked polysaccharides.[32]

3.1.3 Effects of molecular weight on anti-coagulant action and elimination mechanisms

The influence of molecular weight (MW) on the anti-coagulant 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 anti-coagulation 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. [48, 50, 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 and coworkers 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 anti-coagulant activity usually employ doses below the critical polymer concentrations, there are presumably few intra- and intermolecular entanglements, but aggregation can potentially occur in sulfated chitosans or polymers with hydrophobic functional groups. For *in vivo* studies, the MW will additionally influence elimination mechanisms and thus prolong or shorten anti-coagulating effects. This was observed in a study on microcrystalline cellulose sulfate (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 approximately 60-90 minutes 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 desulfatases.[131] While this process is still not fully understood, the enzymes are presumably highly specific for heparin, indicating that other sulfated polysaccharides are eliminated by renal excretion alone or by other slower-acting mechanisms. Wang and coworkers subcutaneously injected mice with 0.3-1.5 mg/kg of MCS or heparin and withdrew intracardial blood over a period of 12 hours, 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, alginate sulfates 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 non-coated controls. These inhibitory effects were also found to be correlated with an increasing sulfation degree of the alginate sulfates.[132] In a different study PET surfaces were coated with anti-coagulating cellulose sulfates with varying sulfation degrees, resulting in increased blood compatibility compared with non-coated materials.[38]

3.1.4 Mechanisms of anti-coagulating properties in ESPs

Engineered sulfated polysaccharides can prolong coagulation through non-specific binding and sequestration of various factors in the coagulation cascade. Most of the published studies on anti-coagulating 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 "neoheparin" based on the E. coli K5 polysaccharide sharing the same fundamental backbone structure as heparin and heparan sulfate.[133] Yet, several of the reviewed studies on analogous sulfated polysaccharides have showed a dependence on having AT in the plasma for exerting their anti-coagulant effects. Interactions with antithrombin have been investigated through various affinity assays. For instance, through studies employing surface plasmon resonance (SPR), sulfated chitosans have been found to only interact with AT non-specifically 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 anti-coagulant 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 anti-coagulant 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 glycoglucuronomannans compared with heparin over a large dosing range, despite having a significantly lower sulfation degree (DS=0.2).[135] Activation of HCII can thus have a significant role in the anti-coagulant activity of the sulfated polysaccharides studied (Figure 6). Sulfated chitosans,[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 Anti-viral properties

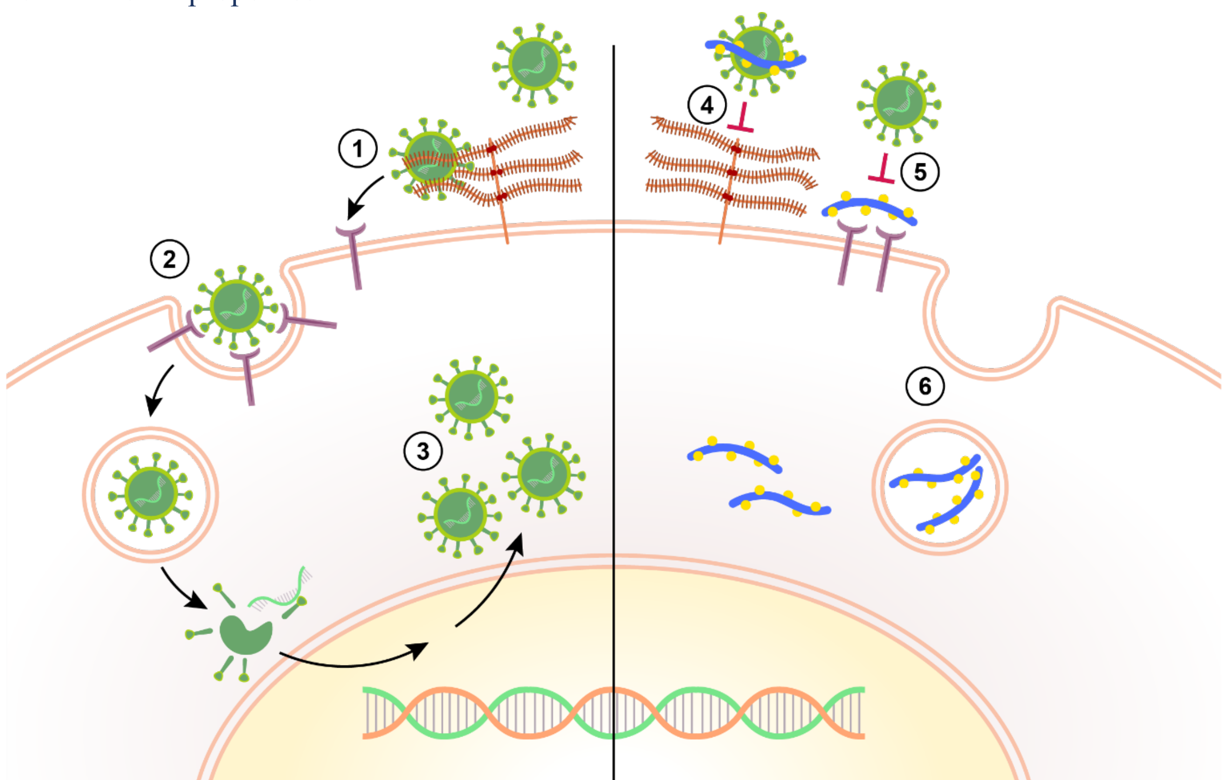

Figure 7: Viral infection of cells and proposed anti-viral mechanisms of engineered sulfated polysaccharides (ESPs). **1)** Initial attachment of viral capsid proteins to cell surface proteoglycans and subsequent binding to adhesion and entry receptor proteins. **2)** Receptor-mediated endocytosis of 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.

Many viruses depend on interactions with sulfated glycosaminoglycans 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 glycosaminoglycans or through addition of soluble glycosaminoglycans, 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 anti-viral 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 anti-viral 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 anti-viral 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 anti-viral properties of various semi-synthetic as well as natural sulfated polysaccharides and have proposed that their inhibiting properties generally increase with increasing sulfation degree 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 between sulfated polysaccharides and soluble proteins, as previously described in the present review. The dependence on sulfation degree in ESPs has been demonstrated for sulfated dextrans, where anti-HIV actions were observed at DS=1.0 but not at DS=0.7.[57] Furthermore, over-sulfation 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 anti-viral activity.[146]

Heparin-like K5 polysaccharides with different sulfation degrees 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 sulfation degree 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 anti-viral 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 anti-viral 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 sulfation degree 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 anti-viral activities of polysaccharides, including sulfated derivatives, the reader is additionally referred to the recent review by Chen and Huang.[152]

3.3 Anti-microbial properties

Only a few studies have demonstrated direct anti-microbial 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 co-administered 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. Anti-microbial and anti-fouling 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 anti-bacterial activity toward *Escherichia 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).[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 minimum inhibitory concentration (MIC), a correspondingly high presence of contaminants can cause inaccuracies in observed growth inhibiting or potentiating effects. Engineered sulfated polysaccharides can allow utilization of more pure and well-characterized fractions. Furthermore, specific non-charged polysaccharides may exhibit anti-microbial properties but little to no solubility in water, where sulfation can vastly improve solubility and allow their use in therapeutic formulations.

Of the ESPs, sulfated chitosans (sChit) have demonstrated anti-microbial 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 *Botrytys 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 non-sulfated control.[25] In a different study 1→3-linked β-glucans were sulfated (DS=0.9), resulting in improved solubility and anti-microbial properties against multiple bacterial strains with a MIC in the range of 1-5 mg/mL.[158] Sulfated dextrans (sDex) have been demonstrated to block proteoglycan-medicated cell attachment of both gram negative and positive bacteria within the genera *Helicobacter*,[159] *Staphylococci*,[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 sulfation degree 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.[162] *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 extracellular matrix.[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 non-treated 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] Cellulose sulfates were in the early 2000s developed as a topical contraceptive and microbicide, 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. Pre-clinical studies of cellulose sulfates showed anti-bacterial 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.[165, 166] These studies were however performed with relatively high dose concentrations

(up to 10 mg/ml) indicating little specificity in the anti-microbial actions. Moreover, the cellulose sulfates were not compared with a non-sulfated 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 (pro-inflammatory) 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.[168-172] Interactions between ESPs and these cytokines can contribute to the regulation of the immunological response, where ESPs potentially act as co-receptors or antagonists.

It has been shown that both natural polysaccharides and semi-synthetic 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 immunoactivating or immunosuppressing effects (Figure 8).[179] 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.

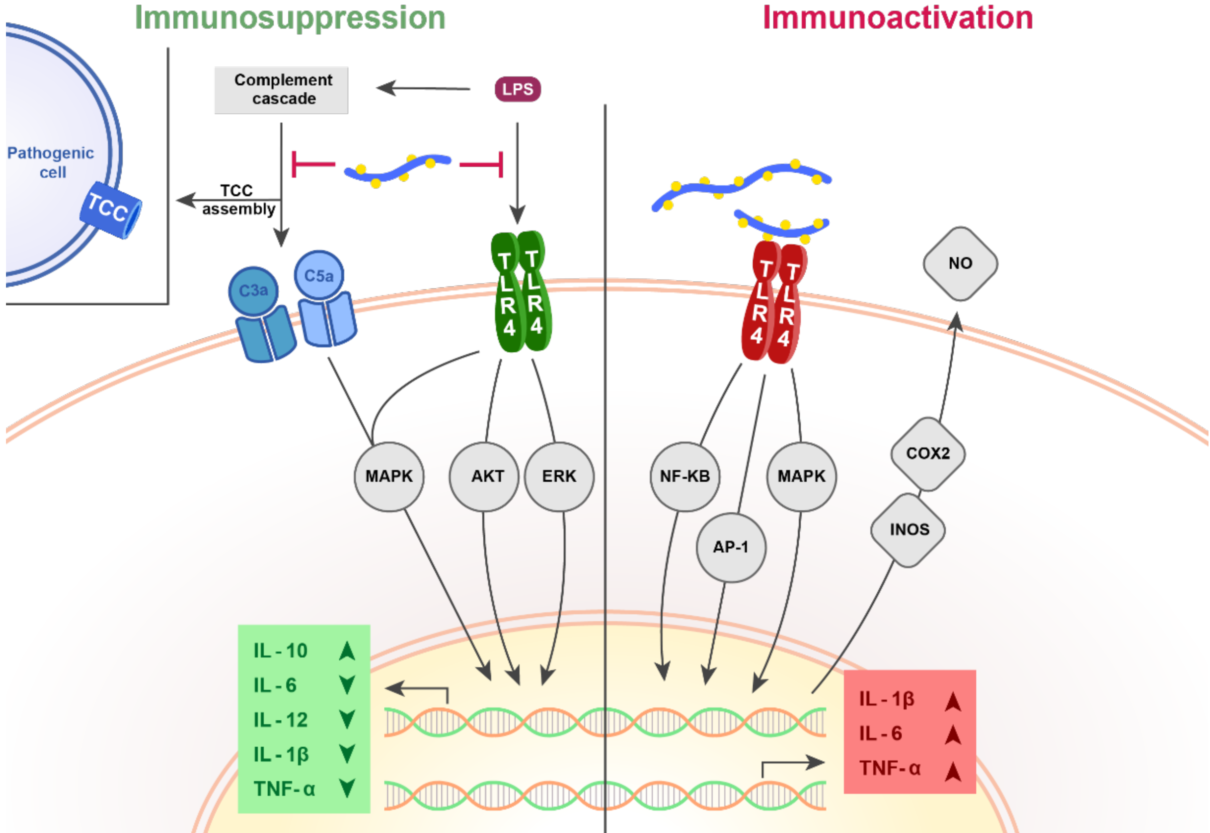

Figure 8: ESPs show immunosuppressing and immunoactivating effects on lymphocytes, depending on the structural nature of the ESP and the model system employed. **Left:** ESPs exhibit imunnosuppressing/anti-inflammatory properties through interference with the complement cascade (including assembly of the terminal complement complex (TCC))[31, 180, 181] and LPS-induced TLR4 activation.[182, 183] 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).[184, 185]

3.4.1 Immunoactivation

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.[170] Multiple studies have shown that ESPs can induce expression and production of NO, ROS, and pro-inflammatory cytokines by macrophages, compared to their non-sulfated counterparts.[184, 191-196] Yu and coworkers 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 non-sulfated control.[194] Similar immunoactivating 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*.[191] In an *in vivo* experiment, mice were treated with an intragastrical-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 non-sulfated polysaccharides enhances their immunoactivating effect on other cell types involved in innate immunity, including DCs[197] and NK cells.[196] Yao and coworkers 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.[198] Stimulation of macrophages by sulfated polysaccharides presumably occurs through their interaction with multiple pattern recognition receptors.[184] The most notable example is the toll-like receptor (TLR)-4, present on macrophages, neutrophils, DCs, and several other cell types during inflammation.[199] TLR-4 is the primary receptor for lipopolysaccharide (LPS) but can also be activated by several polysaccharides.[200] Moreover, TLR-4 has been shown to play a key role in the immunomodulatory effects of both natural and engineered sulfated polysaccharides.[184, 201-204] According to one study, sChit treatment increases TLR-4 expression on RAW 264.7 macrophages, and pre-treatment 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.[184] Similarly, treatment of DCs with an ESP from *C. paliurus* resulted in significant TLR-4 and -2 upregulation compared to the non-sulfated control, mediated by NF-κB and MAPK pathways. Blocking TLR-4 and -2 reduced this observed immunoactivating effect.[202] Interestingly, a recent study showed that non-sulfated polysaccharides from *C. paliurus* downregulated TLR-4 expression in a mouse model of carbon tetrachloride (CCl4)-induced liver inflammation, resulting in decreased inflammatory response.[205]

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,[175] and it has been shown that ESPs alone or in combination with ConA or LPS can enhance lymphocyte proliferation significantly more than the non-sulfated polysaccharide control.[195, 196, 206-212] 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.[195] 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.[196] 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.[196, 209, 211] 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 non-sulfated 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.[211] 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.[207] Guo and coworkers showed that sulfation of lentinan, licensed as potent immunoactivating drug in Japan,[213] 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) sulfation degrees.[206] In a similar experiment, polysaccharides derived from *Lycium barbarum* were sulfated to obtain ESPs with different DS. The ESPs showed enhanced immunoactivating effect on lymphocyte proliferation and serum antibody levels compared to the non-sulfated control, and the ESP with an intermediate DS (1.5) was more effective than the one with a higher D (1.9).[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,[195, 207, 214] while other studies have achieved the highest efficacy at an intermediate DS.[191, 206, 210, 211] As an example immunomodulatory effect of three ESPs derived from *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-α production. As the DS was further increased to 3.4, the TNF-α secretion was dramatically decreased.[191] 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 molecular weight (MW), monosaccharide composition and linkage type, can strongly affect immunoregulatory properties of the ESP. While both non-sulfated and sulfated chitosan oligosaccharides with a degree of polymerization between 2 and 6 have shown immunosuppressing effects on LPS-induced RAW 264.7 macrophage cells,[215-217] non-sulfated higher molecular weight chitosan (3 kDa and 50 kDa) exhibited immunoactivating activity.[218] In a study to investigate the effect of sulfation on immunomodulatory properties of chitosan, α-chitosan and β-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 by cells, β-chitosan sulfate had a more stimulatory effect than α-chitosan sulfate, presumably due to its higher solubility and reactivity, and among its different MWs (3, 5, 15 kDa), the 5 kDa β-chitosan sulfate exhibited the highest stimulatory effect.[184]

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.

Table 3: Reported immunoactivating and immunosuppressing effects of selected engineered sulfated polysaccharides (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.

ESP	DS	Model system	Effect of ESP	Ref
Immunoactivation				
sChit	30 %	RAW 264.7 macrophages	Increased NO, TNF-α, IL-1β, IL-6 and PGE2 secretion	[184]
sK5	0.1-0.5	RAW 264.7 macrophages and mice spleen lymphocytes	Increased macrophage viability, TNF- α and IL-1β secretion, spleen lymphocyte proliferation and IL-2, IFN-, IgG1a, and IgG2b secretion	[196]
***P. albicans* ESP**	0.5 0.6 0.8	Intragastrical administration to mice	Increased splenocyte proliferation Increased NO and ROS production	[195]
***Ganoderma* ESP**	0.7 0.8 3.4	Mice peritoneal macrophages	Increased TNF secretion and phagocytic activity	[191]
***C. paliurus* ESP**	0.1	RAW 264.7 macrophages	Increased NO, TNF-α, IL-1β and IL-6 secretion and phagocytic activities	[194]
***C. paliurus* ESP**	0.1 0.5	Spleen lymphocytes	Increased TNF-α, IL-2 and IL-4 secretion Increased spleen lymphocyte proliferation	[211]
Immunosuppression				

sAlg	0.3	Human whole blood	Decreased IL-1β, TNF, IL-6, IL-8, MIP-1a secretion	[180]
sAlg	0.9	Chondrocytes	Decreased IL-6, IL-8, COX-2 and ADAMTS-5 expression	[219]
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-α in M1-like macrophages	[220]
sHA	1.7	Human macrophages	Decreased TNFα, IL-12(p40), IL-6 and MCP-1 secretion	[221]
sHA	0.5 1.6	Human blood monocytes	Decreased IL-1β, IL-8, IL-12p40, TNF-α and RANTES secretion and increased IL-10 and MCP-1 secretion	[222]
sK5	N.a.	Human mononuclear cells	Decreased IL-1β, IL-6 and TNF-a secretion	[223]
S. cristaefolium ESP	1% 9% 51%	RAW 264.7 macrophages	Decreased NO production	[182]
C. paliurus ESP	0.4	RAW 264.7 macrophages Balb/c mice	Decreased NO production, phagocytic activity, and IL-6 and IL-1β secretion *in vitro* Decreased IL-6 and TNF-α secretion, and increased IL-10 secretion *in vivo*	[224]
Astragalus ESP	1.4	Caco2 cells	Decreased TNF-, IL-1 and IL-8 expression	[225]

3.4.2 Immunosuppression

Inspired by the anti-inflammatory properties of naturally sulfated polysaccharides such as heparin,[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 anti-coagulating 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.[180, 220]

ESPs have been demonstrated to inhibit (LPS)-stimulated inflammation in immune cells. Pre-incubation 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-α and IL-6 production as compared to the non-sulfated polysaccharide.[229] ESPs with different sulfate contents (0.8-50%) based on a naturally sulfated polysaccharide from the brown alga, *Sargassum cristaefolium,* were produced via over-sulfation 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 macrophages 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.[182] Wang and coworkers showed that pre-incubation of macrophage with ESPs from *C. paliurus* polysaccharide with DS~0.4 suppressed phagocytosis, NO production, and the release of IL-6 and IL-1β after LPS-stimulation. These effects were also observed for the non-sulfated polysaccharide, but with a much lower potency compared with the ESP. Moreover, the ESP improved the thymus and spleen

index values, decreased the levels of TNF-α and IL-6 and increased the level of IL-10 in serum and liver homogenate in LPS-treated mice.[224] Inhibition of LPS-induced inflammation has also been shown to be dependent on sulfation pattern. Using four different regioselectively sulfated K5 polysaccharides, Gori and coworkers showed that simultaneous addition of LPS and non-sulfated K5 as well as *N, O*-sulfated and epimerized *N*-sulfated K5 polysaccharide to mononuclear cells did not exert any immunosuppressive effect, whereas the addition of epimerized *N, O*-sulfated K5 polysaccharide and *O*-sulfated K5 polysaccharide to LPS-stimulated cells caused a significant dose-dependent inhibition of IL-1 β, IL-6 or TNF- α cytokines.[223] 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-κB activation pathway through restoration of LPS-induced IκB protein degradation and suppression of AKT are involved in inhibitory effect of sulfated polysaccharides on LPS-stimulated macrophages, as are ERK-signaling pathways.[183] Moreover, it has been shown that ESP treatment of macrophages can suppress LPS-induced p38, ERK1/2, and JNK phosphorylation in a concentration-dependent manner.[182] Furthermore, decreased expression of the TLR-4 receptor has been reported for an LPS-stimulated human epithelial cell line when pre-incubated with sulfated polysaccharide.[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 (ARDS).[230] Heparin shows profound anti-complement activity, but its undesirable anti-coagulating effect limits its application in the treatment of complement-associated diseases.[231] Here, ESPs with tailored structures have been synthesized, which have potent anti-complement activities with few or no anti-coagulating effects.[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 anti-complement 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 anti-coagulating effects, proposed to be related to their low MW (3.6 KDa) and lack of specific antithrombin activation.[233] Sulfated alginates (sAlg) have also shown anti-complement effects.[31, 219] Co-incubation 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 polymannuronic acid and polyguluronic acid.[31] Chemical sulfation of a *Ganoderma lucidum*-derived polysaccharide conferred anti-complement 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.[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.[167] While high-molecular weight hyaluronan (HMW-HA, >1,000 kDa) has previously demonstrated anti-inflammatory properties, low-molecular weight HA (LMW-HA, ~250 kDa) exhibits immunoactivating and inflammatory activities.[235] 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 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.[222] 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 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 NFB, STAT1, and IRF5 (Figure 9).[221] 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.

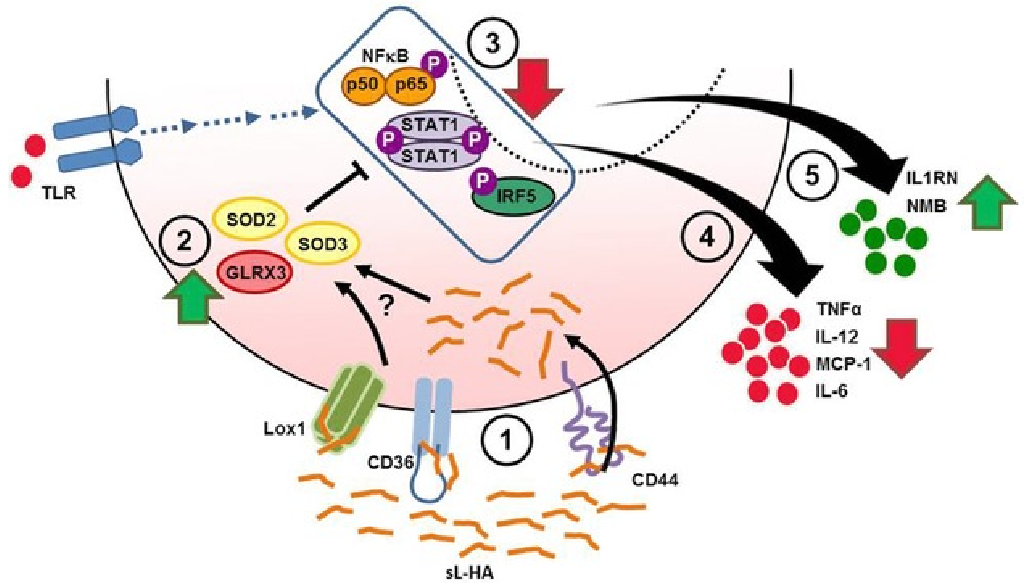

Figure 9: Proposed mechanisms contributing to the anti-inflammatory activity of sulfated hyaluronan (sL-HA) on macrophages. (1) Uptake of SHA via CD44 and the scavenger receptors CD36 and Lox1. (2) Upregulation of antioxidants SOD2, SOD3, and GLRX3 (unknown mechanism). (3) Inhibition of TLR-induced activation of transcription factors pNFkB, pSTAT1, and IRF5 via redox-dependent regulations, resulting in (4) (5) reduced expression and release of pro-inflammatory cytokines (TNF, IL-12(p40), MCP-1, IL-6) and upregulated anti-inflammatory proteins (IL1RN, NMB). Figure reproduced with permission.[221] Copyright 2020, John Wiley & Sons.

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 alginate microspheres, where sulfated alginates (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-1a) 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.[180]

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.[219] Gene expression of pro-inflammatory markers IL-6, IL-8, COX-2 and aggrecanase were significantly lower in the sAlg gels compared with the non-sulfated control. Moreover, matrix metalloprotease-13 (MMP-13) expression was suppressed by sAlg under non-stimulated conditions but not following IL-1β stimulation. The observed effects were hypothesized to be caused by sequestration of IL-1β in the hydrogel network which prevented activation of the encapsulated cells (Figure 10). 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.[219] In a follow-up study, it was shown that sulfated alginate 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 sulfation degree of the sAlg.[220]

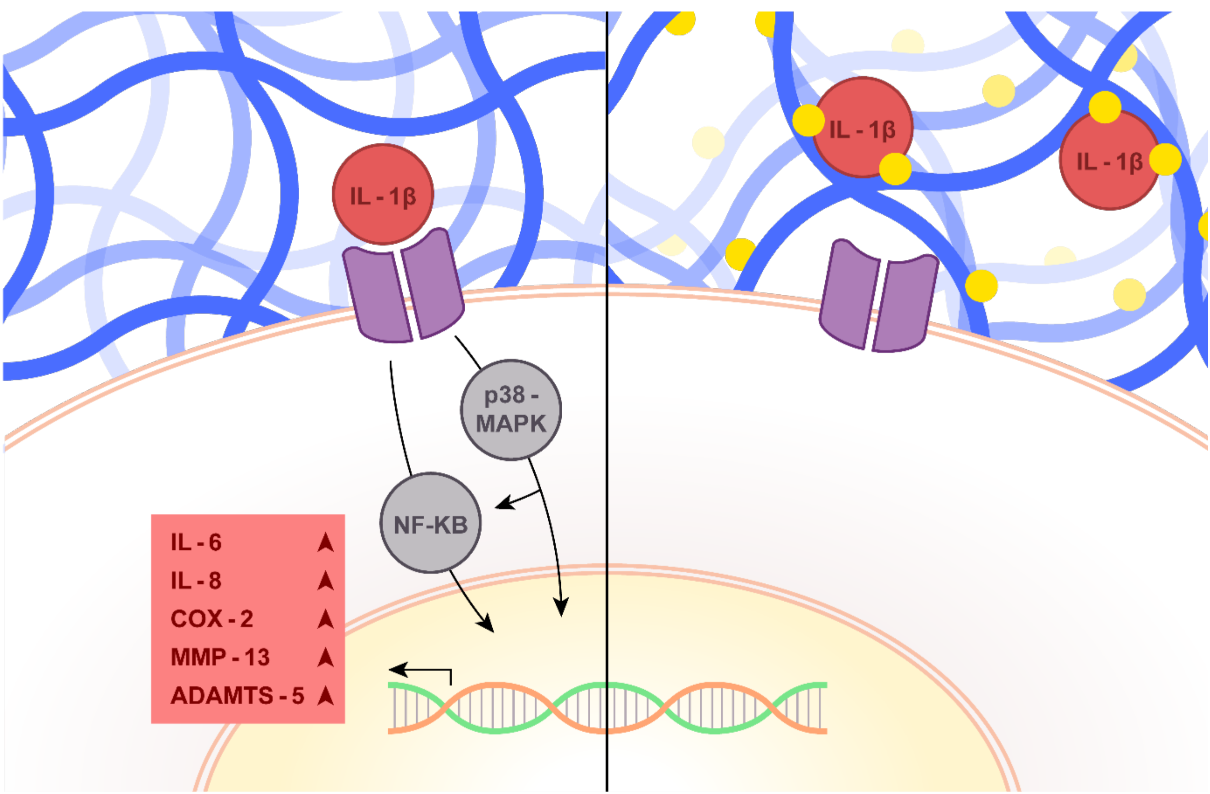

Figure 10: Schematic depiction of the proposed anti-inflammatory effects of sulfated alginate hydrogels on encapsulated chondrocytes. Stimulation of chondrocytes with IL-1β induces gene expression of inflammatory and catabolic markers, mediated by NF-κB and p38-MAPK signaling pathways. Sulfation promotes interaction with IL-1β and thus provides a protective microenvironment by sequestering the cytokine in the gel network and inhibiting receptor activation.[219]

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, sulfation of a polysaccharide from *Sphallerocarpus gracilis* at DS=0.9 not only increased DPPH-, hydroxyl-, and superoxide-scavenging activity, but also improved its reducing power as tested by the reduction of Fe^{3+}/ferricyanide complex to the ferrous form.[243] High-molecular-weight chitosan sulfate has also demonstrated scavenging activity against superoxide radicals in a concentration-dependent manner.[244] Moreover, a different study showed that hydroxyethyl chitosan sulfate 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-caroteneelinoleic acid assays.[251] In one study, regioselective sulfation of chitosan 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 non-sulfated 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 (H_2O_2) 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 H_2O_2-induced oxidative stress compared to the non-sulfated polysaccharide. Specifically, the authors found that the low-DS ESPs maintained the viability of the H_2O_2-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.[253] A later study proposed that the underlying protective effect on RAW264.7 cells from H_2O_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.[254] Since there is a strong correlation between oxidative stress and diabetes,[255] 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 non-sulfated polysaccharide displayed much lower protective effects.[256]

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.[257] 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.[258] Thus, many of the affinity-based systems include heparin or other sulfated GAGs in GF delivery systems by physical incorporation or chemical conjugation.[259-262] However, aside from the adverse clinical effects of heparin, its heterogeneity makes it challenging to reproduce and to control its binding.[263, 264] 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 co-receptors and/or reservoirs for growth factors to facilitate signaling pathways and stabilize proteins against enzymatic hydrolysis.[265]

Desulfation of chondroitin sulfate (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.[266] 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-polyethylene 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 3-fold 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 5-fold 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.[267] Thus, the study provided a good example of how tuning the sulfation degree and sulfation pattern can contribute to control of growth factor affinity and subsequent release kinetics from ESP-based biomaterials.

Freeman and coworkers found that sulfated alginate (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 alginate or for non-heparin-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 alginate or its sulfated form.[51] 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 non-sulfated scaffold over an 8-day period. Moreover, the kinetics of release from alginate-sulfate/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 non-sulfated 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 sulfated alginate hydrogels was three times lower than for unmodified alginate hydrogels over an incubation period of one week.[265] Sequential delivery of HGF and insulin-like growth factor-1 (IGF-1) from sulfated alginate has been achieved for promotion of myocardial repair. Despite similar affinity binding and even stronger binding of IGF-1 to sulfated alginate, 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.[269] 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 non-sulfated 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 sulfated alginate 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 sulfation degree 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 alginate/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 and coworkers found that sulfation of HA increases its interaction with BMP-4 and TGF-β1 in a DS-dependent manner.[59, 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] Photocrosslinked 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 non-sulfated hydrogels after 7 days of incubation at 37 °C.[109] In a recent study, Hintze and coworkers fabricated sHA/Coll I photocrosslinked microgels by a microfluidic technique, incorporated them into bulk HA/Coll I hydrogels and performed a second crosslinking 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 crosslinked microgel structure.[277] In a different study, release of stromal cell-derived factor-1 (SDF-1) from HA/sHA photocrosslinked 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 photocrosslinked sHA/Coll I scaffolds compared to non-sulfated 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 crosslinking, 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 non-sulfated 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 sulfation degree.[282] In a different study, similarly modified dextran demonstrated binding to TGFβ1 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 sulfated alginates 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 counter-ions. 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 four-fold increase in relative interaction strength for sulfated polymannuronic and polyguluronic 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.[31] 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 sulfated alginates displacing HGF and FGF-2 from heparan sulfate, and to achieve interaction strengths equivalent to the polysaccharides of the same sulfation degree.[78] The same methodology was earlier employed to study the binding of chemically synthesized heparan sulfate oligosaccharides to FGF-2 and VEGF.[284] For native or engineered sulfated polysaccharides 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]

Table 4: Immobilization of growth factors in engineered sulfated polysaccharide (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%	Ionically crosslinked microspheres	HGF	[265]
sAlg	7.9%	Ionically crosslinked microspheres	bFGF	[51]
sAlg	7.9%	Ionically crosslinked freeze-dried scaffold	VEGF, PDGF, TGFβ1	[268]
sAlg	7.9%	Ionically crosslinked microspheres	HGF, IGF-1	[269]
sAlg	0.6	Ionically crosslinked 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 crosslinked scaffolds with gelatin	TGF-β1	[280]
sCMC	1.4	HRP crosslinked hydrogel	TGF-β1	[281]
sHA	0.5, 1.5	Collagen I-based scaffold	TGF-β1	[276]
sHA	1.6, 2.9%	Photocrosslinked hydrogel	TGF-β1	[109]
sHA	1.5	Collagen I-based microgels	TGF-β1	[286]
sHA	1.4	Photo-crosslinked hydrogel	SDF-1α	[287]
sHA	0.6	Collagen I-based scaffold	EGF	[279]

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.[288] 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 asparatate-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.[289] Moreover, sDex complexes with poly(methylaminophosphazene) hydrochloride (PMAP) have been shown to be effective in immobilization of insulin and its protection against proteolytic degradation.[290] Sulfated cellulose (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(dimethyldiallyammonium 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).[291]

Lysozyme is a natural enzyme involved in the innate immune response, and its application as an anti-cancer drug is also under investigation.[292, 293] 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 non-sulfated 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 sulfated chitosan with lysozyme.[294] Lysozyme release from photocrosslinked scaffolds containing sHA has been investigated, demonstrating enhanced binding and retention and slower release of lysozyme over 8 days compared to non-sulfated HA. Here, there was no significant difference in the release profile for scaffolds containing sulfated HA with or without 6-*O*-sulfation.[277]

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.[295, 296] Such micelles have been evaluated as drug delivery carriers, e.g., for the anti-cancer Paclitaxel (PTX).[297, 298] Here, *in vivo* studies in rats showed that the oral bioavailability of Paclitaxel loaded in NOSC micelles was increased six-fold 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.[299, 300] Murali and coworkers prepared sChit nanoparticles loaded with an anti-fungal, Amphotericin B (AmpB), to treat intracellular *Candida glabrata* infections. The prepared particles, with a diameter of approximately 300-310 nm, showed superior intracellular killing of *C. glabrata* in infected RAW264.7 macrophage cell lines compared to the drug alone and non-sulfated nanoparticles. The more potent anti-fungal 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 non-sulfated nanoparticles through a general endocytic process and simple diffusion of AmpB alone.[301] Alginate sulfate has been used for ionic complex formation with small molecule cationic drugs as a potential delivery system. One study found that sulfated alginate forms complexes with the cationic drug tetracycline hydrochloride (TCH), while no TCH-polymer complexes were observed using unmodified alginates.[302] In a different study, sulfated dextran-based nanocarriers were developed for targeted delivery of methotrexate (MTX) as an anti-rheumatic 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.[303]

3.7 Regenerative Medicine

3.7.1 ESPs in bio- and nanomaterials

Engineered sulfated polysaccharides 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 extracellular matrix. 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 three-dimensional hydrogel networks, fibers, layer-by-layer structures, particles/micelles and other types of structures (Figure 11).

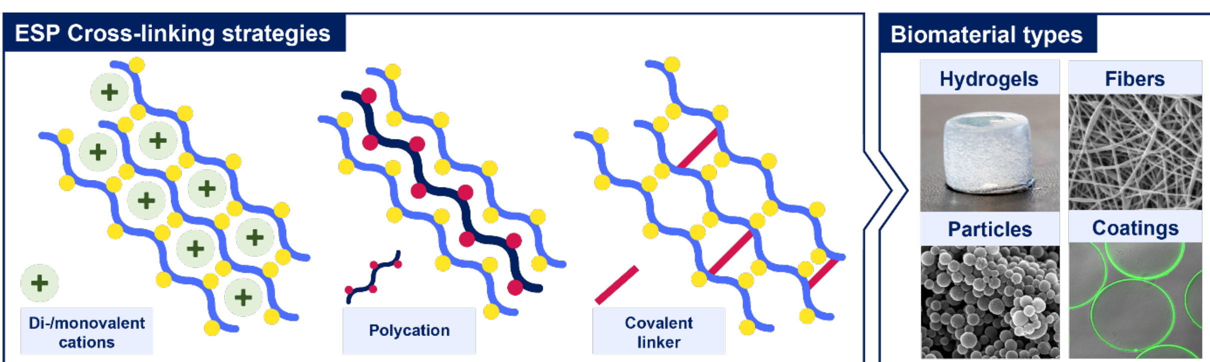

Figure 11: Strategies for ionic and covalent cross-linking of engineered sulfated polysaccharides (ESPs) and common biomaterial types incorporating ESPs.

Alginates 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 sulfation degree increases, the hydrogel stiffness decreases to the point where the sulfated alginates are unable to form stable gels, as the bulky sulfate groups prevent sufficient intermolecular cross-linking.[219, 270, 304] For sulfated alginates 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.[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.[219] Interestingly, the combination of hydrogel beads with a low relative content of sulfated alginates mixed with unmodified alginates (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.[219] Specific natural and engineered carrageenans form thermoreversible hydrogels in the presence of K^+.[305] Sulfation of other polysaccharides with inherent gel-forming properties such as agarose and gellan is described only in a few studies,[306-308] 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.[109] An alternative strategy employs modifying polysaccharides with peptides and forming isopeptide bonds catalyzed by transglutaminase.[309-311] 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.[276, 312] 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.[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 fiber-forming polymers. In one study, sulfated alginate (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.[313, 314] Chitosan and synthetic cationic polymers have additionally been used for layer-by-layer combination with cellulose sulfate to prepare thin films or encapsulation complexes for cell cultivation and drug delivery applications.[315-317] 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, sulfated alginates have been applied as a secondary coat on poly-L-lysine (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 alginate microsphere promoting detachment rather than layering in sandwich-like structures.

Presumably, these interactions can be tuned by altering the sulfation degree 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 sulfated chitosan 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

Engineered sulfated polysaccharides 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. Different native tissues have varying glycosaminoglycan contents that also change temporally, i.e., glycosaminoglycan 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 glycosaminoglycan 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.[318] 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 signaling mechanisms involving differentiation, migration and malignancy.[319, 320] 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 stabile tissue-engineering matrices.[321] These advantages, combined with their ability to mimic the native glycosaminoglycans in interacting with protein signal factors as reservoirs, co-receptors 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.

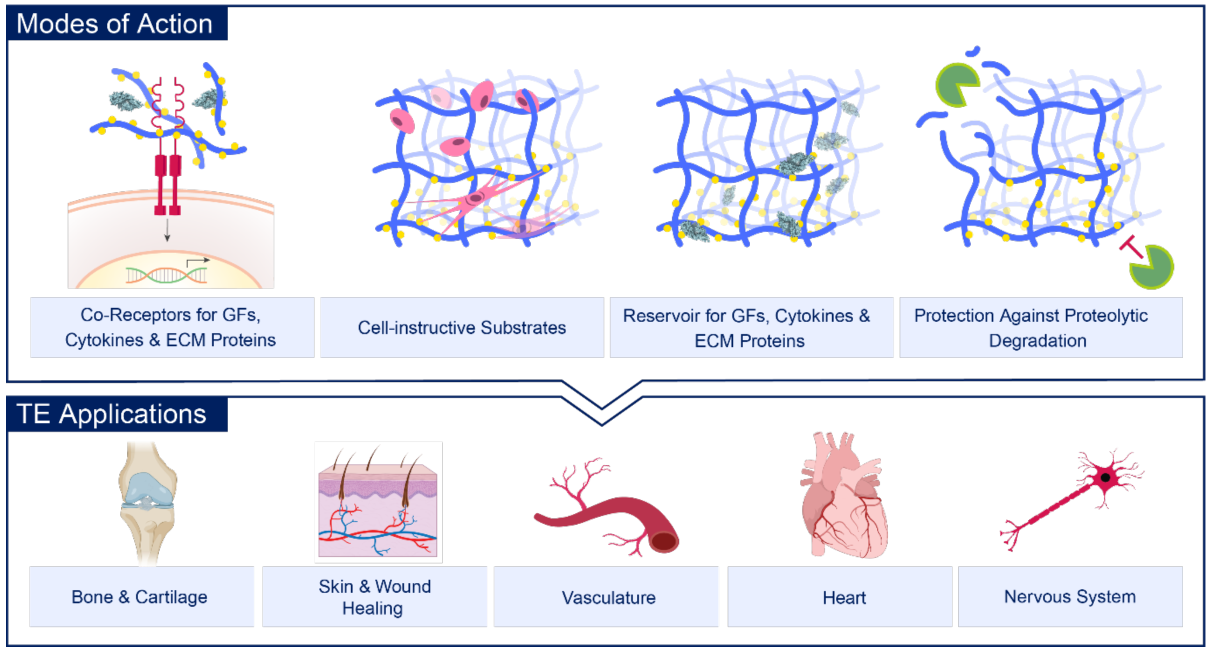

Figure 12: Modes of action of engineered sulfated polysaccharides in tissue engineering (TE) with corresponding applications. Illustrations in bottom panel generated from BioRender.com.

Cartilage

Articular cartilage is frequently damaged by trauma and degenerative joint diseases such as osteoarthritis (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.[322, 323] Articular cartilage has a low cell density embedded within an elegantly organized ECM composed of collagen and proteoglycans.[324] 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.[325] 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.[323] Natural cartilage is rich in sulfated GAGs, including chondroitin sulfate, keratan sulfate, and dermatan sulfate, resulting in a high fixed negative charge of the extracellular matrix. This negative charge gives the tissue its compressive strength and osmotic swelling properties by entrapping water, and it also plays an important role in growth factor sequestration and bioavailability.[322] 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.[270, 304] Zenobi-Wong and colleagues fabricated ionically crosslinked hydrogels based on sulfated alginate for 3D culture of chondrocytes, resulting in a significant promotion of the proliferation and viability of encapsulated chondrocytes compared to non-sulfated hydrogels.[304] 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 (Figure 13).[270] Such FGF-2-mediated mitogenic activity has additionally been reported for sulfated chitosan and cellulose.[326] 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.[304]

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 sulfation degree.[270] Recently, sAlg was modified with tyramine to facilitate tyrosinase-mediated cross-linking *in situ* and demonstrated strong adhesion to native cartilage tissue.[310] Aggrecan and SOX9 gene expressions, as two important chondrogenic markers, were significantly upregulated in chondrocytes in sulfated hydrogels compared to those in the non-sulfated 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.[310] 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.[327] Sulfation of other polysaccharides has also shown enhanced chondrogenesis and enhanced cartilaginous matrix production by chondrocytes. Lord and coworkers 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 sulfated chitosan-arginine promoted a chondrogenic phenotype in the same cells, as indicated by significant upregulation of Coll II, aggrecan, perlecan and SOX9 genes.[328]

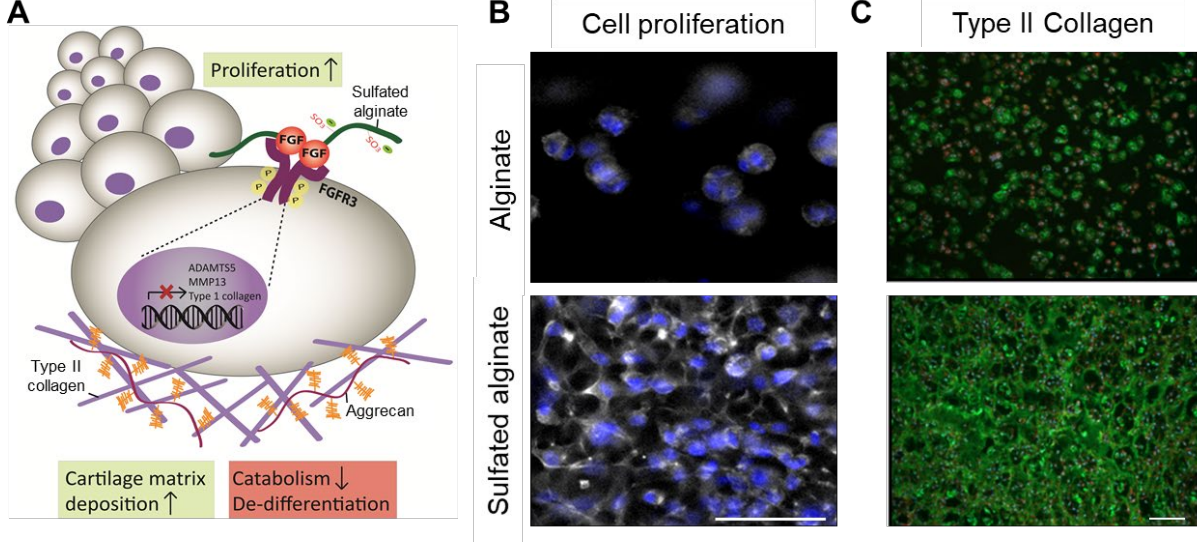

Figure 13: A) Schematic depiction of the mechanism of activation of FGF signaling in sulfated alginate (sAlg) hydrogels. sAlg exerts high affinity for FGFs, mediating growth factor-receptor interactions that leads to initiation of downstream signaling, promoting potent proliferation, cartilage matrix deposition, and suppression of dedifferentiation and catabolism. **B)** Fluorescence imaging of phalloidin-rhodamine (gray) and DAPI (blue) stained aggregates of chondrocytes in alginate sulfate and alginate hydrogels, Scale bar: 50 µm. **C)** Immunofluorescence imaging of Col2 (green) antibody staining of chondrocytes encapsulated in alginate and alginate sulfate hydrogels after 42 d of culture. Rhodamine Phalloidin (red) and DAPI (blue) were used as counterstains, Scale bar: 100 µm. Reproduced and modified with permission.[270] Copyright 2020, John Wiley & Sons.

TGF-β signaling has been shown to be critical for cartilage morphogenesis and chondrogenic differentiation of stem cells.[329] However, optimal GF dosage with sufficiently long and sustained exposure to stem cells are two important prerequisites for stem cell differentiation.[330] 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 and coworkers showed that freeze-dried 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.[331]

In a different study, photocrosslinked injectable hydrogels were fabricated from sulfated hyaluronan 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 sulfation degree. 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 non-sulfated controls was reported.[109] Waghmare and coworkers recently fabricated macroporous scaffolds based on sulfated carboxymethyl cellulose (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 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 coworkers showed that adding sulfated cellulose (sCel) to an induction media containing TGF-β3 significantly enhanced chondrogenic differentiation of hMSCs.[332] 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-β3 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.[332, 333]

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 (PEG-DA) with either chondroitin sulfate (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.[266]

Bone

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.[334] 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.[335] 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,[336] where the family of bone morphogenic proteins (BMP) is the most potent and well-known stimulating factor. Among them, BMP-2 plays critical roles in osteogenesis and bone metabolism and has a remarkable ability to induce bone formation and tissue reconstruction.[337] 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.[338] 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.[339] However, using heparin for bone treatment has shown adverse effects, including delayed healing in short term and increased risk of osteoporosis in long term.[340, 341] 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.[342] Sulfated chitosan (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.[342, 343] 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.[343] 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 2SCS 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.[343] 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.[342] Furthermore, simultaneous administration of BMP-2 and 26SCS *in vivo* dose-dependently induced larger amounts of mineralized bone tissue compare with BMP-2 alone.[343] 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.[344] To obtain a more robust scaffold, the authors incorporated the BMP-2 loaded 26SCS nanoparticles in photocrosslinked 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.[345] In another approach, 26SCS was electrostatically self-assembled on the surface of aminolyzed PLGA scaffolds. The immobilized 26SCS increased surface hydrophilicity, C2C12 cell attachment, and

facilitated adsorption and sustained release of BMP-2.[346] 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.[274]

Since angiogenesis is a critical factor for bone tissue regeneration,[347] 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 pro-angiogenic 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 (EC) in nearby blood vessels, leading to the migration of ECs out of the vessel and the formation of growing sprouts (Figure 14).[344] 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.[348] 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 photocrosslinked 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.[349]

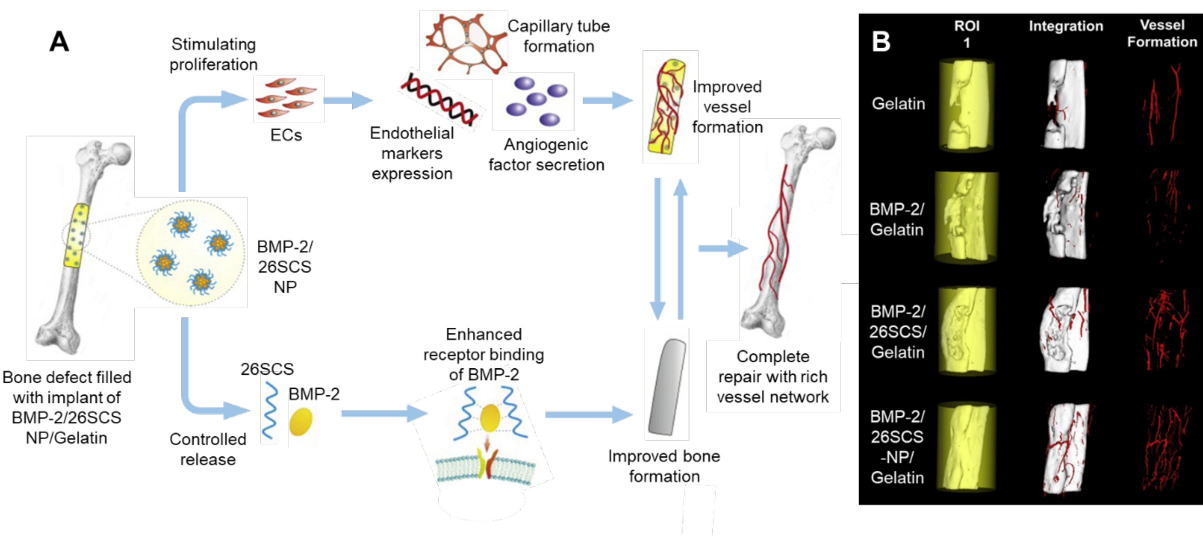

Figure 14: A) Schematic diagram of the effect of BMP-2/26SCS-NP/Gelatin on bone repair. Top: Enhanced angiogenesis via stimulating the proliferation of endothelial cells (ECs), and further promoting cell migration, angiogenic factors (such as VEGF) secretion, and gene expression of endothelial markers (such as CD31), resulting in improved new vessel formation. Bottom: Enhanced osteogenesis via synergistic effect of released 26SCS and BMP-2 resulting in improved bone formation. Finally, the mutual promotion of angiogenesis and osteogenesis helps the implant reach a complete repair of the critical sized defect with rich vessel networks **B)** μCT images of representative radiuses showing bone regeneration and vessel formation at 8 weeks post-implantation for different implants (ROI=Region of interest). Reproduced and modified with permission.[344] Copyright 2020, Elsevier Science & Technology.

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 heparan sulfate proteoglycans,

presumably involved in regulating the osteogenic differentiation processes.[350] Hempel and coworkers investigated the effect of sulfated hyaluronan (sHA) on osteogenic differentiation of hMSCs.[351] The researchers fabricated scaffolds composed of Col I and either non-sulfated HA, sHA with varying DS (1.0-3.0) or chondroitin sulfate (CS). Compared with scaffolds containing chondroitin sulfate (CS) or Col I alone, scaffolds with sHA-induced calcium phosphate deposition, tissue non-specific 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.[351] Later, the researchers showed that combined usage of sHA and dexamethasone had synergistic effects for osteogenic differentiation of hMSCs.[352] 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.[353] 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 MSCs to osteoblast lineage,[354] has been investigated.[355] It has previously been shown that heparin-induced conformational changes of fibronectin within the ECM promote hMSC osteogenic differentiation.[354] 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 up-regulation of proteins associated with important cellular functions such as cell adhesion, cell signaling, matrix remodeling, and endocytosis of hMSCs.[356] 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.[357, 358] Moreover, sulfation of HA and over-sulfation 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.[359]

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 polyether ether ketone plates were coated with Col I supplemented with either non-sulfated 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 over-sulfation increased the osteogenic effect of HA but reduced that of CS.[360] 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 non-mineralized bone matrix. The proposed underlying mechanisms were improved osteoblast function and enhanced binding of sHA to the Wnt inhibitor, sclerostin.[361, 362] 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. Pre-incubation of sclerostin with sHA led to a sulfate- and dose-dependent loss of its bioactivity.[362]

Table 5: Growth factor-potentiating effects of engineered sulfated polysaccharides (ESPs) in cartilage and bone engineering

ESP	Scaffold / Carrier	Growth factor	Model system	Effect of ESP	Ref.
			Cartilage		
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	[304]

sAlg	Covalently X-linked gel	TGF-β3	Chondrocytes	Increased ECM gene expression Induced ECM production *in vivo*	[310]
sAlg	Nanocellulose bioink	-	Chondrocytes	Improved chondrogenesis	[327]
sAlg	Ionically X-linked gel	TGF-B1	hMSCs	Improved chondrogenesis	[331]
sChit	Dissolved in medium	-	Chondroprogenito r cells	Improved chondrogenesis	[328]
sCMC	Gelatin nanofibres	TGF-β3	hMSCs	Improved chondrogenesis	[332]
sCMC	Gelatin nanofibres	TGF-β3	hMSCs	Improved chondrogenesis	[333]
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 Improved regeneration in OA rat	[109]
			Bone		
sAlg	Agarose, Pluronic F127	BMP-2	MC3T3-E1 osteoblasts	Improved osteogenesis	[274]
sChit **sCel**	Dissolved in medium	BMP-2	C2C12 cells	Improved ALP activity	[342]
sChit	Dissolved in medium	BMP-2	C2C12 cells *In vivo* implantation	Increased ALP activity Increased bone formation in mice	[343]
sChit	Nanoparticles in gelatin sponges	BMP-2	HUVECs Implantation in rabbit bone defect	Improved angiogenesis Improved bone regeneration and angiogenesis *in vivo*	[344]
sChit	Nanoparticles in photo-linked gelatin	BMP-2	Implantation in rabbit bone defect	Improved bone regeneration and angiogenesis *in vivo*	[345]
sChit	Immobilized on PLGA	BMP-2	C2C12 cells	Improved cell attachment	[346]
sChit	PLGA particles in gelatin/26SCS photo-X-linked gel	BMP-2 VEGF	HUVECs/ MC3T3-E1 Mice implantation	Improved angiogenesis and osteogenesis Improved bone formation *in vivo*	[349]
sHA	Collagen I gel	-	hMSCs	Improved osteogenesis	[353]
sHA	Collagen I gel	-	hMSCs	Improved osteogenesis	[352]
sHA	Collagen I gel	-	hMSCs	Improved osteogenesis	[355]

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 (LV) remodeling leading to aberrant contractility and congestive heart failure.[363, 364] 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.[364, 365] Recently described tissue engineering approaches commonly involve the combination of biomaterials with cells and bioactive molecules with the aim of achieving myocardial regeneration.[365-367] 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.[368-374] 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.[364, 368-374] 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.[364] Among the bioactive molecules that are involved in myocardial regeneration and neovascularization are FGF,[375] IGF-1,[376] granulocyte colony-stimulating factor,[377] SDF-1,[378] VEGF,[379] and HGF.[380]

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 alginate/alginate sulfate (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.[381] These scaffolds, when re-implanted into infarcted rat 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.[380] Alg/sAlg scaffolds were shown to facilitate sustained release of HGF and provide cytoprotection in rat neonatal cardiomyocytes via an ERK1/2-mediated mechanism.[265] An injectable form of Alg/sAlg loaded with IGF-1 and HGF was used in an acute myocardial infarct model.[269] 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.[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.[382] In a separate study, complexes of alginate sulfate with VEFG 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.[383] Another strategy for using alginate sulfates 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 alginate sulfate have been shown to successfully enable LDL apheresis.[384]

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 complexed with sulfated hyaluronan into nanoparticles and delivered to infarct sites to control macrophage-mediated inflammation.[385, 386] miRNA-21-sHA nanoparticles were found to target cardiac macrophages at the infarct site, inducing a change in their phenotype from pro-inflammatory to regenerative, which ultimately resulted in angiogenesis and a reduction in cardiomyocyte death and fibrotic remodeling.[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;[387, 388] such protection has been revealed to prevent ischemia/reperfusion-induced organ damage in a porcine model.[389] 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.[390] 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.[391] 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 under-oxygenation, and inflammation. A hyaluronan-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.[392] 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.[393]

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,[394-398] CS,[395, 398-404] DS,[395, 398, 403, 405, 406] and KS[403, 407] promote neural survival and neurite outgrowth. However, the specific sulfation patterns of GAGs have also been linked to inhibitory effects on neurite outgrowth and central neural system (CNS) repair.[408-413] For neuronal TE applications, HS/heparin and CS seem particularly promising. The interactions of HS/heparin chains with GFs (pleiotropin,[414, 415] various FGFs,[415] midkine[415-417]), axon guidance molecules (ephrins,[418] netrin1,[419] semaphorin 5A[415-417, 420]), axon guidance molecules (ephrins,[418] netrin1,[419] semaphorin 5A[420]), morphogens (WNT[421-423]) and chemokines (chemokine ligand 2[424]) 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.[425] An overview of developmentally and physiologically relevant processes is provided in reviews elsewhere.[395, 424, 426]) 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.[425] An overview of developmentally and physiologically relevant processes is provided in reviews elsewhere.[395, 426] 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.[410, 426] This versatility of sulfated glycosaminoglycans 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.[427-432] 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.[432]

In one study addressing spinal cord injury, partially (DS = 0.8) or fully (DS = 2.3) sulfated cellulose was mixed with gelatin to produce aligned fibrous hydrogel scaffolds using electrospinning. Native CS variants CS-A and CS-C were used as controls.[433] 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.[433] McCanney and coworkers reported another approach to tackling the lack of endogenous repair and glial scar formation by elevated astrocyte recruitment following a spinal cord injury.[434] 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.[434, 435]

To assess whether the heparin ESPs supported re-myelination and neurite outgrowth required for treating spinal cord injuries, the authors created lesioned and demyelinated neural cell co-cultures, 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 co-cultures, 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.[434] This finding is in agreement with previous reports regarding the attenuative role played by native low-sulfated heparin moieties towards astrocytes in the CNS.[409, 434] This finding is in agreement with previous reports regarding the attenuative role played by native low-sulfated heparin moieties towards astrocytes in the CNS.[409]

A recent study employed sulfated alginate (sAlg) for controlled growth factor binding and delivery for engineering of neural tissues. Biotinylated sulfated alginates (b-$sAlg_{DS}$) 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-hepairn, b-$sAlg_{0.0}$, b-$sAlg_{0.8}$, and b-$sAlg_{2.0}$, 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 and coworkers made similar observations for astrocytes cultured in the presence of sulfated hyaluronic acid (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 co-cultures, 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).[432]

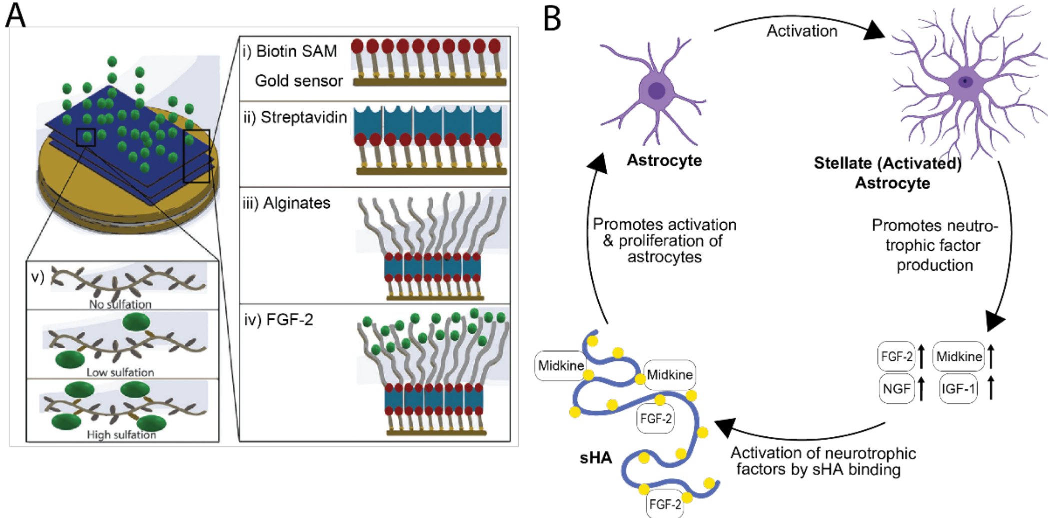

Figure 15: (A) Molecular assembly and binding mechanism of a biotinylated sulfated alginate (b-sAlg) coated substrate. The gold sensor is part of the QCM-D quartz crystal microbalance setup to measure the amount of deposited material. (i) Assembly of biotinylated oligoethylene glycol subunits as self-assembled 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. (v) Increasing sulfation yields higher FGF-2 binding.[271] **(B)** Mechanism of the effect of hyaluronan sulfate (sHA) on astrocyte activation and neurotrophic factor production.[432] Figure A reproduced with permission. Copyright 2020, Royal Society of Chemistry.

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 towards 6-*O*-sulfated HA was 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 poly-L-lysine (PLL) substrates. In contrast, no binding was observed for sHA monosulfated at 4-*O* and disulfated at the 4-*O* and 6-*O* 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-*O* 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*.[436]

Tully and coworkers 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-DL-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.[437]

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.[435] Tissue repair following injury involves a complex series of events mediated by the interactions between cells and signaling molecules within their three-dimensional ECM. Heparan sulfate (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 from the protective environment of the ECM and tissue integrity quickly becomes compromised.[438, 439] Following an injury, HS is upregulated, as can be observed from elevated levels in acute wound fluid during the following 24-72 hours. Here, one of its primary functions is to bind heparin-binding EGF-like growth factor (HB-EGF), which acts as a mitogenic factor for fibroblasts and epithelial cells, among others.[440] Thönes and coworkers found that HB-EGF was able to bind to sulfated hyaluronic acid (sHA) with higher affinity compared to native HA, resulting in keratinocyte migration and an upregulation in EGFR-signaling in dermal fibroblasts.[279] The authors further showed that HB-EGF was more effective in inducing epithelial tip growth within epithelial wounds than soluble HB-EGF alone, implying a benefit in using HA and sHA in wound dressings.[279] This result is in line with previous findings where the sulfation of HA demonstrated positive effects on wound healing. Several studies focusing on the underlying mechanisms found that enhanced interaction with proteins led to changes in inflammatory processes and matrix remodeling.[441-443]

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 dextrans, designated "ReGeneraTing Agents" (RGTAs).[34, 441] Already before that, the role of functionalized dextran RGTAs as a mimic of heparin in skin had been well established.[442, 443] 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.[439, 444-450] The most prominent example of these ESPs is the already-mentioned OTR4120 (c.f. Figure 17), which localizes to the injured tissue, where it binds to structural proteins present in the ECM, for instance collagen, fibronectin and laminin.[441] 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 towards glycanase (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.[441] In addition to its application in chronic wounds, OTR4120 has also been identified as a possible treatment for skin burns.[451] The positive effects of RGTA OTR4120 on dermal wound healing have been highlighted in several animal models, including rats (burn wounds,[451] surgical excision wounds,[452, 453] dermal ischemia ulcers,[454] diabetic wounds[455]) and mice (necrotic skin ulcers[441]).[454] diabetic wounds[455]) and mice (necrotic skin ulcers[441]). 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 Barritault *et al.*[444] The effectiveness of such RGTAs as OTR4120 in wound healing is proposed to be related to stimulation of neovascularization 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.[452] Rouet and coworkers studied another RGTA 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 GFs.[447]

Carboxymethyl Benzylamide Sulfonate Dextrans (CMDBS; Figure 16) are another important group of functionalized dextrans 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.[456] Interaction of CMDBS with various heparin binding growth factors such as EGF, FGFs, PDGF and TGF-βs 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.[456] Cutaneous wounds treated with CMDBS demonstrated improved wound healing kinetics and quality of the restored skin compared to a pure collagen-based wound dressing.[442]

Figure 16: Chemical structure of carboxymethyl benzylamide sulfonate dextrans (CMDBS).[457]

Some studies have specifically focused on growth factor-binding and -concentrating properties of ESPs. Van der Smissen and coworkers 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.[458] The study showed that initial adhesion and cell proliferation of dermal fibroblasts increased with the sulfation degree, 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 towards increased cell attachment. The trends observed for adhesion were again seen in proliferation rates, with high DS consistently outperforming variants with lower DS.[458]

Tissue-degrading matrix metalloproteinases (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 non-sulfated counterpart has little influence on MMP activity, sHA and CS can actively control ECM remodeling via the stabilization and accumulation of TIMP.[459] 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.[458] Similarly, reduced MMP-2 activity was reported for MSCs cultured on highly sulfated HA-containing collagen type I-based substrates.[356] MMP-2 is a critical player in the interaction between fibroblasts and keratinocytes during late stage wound healing and tissue remodeling processes.[460] Other groups have reported promising results of artificial ECMs based on sulfated GAGs and collagen type I for wound healing applications,[276, 458, 460] Other groups have reported promising results of artificial ECMs based on sulfated GAGs and collagen type I for wound healing applications,[276, 458] 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 & Vascularization

Proangiogenic effects

Several studies have implicated heparin and heparan sulfate in angiogenesis, i.e. the development of new blood vessels from an existing vasculature.[461-466] 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 non-exhaustive overview of the different areas where ESPs have been used to support vascularization *in vitro* or angiogenesis *in vivo*. Similar to their natural analogs,[467] 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 and coworkers, increased retention of heparin/HS binding proteins by sulfated alginate (sAlg) was demonstrated.[51] 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, alginate sulfate 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.[456] 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 ovo* assay.[34, 446, 456] 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, 446] Thus, the role of OTR4120 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 $\alpha v\beta 3$ integrin and involve the activation of ERK1/2 (MAPK signaling pathway).[468]

Sulfated chitosans 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 (c.f. section 3.7.2, Bone). Han and coworkers 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 (VWF) and CD31 in the case of 2-*N*,6-*O*-sulfation.[469]

Anti-angiogenic effects

VEGF has two known isoforms, i.e. pro-angiogenic VEGF$_{165a}$ and anti-angiogenic VEGF$_{165b}$. Some angiogenesis-related pathologies such as cancer or macular degeneration may require selective inhibition of VEGF$_{165a}$, 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$_{165a}$ (K$_D$~1nM) ultimately inhibited endothelial cell survival and proliferation by sequestering available VEGF$_{165a}$.[470] This differential binding was explained by differences in the heparin-binding domain structures of both isoforms.[471] A recent study describes chitosan sulfate as another potent inhibitor of angiogenesis *in vivo*, where an increase 6-*O* sulfation was shown to increase the inhibiting effect on angiogenesis. [470] This differential binding was explained by differences in the heparin-binding domain structures of both isoforms.[471] A recent study describes chitosan sulfate 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.[472]

Whereas heparin has long been known to exhibit proangiogenic activity,[473, 474] inhibiting effects have been described for the oral administration of heparin or heparin fragments.[475] Here, the accompanying release of non-anticoagulant heparin fragments in the serum in the presence of cortisone resulted in anti-angiogenic effects. Similar to LMW heparin, sulfated K5 polysaccharides are able to bind FGF-2 and prevent the formation of HSPG/FGF-2/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*.[476] Inhibitory effects even stronger than those observed for heparin were described for sulfated chitosan. 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 towards stronger inhibitory effects at lower molecular weights. As an important advantage over native heparin, sulfated chitosan did not show anticoagulant activity *in vivo*. 6-*O* sulfation was again identified as the main contributing factor affecting VEGF binding.[472] Antiangiogenic effects of chitosan and various derivatives were reported in several instances, in particular in regards to tumor-induced angiogenesis.[477, 478] In one study, oppositely charged chitosan and dextran sulfate were allowed to self-assemble into nanoparticles and serve as degradable delivery vehicles for an anti-angiogenic hexapeptide.Whereas heparin has long been known to exhibit proangiogenic activity,[473, 474] inhibiting effects have been described for the oral administration of heparin or heparin fragments.[475] Here, the accompanying release of non-anticoagulant heparin fragments in the serum in the presence of cortisone resulted in anti-angiogenic effects. Similar to LMW heparin, sulfated K5 polysaccharides are able to bind FGF-2 and prevent the formation of HSPG/FGF-2/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*.[476] Inhibitory effects

even stronger than those observed for heparin were described for sulfated chitosan. 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 towards stronger inhibitory effects at lower molecular weights. As an important advantage over native heparin, sulfated chitosan did not show anticoagulant activity *in vivo*. 6-*O* sulfation was again identified as the main contributing factor affecting VEGF binding.[472] Antiangiogenic effects of chitosan and various derivatives were reported in several instances, in particular in regards to tumor-induced angiogenesis.[477, 478] In one study, oppositely charged chitosan and dextran sulfate were allowed to self-assemble into nanoparticles and serve as degradable delivery vehicles for an anti-angiogenic hexapeptide.[313]

An interesting approach towards generating a non-anticoagulant anti-angiogenic heparin derivative (ST1514) was presented by Casu and coworkers.[479] Selectively 2-*O* de-sulfated 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 acitivity. More recently, an LMW variant of the above glycol-split heparin derivative with negligible anticoagulant and strong antiangiogenic properties was reported.[480] Finally, various sulfated oligosaccharide derivatives have been shown to inhibit angiogenesis, e.g. sulfated mannopentaose phosphate (PI-88),[481, 482] maltotetraose and maltohexaose sulfate,[483] or the heparanase inhibitor oligomannurarate sulfate.[416, 481, 482] maltotetraose and maltohexaose sulfate,[483] or the heparanase inhibitor oligomannurarate sulfate.[416]An interesting approach towards generating a non-anticoagulant anti-angiogenic heparin derivative (ST1514) was presented by Casu and coworkers.[479] Selectively 2-*O* de-sulfated 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 acitivity. More recently, an LMW variant of the above glycol-split heparin derivative with negligible anticoagulant and strong antiangiogenic properties was reported.[480] Finally, various sulfated oligosaccharide derivatives have been shown to inhibit angiogenesis, e.g. sulfated mannopentaose phosphate (PI-88),[481, 482] maltotetraose and maltohexaose sulfate,[483] or the heparanase inhibitor oligomannurarate sulfate.[416, 481, 482] maltotetraose and maltohexaose sulfate,[483] or the heparanase inhibitor oligomannurarate sulfate.[416]

3.7.5 Stem Cell Maintenance & Differentiation

Embryonic stem cells (ESCs) 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.[484-486]

Evidence suggests that GAGs play a crucial role in homeostasis and lineage commitment of pluripotent stem cells.[86, 487-491] 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.[85, 492-494] 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 towards 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.[85, 487, 495] The

following sections are focused on the effects of several ESPs on the behavior of various pluri- and multipotent stem cells. Whereas a large part of the literature focuses on neural differentiation of ESCs or chondrogenic and osteogenic differentiation of mesenchymal stem cells (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.[493]

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 co-receptor and reservoir for various growth factors and morphogens (e.g. BMP, Wnt, FGF) involved in self-renewal.[85, 490, 496-499] Early studies have shown that sustained and elevated levels of FGF-2 support long-term maintenance of ESCs while suppressing BMP signaling-mediated differentiation.[497, 500-502] 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.[488, 490] Besides the specific sulfation patterns, Pickford and coworkers demonstrated that length and structure of the polysaccharide backbone are important factors in stem cell maintenance and differentiation.[503] In particular, under-sulfated HS appears to be characteristic of pluripotent cells.[504]

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 and coworkers developed a novel feeder-free and FGF-2-free culture system.[505] Although the specific regulation of GAG-mediated signaling in human iPSCs has not been investigated so far, evidence presented by the authors suggests that sulfated hyaluronan (sHA) maintains stemness by facilitating FGF-2 signaling. To investigate the effect of sulfation on stem cell maintenance, sHA with different DS were 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 non-sulfated 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 sHA. Moreover, embryoid bodies (EB) 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.[505]

Mhanna and coworkers synthesized sulfated alginate (sAlg) derivatives with different DS.[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 multi-layered 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 (ADSC). The study showed that FGF-2 binding improved at higher DS, such that the following trend could be observed for $sAlg_{DS}$: $sAlg_{1.3} > sAlg_{0.4} > sAlg_{0.0}$ (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 $sAlg_{1.3}$ and decreased at lower levels of sulfation.[272, 506] 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.[507]

Glycosaminoglycan-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 and coworkers employed a co-polymerization strategy that yielded various GAG analogs by reversible addition-fragmentation chain transfer (RAFT) radical polymerization (c.f. 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 cultured under feeder-free conditions

and in the presence of the co-polymer 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 linker 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-renewal. 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.[508]

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 sulfation degree has been observed during differentiation.[85, 488, 490, 496-499] Here, HS acts as a co-receptor mediating the interaction between fibroblast growth factor receptors (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.[509] 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, 487, 488, 490, 496-499] Here, HS acts as a co-receptor mediating the interaction between fibroblast growth factor receptors (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.[509] 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.[487] 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.[488, 504]

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 hBMSC cultures was demonstrated. Here, the authors treated hBMSCs with various sulfated and non-sulfated HA derivatives. sHA treatment led to increased fibronectin (FN) secretion and changes in FN fibril assembly. Analysis of tissue-nonspecific alkaline phosphatase (TNAP) activity, a marker for early osteogenic differentiation, was significantly increased for sHA treated cultures.[355] This result was in agreement with earlier studies that described the pro-osteogenic effect of sHA on hBMSCs within collagen-based matrices.[353, 357] 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 co-cultures. Proteomic analysis highlighted several pathways involved in the osteogenic differentiation (EXCR4, ERK/MAPK, EIF2).[352] Several studies utilized sHA to promote the differentiation of mesenchymal stem cells. Feng and coworkers 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 and co-workers analyzed synthetic sulfated oligosaccharide mimetics of heparan sulfate.[510] EP80031, a synthetic octosaccharide was analyzed with respect to its ability to mobilize hematopoietic stem cells (HSC) in peripheral blood. Following intravenous injections into C57BL/6 mice, the authors observed a maximum 5-fold 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 heparan sulfate.[510]

Sulfated alginate has been shown to indirectly induce chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells (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.[331] 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.[511] In addition to bulk hydrogels, sAlg was also combined with polyvinyl alcohol (PVA) to form complex electrospun nanofibrous scaffolds.[512] 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.[512] Ding and coworkers demonstrated the ability of murine ESCs (mESCs) to differentiate into nerve cells using differentiation media supplemented with various sulfated chitosan derivatives.[513] 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 sulfated chitosan promotes neural differentiation of ESC is similar to heparin/HS.[513]

Sulfated cellulose (sCel) has been investigated for use in chondrogenic differentiation of MSCs through incorporation into a fibrous gelatin scaffold using electrospinning.[514] The degree of sulfation (DS=0.8) for sCel was tailored to more closely mimic naturally occurring 6-*O*-sulfated chondroitin sulfate (C6S). Scaffolds containing sCel retained TGF-β3 more effectively than gelatin alone, although no significant difference between high (5.0 wt%) and low concentrations (0.1 wt%) of sCel was observed. In 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.[515] sCel further exhibited increased binding affinity towards FGF-2. Zhang and coworkers 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.[53] Even though overall binding only reached approximately 60% of that observed for heparin, sulfated cellulose appears to be an interesting scaffolding material for stem cell differentiation due to its structural properties and potential for regioselective modification.[53]

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 approximately 2500 glucosidic units linked by α-1→6 bonds.[34]

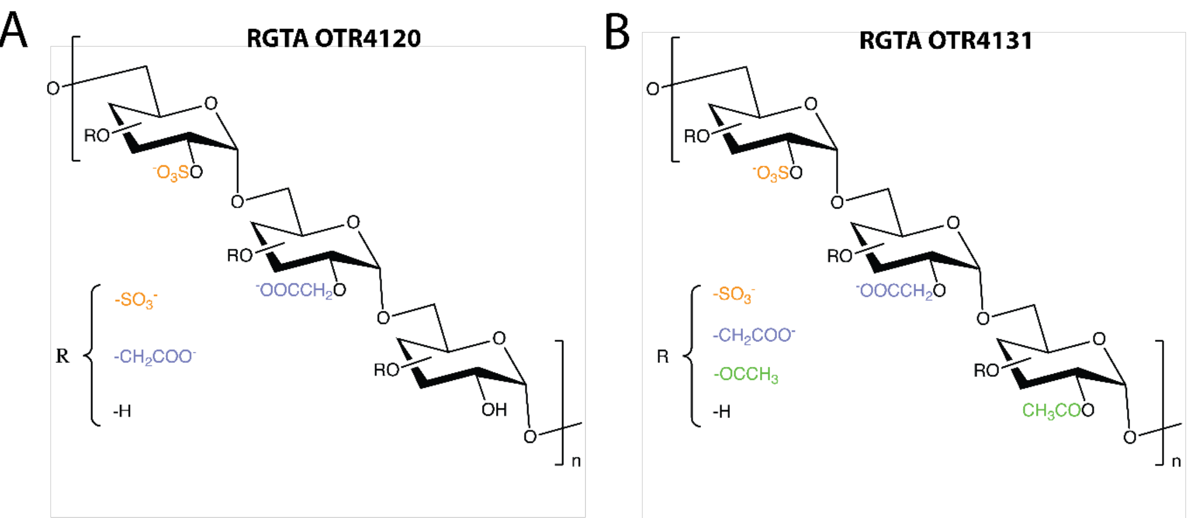

Figure 17: Structures of RGTAs (A) OTR4120 and (B) OTR4131[445, 448]

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.[516] These findings agree with previous results suggesting that RGTAs can provide a beneficial matrix environment for the migration of osteoblastic progenitors.[517] 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.[516] 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 and coworkers.[518] Subsequent sulfation yielded its over-sulfated 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.[73] Another study showed that algal fucoidans (DS = 0.8-1.0)

were more efficient in mobilizing hematopoietic stem cells compared with both RGTA variants,[519] demonstrating again that higher DS does not always correlate with higher potentials in the regulation of stem cells, as described above for regioselectively sulfated chitosans.

As previously described, membrane-associated HS have central roles in modulating stem cell behavior, and recent research has described various strategies for integrating ESPs in the plasma membrane. Huang and coworkers 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.[509] 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).[520] 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.[520] A similar strategy was employed to incorporate lipid-anchored biomimetics (co-polymerized from synthetic GAG analogues) into ESC membranes (Figure 18C).[521]Upon cell-surface modification, embryonic PC12 progenitor cells upregulated NGF-mediated signaling and increased neurite outgrowth.[520] A similar strategy was employed to incorporate lipid-anchored biomimetics (co-polymerized from synthetic GAG analogues) into ESC membranes (Figure 18C).[521] To prolong the display time of the ESPs, an anchoring strategy involving HaloTag proteins (HTP) 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.[522]

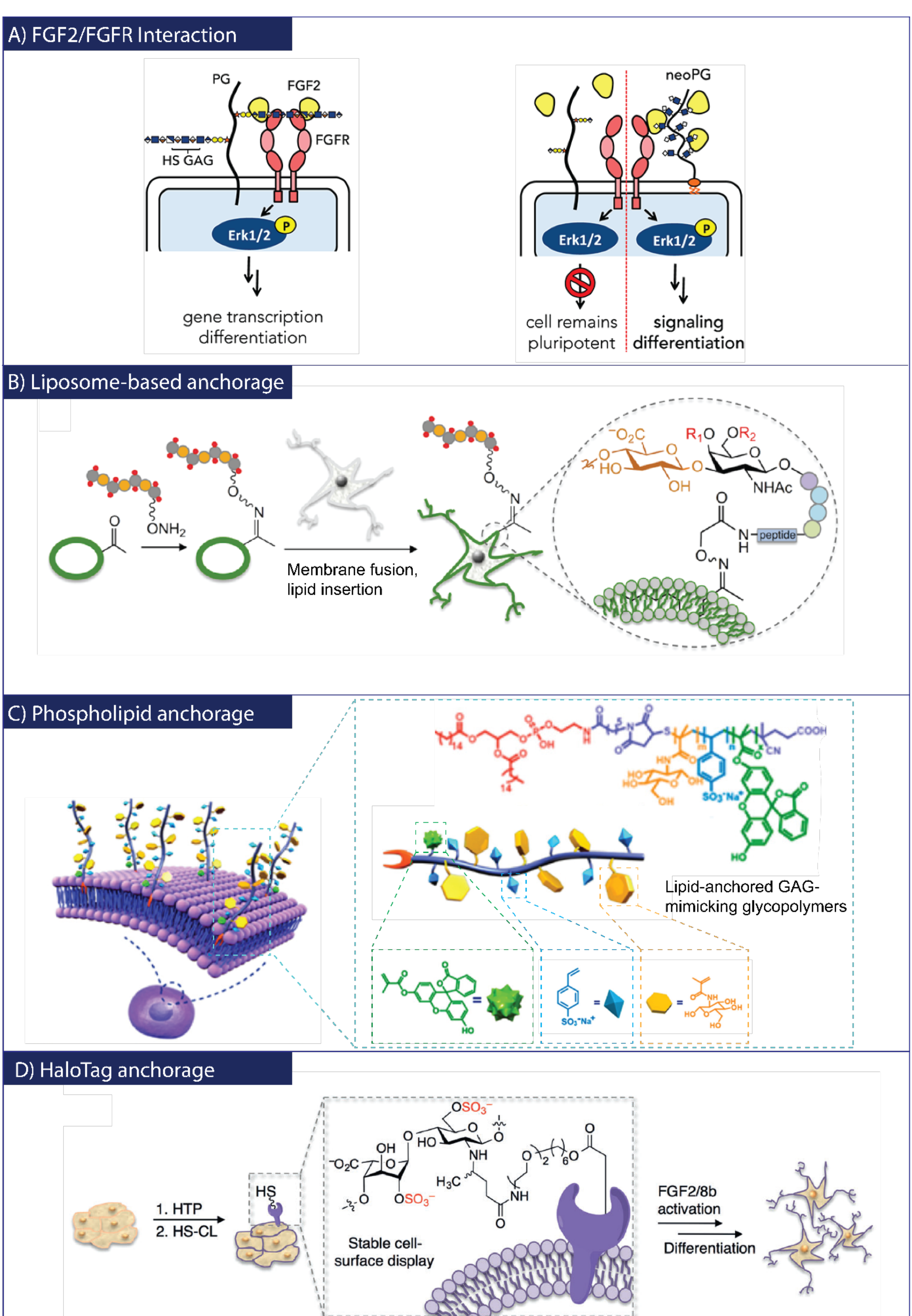

Figure 18: Stem cell specification based on cell surface engineering strategies. (A) The HS-mediated interaction between FGF-2 and FGFR in ESCs can be mimicked by the insertion of HS mimetics (neoPG) into the plasma membrane using various anchoring strategies.[509] (B) Sulfated CS chains (n~110 disaccharide units) are coupled to lipid anchors and indirectly

incoporated into the plasma membrane using liposomes.[520] (C) Synthetic GAG analogues are functionalized with phospholipid anchors and incorporated into cell membranes of ESCs to promote short-term differentiation into neurons.[521]: 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.[509] (B) Sulfated CS chains (n~110 disaccharide units) are coupled to lipid anchors and indirectly incoporated into the plasma membrane using liposomes.[520] (C) Synthetic GAG analogues are functionalized with phospholipid anchors and incorporated into cell membranes of ESCs to promote short-term differentiation into neurons.[521] (D) HaloTag proteins (HTP) allow the long-term presentation of HS-derived chains on the cell surface of mESCs.[522] Figures reproduced and modified with permission. Copyright 2020, American Chemical Society (Figure A, B, C), John Wiley & Sons (Figure D)

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 glycopolymers, the reader is referred to the comprehensive reviews by Wang *et al*.,[88] Miura *et al.,*[89] and Paluck *et al.*[92]

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 sulfation degree. 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 multi-step biological processes, additionally avoiding non-physiological 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 sulfation degree 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 non-mammalian 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 co-receptors 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 non-regular 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 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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[1.] R. Raman; V. Sasisekharan; R. Sasisekharan, Structural insights into biological roles of protein-glycosaminoglycan interactions. *Chem Biol* **2005,** *12* (3), 267-277.
[2.] D. R. Coombe, Parish, C. R., Sulfated polysaccharide-mediated sponge cell aggregation: The clue to invertebrate self/nonself-regognition? In *Invertebrate Historecognition*, Grosberg, R. K., Hedgecock, D., Nelson, K., Ed. Springer Science & Business Media: Boston, MA, USA, 1988; pp 31-54.
[3.] G. F. Medeiros; A. Mendes; R. A. B. Castro; E. C. Bau; H. B. Nader; C. P. Dietrich, Distribution of sulfated glycosaminoglycans in the animal kingdom: widespread occurrence of heparin-like compounds in invertebrates. *Bba-Gen Subjects* **2000,** *1475* (3), 287-294.
[4.] O. Berteau; B. Mulloy, Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* **2003,** *13* (6), 29r-40r.
[5.] P. A. S. Mourao; M. S. Pereira, Searching for alternatives to heparin - Sulfated fucans from marine invertebrates. *Trends Cardiovas Med* **1999,** *9* (8), 225-232.
[6.] R. Harel; M. L. Tanzer, Extracellular-Matrix .3. Evolution of the Extracellular-Matrix in Invertebrates. *Faseb J* **1993,** *7* (12), 1115-1123.
[7.] A. V. Skriptsova, Fucoidans of brown algae: Biosynthesis, localization, and physiological role in thallus. *Russ J Mar Biol+* **2015,** *41* (3), 145-156.
[8.] M. Lahaye; M. A. V. Axelos, Gelling Properties of Water-Soluble Polysaccharides from Proliferating Marine Green Seaweeds (Ulva Spp). *Carbohyd Polym* **1993,** *22* (4), 261-265.
[9.] S. Mabeau; B. Kloareg; J. P. Joseleau, Fractionation and Analysis of Fucans from Brown-Algae. *Phytochemistry* **1990,** *29* (8), 2441-2445.
[10.] N. Soanen; E. Da Silva; C. Gardarin; P. Michaud; C. Laroche, Improvement of exopolysaccharide production by Porphyridium marinum. *Bioresource Technol* **2016,** *213*, 231-238.
[11.] G. Squillaci; R. Finamore; P. Diana; O. F. Restaino; C. Schiraldi; S. Arbucci; E. Ionata; F. La Cara; A. Morana, Production and properties of an exopolysaccharide synthesized by the extreme halophilic archaeon Haloterrigena turkmenica. *Appl Microbiol Biot* **2016,** *100* (2), 613-623.
[12.] L. Richert; S. Golubic; R. Le Guedes; J. Ratiskol; C. Payri; J. Guezennec, Characterization of exopolysaccharides produced by cyanobacteria isolated from polynesian microbial mats. *Curr Microbiol* **2005,** *51* (6), 379-384.
[13.] R. S. Aquino; C. Grativol; P. A. S. Mourao, Rising from the Sea: Correlations between Sulfated Polysaccharides and Salinity in Plants. *Plos One* **2011,** *6* (4).
[14.] N. Dantas-Santos; D. L. Gomes; L. S. Costa; S. L. Cordeiro; M. S. S. P. Costa; E. S. Trindade; C. R. C. Franco; K. C. Scortecci; E. L. Leite; H. A. O. Rocha, Freshwater Plants Synthesize Sulfated Polysaccharides: Heterogalactans from Water Hyacinth (Eicchornia crassipes). *Int J Mol Sci* **2012,** *13* (1), 961-976.
[15.] D. Wardrop; D. Keeling, The story of the discovery of heparin and warfarin. *Brit J Haematol* **2008,** *141* (6), 757-763.
[16.] A. Kowitsch; G. Y. Zhou; T. Groth, Medical application of glycosaminoglycans: a review. *J Tissue Eng Regen M* **2018,** *12* (1), E23-E41.

[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, Sulfated polysaccharides effectively inhibit SARS-CoV-2 in vitro. *Cell Discov* **2020,** *6* (1).
[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, Homogeneous low-molecular-weight heparins with reversible anticoagulant activity. *Nat Chem Biol* **2014,** *10* (4), 248-+.
[19.] N. Sugiura; T. Shioiri; M. Chiba; T. Sato; H. Narimatsu; K. Kimata; H. Watanabe, Construction of a Chondroitin Sulfate Library with Defined Structures and Analysis of Molecular Interactions. *J Biol Chem* **2012,** *287* (52), 43390-43400.
[20.] A. S. Saribas; A. Mobasseri; P. Pristatsky; X. Chen; R. Barthelson; D. Hakes; J. Wang, Production of N-sulfated polysaccharides using yeast-expressed N-deacetylase/N-sulfotransferase-1 (NDST-1). *Glycobiology* **2004,** *14* (12), 1217-1228.
[21.] A. Prechoux; C. Halimi; J. P. Simorre; H. Lortat-Jacob; C. Laguri, C5-epimerase and 2-O-sulfotransferase associate in vitro to generate contiguous epimerized and 2-O-sulfated heparan sulfate domains. *ACS Chem Biol* **2015,** *10* (4), 1064-71.
[22.] E. Tykesson; A. Hassinen; K. Zielinska; M. A. Thelin; G. Frati; U. Ellervik; G. Westergren-Thorsson; A. Malmstrom; S. Kellokumpu; M. Maccarana, Dermatan sulfate epimerase 1 and dermatan 4-O-sulfotransferase 1 form complexes that generate long epimerized 4-O-sulfated blocks. *J Biol Chem* **2018,** *293* (35), 13725-13735.
[23.] E. C. a. B. Sherrard, G.W., Some of the products obtained in the hydrolysis of White spruce wood with dilute sulfuric acid under steam pressure. *Industrial and Engineering Chemistry* **1923,** *15* (6), 611-616.
[24.] R. A. Al-Horani; U. R. Desai, Chemical sulfation of small molecules-advances and challenges. *Tetrahedron* **2010,** *66* (16), 2907-2918.
[25.] G. C. Calegari; V. A. Q. Santos; A. M. Barbosa-Dekker; C. Busso; R. F. H. Dekker; M. A. A. da Cunha, Sulfonated (1 -> 6)-beta-D-Glucan (Lasiodiplodan): Preparation, Characterization and Bioactive Properties. *Food Technol Biotech* **2019,** *57* (4), 490-502.
[26.] L. Fan; Y. Gong; M. Cao; S. Gao; Y. Sun; L. Chen; H. Zheng; W. Xie, Synthesis, characterization, and anticoagulant activity of carboxymethyl starch sulfates. *Journal of Applied Polymer Science* **2013,** *127* (6), 4865-4872.
[27.] K. R. Holme; A. S. Perlin, Chitosan N-sulfate. A water-soluble polyelectrolyte. *Carbohyd Res* **1997,** *302* (1-2), 7-12.
[28.] V. Crescenzi; M. Dentini; D. Risica; S. Spadoni; G. Skjak-Braek; D. Capitani; L. Mannina; S. Viel, C(6)-oxidation followed by C(5)-epimerization of guar gum studied by high field NMR. *Biomacromolecules* **2004,** *5* (2), 537-546.
[29.] K. Lidholt; M. Fjelstad; K. Jann; U. Lindahl, Substrate Specificities of Glycosyltransferases Involved in the Formation of Heparin and of Escherichia-Coli K5 Capsular Polysaccharide. *Glycoconjugate J* **1993,** *10* (4), 221-221.
[30.] J. Becher; S. Möller; D. Weiss; J. Schiller; M. Schnabelrauch, Synthesis of New Regioselectively Sulfated Hyaluronans for Biomedical Application. *Macromolecular Symposia* **2010,** *296* (1), 446-452.

[31.] Ø. Arlov; F. L. Aachmann; A. Sundan; T. Espevik; G. Skjåk-Bræk, Heparin-like properties of sulfated alginates with defined sequences and sulfation degrees. *Biomacromolecules* **2014,** *15* (7), 2744-2750.
[32.] A. Chaidedgumjorn; H. Toyoda; E. R. Woo; K. B. Lee; Y. S. Kim; T. Toida; T. Imanari, Effect of (1 -> 3)- and (1 -> 4)-linkages of fully sulfated polysaccharides on their anticoagulant activity. *Carbohyd Res* **2002,** *337* (10), 925-933.
[33.] Z. M. Wang; L. Li; K. J. Xiao; J. Y. Wu, Homogeneous sulfation of bagasse cellulose in an ionic liquid and anticoagulation activity. *Bioresource Technol* **2009,** *100* (4), 1687-1690.
[34.] D. Papy-Garcia; V. Barbier-Chassefiere; V. Rouet; M. E. Kerros; C. Klochendler; M. C. Tournaire; D. Barritault; J. P. Caruelle; E. Petit, Nondegradative sulfation of polysaccharides. synthesis and structure characterization of biologically active heparan sulfate mimetics. *Macromolecules* **2005,** *38* (11), 4647-4654.
[35.] N. Chopin; C. Sinquin; J. Ratiskol; A. Zykwinska; P. Weiss; S. Cerantola; J. Le Bideau; S. Colliec-Jouault, A Direct Sulfation Process of a Marine Polysaccharide in Ionic Liquid. *Biomed Res Int* **2015**.
[36.] T. Liebert; J. Wotschadlo; M. Gericke; S. Kohler; P. Laudeley; T. Heinze, Modification of cellulose in ionic liquids towards biomedical applications. *Acs Sym Ser* **2009,** *1017*, 115-132.
[37.] A. Magnani; S. Lamponi; R. Rappuoli; R. B. Barbucci, Sulphated hyaluronic acids: a chemical and biological characterisation. *Polym Int* **1998,** *46* (3), 225-240.
[38.] M. Gericke; A. Doliska; J. Stana; T. Liebert; T. Heinze; K. Stana-Kleinschek, Semi-Synthetic Polysaccharide Sulfates as Anticoagulant Coatings for PET, 1-Cellulose Sulfate. *Macromolecular Bioscience* **2011,** *11* (4), 549-556.
[39.] C. B. S. 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, Sulfation of the extracellular polysaccharide produced by the edible mushroom Pleurotus sajor-caju alters its antioxidant, anticoagulant and antiproliferative properties in vitro. *Carbohyd Polym* **2011,** *85* (3), 514-521.
[40.] T. Groth; W. Wagenknecht, Anticoagulant potential of regioselective derivatized cellulose. *Biomaterials* **2001,** *22* (20), 2719-2729.
[41.] J. L. Wang; W. Yang; J. C. Wang; X. Wang; F. Wu; J. Yao; J. Zhang; Z. Q. Lei, Regioselective sulfation of Artemisia sphaerocephala polysaccharide: Characterization of chemical structure. *Carbohyd Polym* **2015,** *133*, 320-327.
[42.] Z. S. Zhang; Q. B. Zhang; J. Wang; H. F. Song; H. Zhang; X. Z. Niu, Regioselective syntheses of sulfated porphyrans from Porphyra haitanensis and their antioxidant and anticoagulant activities in vitro. *Carbohyd Polym* **2010,** *79* (4), 1124-1129.
[43.] C. Mahner; M. D. Lechner; E. Nordmeier, Synthesis and characterisation of dextran and pullulan sulphate. *Carbohyd Res* **2001,** *331* (2), 203-208.
[44.] G. Skjakbraek; S. Paoletti; T. Gianferrara, Selective Acetylation of Mannuronic Acid Residues in Calcium Alginate Gels. *Carbohyd Res* **1989,** *185* (1), 119-129.

[45.] T. Yamagaki; Y. Tsuji; M. Maeda; H. Nakanishi, NMR spectroscopic analysis of sulfated beta-1,3-xylan and sulfation stereochemistry. *Biosci Biotech Bioch* **1997,** *61* (8), 1281-1285.
[46.] S. Nishimura; H. Kai; K. Shinada; T. Yoshida; S. Tokura; K. Kurita; H. Nakashima; N. Yamamoto; T. Uryu, Regioselective syntheses of sulfated polysaccharides: specific anti-HIV-1 activity of novel chitin sulfates. *Carbohyd Res* **1998,** *306* (3), 427-433.
[47.] V. Crescenzi; A. Francescangeli; D. Renier; D. Bellini, New cross-linked and sulfated derivatives of partially deacetylated hyaluronan: Synthesis and preliminary characterization. *Biopolymers* **2002,** *64* (2), 86-94.
[48.] Y. Q. Zou; E. Khor, Preparation of sulfated-chitins under homogeneous conditions. *Carbohyd Polym* **2009,** *77* (3), 516-525.
[49.] A. V. Pestov; Y. S. Petrova; A. V. Bukharova; L. K. Neudachina; O. V. Koryakova; E. G. Matochkina; M. I. Kodess; Y. G. Yatluk, Synthesis in a gel and sorption properties of N-2-sulfoethyl chitosan. *Russ J Appl Chem+* **2013,** *86* (2), 269-272.
[50.] L. H. Fan; L. Jiang; Y. M. Xu; Y. Zhou; Y. A. Shen; W. G. Xie; Z. H. Long; J. P. Zhou, Synthesis and anticoagulant activity of sodium alginate sulfates. *Carbohyd Polym* **2011,** *83* (4), 1797-1803.
[51.] I. Freeman; A. Kedem; S. Cohen, The effect of sulfation of alginate hydrogels on the specific binding and controlled release of heparin-binding proteins. *Biomaterials* **2008,** *29* (22), 3260-3268.
[52.] H. Ronghua; D. Yumin; Y. Jianhong, Preparation and in vitro anticoagulant activities of alginate sulfate and its quaterized derivatives. *Carbohyd Polym* **2003,** *52* (1), 19-24.
[53.] K. Zhang; D. Peschel; E. Brendler; T. Groth; S. Fischer, Synthesis and Bioactivity of Cellulose Derivatives. *Macromolecular Symposia* **2009,** *280*, 28-35.
[54.] K. Zhang; D. Peschel; E. Baucker; T. Groth; S. Fischer, Synthesis and characterisation of cellulose sulfates regarding the degrees of substitution, degrees of polymerisation and morphology. *Carbohyd Polym* **2011,** *83* (4), 1659-1664.
[55.] E. Bedini; A. Laezza; M. Parrilli; A. Iadonisi, A review of chemical methods for the selective sulfation and desulfation of polysaccharides. *Carbohydr Polym* **2017,** *174*, 1224-1239.
[56.] E. Doncel-Perez; I. Aranaz; A. Bastida; J. Revuelta; C. Camacho; N. Acosta; L. Garrido; C. Civera; E. Garcia-Junceda; A. Heras; A. Fernandez-Mayoralas, Synthesis, physicochemical characterization and biological evaluation of chitosan sulfate as heparan sulfate mimics. *Carbohyd Polym* **2018,** *191*, 225-233.
[57.] T. Yoshida; H. Nakashima; N. Yamamoto; T. Uryu, Anti-Aids Virus Activity in-Vitro of Dextran Sulfates Obtained by Sulfation of Synthetic and Natural Dextrans. *Polym J* **1993,** *25* (10), 1069-1077.
[58.] R. B. Barbucci; A. Magnani; N. Casolaro; N. Marchettini; C. Rossi; M. Bosco, Modification of hyaluronic acid by insertion of sulphate groups to obtain a heparin-like molecule. Part I. Characterisation and behaviour in aqueous solution towards H+ and Cu2+ ions. *Gazzetta Chimica Italiana* **1995,** *125*, 169-180.

[59.] V. Hintze; S. Moeller; M. Schnabelrauch; S. Bierbaum; M. Viola; H. Worch; D. Scharnweber, Modifications of Hyaluronan Influence the Interaction with Human Bone Morphogenetic Protein-4 (hBMP-4). *Biomacromolecules* **2009,** *10* (12), 3290-3297.
[60.] S. Möller; M. Schmidtke; D. Weiss; J. Schiller; K. Pawlik; P. Wutzler; M. Schnabelrauch, Synthesis and antiherpetic activity of carboxymethylated and sulfated hyaluronan derivatives. *Carbohyd Polym* **2012,** *90*, 608-615.
[61.] B. Casu; G. Grazioli; N. Razi; M. Guerrini; A. Naggi; G. Torri; P. Oreste; F. Tursi; G. Zoppetti; U. Lindahl, Heparin-like compounds prepared by chemical modification of capsular polysaccharide from E. coli K5. *Carbohydr Res* **1994,** *263* (2), 271-84.
[62.] W. Helbert, Marine Polysaccharide Sulfatases. *Front Mar Sci* **2017,** *4*.
[63.] K. Nagasawa; Y. Inoue; T. Kamata, Solvolytic desulfation of glycosaminoglycuronan sulfates with dimethyl sulfoxide containing water or methanol. *Carbohydr Res* **1977,** *58* (1), 47-55.
[64.] Y. Inoue; K. Nagasawa, Selective N-desulfation of heparin with dimethyl sulfoxide containing water or methanol. *Carbohydr Res* **1976,** *46* (1), 87-95.
[65.] M. Matsuo; R. Takano; K. Kameihayashi; S. Hara, A Novel Regioselective Desulfation of Polysaccharide Sulfates - Specific 6-O-Desulfation with N,O-Bis(Trimethylsilyl)Acetamide. *Carbohyd Res* **1993,** *241*, 209-215.
[66.] R. Takano; T. Kanda; K. Hayashi; K. Yoshida; S. Hara, Desulfation of Sulfated Carbohydrates Mediated by Silylating Reagents. *J Carbohyd Chem* **1995,** *14* (6), 885-888.
[67.] S. J. Wiliams; E. Denehy; E. H. Krenske, Experimental and Theoretical Insights into the Mechanisms of Sulfate and Sulfamate Ester Hydrolysis and the End Products of Type I Sulfatase Inactivation by Aryl Sulfamates. *J Org Chem* **2014,** *79* (5), 1995-2005.
[68.] J. Hogwood; A. Naggi; G. Torri; C. Page; P. Rigsby; B. Mulloy; E. Gray, The effect of increasing the sulfation level of chondroitin sulfate on anticoagulant specific activity and activation of the kinin system. *Plos One* **2018,** *13* (3).
[69.] S. Miyata; H. Kitagawa, Formation and remodeling of the brain extracellular matrix in neural plasticity: Roles of chondroitin sulfate and hyaluronan. *Bba-Gen Subjects* **2017,** *1861* (10), 2420-2434.
[70.] H. M. Yuan; W. W. Zhang; X. G. Li; X. X. Lu; N. Li; X. L. Gao; J. M. Song, Preparation and in vitro antioxidant activity of kappa-carrageenan oligosaccharides and their oversulfated, acetylated, and phosphorylated derivatives. *Carbohyd Res* **2005,** *340* (4), 685-692.
[71.] X. D. Qiu; A. Amarasekara; V. Doctor, Effect of oversulfation on the chemical and biological properties of fucoidan. *Carbohyd Polym* **2006,** *63* (2), 224-228.
[72.] R. M. Maaroufi; P. Giordano; P. Triadou; J. Tapon-Bretaudiere; M. D. Dautzenberg; A. M. Fischer, Effect of oversulfated dermatan sulfate derivatives on platelet aggregation. *Thrombosis Research* **2007,** *120* (4), 615-621.
[73.] C. Merceron; S. Portron; C. Vignes-Colombeix; E. Rederstorff; M. Masson; J. Lesoeur; S. Sourice; C. Sinquin; S. Colliec-Jouault; P. Weiss; C. Vinatier; J. Guicheux, Pharmacological Modulation of Human Mesenchymal Stem Cell Chondrogenesis by a

Chemically Oversulfated Polysaccharide of Marine Origin: Potential Application to Cartilage Regenerative Medicine. *Stem Cells* **2012,** *30* (3), 471-480.
[74.] A. Naggi; G. Torri; B. Casu; P. Oreste; G. Zoppetti; J. P. Li; U. Lindahl, Toward a biotechnological heparin through combined chemical and enzymatic modification of the Escherichia coli K5 polysaccharide. *Semin Thromb Hemost* **2001,** *27* (5), 437-43.
[75.] U. Lindahl; G. Backstrom; L. Thunberg, The antithrombin-binding sequence in heparin. Identification of an essential 6-O-sulfate group. *J Biol Chem* **1983,** *258* (16), 9826-30.
[76.] H. Ertesvag; H. K. Hoidal; H. Schjerven; B. I. G. Svanem; S. Valla, Mannuronan C-5-Epimerases and Their Application for in Vitro and in Vivo Design of New Alginates Useful in Biotechnology. *Metab Eng* **1999,** *1* (3), 262-269.
[77.] E. Buchinger; D. H. Knudsen; M. A. Behrens; J. S. Pedersen; O. A. Aarstad; A. Tondervik; S. Valla; G. Skjak-Braek; R. Wimmer; F. L. Aachmann, Structural and functional characterization of the R-modules in alginate C-5 epimerases AlgE4 and AlgE6 from Azotobacter vinelandii. *J Biol Chem* **2014,** *289* (45), 31382-96.
[78.] Ø. Arlov; F. L. Aachmann; E. Feyzi; A. Sundan; G. Skjåk-Bræk, The impact of chain length and flexibility in the interaction between sulfated alginates and HGF and FGF-2. *Biomacromolecules* **2015,** *16* (11), 3417-3424.
[79.] M. Singh; A. R. Ray; P. Vasudevan, Biodegradation studies on periodate oxidized cellulose. *Biomaterials* **1982,** *3* (1), 16-20.
[80.] B. E. Christensen; I. M. N. Vold; K. M. Varum, Chain stiffness and extension of chitosans and periodate oxidised chitosans studied by size-exclusion chromatography combined with light scattering and viscosity detectors. *Carbohyd Polym* **2008,** *74* (3), 559-565.
[81.] K. H. Bouhadir; K. Y. Lee; E. Alsberg; K. L. Damm; K. W. Anderson; D. J. Mooney, Degradation of partially oxidized alginate and its potential application for tissue engineering. *Biotechnol Progr* **2001,** *17* (5), 945-950.
[82.] M. Petitou; C. A. van Boeckel, A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? *Angew Chem Int Ed Engl* **2004,** *43* (24), 3118-33.
[83.] R. Liu; Y. Xu; M. Chen; M. Weiwer; X. Zhou; A. S. Bridges; P. L. DeAngelis; Q. Zhang; R. J. Linhardt; J. Liu, Chemoenzymatic design of heparan sulfate oligosaccharides. *J Biol Chem* **2010,** *285* (44), 34240-9.
[84.] B. Kuberan; M. Z. Lech; D. L. Beeler; Z. L. Wu; R. D. Rosenberg, Enzymatic synthesis of antithrombin III-binding heparan sulfate pentasaccharide. *Nat Biotechnol* **2003,** *21* (11), 1343-6.
[85.] R. A. Smith; K. Meade; C. E. Pickford; R. J. Holley; C. L. Merry, Glycosaminoglycans as regulators of stem cell differentiation. *Biochem Soc Trans* **2011,** *39* (1), 383-7.
[86.] D. C. Kraushaar; S. Dalton; L. Wang, Heparan sulfate: a key regulator of embryonic stem cell fate. *Biol Chem* **2013,** *394* (6), 741-51.
[87.] Y. Gu; B. Liu; Q. Liu; Y. Hang; L. Wang; J. L. Brash; G. Chen; H. Chen, Modular Polymers as a Platform for Cell Surface Engineering: Promoting Neural Differentiation

and Enhancing the Immune Response. *ACS Appl Mater Interfaces* **2019,** *11* (51), 47720-47729.
[88.] M. Wang; X. Liu; Z. Lyu; H. Gu; D. Li; H. Chen, Glycosaminoglycans (GAGs) and GAG mimetics regulate the behavior of stem cell differentiation. *Colloids Surf B Biointerfaces* **2017,** *150*, 175-182.
[89.] Y. Miura; T. Fukuda; H. Seto; Y. Hoshino, Development of glycosaminoglycan mimetics using glycopolymers. *Polym J* **2015,** *48* (3), 229-237.
[90.] Y. Suda; A. Arano; Y. Fukui; S. Koshida; M. Wakao; T. Nishimura; S. Kusumoto; M. Sobel, Immobilization and clustering of structurally defined oligosaccharides for sugar chips: an improved method for surface plasmon resonance analysis of protein-carbohydrate interactions. *Bioconjugate Chemistry* **2006,** *17*, 1125-1135.
[91.] J. Hirsh; R. Raschke, Heparin and low-molecular-weight heparin: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest* **2004,** *126*, 188S-203S.
[92.] S. J. Paluck; T. H. Nguyen; H. D. Maynard, Heparin-Mimicking Polymers: Synthesis and Biological Applications. *Biomacromolecules* **2016,** *17* (11), 3417-3440.
[93.] M. Wang; Z. Lyu; G. Chen; H. Wang; Y. Yuan; K. Ding; Q. Yu; L. Yuan; H. Chen, A new avenue to the synthesis of GAG-mimicking polymers highly promoting neural differentiation of embryonic stem cells. *Chem Commun (Camb)* **2015,** *51* (84), 15434-7.
[94.] V. N. Rakhmanova; L. A. Nud'ga; R. Y. Milusheva; B. Z. Volchek; A. A. Kholmuminov; Y. G. Baklagina; S. S. Rashidova, Determination of the degree of sulfation of Bombyx mori chitosan by conductometric titration. *Russ J Appl Chem+* **2009,** *82* (12), 2192-2196.
[95.] K. Zhang; E. Brendler; A. Geissler; S. Fischer, Synthesis and spectroscopic analysis of cellulose sulfates with regulable total degrees of substitution and sulfation patterns via C-13 NMR and FT Raman spectroscopy. *Polymer* **2011,** *52* (1), 26-32.
[96.] R. R. Huang; V. H. Pomin; J. S. Sharp, LC-MS (n) Analysis of Isomeric Chondroitin Sulfate Oligosaccharides Using a Chemical Derivatization Strategy. *J Am Soc Mass Spectr* **2011,** *22* (9), 1577-1587.
[97.] M. Lei; Y. Mechref; M. V. Novotny, Structural Analysis of Sulfated Glycans by Sequential Double-Permethylation Using Methyl Iodide and Deuteromethyl Iodide. *J Am Soc Mass Spectr* **2009,** *20* (9), 1660-1671.
[98.] M. Guerrini; A. Bisio; G. Torri, Combined quantitative (1)H and (13)C nuclear magnetic resonance spectroscopy for characterization of heparin preparations. *Semin Thromb Hemost* **2001,** *27* (5), 473-82.
[99.] P. Saboural; F. Chaubet; F. Rouzet; F. Al-Shoukr; R. Ben Azzouna; N. Bouchemal; L. Picton; L. Louedec; M. Maire; L. Rolland; G. Potier; D. Le Guludec; D. Letourneur; C. Chauvierre, Purification of a Low Molecular Weight Fucoidan for SPECT Molecular Imaging of Myocardial Infarction. *Mar Drugs* **2014,** *12* (9), 4851-4867.
[100.] M. Yamamoto, Tadokoro, Y., Imai, H., Mita, K., Physicochemical Characterization of Sulfated Polysaccharides from Green Seaweeds: Ulva pertusa and Ulva conglobata. *Agricultural and Biological Chemistry* **1980,** *44* (4), 723-729.

[101.] H. M. Wang; D. Loganathan; R. J. Linhardt, Determination of the Pka of Glucuronic-Acid and the Carboxy Groups of Heparin by C-13-Nuclear-Magnetic-Resonance Spectroscopy. *Biochem J* **1991,** *278*, 689-695.
[102.] A. Robic; C. Rondeau-Mouro; J. F. Sassi; Y. Lerat; M. Lahaye, Structure and interactions of ulvan in the cell wall of the marine green algae Ulva rotundata (Ulvales, Chlorophyceae). *Carbohyd Polym* **2009,** *77* (2), 206-216.
[103.] M. J. Clancy, Turvey, J. R., Sulphates of Monosaccharides and Derivatives. Part III. Acid Hydrolysis. *Journal of the Chemical Society* **1961**, 2935-2938.
[104.] O. Smidsrod; A. Haug; B. Larsen, The influence of pH on the rate of hydrolysis of acidic polysaccharides. *Acta Chem Scand* **1966,** *20* (4), 1026-34.
[105.] A. Karlsson; S. K. Singh, Acid hydrolysis of sulphated polysaccharides. Desulphation and the effect on molecular mass. *Carbohyd Polym* **1999,** *38* (1), 7-15.
[106.] T. Hjerde; O. Smidsrod; B. E. Christensen, The influence of the conformational state of kappa- and iota-carrageenan on the rate of acid hydrolysis. *Carbohyd Res* **1996,** *288*, 175-187.
[107.] V. H. *e. a.* Pomin, Mild acid hydrolysis of sulfated fucans: a selective 2-desulfation reaction and an alternative approach for preparing tailored sulfated oligosaccharides. *Glycobiology* **2005,** *15* (12), 1376-1385.
[108.] M. Roman; W. T. Winter, Effect of sulfate groups from sulfuric acid hydrolysis on the thermal degradation behavior of bacterial cellulose. *Biomacromolecules* **2004,** *5* (5), 1671-1677.
[109.] Q. Feng; S. Lin; K. Zhang; C. Dong; T. Wu; H. Huang; X. Yan; L. Zhang; G. Li; L. Bian, Sulfated hyaluronic acid hydrogels with retarded degradation and enhanced growth factor retention promote hMSC chondrogenesis and articular cartilage integrity with reduced hypertrophy. *Acta Biomater* **2017,** *53*, 329-342.
[110.] E. W. Davie; K. Fujikawa; W. Kisiel, The Coagulation Cascade - Initiation, Maintenance, and Regulation. *Biochemistry-Us* **1991,** *30* (43), 10363-10370.
[111.] T. Koide, Antithrombin and Heparin Cofactor II: Structure and Functions. In *Recent Advances in Thrombosis and Hemostasis*, Tanaka, K., Ed. Springer: Tokyo, 2008; pp 177-189.
[112.] C. Nelson-Piercy, Hazards of heparin: allergy, heparin-induced thrombocytopenia and osteoporosis. *Baillieres Clin Obstet Gynaecol* **1997,** *11* (3), 489-509.
[113.] N. M. Mestechkina; V. D. Shcherbukhin, Sulfated polysaccharides and their anticoagulant activity: A review. *Applied Biochemistry and Microbiology* **2010,** *46* (3), 267-273.
[114.] S. A. Smith; R. J. Travers; J. H. Morrissey, How it all starts: Initiation of the clotting cascade. *Crit Rev Biochem Mol Biol* **2015,** *50* (4), 326-36.
[115.] A. S. Amarasekara; G. Opoku; X. D. Qiu; V. Doctor, Effect of oversulfation on the chemical and biological properties of chondroitin-6-sulfate. *Carbohyd Polym* **2007,** *68* (1), 116-121.
[116.] Y. E. Carranza; C. Durand-Rougley; V. Doctor, Effect of oversulfation on the chemical and biological properties of chondroitin-4-sulfate. *Blood Coagul Fibrin* **2008,** *19* (6), 483-487.

[117.] G. Opoku; X. D. Qiu; V. Doctor, Effect of oversulfation on the chemical and biological properties of kappa carrageenan. *Carbohyd Polym* **2006,** *65* (2), 134-138.
[118.] T. Nishino; T. Nagumo, Anticoagulant and antithrombin activities of oversulfated fucans. *Carbohydr Res* **1992,** *229* (2), 355-62.
[119.] S. Alban; A. Schauerte; G. Franz, Anticoagulant sulfated polysaccharides: Part I. Synthesis and structure-activity relationships of new pullulan sulfates. *Carbohyd Polym* **2002,** *47* (3), 267-276.
[120.] R. Dace; E. McBride; K. Brooks; J. Gander; M. Buszko; V. M. Doctor, Comparison of the anticoagulant action of sulfated and phosphorylated polysaccharides. *Thrombosis Research* **1997,** *87* (1), 113-121.
[121.] R. H. Huang; Y. M. Du; J. H. Yang, Preparation and in vitro anticoagulant activities of alginate sulfate and its quaterized derivatives. *Carbohyd Polym* **2003,** *52* (1), 19-24.
[122.] Z. M. Wang; L. Li; B. S. Zheng; N. Normakhamatov; S. Y. Guo, Preparation and anticoagulation activity of sodium cellulose sulfate. *Int J Biol Macromol* **2007,** *41* (4), 376-82.
[123.] S. G. Chen; G. Y. Li; N. A. Wu; X. Guo; N. B. Liao; X. Q. Ye; D. H. Liu; C. H. Xue; W. G. Chai, Sulfation pattern of the fucose branch is important for the anticoagulant and antithrombotic activities of fucosylated chondroitin sulfates. *Bba-Gen Subjects* **2013,** *1830* (4), 3054-3066.
[124.] N. C. Maas; A. H. P. Gracher; G. L. Sassaki; P. A. J. Gorin; M. Iacomini; T. R. Cipriani, Sulfation pattern of citrus pectin and its carboxy-reduced derivatives: Influence on anticoagulant and antithrombotic effects. *Carbohyd Polym* **2012,** *89* (4), 1081-1087.
[125.] C. A. de Araujo; M. D. Noseda; T. R. Cipriani; A. G. Goncalves; M. E. R. Duarte; D. R. B. Ducatti, Selective sulfation of carrageenans and the influence of sulfate regiochemistry on anticoagulant properties. *Carbohyd Polym* **2013,** *91* (2), 483-491.
[126.] N. N. Drozd; Y. S. Logvinova; M. A. Torlopov; E. V. Udoratina, Effect of Sulfation and Molecular Weight on Anticoagulant Activity of Dextran. *B Exp Biol Med+* **2017,** *162* (4), 462-465.
[127.] C. Z. Lin; H. S. Guan; H. H. Li; G. L. Yu; C. X. Gu; G. Q. Li, The influence of molecular mass of sulfated propylene glycol ester of low-molecular-weight alginate on anticoagulant activities. *Eur Polym J* **2007,** *43* (7), 3009-3015.
[128.] J. H. Yang; K. Luo; D. L. Li; S. S. Yu; J. Cai; L. Y. Chen; Y. M. Du, Preparation, characterization and in vitro anticoagulant activity of highly sulfated chitosan. *Int J Biol Macromol* **2013,** *52*, 25-31.
[129.] F. R. Melo; M. S. Pereira; D. Foguel; P. A. S. Mourao, Antithrombin-mediated anticoagulant activity of sulfated polysaccharides - Different mechanisms for heparin and sulfated galactans. *J Biol Chem* **2004,** *279* (20), 20824-20835.
[130.] Z.-M. Wang; K.-J. Xiao; L. Li; J.-Y. Wu, Molecular weight-dependent anticoagulation activity of sulfated cellulose derivatives. *Cellulose* **2010,** *17* (5), 953-961.
[131.] R. J. Kandrotas, Heparin pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet* **1992,** *22* (5), 359-74.

[132.] L. Ma; C. Cheng; C. Nie; C. He; J. Deng; L. Wang; Y. Xia; C. Zhao, Anticoagulant sodium alginate sulfates and their mussel-inspired heparin-mimetic coatings. *J Mater Chem B* **2016,** *4* (19), 3203-3215.
[133.] U. Lindahl; J. P. Li; M. Kusche-Gullberg; M. Salmivirta; S. Alaranta; T. Veromaa; J. Emeis; I. Roberts; C. Taylor; P. Oreste; G. Zoppetti; A. Naggi; G. Torri; B. Casu, Generation of "neoheparin" from E. coli K5 capsular polysaccharide. *J Med Chem* **2005,** *48* (2), 349-52.
[134.] J. Suwan; Z. Q. Zhang; B. Y. Z. Li; P. Vongchan; P. Meepowpan; F. M. Zhang; S. A. Mousa; S. Mousa; B. Premanode; P. Kongtawelert; R. J. Linhardt, Sulfonation of papain-treated chitosan and its mechanism for anticoagulant activity. *Carbohyd Res* **2009,** *344* (10), 1190-1196.
[135.] H. P. D. Barddal; A. H. P. Gracher; F. F. Simas-Tosin; M. Iacomini; T. R. Cipriani, Anticoagulant activity of native and partially degraded glycoglucuronomannan after chemical sulfation. *Int J Biol Macromol* **2015,** *80*, 328-333.
[136.] K. Heise; M. Hobisch; L. Sacarescu; U. Maver; J. Hobisch; T. Reichelt; M. Sega; S. Fischer; S. Spirk; M. EPNOE; N. Graz, Low-molecular-weight sulfonated chitosan as template for anticoagulant nanoparticles. *Int J Nanomed* **2018,** *13*, 4881-4894.
[137.] B. F. Glauser; R. M. Rezendel; F. R. Melo; M. S. Pereira; I. M. B. Francischetti; R. Q. Monteiro; A. R. Rezaie; P. A. S. Mourao, Anticoagulant activity of a sulfated galactan: Serpin-independent effect and specific interaction with factor Xa. *Thromb Haemostasis* **2009,** *102* (6), 1183-1193.
[138.] E. M. Munoz; R. J. Linhardt, Heparin-binding domains in vascular biology. *Arterioscl Throm Vas* **2004,** *24* (9), 1549-1557.
[139.] D. Wudunn; P. G. Spear, Initial Interaction of Herpes-Simplex Virus with Cells Is Binding to Heparan-Sulfate. *Journal of Virology* **1989,** *63* (1), 52-58.
[140.] Y. P. Chen; T. Maguire; R. E. Hileman; J. R. Fromm; J. D. Esko; R. J. Linhardt; R. M. Marks, Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nature Medicine* **1997,** *3* (8), 866-871.
[141.] M. Koenighofer; T. Lion; A. Bodenteich; E. Prieschl-Grassauer; A. Grassauer; H. Unger; C. A. Mueller; T. Fazekas, Carrageenan nasal spray in virus confirmed common cold: individual patient data analysis of two randomized controlled trials. *Multidiscip Respir Med* **2014,** *9* (1), 57.
[142.] C. Urbinati; P. Chiodelli; M. Rusnati, Polyanionic drugs and viral oncogenesis: a novel approach to control infection, tumor-associated inflammation and angiogenesis. *Molecules* **2008,** *13* (11), 2758-85.
[143.] L. K. Hallak; P. L. Collins; W. Knudson; M. E. Peeples, Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. *Virology* **2000,** *271* (2), 264-275.
[144.] R. K. Williams; S. E. Straus, Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans. *J Virol* **1997,** *71* (2), 1375-80.
[145.] M. Witvrouw; E. DeClercq, Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *Gen Pharmacol-Vasc S* **1997,** *29* (4), 497-511.

[146.] M. P. Recalde; M. J. Carlucci; M. D. Noseda; M. C. Matulewicz, Chemical modifications of algal mannans and xylomannans: Effects on antiviral activity. *Phytochemistry* **2012,** *73*, 57-64.
[147.] E. Vicenzi; A. Gatti; S. Ghezzi; P. Oreste; G. Zoppetti; G. Poli, Broad spectrum inhibition of HIV-1 infection by sulfated K5 Escherichia coli polysaccharide derivatives. *AIDS* **2003,** *17* (2), 177-81.
[148.] V. Cagno; M. Donalisio; A. Civra; M. Volante; E. Veccelli; P. Oreste; M. Rusnati; D. Lembo, Highly Sulfated K5 Escherichia coli Polysaccharide Derivatives Inhibit Respiratory Syncytial Virus Infectivity in Cell Lines and Human Tracheal-Bronchial Histocultures. *Antimicrob Agents Ch* **2014,** *58* (8), 4782-4794.
[149.] B. Mercorelli; P. Oreste; E. Sinigalia; G. Muratore; D. Lembo; G. Palu; A. Loregian, Sulfated Derivatives of Escherichia coli K5 Capsular Polysaccharide Are Potent Inhibitors of Human Cytomegalovirus. *Antimicrob Agents Ch* **2010,** *54* (11), 4561-4567.
[150.] E. Lee; M. Pavy; N. Young; C. Freeman; M. Lobigs, Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. *Antiviral Res* **2006,** *69* (1), 31-8.
[151.] Y. Y. Gao; W. Liu; W. Wang; X. S. Zhang; X. Zhao, The inhibitory effects and mechanisms of 3,6-O-sulfated chitosan against human papillomavirus infection. *Carbohyd Polym* **2018,** *198*, 329-338.
[152.] L. Chen; G. L. Huang, The antiviral activity of polysaccharides and their derivatives. *Int J Biol Macromol* **2018,** *115*, 77-82.
[153.] S. Khan; A. Tondervik; H. Sletta; G. Klinkenberg; C. Emanuel; E. Onsoyen; R. Myrvold; R. A. Howe; T. R. Walsh; K. E. Hill; D. W. Thomas, Overcoming Drug Resistance with Alginate Oligosaccharides Able To Potentiate the Action of Selected Antibiotics. *Antimicrob Agents Ch* **2012,** *56* (10), 5134-5141.
[154.] K. Y. Lee; M. R. Jeong; S. M. Choi; S. S. Na; J. D. Cha, Synergistic effect of fucoidan with antibiotics against oral pathogenic bacteria. *Arch Oral Biol* **2013,** *58* (5), 482-92.
[155.] M. Liu; Y. Liu; M. J. Cao; G. M. Liu; Q. Chen; L. Sun; H. Chen, Antibacterial activity and mechanisms of depolymerized fucoidans isolated from Laminaria japonica. *Carbohydr Polym* **2017,** *172*, 294-305.
[156.] G. Pierre; V. Sopena; C. Juin; A. Mastouri; M. Graber; T. Maugard, Antibacterial Activity of a Sulfated Galactan Extracted from the Marine Alga Chaetomorpha aerea Against Staphylococcus aureus. *Biotechnol Bioproc E* **2011,** *16* (5), 937-945.
[157.] Z. M. Sun; C. G. Shi; X. Y. Wang; Q. Fang; J. Y. Huang, Synthesis, characterization, and antimicrobial activities of sulfonated chitosan. *Carbohyd Polym* **2017,** *155*, 321-328.
[158.] W. A. I. Wan-Mohtar; L. Young; G. M. Abbott; C. Clements; L. M. Harvey; B. McNeil, Antimicrobial Properties and Cytotoxicity of Sulfated (1,3)-beta-D-Glucan from the Mycelium of the Mushroom Ganoderma lucidum. *J Microbiol Biotechn* **2016,** *26* (6), 999-1010.

[159.] M. Utt; T. Wadstrom, Identification of heparan sulphate binding surface proteins of Helicobacter pylori: Inhibition of heparan sulphate binding with sulphated carbohydrate polymers. *J Med Microbiol* **1997,** *46* (7), 541-546.
[160.] C. Fallgren; A. Andersson; A. Ljungh, The role of glycosaminoglycan binding of staphylococci in attachment to eukaryotic host cells. *Curr Microbiol* **2001,** *43* (1), 57-63.
[161.] J. M. Leong; P. E. Morrissey; E. Ortegabarria; M. E. A. Pereira; J. Coburn, Hemagglutination and Proteoglycan Binding by the Lyme-Disease Spirochete, Borrelia-Burgdorferi. *Infection and Immunity* **1995,** *63* (3), 874-883.
[162.] R. Thomas; T. Brooks, Common oligosaccharide moieties inhibit the adherence of typical and atypical respiratory pathogens. *J Med Microbiol* **2004,** *53* (9), 833-840.
[163.] E. Ortega-Barria; J. C. Boothroyd, A Toxoplasma lectin-like activity specific for sulfated polysaccharides is involved in host cell infection. *J Biol Chem* **1999,** *274* (3), 1267-1276.
[164.] Y. Adams; C. Freeman; R. Schwartz-Albiez; V. Ferro; C. R. Parish; K. T. Andrews, Inhibition of Plasmodium falciparum growth in vitro and adhesion to chondroitin-4-sulfate by the heparan sulfate mimetic PI-88 and other sulfated oligosaccharides. *Antimicrob Agents Ch* **2006,** *50* (8), 2850-2852.
[165.] R. A. Anderson; K. A. Feathergill; X. H. Diao; M. D. Cooper; R. Kirkpatrick; B. C. Herold; G. F. Doncel; C. J. Chany; D. P. Waller; W. F. Rencher; L. J. Zaneveld, Preclinical evaluation of sodium cellulose sulfate (Ushercell) as a contraceptive antimicrobial agent. *J Androl* **2002,** *23* (3), 426-38.
[166.] J. A. Simoes; D. M. Citron; A. Aroutcheva; R. A. Anderson, Jr.; C. J. Chany, 2nd; D. P. Waller; S. Faro; L. J. Zaneveld, Two novel vaginal microbicides (polystyrene sulfonate and cellulose sulfate) inhibit Gardnerella vaginalis and anaerobes commonly associated with bacterial vaginosis. *Antimicrob Agents Chemother* **2002,** *46* (8), 2692-5.
[167.] S. Franz; S. Rammelt; D. Scharnweber; J. C. J. B. Simon, Immune responses to implants–a review of the implications for the design of immunomodulatory biomaterials. **2011,** *32* (28), 6692-6709.
[168.] J. S. Lewis; K. Roy; B. G. Keselowsky, Materials that harness and modulate the immune system. *MRS bulletin* **2014,** *39* (1), 25-34.
[169.] J. I. Andorko; C. M. J. B. Jewell; t. medicine, Designing biomaterials with immunomodulatory properties for tissue engineering and regenerative medicine. **2017,** *2* (2), 139-155.
[170.] Z. Julier; A. J. Park; P. S. Briquez; M. M. J. A. b. Martino, Promoting tissue regeneration by modulating the immune system. **2017,** *53*, 13-28.
[171.] S. K. Mallapragada; B. J. I. j. o. p. Narasimhan, Immunomodulatory biomaterials. **2008,** *364* (2), 265-271.
[172.] K. Garg; N. A. Pullen; C. A. Oskeritzian; J. J. Ryan; G. L. Bowlin, Macrophage functional polarization (M1/M2) in response to varying fiber and pore dimensions of electrospun scaffolds. *Biomaterials* **2013,** *34* (18), 4439-4451.
[173.] F. Chen; G. Huang, Preparation and immunological activity of polysaccharides and their derivatives. *Int J Biol Macromol* **2018,** *112*, 211-216.

[174.] S. S. Ferreira; C. P. Passos; P. Madureira; M. Vilanova; M. A. J. C. p. Coimbra, Structure–function relationships of immunostimulatory polysaccharides: A review. **2015,** *132*, 378-396.
[175.] L. Huang; M. Shen; G. A. J. T. i. F. S. Morris; Technology, Sulfated polysaccharides: Immunomodulation and signaling mechanisms. **2019**.
[176.] S. R. Khil'chenko; T. S. Zaporozhets; N. M. Shevchenko; T. N. Zvyagintseva; U. Vogel; P. Seeberger; B. J. J. o. C. C. Lepenies, Immunostimulatory activity of fucoidan from the brown alga Fucus evanescens: role of sulfates and acetates. **2011,** *30* (4-6), 291-305.
[177.] J. M. Leiro; R. Castro; J. A. Arranz; J. Lamas, Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed Ulva rigida C. Agardh. *International immunopharmacology* **2007,** *7* (7), 879-888.
[178.] C. Li; N. Talapphet; S. Palanisamy; N. Ma; M. L. Cho; S. You, The relationship between structural properties and activation of RAW264. 7 and natural killer (NK) cells by sulfated polysaccharides extracted from Astragalus membranaceus roots. *Process Biochemistry* **2020**.
[179.] D. Chen; X. Wu; Z. J. P. m. Wen, Sulfated polysaccharides and immune response: promoter or inhibitor? **2008,** *50* (2), 177-183.
[180.] Ø. Arlov; G. Skjåk-Bræk; A. M. J. A. b. Rokstad, Sulfated alginate microspheres associate with factor H and dampen the inflammatory cytokine response. **2016,** *42*, 180-188.
[181.] X. Zhang; Y. Kimura; C. Fang; L. Zhou; G. Sfyroera; J. D. Lambris; R. A. Wetsel; T. Miwa; W. C. Song, Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. *Blood* **2007,** *110* (1), 228-36.
[182.] G. J. Wu; S. M. Shiu; M. C. Hsieh; G. J. Tsai, Anti-inflammatory activity of a sulfated polysaccharide from the brown alga Sargassum cristaefolium. *Food Hydrocolloid* **2016,** *53*, 16-23.
[183.] M. K. Lu; M. H. Lee; C. H. Chao; Y. C. Hsu, Physiochemical changes and mechanisms of anti-inflammation effect of sulfated polysaccharides from ammonium sulfate feeding of Antrodia cinnamomea. *Int J Biol Macromol* **2020,** *148*, 715-721.
[184.] Y. Yang; R. Xing; S. Liu; Y. Qin; K. Li; H. Yu; P. Li, Immunostimulatory effects of sulfated chitosans on RAW 264.7 mouse macrophages via the activation of PI3K/Akt signaling pathway. *Int J Biol Macromol* **2018,** *108*, 1310-1321.
[185.] X. Wang; S. Wang; Y. Li; F. Wang; X. Yang; J. J. I. j. o. b. m. Yao, Sulfated Astragalus polysaccharide can regulate the inflammatory reaction induced by LPS in Caco2 cells. **2013,** *60*, 248-252.
[186.] J. d. S. Barbosa; M. S. S. P. Costa; L. F. M. d. Melo; M. J. C. d. Medeiros; D. d. L. Pontes; K. C. Scortecci; H. A. O. Rocha, In vitro immunostimulating activity of sulfated polysaccharides from Caulerpa cupressoides var. Flabellata. *Mar Drugs* **2019,** *17* (2), 105.
[187.] J. K. Kim; M. L. Cho; S. Karnjanapratum; I. S. Shin; S. G. You, In vitro and in vivo immunomodulatory activity of sulfated polysaccharides from Enteromorpha prolifera. *Int J Biol Macromol* **2011,** *49* (5), 1051-8.

[188.] M. Wang; X.-b. Yang; J.-w. Zhao; C.-j. Lu; W. Zhu, Structural characterization and macrophage immunomodulatory activity of a novel polysaccharide from Smilax glabra Roxb. *Carbohyd Polym* **2017,** *156*, 390-402.
[189.] Q. Yuan; L. Zhao; Q. Cha; Y. Sun; H. Ye; X. Zeng, Structural characterization and immunostimulatory activity of a homogeneous polysaccharide from Sinonovacula constricta. *Journal of agricultural and food chemistry* **2015,** *63* (36), 7986-7994.
[190.] X. Zhao; G. Jiao; Y. Yang; M. Li; Q. Li; X. Wang; C. Cai; G. Li; J. Hao; G. Yu, Structure and immunomodulatory activity of a sulfated agarose with pyruvate and xylose substitutes from Polysiphonia senticulosa Harvey. *Carbohydr Polym* **2017,** *176*, 29-37.
[191.] Y. Chen; H. Zhang; Y. Wang; S. Nie; C. Li; M. Xie, Sulfated modification of the polysaccharides from Ganoderma atrum and their antioxidant and immunomodulating activities. *Food Chem* **2015,** *186*, 231-8.
[192.] J. Jiang; F.-Y. Meng; Z. He; Y.-L. Ning; X.-H. Li; H. Song; J. Wang; R. J. I. j. o. b. m. Zhou, Sulfated modification of longan polysaccharide and its immunomodulatory and antitumor activity in vitro. **2014,** *67*, 323-329.
[193.] L. Teng; H. Fu; M. Wang; C. Deng; J. Chen, Stimulation of RAW264. 7 macrophages by sulfated Escherichia coli K5 capsular polysaccharide in vitro. *Molecular Medicine Reports* **2015,** *12* (4), 5545-5553.
[194.] Y. Yu; M. Shen; Z. Wang; Y. Wang; M. Xie; J. Xie, Sulfated polysaccharide from Cyclocarya paliurus enhances the immunomodulatory activity of macrophages. *Carbohydr Polym* **2017,** *174*, 669-676.
[195.] Y. Sun; H. Liang; G. Cai; S. Guan; H. Tong; X. Yang; J. Liu, Sulfated modification of the water-soluble polysaccharides from Polyporus albicans mycelia and its potential biological activities. *Int J Biol Macromol* **2009,** *44* (1), 14-7.
[196.] L. Teng; H. Fu; M. Wang; C. Deng; Z. Song; J. J. C. p. Chen, Immunomodulatory activity of heparan sulfate mimetics from Escherichia coli K5 capsular polysaccharide in vitro. **2015,** *115*, 643-650.
[197.] L. Yao; L. Bai; Y. Tan; J. Sun; Q. Qu; D. Shi; S. Guo; C. Liu, The immunoregulatory effect of sulfated Echinacea purpurea polysaccharide on chicken bone marrow-derived dendritic cells. *Int J Biol Macromol* **2019,** *139*, 1123-1132.
[198.] S. Gordon, Pattern recognition receptors: doubling up for the innate immune response. *Cell* **2002,** *111* (7), 927-30.
[199.] I. Sabroe; E. C. Jones; L. R. Usher; M. K. Whyte; S. K. Dower, Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol* **2002,** *168* (9), 4701-10.
[200.] X. Zhang; C. Qi; Y. Guo; W. Zhou; Y. Zhang, Toll-like receptor 4-related immunostimulatory polysaccharides: Primary structure, activity relationships, and possible interaction models. *Carbohydr Polym* **2016,** *149*, 186-206.
[201.] Y. Geng; L. Xing; M. Sun; F. Su, Immunomodulatory effects of sulfated polysaccharides of pine pollen on mouse macrophages. *Int J Biol Macromol* **2016,** *91*, 846-855.
[202.] Y. Han; K. Ouyang; J. Li; X. Liu; Q. An; M. Zhao; S. Chen; X. Li; X. Ye; Z. Zhao, Sulfated modification, characterization, immunomodulatory activities and mechanism

of the polysaccharides from Cyclocarya paliurus on dendritic cells. *Int J Biol Macromol* **2020**.
[203.] F. Wu; C. Zhou; D. Zhou; S. Ou; Z. Liu; H. Huang, Immune-enhancing activities of chondroitin sulfate in murine macrophage RAW 264.7 cells. *Carbohyd Polym* **2018,** *198*, 611-619.
[204.] K. J. Goodall; I. K. Poon; S. Phipps; M. D. Hulett, Soluble heparan sulfate fragments generated by heparanase trigger the release of pro-inflammatory cytokines through TLR-4. *Plos One* **2014,** *9* (10), e109596.
[205.] T. Wu; M. Shen; X. Guo; L. Huang; J. Yang; Q. Yu; Y. Chen; J. Xie, Cyclocarya paliurus polysaccharide alleviates liver inflammation in mice via beneficial regulation of gut microbiota and TLR4/MAPK signaling pathways. *Int J Biol Macromol* **2020**.
[206.] Z. Guo; Y. Hu; D. Wang; X. Ma; X. Zhao; B. Zhao; J. Wang; P. Liu, Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. *Vaccine* **2009,** *27* (5), 660-5.
[207.] X. Huang; Y. Hu; X. Zhao; Y. Lu; J. Wang; F. Zhang; J. J. C. p. Sun, Sulfated modification can enhance the adjuvant activity of astragalus polysaccharide for ND vaccine. **2008,** *73* (2), 303-308.
[208.] T. L. Nguyen; D. Wang; Y. Hu; Y. Fan; J. Wang; S. Abula; L. Guo; J. Zhang; B. K. J. C. p. Dang, Immuno-enhancing activity of sulfated Auricularia auricula polysaccharides. **2012,** *89* (4), 1117-1122.
[209.] J. Wang; B. Ge; C. Du; J. Xue; Y. Zhuang; K. Xue, Sulfated modification promotes the immunomodulatory bioactivities of Lyciumbarbarum polysaccharides in vitro. *International journal of clinical and experimental medicine* **2015,** *8* (11), 20380.
[210.] J. Wang; Y. Hu; D. Wang; J. Liu; J. Zhang; S. Abula; B. Zhao; S. J. C. i. Ruan, Sulfated modification can enhance the immune-enhancing activity of Lycium barbarum polysaccharides. **2010,** *263* (2), 219-223.
[211.] Y. Yu; Q. Song; L. Huang; M. Shen; Q. Yu; Y. Chen; J. J. J. o. F. F. Xie, Immunomodulatory activities of sulfated Cyclocarya paliurus polysaccharides with different degrees of substitution on mouse spleen lymphocytes. **2020,** *64*, 103706.
[212.] J. Zhang; J. Chen; D. Wang; Y. Hu; C. Zhang; T. Qin; C. Liu; X. Sheng; T. L. Nguyen, Immune-enhancing activity comparison of sulfated ophiopogonpolysaccharide and sulfated jujube polysaccharide. *Int J Biol Macromol* **2013,** *52*, 212-217.
[213.] C. Kupfahl; G. Geginat; H. J. I. i. Hof, Lentinan has a stimulatory effect on innate and adaptive immunity against murine Listeria monocytogenes infection. **2006,** *6* (4), 686-696.
[214.] U. Surayot; S. Lee; S. You, Effects of sulfated fucan from the sea cucumber stichopus japonicus on natural killer cell activation and cytotoxicity. *Int J Biol Macromol* **2018,** *108*, 177-184.
[215.] J.-H. Kim; Y.-S. Kim; J.-W. Hwang; Y.-K. Han; J.-S. Lee; S.-K. Kim; Y.-J. Jeon; S.-H. Moon; B.-T. Jeon; Y. Y. J. M. Bahk, Sulfated chitosan oligosaccharides suppress LPS-induced NO production via JNK and NF-κB inactivation. **2014,** *19* (11), 18232-18247.
[216.] P. Ma; H.-T. Liu; P. Wei; Q.-S. Xu; X.-F. Bai; Y.-G. Du; C. J. C. p. Yu, Chitosan oligosaccharides inhibit LPS-induced over-expression of IL-6 and TNF-α in RAW264. 7

macrophage cells through blockade of mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways. **2011,** *84* (4), 1391-1398.
[217.] H. J. Yoon; M. E. Moon; H. S. Park; S. Y. Im; Y. H. J. B. Kim; b. r. communications, Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. **2007,** *358* (3), 954-959.
[218.] N. Wu; Z.-S. Wen; X.-W. Xiang; Y.-N. Huang; Y. Gao; Y.-L. J. M. d. Qu, Immunostimulative activity of low molecular weight chitosans in RAW264. 7 macrophages. **2015,** *13* (10), 6210-6225.
[219.] Ø. Arlov; E. Öztürk; M. Steinwachs; G. Skjåk-Bræk; M. Zenobi-Wong, Biomimetic sulphated alginate hydrogels suppress IL-1B-induced inflammatory responses in human chondrocytes. **2017**.
[220.] A. Kerschenmeyer; Ø. Arlov; V. Malheiro; M. Steinwachs; M. Rottmar; K. Maniura-Weber; G. Palazzolo; M. J. B. s. Zenobi-Wong, Anti-oxidant and immune-modulatory properties of sulfated alginate derivatives on human chondrocytes and macrophages. **2017,** *5* (9), 1756-1765.
[221.] F. Jouy; N. Lohmann; E. Wandel; G. Ruiz-Gomez; M. T. Pisabarro; A. G. Beck-Sickinger; M. Schnabelrauch; S. Moller; J. C. Simon; S. Kalkhof; M. von Bergen; S. Franz, Sulfated hyaluronan attenuates inflammatory signaling pathways in macrophages involving induction of antioxidants. *Proteomics* **2017,** *17* (10), e1700082.
[222.] J. Kajahn; S. Franz; E. Rueckert; I. Forstreuter; V. Hintze; S. Moeller; J. C. Simon, Artificial extracellular matrices composed of collagen I and high sulfated hyaluronan modulate monocyte to macrophage differentiation under conditions of sterile inflammation. *Biomatter* **2012,** *2* (4), 226-36.
[223.] A. M. Gori; M. Attanasio; A. Gazzini; L. Rossi; L. Lucarini; S. Miletti; J. Chini; M. Manoni; R. Abbate; G. J. J. o. T. Gensini; Haemostasis, Cytokine gene expression and production by human LPS‐stimulated mononuclear cells are inhibited by sulfated heparin‐like semi‐synthetic derivatives. **2004,** *2* (9), 1657-1662.
[224.] Z. Wang; J. Xie; Y. Yang; F. Zhang; S. Wang; T. Wu; M. Shen; M. J. S. r. Xie, Sulfated Cyclocarya paliurus polysaccharides markedly attenuates inflammation and oxidative damage in lipopolysaccharide-treated macrophage cells and mice. **2017,** *7* (1), 1-12.
[225.] X. Wang; S. Wang; Y. Li; F. Wang; X. Yang; J. Yao, Sulfated Astragalus polysaccharide can regulate the inflammatory reaction induced by LPS in Caco2 cells. *Int J Biol Macromol* **2013,** *60*, 248-252.
[226.] L. E. Collins; L. Troeberg, Heparan sulfate as a regulator of inflammation and immunity. *Journal of Leukocyte Biology* **2019,** *105* (1), 81-92.
[227.] C. R. Parish, The role of heparan sulphate in inflammation. *Nature Reviews Immunology* **2006,** *6* (9), 633-643.
[228.] E. Young, The anti-inflammatory effects of heparin and related compounds. *Thrombosis research* **2008,** *122* (6), 743-752.
[229.] J.-J. Cheng; C.-H. Chao; P.-C. Chang; M.-K. Lu, Studies on anti-inflammatory activity of sulfated polysaccharides from cultivated fungi Antrodia cinnamomea. *Food Hydrocolloid* **2016,** *53*, 37-45.

[230.] S. C. Makrides, Therapeutic inhibition of the complement system. *Pharmacological Reviews* **1998,** *50* (1), 59-88.
[231.] R. J. Ormsby; T. S. Jokiranta; T. G. Duthy; K. M. Griggs; T. A. Sadlon; E. Giannakis; D. L. Gordon, Localization of the third heparin-binding site in the human complement regulator factor H1. *Molecular immunology* **2006,** *43* (10), 1624-1632.
[232.] H. Wang; H. Wang; S. Shi; J. Duan; S. Wang, Structural characterization of a homogalacturonan from Capparis spinosa L. fruits and anti-complement activity of its sulfated derivative. *Glycoconjugate J* **2012,** *29* (5-6), 379-387.
[233.] H. Fan; F. Liu; S. A. Bligh; S. Shi; S. J. C. p. Wang, Structure of a homofructosan from Saussurea costus and anti-complementary activity of its sulfated derivatives. **2014,** *105*, 152-160.
[234.] K. Zhang; Y. Liu; X. Zhao; Q. Tang; J. Dernedde; J. Zhang; H. Fan, Anti-inflammatory properties of GLPss58, a sulfated polysaccharide from Ganoderma lucidum. *Int J Biol Macromol* **2018,** *107*, 486-493.
[235.] F. Zamboni; S. Vieira; R. L. Reis; J. M. Oliveira; M. N. J. P. i. M. S. Collins, The potential of hyaluronic acid in immunoprotection and immunomodulation: chemistry, processing and function. **2018,** *97*, 97-122.
[236.] C. Cheignon; M. Tomas; D. Bonnefont-Rousselot; P. Faller; C. Hureau; F. J. R. b. Collin, Oxidative stress and the amyloid beta peptide in Alzheimer's disease. **2018,** *14*, 450-464.
[237.] P. Kovacic; R. J. C. n. Somanathan, Redox processes in neurodegenerative disease involving reactive oxygen species. **2012,** *10* (4), 289-302.
[238.] S. Dimassi; N. Tabary; F. Chai; N. Blanchemain; B. J. C. p. Martel, Sulfonated and sulfated chitosan derivatives for biomedical applications: A review. **2018,** *202*, 382-396.
[239.] G. Huang; X. Mei; J. J. C. d. t. Hu, The antioxidant activities of natural polysaccharides. **2017,** *18* (11), 1296-1300.
[240.] J. Wang; S. Hu; S. Nie; Q. Yu; M. J. O. M. Xie; C. Longevity, Reviews on mechanisms of in vitro antioxidant activity of polysaccharides. **2016,** *2016*.
[241.] J. Wang; H. Guo; J. Zhang; X. Wang; B. Zhao; J. Yao; Y. J. C. P. Wang, Sulfated modification, characterization and structure–antioxidant relationships of Artemisia sphaerocephala polysaccharides. **2010,** *81* (4), 897-905.
[242.] C. Liu; J. Chen; E. Li; Q. Fan; D. Wang; P. Li; X. Li; X. Chen; S. Qiu; Z. J. I. i. Gao, The comparison of antioxidative and hepatoprotective activities of Codonopsis pilosula polysaccharide (CP) and sulfated CP. **2015,** *24* (2), 299-305.
[243.] Y. Xu; S. Song; Y. Wei; F. Wang; M. Zhao; J. Guo; J. J. I. j. o. b. m. Zhang, Sulfated modification of the polysaccharide from Sphallerocarpus gracilis and its antioxidant activities. **2016,** *87*, 180-190.
[244.] R. Xing; S. Liu; H. Yu; Z. Guo; Z. Li; P. J. C. p. Li, Preparation of high-molecular weight and high-sulfate content chitosans and their potential antioxidant activity in vitro. **2005,** *61* (2), 148-154.
[245.] R. Huang; E. Mendis; S.-K. J. I. j. o. b. m. Kim, Factors affecting the free radical scavenging behavior of chitosan sulfate. **2005,** *36* (1-2), 120-127.

[246.] L. Chen; G. J. I. j. o. b. m. Huang, Antioxidant activities of sulfated pumpkin polysaccharides. **2019,** *126*, 743-746.
[247.] J. Li; Z. Chi; L. Yu; F. Jiang; C. J. I. j. o. b. m. Liu, Sulfated modification, characterization, and antioxidant and moisture absorption/retention activities of a soluble neutral polysaccharide from Enteromorpha prolifera. **2017,** *105*, 1544-1553.
[248.] X. Liu; T. Chen; Y. Hu; K. Li; L. J. B. Yan, Catalytic synthesis and antioxidant activity of sulfated polysaccharide from Momordica charantia L. **2014,** *101* (3), 210-215.
[249.] J. Wang; S. Niu; B. Zhao; T. Luo; D. Liu; J. J. C. p. Zhang, Catalytic synthesis of sulfated polysaccharides. II: Comparative studies of solution conformation and antioxidant activities. **2014,** *107*, 221-231.
[250.] C. Zou; Y. Du; Y. Li; J. Yang; L. J. I. j. o. b. m. Zhang, Preparation and in vitro antioxidant activity of lacquer polysaccharides with low molecular weights and their sulfated derivatives. **2010,** *46* (2), 140-144.
[251.] J.-H. Xie; Z.-J. Wang; M.-Y. Shen; S.-P. Nie; B. Gong; H.-S. Li; Q. Zhao; W.-J. Li; M.-Y. J. F. H. Xie, Sulfated modification, characterization and antioxidant activities of polysaccharide from Cyclocarya paliurus. **2016,** *53*, 7-15.
[252.] R. Xing; H. Yu; S. Liu; W. Zhang; Q. Zhang; Z. Li; P. J. B. Li; m. chemistry, Antioxidant activity of differently regioselective chitosan sulfates in vitro. **2005,** *13* (4), 1387-1392.
[253.] Z.-J. Wang; J.-H. Xie; L.-J. Kan; J.-Q. Wang; M.-Y. Shen; W.-J. Li; S.-P. Nie; M.-Y. J. I. j. o. b. m. Xie, Sulfated polysaccharides from Cyclocarya paliurus reduce H2O2-induced oxidative stress in RAW264. 7 cells. **2015,** *80*, 410-417.
[254.] M. Jin; Y. Wang; M. Huang; Z. Lu; Y. J. C. p. Wang, Sulphation can enhance the antioxidant activity of polysaccharides produced by Enterobacter cloacae Z0206. **2014,** *99*, 624-629.
[255.] J. S. Johansen; A. K. Harris; D. J. Rychly; A. J. C. d. Ergul, Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. **2005,** *4* (1), 5.
[256.] S. Xue; X. Chen; J. Lu; L. J. C. p. Jin, Protective effect of sulfated Achyranthes bidentata polysaccharides on streptozotocin-induced oxidative stress in rats. **2009,** *75* (3), 415-419.
[257.] K. Vulic; M. S. Shoichet, Affinity-based drug delivery systems for tissue repair and regeneration. *Biomacromolecules* **2014,** *15* (11), 3867-80.
[258.] S. E. Sakiyama-Elbert, Incorporation of heparin into biomaterials. *Acta Biomater* **2014,** *10* (4), 1581-7.
[259.] B. Casu; U. Lindahl, Structure and biological interactions of heparin and heparan sulfate. *Advances in carbohydrate chemistry and biochemistry* **2001,** *57*, 159-206.
[260.] M. Guerrini; T. Agulles; A. Bisio; M. Hricovini; L. Lay; A. Naggi; L. Poletti; L. Sturiale; G. Torri; B. Casu, Minimal heparin/heparan sulfate sequences for binding to fibroblast growth factor-1. *Biochemical and biophysical research communications* **2002,** *292* (1), 222-230.
[261.] M. Maccarana; B. Casu; U. Lindahl, Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J Biol Chem* **1993,** *268* (32), 23898-23905.

[262.] M. C. Meneghetti; A. J. Hughes; T. R. Rudd; H. B. Nader; A. K. Powell; E. A. Yates; M. A. Lima, Heparan sulfate and heparin interactions with proteins. *Journal of the Royal Society Interface* **2015,** *12* (110), 20150589.
[263.] M. M. Pakulska; S. Miersch; M. S. Shoichet, Designer protein delivery: From natural to engineered affinity-controlled release systems. *Science* **2016,** *351* (6279), aac4750.
[264.] M. D. Freedman, Pharmacodynamics, clinical indications, and adverse effects of heparin. *The Journal of Clinical Pharmacology* **1992,** *32* (7), 584-596.
[265.] E. Ruvinov; J. Leor; S. Cohen, The effects of controlled HGF delivery from an affinity-binding alginate biomaterial on angiogenesis and blood perfusion in a hindlimb ischemia model. *Biomaterials* **2010,** *31* (16), 4573-4582.
[266.] J. J. Lim; J. S. J. B. Temenoff, The effect of desulfation of chondroitin sulfate on interactions with positively charged growth factors and upregulation of cartilaginous markers in encapsulated MSCs. **2013,** *34* (21), 5007-5018.
[267.] A. Zieris; R. Dockhorn; A. Röhrich; R. Zimmermann; M. Müller; P. B. Welzel; M. V. Tsurkan; J.-U. Sommer; U. Freudenberg; C. Werner, Biohybrid networks of selectively desulfated glycosaminoglycans for tunable growth factor delivery. *Biomacromolecules* **2014,** *15* (12), 4439-4446.
[268.] I. Freeman; S. J. B. Cohen, The influence of the sequential delivery of angiogenic factors from affinity-binding alginate scaffolds on vascularization. **2009,** *30* (11), 2122-2131.
[269.] E. Ruvinov; J. Leor; S. Cohen, The promotion of myocardial repair by the sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial in a model of acute myocardial infarction. *Biomaterials* **2011,** *32* (2), 565-78.
[270.] E. Öztürk; Ø. Arlov; S. Aksel; L. Li; D. M. Ornitz; G. Skjåk‐Bræk; M. J. A. f. m. Zenobi‐Wong, Sulfated hydrogel matrices direct mitogenicity and maintenance of chondrocyte phenotype through activation of FGF signaling. **2016,** *26* (21), 3649-3662.
[271.] W. Malaeb; H. F. Bahmad; W. Abou-Kheir; R. Mhanna, The sulfation of biomimetic glycosaminoglycan substrates controls binding of growth factors and subsequent neural and glial cell growth. *Biomater Sci-Uk* **2019,** *7* (10), 4283-4298.
[272.] R. Mhanna; J. Becher; M. Schnabelrauch; R. L. Reis; I. Pashkuleva, Sulfated alginate as a mimic of sulfated glycosaminoglycans: binding of growth factors and effect on stem cell behavior. *Adv Biosyst* **2017,** *1* (7), 1700043.
[273.] S. Mohammadi; S. Ramakrishna; S. Laurent; M. A. Shokrgozar; D. Semnani; D. Sadeghi; S. Bonakdar; M. Akbari, Fabrication of Nanofibrous PVA/Alginate-Sulfate Substrates for Growth Factor Delivery. *J Biomed Mater Res A* **2019,** *107* (2), 403-413.
[274.] J. Park; S. J. Lee; H. Lee; S. A. Park; J. Y. Lee, Three dimensional cell printing with sulfated alginate for improved bone morphogenetic protein-2 delivery and osteogenesis in bone tissue engineering. *Carbohydr Polym* **2018,** *196*, 217-224.
[275.] V. Hintze; A. Miron; S. Moeller; M. Schnabelrauch; H. P. Wiesmann; H. Worch; D. Scharnweber, Sulfated hyaluronan and chondroitin sulfate derivatives interact differently with human transforming growth factor-beta1 (TGF-beta1). *Acta Biomater* **2012,** *8* (6), 2144-52.

[276.] U. Hempel; V. Hintze; S. Moller; M. Schnabelrauch; D. Scharnweber; P. Dieter, Artificial extracellular matrices composed of collagen I and sulfated hyaluronan with adsorbed transforming growth factor beta1 promote collagen synthesis of human mesenchymal stromal cells. *Acta Biomater* **2012,** *8* (2), 659-66.
[277.] S. Rother; V. D. Galiazzo; D. Kilian; K. M. Fiebig; J. Becher; S. Moeller; U. Hempel; M. Schnabelrauch; J. Waltenberger; D. Scharnweber; V. Hintze, Hyaluronan/Collagen Hydrogels with Sulfated Hyaluronan for Improved Repair of Vascularized Tissue Tune the Binding of Proteins and Promote Endothelial Cell Growth. *Macromol Biosci* **2017,** *17* (11).
[278.] B. P. Purcell; I. L. Kim; V. Chuo; T. Guinen; S. M. Dorsey; J. A. Burdick, Incorporation of Sulfated Hyaluronic Acid Macromers into Degradable Hydrogel Scaffolds for Sustained Molecule Delivery. *Biomater Sci* **2014,** *2*, 693-702.
[279.] S. Thönes; S. Rother; T. Wippold; J. Blaszkiewicz; K. Balamurugan; S. Moeller; G. Ruiz-Gómez; M. Schnabelrauch; D. Scharnweber; A. Saalbach, Hyaluronan/collagen hydrogels containing sulfated hyaluronan improve wound healing by sustained release of heparin-binding EGF-like growth factor. *Acta biomaterialia* **2019,** *86*, 135-147.
[280.] N. A. Waghmare; A. Arora; A. Bhattacharjee; D. S. Katti, Sulfated polysaccharide mediated TGF-beta1 presentation in pre-formed injectable scaffolds for cartilage tissue engineering. *Carbohydr Polym* **2018,** *193*, 62-72.
[281.] A. Arora; A. Mahajan; D. S. Katti, TGF-β1 presenting enzymatically cross-linked injectable hydrogels for improved chondrogenesis. *Colloids and Surfaces B: Biointerfaces* **2017,** *159*, 838-848.
[282.] A. S. Vercoutter-Edouart; G. Dubreucq; B. Vanhoecke; C. Rigaut; F. Renaux; L. Dahri-Correia; J. Lemoine; M. Bracke; J. C. Michalski; J. Correia, Enhancement of PDGF-BB mitogenic activity on human dermal fibroblasts by biospecific dextran derivatives. *Biomaterials* **2008,** *29* (14), 2280-2292.
[283.] D. Logeart-Avramoglou; R. Huynh; F. Chaubet; L. Sedel; A. Meunier, Interaction of specifically chemically modified dextrans with transforming growth factor beta 1: potentiation of its biological activity. *Biochem Pharmacol* **2002,** *63* (2), 129-137.
[284.] C. L. Cole; S. U. Hansen; M. Barath; G. Rushton; J. M. Gardiner; E. Avizienyte; G. C. Jayson, Synthetic Heparan Sulfate Oligosaccharides Inhibit Endothelial Cell Functions Essential for Angiogenesis. *Plos One* **2010,** *5* (7).
[285.] Z. J. Wang; P. H. Hsieh; Y. M. Xu; D. Thieker; E. J. E. Chai; S. S. Xie; B. Cooley; R. J. Woods; L. L. Chi; J. Liu, Synthesis of 3-O-Sulfated Oligosaccharides to Understand the Relationship between Structures and Functions of Heparan Sulfate. *J Am Chem Soc* **2017,** *139* (14), 5249-5256.
[286.] S. Rother; V. D. Galiazzo; D. Kilian; K. M. Fiebig; J. Becher; S. Moeller; U. Hempel; M. Schnabelrauch; J. Waltenberger; D. Scharnweber, Hyaluronan/collagen hydrogels with sulfated hyaluronan for improved repair of vascularized tissue tune the binding of proteins and promote endothelial cell growth. *Macromolecular bioscience* **2017,** *17* (11), 1700154.

[287.] B. P. Purcell; I. L. Kim; V. Chuo; T. Guenin; S. M. Dorsey; J. A. Burdick, Incorporation of sulfated hyaluronic acid macromers into degradable hydrogel scaffolds for sustained molecule delivery. *Biomater Sci-Uk* **2014,** *2* (5), 693-702.
[288.] S. Lankalapalli; V. Kolapalli, Polyelectrolyte complexes: A review of their applicability in drug delivery technology. *Indian journal of pharmaceutical sciences* **2009,** *71* (5), 481.
[289.] B. C. Pessela; C. Mateo; A. V. Carrascosa; R. Fernandez-Lafuente; J. M. Guisán, Reversible immobilization of a hexameric α-galactosidase from Thermus sp. strain T2 on polymeric ionic exchangers. *Process Biochemistry* **2008,** *43* (10), 1142-1146.
[290.] T. V. Burova; N. V. Grinberg; D. R. Tur; V. S. Papkov; A. S. Dubovik; E. D. Shibanova; D. I. Bairamashvili; V. Y. Grinberg; A. R. Khokhlov, Ternary interpolyelectrolyte complexes insulin-poly (methylaminophosphazene)-dextran sulfate for oral delivery of insulin. *Langmuir* **2013,** *29* (7), 2273-2281.
[291.] M. Gericke; T. Liebert; T. Heinze, Polyelectrolyte synthesis and in situ complex formation in ionic liquids. *J Am Chem Soc* **2009,** *131* (37), 13220-13221.
[292.] T. Guo; X. Zhao; X. Xie; Z. Chen; C. Zhou; L. Wei; H. Zhang, The anti-proliferative effects of recombinant human lysozyme on human gastric cancer cells. *Journal of international medical research* **2007,** *35* (3), 353-360.
[293.] R. Huopalahti; M. Anton; R. López-Fandiño; R. Schade, *Bioactive egg compounds*. Springer: 2007.
[294.] L. Yuan; Z. Yue; H. Chen; H. Huang; T. Zhao, Biomacromolecular affinity: interactions between lysozyme and regioselectively sulfated chitosan. *Colloids Surf B Biointerfaces* **2009,** *73* (2), 346-50.
[295.] Z. Yao; C. Zhang; Q. Ping; L. L. J. C. P. Yu, A series of novel chitosan derivatives: synthesis, characterization and micellar solubilization of paclitaxel. **2007,** *68* (4), 781-792.
[296.] C. Zhang; Q. Ping; H. Zhang; J. J. C. P. Shen, Preparation of N-alkyl-O-sulfate chitosan derivatives and micellar solubilization of taxol. **2003,** *54* (2), 137-141.
[297.] G. Qu; Z. Yao; C. Zhang; X. Wu; Q. J. E. J. o. P. S. Ping, PEG conjugated N-octyl-O-sulfate chitosan micelles for delivery of paclitaxel: in vitro characterization and in vivo evaluation. **2009,** *37* (2), 98-105.
[298.] C. Zhang; G. Qu; Y. Sun; X. Wu; Z. Yao; Q. Guo; Q. Ding; S. Yuan; Z. Shen; Q. J. B. Ping, Pharmacokinetics, biodistribution, efficacy and safety of N-octyl-O-sulfate chitosan micelles loaded with paclitaxel. **2008,** *29* (9), 1233-1241.
[299.] X. Jin; R. Mo; Y. Ding; W. Zheng; C. J. M. p. Zhang, Paclitaxel-loaded N-octyl-O-sulfate chitosan micelles for superior cancer therapeutic efficacy and overcoming drug resistance. **2014,** *11* (1), 145-157.
[300.] R. Mo; X. Jin; N. Li; C. Ju; M. Sun; C. Zhang; Q. J. B. Ping, The mechanism of enhancement on oral absorption of paclitaxel by N-octyl-O-sulfate chitosan micelles. **2011,** *32* (20), 4609-4620.
[301.] M. Sandhya; V. Aparna; B. Raja; R. Jayakumar; S. J. I. j. o. b. m. Sathianarayanan, Amphotericin B loaded sulfonated chitosan nanoparticles for targeting macrophages to treat intracellular Candida glabrata infections. **2018,** *110*, 133-139.

[302.] Z. Nazemi; M. S. Nourbakhsh; S. Kiani; H. Daemi; M. K. Ashtiani; H. Baharvand, Effect of chemical composition and sulfated modification of alginate in the development of delivery systems based on electrostatic interactions for small molecule drugs. *Mater Lett* **2020,** *263*, 127235.
[303.] R. Heo; D. G. You; W. Um; K. Y. Choi; S. Jeon; J. S. Park; Y. Choi; S. Kwon; K. Kim; I. C. Kwon; D. G. Jo; Y. M. Kang; J. H. Park, Dextran sulfate nanoparticles as a theranostic nanomedicine for rheumatoid arthritis. *Biomaterials* **2017,** *131*, 15-26.
[304.] R. Mhanna; A. Kashyap; G. Palazzolo; Q. Vallmajo-Martin; J. Becher; S. Moller; M. Schnabelrauch; M. Zenobi-Wong, Chondrocyte Culture in Three Dimensional Alginate Sulfate Hydrogels Promotes Proliferation While Maintaining Expression of Chondrogenic Markers. *Tissue Eng Pt A* **2014,** *20* (9-10), 1454-1464.
[305.] T. T. T. Thanh; H. Yasunaga; R. Takano; H. Urakawa; K. Kajiwara, Molecular characteristics and gelling properties of carrageenan family - 2. Tri-sulfated and tetra-sulfated carrageenans. *Polym Bull* **2001,** *47* (3-4), 305-312.
[306.] K. Miyamoto; Y. Asakawa; Y. Arai; T. Shimizu; M. Tokita; T. Komai, Preparation of gellan sulfate as an artificial ligand for removal of extra domain A containing fibronectin. *Int J Biol Macromol* **2001,** *28* (5), 381-385.
[307.] F. C. Recuenco; K. Kobayashi; A. Ishiwa; Y. Enomoto-Rogers; N. G. V. Fundador; T. Sugi; H. Takemae; T. Iwanaga; F. Murakoshi; H. Y. Gong; A. Inomata; T. Horimoto; T. Iwata; K. Kato, Gellan sulfate inhibits Plasmodium falciparum growth and invasion of red blood cells in vitro. *Sci Rep-Uk* **2014,** *4*.
[308.] B. Matsuhiro; T. Barahona; M. V. Encinas; A. Mansilla; J. A. Ortiz, Sulfation of agarose from subantarctic Ahnfeltia plicata (Ahnfeltiales, Rhodophyta): studies of its antioxidant and anticoagulant properties in vitro and its copolymerization with acrylamide. *J Appl Phycol* **2014,** *26* (5), 2011-2019.
[309.] N. Broguiere; L. Isenmann; M. Zenobi-Wong, Novel enzymatically cross-linked hyaluronan hydrogels support the formation of 3D neuronal networks. *Biomaterials* **2016,** *99*, 47-55.
[310.] E. Öztürk; T. Stauber; C. Levinson; E. Cavalli; Ø. Arlov; M. Zenobi-Wong, Tyrosinase-crosslinked, tissue adhesive and biomimetic alginate sulfate hydrogels for cartilage repair. *Biomedical Materials* **2020,** *15* (4), 045019.
[311.] A. Ranga; M. P. Lutolf; J. Hilborn; D. A. Ossipov, Hyaluronic Acid Hydrogels Formed in Situ by Transglutaminase-Catalyzed Reaction. *Biomacromolecules* **2016,** *17* (5), 1553-1560.
[312.] S. Thones; S. Rother; T. Wippold; J. Blaszkiewicz; K. Balamurugan; S. Moeller; G. Ruiz-Gomez; M. Schnabelrauch; D. Scharnweber; A. Saalbach; J. Rademann; M. T. Pisabarro; V. Hintze; U. Anderegg, Hyaluronan/collagen hydrogels containing sulfated hyaluronan improve wound healing by sustained release of heparin-binding EGF-like growth factor. *Acta Biomater* **2019,** *86*, 135-147.
[313.] Y. Chen; V. J. Mohanraj; J. E. Parkin, Chitosan-dextran sulfate nanoparticles for delivery of an anti-angiogenesis peptide. *Lett Pept Sci* **2003,** *10* (5-6), 621-629.
[314.] M. R. Saboktakin; R. Tabatabaie; A. Maharramov; M. A. Ramazanov, Synthesis and characterization of superparamagnetic chitosan-dextran sulfate hydrogels as nano carriers for colon-specific drug delivery. *Carbohyd Polym* **2010,** *81* (2), 372-376.

[315.] N. Aggarwal; N. Altgarde; S. Svedhem; K. Zhang; S. Fischer; T. Groth, Study on multilayer structures prepared from heparin and semi-synthetic cellulose sulfates as polyanions and their influence on cellular response. *Colloid Surface B* **2014,** *116*, 93-103.
[316.] Y. L. Xie; M. J. Wang; S. J. Yao, Preparation and Characterization of Biocompatible Microcapsules of Sodium Cellulose Sulfate/Chitosan by Means of Layer-by-Layer Self-Assembly. *Langmuir* **2009,** *25* (16), 8999-9005.
[317.] O. W. Merten; H. Dautzenberg; G. E. Palfi, A New Method for the Encapsulation of Mammalian-Cells. *Cytotechnology* **1991,** *7* (2), 121-130.
[318.] G. Burgstaller; B. Oehrle; M. Gerckens; E. S. White; H. B. Schiller; O. Eickelberg, The instructive extracellular matrix of the lung: basic composition and alterations in chronic lung disease. *Eur Respir J* **2017,** *50* (1).
[319.] O. Chaudhuri; L. Gu; D. Klumpers; M. Darnell; S. A. Bencherif; J. C. Weaver; N. Huebsch; H. P. Lee; E. Lippens; G. N. Duda; D. J. Mooney, Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat Mater* **2016,** *15* (3), 326-34.
[320.] K. M. Wisdom; K. Adebowale; J. Chang; J. Y. Lee; S. Nam; R. Desai; N. S. Rossen; M. Rafat; R. B. West; L. Hodgson; O. Chaudhuri, Matrix mechanical plasticity regulates cancer cell migration through confining microenvironments. *Nat Commun* **2018,** *9* (1), 4144.
[321.] Q. Feng; S. Lin; K. Zhang; C. Dong; T. Wu; H. Huang; X. Yan; L. Zhang; G. Li; L. Bian, Sulfated hyaluronic acid hydrogels with retarded degradation and enhanced growth factor retention promote hMSC chondrogenesis and articular cartilage integrity with reduced hypertrophy. *Acta biomaterialia* **2017,** *53*, 329-342.
[322.] J. A. Buckwalter; H. J. Mankin; A. J. Grodzinsky, Articular cartilage and osteoarthritis. *Instructional Course Lectures-American Academy of Orthopaedic Surgeons* **2005,** *54*, 465.
[323.] E. A. Makris; A. H. Gomoll; K. N. Malizos; J. C. Hu; K. A. Athanasiou, Repair and tissue engineering techniques for articular cartilage. *Nat Rev Rheumatol* **2015,** *11* (1), 21.
[324.] A. J. Sophia Fox; A. Bedi; S. A. Rodeo, The basic science of articular cartilage: structure, composition, and function. *Sports health* **2009,** *1* (6), 461-468.
[325.] C. Vinatier; J. Guicheux, Cartilage tissue engineering: From biomaterials and stem cells to osteoarthritis treatments. *Annals of physical and rehabilitation medicine* **2016,** *59* (3), 139-144.
[326.] A. Weltrowski; M. L. da Silva Almeida; D. Peschel; K. Zhang; S. Fischer; T. Groth, Mitogenic Activity of Sulfated Chitosan and Cellulose Derivatives is Related to Protection of FGF‐2 from Proteolytic Cleavage. *Macromolecular bioscience* **2012,** *12* (6), 740-750.
[327.] M. Müller; E. Öztürk; Ø. Arlov; P. Gatenholm; M. J. A. o. b. e. Zenobi-Wong, Alginate sulfate–nanocellulose bioinks for cartilage bioprinting applications. **2017,** *45* (1), 210-223.
[328.] M. S. Lord; B. M. Tsoi; B. L. Farrugia; S. S. Ting; S. Baker; W. P. Wiesmann; J. M. J. J. o. M. C. B. Whitelock, Synthesis and characterization of water soluble

biomimetic chitosans for bone and cartilage tissue regeneration. **2014,** *2* (38), 6517-6526.
[329.] A. Spagnoli; L. O'Rear; R. L. Chandler; F. Granero-Molto; D. P. Mortlock; A. E. Gorska; J. A. Weis; L. Longobardi; A. Chytil; K. Shimer, TGF-β signaling is essential for joint morphogenesis. *The Journal of cell biology* **2007,** *177* (6), 1105-1117.
[330.] V. E. Santo; M. E. Gomes; J. F. Mano; R. L. Reis, Controlled release strategies for bone, cartilage, and osteochondral engineering--Part I: recapitulation of native tissue healing and variables for the design of delivery systems. *Tissue Eng Part B Rev* **2013,** *19* (4), 308-26.
[331.] T. Re'em; Y. Kaminer-Israeli; E. Ruvinov; S. Cohen, Chondrogenesis of hMSC in affinity-bound TGF-beta scaffolds. *Biomaterials* **2012,** *33* (3), 751-61.
[332.] G. Portocarrero Huang; R. Menezes; R. Vincent; W. Hammond; L. Rizio; G. Collins; T. L. J. T. E. P. A. Arinzeh, Gelatin scaffolds containing partially sulfated cellulose promote mesenchymal stem cell chondrogenesis. *Tissue Eng Part A* **2017,** *23* (17-18), 1011-1021.
[333.] G. P. Huang; A. Molina; N. Tran; G. Collins; T. L. J. J. o. t. e. Arinzeh; r. medicine, Investigating cellulose derived glycosaminoglycan mimetic scaffolds for cartilage tissue engineering applications. **2018,** *12* (1), e592-e603.
[334.] R. A. Carano; E. H. Filvaroff, Angiogenesis and bone repair. *Drug discovery today* **2003,** *8* (21), 980-989.
[335.] J. R. Porter; T. T. Ruckh; K. C. Popat, Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. *Biotechnol Progr* **2009,** *25* (6), 1539-1560.
[336.] A. J. Salgado; O. P. Coutinho; R. L. Reis, Bone tissue engineering: state of the art and future trends. *Macromolecular bioscience* **2004,** *4* (8), 743-765.
[337.] D. Chen; M. Zhao; G. R. Mundy, Bone morphogenetic proteins. *Growth factors* **2004,** *22* (4), 233-241.
[338.] A. Carreira; F. Lojudice; E. Halcsik; R. Navarro; M. Sogayar; J. Granjeiro, Bone morphogenetic proteins: facts, challenges, and future perspectives. *Journal of dental research* **2014,** *93* (4), 335-345.
[339.] T. Takada; T. Katagiri; M. Ifuku; N. Morimura; M. Kobayashi; K. Hasegawa; A. Ogamo; R. Kamijo, Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins. *J Biol Chem* **2003,** *278* (44), 43229-43235.
[340.] C. Nelson-Piercy, Heparin-induced osteoporosis. *Scandinavian Journal of Rheumatology* **1998,** *27* (sup107), 68-71.
[341.] J. T. Street; M. McGrath; K. O'Regan; A. Wakai; A. McGuinness; H. P. Redmond, Thromboprophylaxis using a low molecular weight heparin delays fracture repair. *Clinical Orthopaedics and Related Research®* **2000,** *381*, 278-289.
[342.] D. Peschel; K. Zhang; S. Fischer; T. Groth, Modulation of osteogenic activity of BMP-2 by cellulose and chitosan derivatives. *Acta biomaterialia* **2012,** *8* (1), 183-193.
[343.] H. Zhou; J. Qian; J. Wang; W. Yao; C. Liu; J. Chen; X. Cao, Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-N, 6-O-sulfated chitosan in vitro and in vivo. *Biomaterials* **2009,** *30* (9), 1715-24.

[344.] L. Cao; J. Wang; J. Hou; W. Xing; C. J. B. Liu, Vascularization and bone regeneration in a critical sized defect using 2-N, 6-O-sulfated chitosan nanoparticles incorporating BMP-2. *Biomaterials* **2014,** *35* (2), 684-698.
[345.] L. Cao; J. A. Werkmeister; J. Wang; V. Glattauer; K. M. McLean; C. J. B. Liu, Bone regeneration using photocrosslinked hydrogel incorporating rhBMP-2 loaded 2-N, 6-O-sulfated chitosan nanoparticles. **2014,** *35* (9), 2730-2742.
[346.] X. Kong; J. Wang; L. Cao; Y. Yu; C. J. C. Liu; S. B. Biointerfaces, Enhanced osteogenesis of bone morphology protein-2 in 2-N, 6-O-sulfated chitosan immobilized PLGA scaffolds. **2014,** *122*, 359-367.
[347.] S. Stegen; N. van Gastel; G. Carmeliet, Bringing new life to damaged bone: the importance of angiogenesis in bone repair and regeneration. *Bone* **2015,** *70*, 19-27.
[348.] Y. Pan; J. Chen; Y. Yu; K. Dai; J. Wang; C. J. B. s. Liu, Enhancement of BMP-2-mediated angiogenesis and osteogenesis by 2-N, 6-O-sulfated chitosan in bone regeneration. **2018,** *6* (2), 431-439.
[349.] L. Cao; X. Kong; S. Lin; S. Zhang; J. Wang; C. Liu; X. Jiang, Synergistic effects of dual growth factor delivery from composite hydrogels incorporating 2-N, 6-O-sulphated chitosan on bone regeneration. *Artificial cells, nanomedicine, and biotechnology* **2018,** *46* (sup3), S1-S17.
[350.] W. Ariyoshi; T. Takahashi; T. Kanno; H. Ichimiya; K. Shinmyouzu; H. Takano; T. Koseki; T. Nishihara, Heparin inhibits osteoclastic differentiation and function. *Journal of cellular biochemistry* **2008,** *103* (6), 1707-1717.
[351.] U. Hempel; S. Möller; C. Noack; V. Hintze; D. Scharnweber; M. Schnabelrauch; P. Dieter, Sulfated hyaluronan/collagen I matrices enhance the osteogenic differentiation of human mesenchymal stromal cells in vitro even in the absence of dexamethasone. *Acta biomaterialia* **2012,** *8* (11), 4064-4072.
[352.] J. R. Schmidt; S. Vogel; S. Moeller; S. Kalkhof; K. Schubert; M. von Bergen; U. Hempel, Sulfated hyaluronic acid and dexamethasone possess a synergistic potential in the differentiation of osteoblasts from human bone marrow stromal cells. *J Cell Biochem* **2018**.
[353.] U. Hempel; S. Moller; C. Noack; V. Hintze; D. Scharnweber; M. Schnabelrauch; P. Dieter, Sulfated hyaluronan/collagen I matrices enhance the osteogenic differentiation of human mesenchymal stromal cells in vitro even in the absence of dexamethasone. *Acta Biomater* **2012,** *8* (11), 4064-72.
[354.] B. Li; Z. Lin; M. Mitsi; Y. Zhang; V. Vogel, Heparin-induced conformational changes of fibronectin within the extracellular matrix promote hMSC osteogenic differentiation. *Biomater Sci-Uk* **2015,** *3* (1), 73-84.
[355.] S. Vogel; S. Arnoldini; S. Moller; M. Schnabelrauch; U. Hempel, Sulfated hyaluronan alters fibronectin matrix assembly and promotes osteogenic differentiation of human bone marrow stromal cells. *Sci Rep* **2016,** *6*, 36418.
[356.] S. Kliemt; C. Lange; W. Otto; V. Hintze; S. Möller; M. von Bergen; U. Hempel; S. Kalkhof, Sulfated hyaluronan containing collagen matrices enhance cell-matrix-interaction, endocytosis, and osteogenic differentiation of human mesenchymal stromal cells. *Journal of Proteome Research* **2013,** *12*, 378-389.

[357.] U. Hempel; C. Preissler; S. Vogel; S. Moller; V. Hintze; J. Becher; M. Schnabelrauch; M. Rauner; L. C. Hofbauer; P. Dieter, Artificial extracellular matrices with oversulfated glycosaminoglycan derivatives promote the differentiation of osteoblast-precursor cells and premature osteoblasts. *Biomed Res Int* **2014,** *2014*, 938368.
[358.] J. Salbach - Hirsch; N. Ziegler; S. Thiele; S. Moeller; M. Schnabelrauch; V. Hintze; D. Scharnweber; M. Rauner; L. C. Hofbauer, Sulfated glycosaminoglycans support osteoblast functions and concurrently suppress osteoclasts. *Journal of cellular biochemistry* **2014,** *115* (6), 1101-1111.
[359.] J. Salbach; S. Kliemt; M. Rauner; T. D. Rachner; C. Goettsch; S. Kalkhof; M. von Bergen; S. Möller; M. Schnabelrauch; V. Hintze, The effect of the degree of sulfation of glycosaminoglycans on osteoclast function and signaling pathways. *Biomaterials* **2012,** *33* (33), 8418-8429.
[360.] Y. Forster; R. Bernhardt; V. Hintze; S. Moller; M. Schnabelrauch; D. Scharnweber; S. Rammelt, Collagen/glycosaminoglycan coatings enhance new bone formation in a critical size bone defect - A pilot study in rats. *Mater Sci Eng C Mater Biol Appl* **2017,** *71*, 84-92.
[361.] A. K. Picke; J. Salbach-Hirsch; V. Hintze; S. Rother; M. Rauner; C. Kascholke; S. Moller; R. Bernhardt; S. Rammelt; M. T. Pisabarro; G. Ruiz-Gomez; M. Schnabelrauch; M. Schulz-Siegmund; M. C. Hacker; D. Scharnweber; C. Hofbauer; L. C. Hofbauer, Sulfated hyaluronan improves bone regeneration of diabetic rats by binding sclerostin and enhancing osteoblast function. *Biomaterials* **2016,** *96*, 11-23.
[362.] J. Salbach-Hirsch; S. A. Samsonov; V. Hintze; C. Hofbauer; A.-K. Picke; M. Rauner; J.-P. Gehrcke; S. Moeller; M. Schnabelrauch; D. Scharnweber, Structural and functional insights into sclerostin-glycosaminoglycan interactions in bone. *Biomaterials* **2015,** *67*, 335-345.
[363.] J. J. McMurray, Clinical practice. Systolic heart failure. *N Engl J Med* **2010,** *362* (3), 228-38.
[364.] E. Ruvinov; T. Harel-Adar; S. Cohen, Bioengineering the infarcted heart by applying bio-inspired materials. *J Cardiovasc Transl Res* **2011,** *4* (5), 559-74.
[365.] K. L. Christman; R. J. Lee, Biomaterials for the treatment of myocardial infarction. *J Am Coll Cardiol* **2006,** *48* (5), 907-13.
[366.] S. Miyagawa; M. Roth; A. Saito; Y. Sawa; S. Kostin, Tissue-engineered cardiac constructs for cardiac repair. *Ann Thorac Surg* **2011,** *91* (1), 320-9.
[367.] D. M. Nelson; Z. Ma; K. L. Fujimoto; R. Hashizume; W. R. Wagner, Intra-myocardial biomaterial injection therapy in the treatment of heart failure: Materials, outcomes and challenges. *Acta Biomater* **2011,** *7* (1), 1-15.
[368.] A. Abbate; R. Bussani; M. S. Amin; G. W. Vetrovec; A. Baldi, Acute myocardial infarction and heart failure: role of apoptosis. *Int J Biochem Cell Biol* **2006,** *38* (11), 1834-40.
[369.] A. Leask, TGFbeta, cardiac fibroblasts, and the fibrotic response. *Cardiovasc Res* **2007,** *74* (2), 207-12.
[370.] M. Nian; P. Lee; N. Khaper; P. Liu, Inflammatory cytokines and postmyocardial infarction remodeling. *Circ Res* **2004,** *94* (12), 1543-53.

[371.] M. A. Renault; D. W. Losordo, Therapeutic myocardial angiogenesis. *Microvasc Res* **2007,** *74* (2-3), 159-71.
[372.] E. Ruvinov; T. Dvir; J. Leor; S. Cohen, Myocardial repair: from salvage to tissue reconstruction. *Expert Rev Cardiovasc Ther* **2008,** *6* (5), 669-86.
[373.] R. J. Tomanek; W. Zheng; X. Yue, Growth factor activation in myocardial vascularization: therapeutic implications. *Mol Cell Biochem* **2004,** *264* (1-2), 3-11.
[374.] A. Zampetaki; J. P. Kirton; Q. Xu, Vascular repair by endothelial progenitor cells. *Cardiovasc Res* **2008,** *78* (3), 413-21.
[375.] S. Liao; D. Porter; A. Scott; G. Newman; T. Doetschman; J. Schultz Jel, The cardioprotective effect of the low molecular weight isoform of fibroblast growth factor-2: the role of JNK signaling. *J Mol Cell Cardiol* **2007,** *42* (1), 106-20.
[376.] E. Conti; C. Carrozza; E. Capoluongo; M. Volpe; F. Crea; C. Zuppi; F. Andreotti, Insulin-like growth factor-1 as a vascular protective factor. *Circulation* **2004,** *110* (15), 2260-5.
[377.] M. Harada; Y. Qin; H. Takano; T. Minamino; Y. Zou; H. Toko; M. Ohtsuka; K. Matsuura; M. Sano; J. Nishi; K. Iwanaga; H. Akazawa; T. Kunieda; W. Zhu; H. Hasegawa; K. Kunisada; T. Nagai; H. Nakaya; K. Yamauchi-Takihara; I. Komuro, G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. *Nat Med* **2005,** *11* (3), 305-11.
[378.] X. Hu; S. Dai; W. J. Wu; W. Tan; X. Zhu; J. Mu; Y. Guo; R. Bolli; G. Rokosh, Stromal cell derived factor-1 alpha confers protection against myocardial ischemia/reperfusion injury: role of the cardiac stromal cell derived factor-1 alpha CXCR4 axis. *Circulation* **2007,** *116* (6), 654-63.
[379.] G. Vera Janavel; A. Crottogini; P. Cabeza Meckert; L. Cuniberti; A. Mele; M. Papouchado; N. Fernandez; A. Bercovich; M. Criscuolo; C. Melo; R. Laguens, Plasmid-mediated VEGF gene transfer induces cardiomyogenesis and reduces myocardial infarct size in sheep. *Gene Ther* **2006,** *13* (15), 1133-42.
[380.] V. Jayasankar; Y. J. Woo; L. T. Bish; T. J. Pirolli; S. Chatterjee; M. F. Berry; J. Burdick; T. J. Gardner; H. L. Sweeney, Gene transfer of hepatocyte growth factor attenuates postinfarction heart failure. *Circulation* **2003,** *108 Suppl 1*, II230-6.
[381.] T. Dvir; A. Kedem; E. Ruvinov; O. Levy; I. Freeman; N. Landa; R. Holbova; M. S. Feinberg; S. Dror; Y. Etzion; J. Leor; S. Cohen, Prevascularization of cardiac patch on the omentum improves its therapeutic outcome. *Proc Natl Acad Sci U S A* **2009,** *106* (35), 14990-5.
[382.] E. Ruvinov; I. Freeman; R. Fredo; S. Cohen, Spontaneous Coassembly of Biologically Active Nanoparticles via Affinity Binding of Heparin-Binding Proteins to Alginate-Sulfate. *Nano Lett* **2016,** *16* (2), 883-8.
[383.] J. Schmidt; M. K. Lee; E. Ko; J. H. Jeong; L. A. DiPietro; H. Kong, Alginate Sulfates Mitigate Binding Kinetics of Proangiogenic Growth Factors with Receptors toward Revascularization. *Mol Pharm* **2016,** *13* (7), 2148-54.
[384.] W. Wang; X. J. Huang; J. D. Cao; P. Lan; W. Wu, Immobilization of sodium alginate sulfates on polysulfone ultrafiltration membranes for selective adsorption of low-density lipoprotein. *Acta Biomater* **2014,** *10* (1), 234-43.

[385.] T. Bejerano; S. Etzion; S. Elyagon; Y. Etzion; S. Cohen, Nanoparticle Delivery of miRNA-21 Mimic to Cardiac Macrophages Improves Myocardial Remodeling after Myocardial Infarction. *Nano Lett* **2018,** *18* (9), 5885-5891.
[386.] F. J. Sheedy, Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response. *Front Immunol* **2015,** *6*, 19.
[387.] P. Fiorante; Y. Banz; P. J. Mohacsi; A. Kappeler; W. A. Wuillemin; P. Macchiarini; A. Roos; M. R. Daha; T. Schaffner; A. Haeberli; G. M. Mazmanian; R. Rieben, Low molecular weight dextran sulfate prevents complement activation and delays hyperacute rejection in pig-to-human xenotransplantation models. *Xenotransplantation* **2001,** *8* (1), 24-35.
[388.] T. Laumonier; P. J. Mohacsi; K. M. Matozan; Y. Banz; A. Haeberli; E. Y. Korchagina; N. V. Bovin; B. Vanhove; R. Rieben, Endothelial cell protection by dextran sulfate: a novel strategy to prevent acute vascular rejection in xenotransplantation. *Am J Transplant* **2004,** *4* (2), 181-7.
[389.] Y. Banz; O. M. Hess; S. C. Robson; D. Mettler; P. Meier; A. Haeberli; E. Csizmadia; E. Y. Korchagina; N. V. Bovin; R. Rieben, Locally targeted cytoprotection with dextran sulfate attenuates experimental porcine myocardial ischaemia/reperfusion injury. *Eur Heart J* **2005,** *26* (21), 2334-43.
[390.] T. Gajanayake; B. Sawitzki; K. Matozan; E. Y. Korchagina; M. Lehmann; H. D. Volk; R. Rieben, Dextran sulfate facilitates anti-CD4 mAb-induced long-term rat cardiac allograft survival after prolonged cold ischemia. *Am J Transplant* **2008,** *8* (6), 1151-62.
[391.] L. Hiebert; T. Ping, Protective effect of dextran sulfate and heparin on adult rat cardiomyocytes damaged by free radicals. *J Mol Cell Cardiol* **1997,** *29* (1), 229-35.
[392.] B. P. Purcell; D. Lobb; M. B. Charati; S. M. Dorsey; R. J. Wade; K. N. Zellars; H. Doviak; S. Pettaway; C. B. Logdon; J. A. Shuman; P. D. Freels; J. H. Gorman, 3rd; R. C. Gorman; F. G. Spinale; J. A. Burdick, Injectable and bioresponsive hydrogels for on-demand matrix metalloproteinase inhibition. *Nat Mater* **2014,** *13* (6), 653-61.
[393.] J. M. Chupa; A. M. Foster; S. R. Sumner; S. V. Madihally; H. W. Matthew, Vascular cell responses to polysaccharide materials: in vitro and in vivo evaluations. *Biomaterials* **2000,** *21* (22), 2315-22.
[394.] T. Kinnunen; E. Raulo; R. Nolo; M. Maccarana; U. Lindahl; H. Rauvala, Neurite outgrowth in brain neurons induced by heparin-binding growth-associated molecule (HB-GAM) depends on the specific interaction of HB-GAM with heparan sulfate at the cell surface. *J Biol Chem* **1996,** *271*, 2243-2248.
[395.] V. P. Swarup; C. P. Mencio; V. Hlady; B. Kuberan, Sugar glues for broken neurons. *Biomol Concepts* **2013,** *4* (3), 233-57.
[396.] B. E. Thacker; E. Seamen; R. Lawrence; M. W. Parker; Y. Xu; J. Liu; C. W. Vander Kooi; J. D. Esko, Expanding the 3-O-Sulfate Proteome--Enhanced Binding of Neuropilin-1 to 3-O-Sulfated Heparan Sulfate Modulates Its Activity. *ACS Chem Biol* **2016,** *11* (4), 971-80.
[397.] F. Zhang; H. A. Moniz; B. Walcott; K. W. Moremen; R. J. Linhardt; L. Wang, Characterization of the interaction between Robo1 and heparin and other glycosaminoglycans. *Biochimie* **2013,** *95* (12), 2345-53.

[398.] P. Bovolenta; I. Fernaud-Espinosa, Nervous system proteoglycans as modulators of neurite

outgrowth. *Progress in Neurobiology* **2000,** *61*, 113-132.
[399.] J. W. Fawcett, The extracellular matrix in plasticity and regeneration after CNS injury and neurodegenerative disease. *Prog Brain Res* **2015,** *218*, 213-26.
[400.] J. C. Kwok; F. Afshari; G. Garcia-Alias; J. W. Fawcett, Proteoglycans in the central nervous system: plasticity, regeneration and their stimulation with chondroitinase ABC. *Restor Neurol Neurosci* **2008,** *26* (2-3), 141-145.
[401.] K. Nakanishi; M. Ito; Y. Sato; A. Oohira, A highly-sulfated chondroitin sulfate, CS-E, adsorbs specifically to neurons with nuclear condensation. *Neuroscience Research* **2012,** *74* (3-4), 223-229.
[402.] F. Properzi; D. Carulli; R. A. Asher; E. Muir; L. M. Camargo; T. H. van Kuppevelt; G. B. ten Dam; Y. Furukawa; T. Mikami; K. Sugahara; T. Toida; H. M. Geller; J. W. Fawcett, Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia. *Eur J Neurosci* **2005,** *21* (2), 378-90.
[403.] A. J. Hayes; J. Melrose, Glycans and glycosaminoglycans in neurobiology: key regulators of neuronal cell function and fate. *Biochem J* **2018,** *475* (15), 2511-2545.
[404.] M. Tham; S. Ramasamy; H. T. Gan; A. Ramachandran; A. Poonepalli; Y. H. Yu, CSPG Is a secreted factor that stimulates neural stem cell survival possibly by enhanced EGFR signaling. *Plos One* **2010,** *5* (12), e15341.
[405.] M. Hikino; T. Mikami; A. Faissner, Oversulfated dermatan sulfate exhibits neurite outgrowth-promoting activity toward embryonic mouse hippocampal neurons: Implications of dermatan sulfate in neuritogenesis in the brain. *J Biol Chem* **2003,** *278*, 43744-43754.
[406.] K. Sugahara; T. Mikami, Chondroitin/dermatan sulfate in the central nervous system. *Current Opinion in Structural Biology* **2007,** *17* (5), 536-545.
[407.] J. Melrose, Keratan sulfate (KS)-proteoglycans and neuronal regulation in health and disease: the importance of KS-glycodynamics and interactive capability with neuroregulatory ligands. *J Neurochem* **2019,** *149* (2), 170-194.
[408.] J.-P. Li; M. L. Escobar Galvis; F. Gong; X. Zhang; E. Zcharia; S. Metzger; I. Vlodavsky; R. Kisilevsky; U. Lindahl, In vivo fragmentation of heparan sulfate by heparanase overexpression renders mice resistant to amyloid protein A amyloidosis. *PNAS* **2005,** *102* (18), 6473-6477.
[409.] J. R. Higginson; S. M. Thompson; A. Santos-Silva; S. E. Guimond; J. E. Turnbull; S. C. Barnett, Differential sulfation remodelling of heparan sulfate by extracellular 6-O-sulfatases regulates fibroblast growth factor-induced boundary formation by glial cells: Implications for glial cell transplantation. *Journal of Neuroscience* **2012,** *32* (45), 15902-15912.
[410.] D. Carulli; T. Laabs; H. M. Geller; J. W. Fawcett, Chondroitin sulfate proteoglycans in neural development and regeneration. *Current Opinion in Neurobiology* **2005,** *15* (1), 116-120.

[411.] S. Imagama; K. Sakamoto; R. Tauchi; R. Shinjo; T. Ohgomori; Z. Ito; H. Zhang; Y. Nishida; N. Asami; S. Takeshita; N. Sugiura; H. Watanabe; T. Yamashita; N. Ishiguro; Y. Matsuyama; K. Kadomatsu, Keratan sulfate restricts neural plasticity after spinal cord injury. *J Neurosci* **2011,** *31* (47), 17091-102.
[412.] R. A. Asher; D. A. Morgenstern; L. D. F. Moon; J. W. Fawcett, Chondroitin sulphate proteoglycans: inhibitory components of the glial scar. *Progress in Brain Research* **2001,** *132*, 611-619.
[413.] D. A. Morgenstern; R. A. Asher; J. W. Fawcett, Chondroitin sulphate proteoglycans in the CNS injury response. *Progress in Brain Research* **2002,** *137*, 313-332.
[414.] P. Zhang; H. Lu; R. T. Peixoto; M. K. Pines; Y. Ge; S. Oku; T. J. Siddiqui; Y. Xie; W. Wu; S. Archer-Hartmann; K. Yoshida; K. F. Tanaka; A. R. Aricescu; P. Azadi; M. D. Gordon; B. L. Sabatini; R. O. L. Wong; A. M. Craig, Heparan Sulfate Organizes Neuronal Synapses through Neurexin Partnerships. *Cell* **2018,** *174* (6), 1450-1464 e23.
[415.] S. S. Deepa; S. Yamada; M. Zako; O. Goldberger; K. Sugahara, Chondroitin sulfate chains on syndecan-1 and syndecan-4 from normal murine mammary gland epithelial cells are structurally and functionally distinct and cooperate with heparan sulfate chains to bind growth factors. A novel function to control binding of midkine, pleiotrophin, and basic fibroblast growth factor. *J Biol Chem* **2004,** *279* (36), 37368-76.
[416.] M. Fan; Y. Ma; H. P. Tan; Y. Jia; S. Y. Zou; S. X. Guo; M. Zhao; H. Huang; Z. H. Ling; Y. Chen; X. H. Hu, Covalent and injectable chitosan-chondroitin sulfate hydrogels embedded with chitosan microspheres for drug delivery and tissue engineering. *Mat Sci Eng C-Mater* **2017,** *71*, 67-74.
[417.] T. Muramatsu, Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases. *Proc Jpn Acad Ser B Phys Biol Sci* **2010,** *86* (4), 410-25.
[418.] F. Irie; M. Okuno; K. Matsumoto; E. B. Pasquale; Y. Yamaguchi, Heparan sulfate regulates ephrin-A3/EphA receptor signaling. *Proc Natl Acad Sci U S A* **2008,** *105* (34), 12307-12.
[419.] E. Kastenhuber; U. Kern; J. L. Bonkowsky; C. B. Chien; W. Driever; J. Schweitzer, Netrin-DCC, Robo-Slit, and Heparan Sulfate Proteoglycans Coordinate Lateral Positioning of Longitudinal Dopaminergic Diencephalospinal Axons. *Journal of Neuroscience* **2009,** *29* (28), 8914-8926.
[420.] D. B. Kantor; O. Chivatakarn; K. L. Peer; S. F. Oster; M. Inatani; M. J. Hansen; J. G. Flanagan; Y. Yamaguchi; D. W. Sretavan; R. J. Giger; A. L. Kolodkin, Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron* **2004,** *44* (6), 961-75.
[421.] Y. Mii; T. Yamamoto; R. Takada; S. Mizumoto; M. Matsuyama; S. Yamada; S. Takada; M. Taira, Roles of two types of heparan sulfate clusters in Wnt distribution and signaling in Xenopus. *Nat Commun* **2017,** *8* (1), 1973.
[422.] C. Fuerer; S. J. Habib; R. Nusse, A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. *Dev Dyn* **2010,** *239* (1), 184-90.
[423.] K. Saied-Santiago; R. A. Townley; J. D. Attonito; D. S. da Cunha; C. A. Diaz-Balzac; E. Tecle; H. E. Bulow, Coordination of Heparan Sulfate Proteoglycans with Wnt

Signaling To Control Cellular Migrations and Positioning in Caenorhabditis elegans. *Genetics* **2017,** *206* (4), 1951-1967.
[424.] J. J. van Gemst; M. Kouwenberg; A. Rops; T. H. van Kuppevelt; J. H. Berden; T. J. Rabelink; M. A. Loeven; J. van der Vlag, Differential binding of chemokines CXCL1, CXCL2 and CCL2 to mouse glomerular endothelial cells reveals specificity for distinct heparan sulfate domains. *Plos One* **2018,** *13* (9), e0201560.
[425.] N. B. Schwartz; M. S. Domowicz, Chemistry and function of glycosaminoglycans in the nervous system. *Advances in Neurobiology* **2014,** *9*, 89-115.
[426.] K. Saied-Santiago; H. E. Bulow, Diverse roles for glycosaminoglycans in neural patterning. *Dev Dyn* **2018,** *247* (1), 54-74.
[427.] E. J. Bradbury; E. R. Burnside, Moving beyond the glial scar for spinal cord repair. *Nat Commun* **2019,** *10* (1), 3879.
[428.] B. S. Khakh; B. Deneen, The Emerging Nature of Astrocyte Diversity. *Annu Rev Neurosci* **2019,** *42*, 187-207.
[429.] D. Lukovic; M. Stojkovic; V. Moreno-Manzano; P. Jendelova; E. Sykova; S. S. Bhattacharya; S. Erceg, Concise review: reactive astrocytes and stem cells in spinal cord injury: good guys or bad guys? *Stem Cells* **2015,** *33* (4), 1036-41.
[430.] I. Matias; J. Morgado; F. C. A. Gomes, Astrocyte Heterogeneity: Impact to Brain Aging and Disease. *Front Aging Neurosci* **2019,** *11*, 59.
[431.] S. Wiese; M. Karus; A. Faissner, Astrocytes as a source for extracellular matrix molecules and cytokines. *Frontiers in Pharmacology* **2012,** *3*, 120.
[432.] T. Yamada; R. Sawada; T. Tsuchiya, The effect of sulfated hyaluronan on the morphological transformation and activity of cultured human astrocytes. *Biomaterials* **2008,** *29* (26), 3503-13.
[433.] R. Menezes; S. Hashemi; R. Vincent; G. Collins; J. Meyer; M. Foston; T. L. Arinzeh, Investigation of glycosaminoglycan mimetic scaffolds for neurite growth. *Acta Biomater* **2019,** *90*, 169-178.
[434.] G. A. McCanney; M. A. McGrath; T. D. Otto; R. Burchmore; E. A. Yates; C. D. Bavington; H. J. Willison; J. E. Turnbull; S. C. Barnett, Low sulfated heparins target multiple proteins for central nervous system repair. *Glia* **2019,** *67* (4), 668-687.
[435.] F. Gottrup, A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. *American Journal of Surgery* **2004,** *187* (5A), 38S-43S.
[436.] W. Yao; M. Chen; X. Dou; H. Jin; X. Zhang; Y. Zhu; M. Sha; Z. Liu; X. Meng; L. Zhang; S. Zhu; Z. Li, Unravel a neuroactive sHA sulfation pattern with neurogenesis activity by a library of defined oligosaccharides. *Eur J Med Chem* **2019,** *163*, 583-596.
[437.] S. E. Tully; R. Mabon; C. I. Gama; S. M. Tsai; X. Liu; L. C. Hsieh-Wilson, A chondroitin sulfate small molecule that stimulates neuronal growth. *JACS* **2004,** *126*, 7736-7737.
[438.] S. Sarrazin; W. C. Lamanna; J. D. Esko, Heparan sulfate proteoglycans. *Cold Spring Harbor Perspectives in Biology* **2011,** *3* (7), a004952.
[439.] D. Barritault; M. Gilbert-Sirieix; K. L. Rice; F. Siñeriz; D. Papy-Garcia; C. Baudouin; P. Desgranges; G. Zakine; J.-L. Saffar; J. van Neck, RGTA(®) or

ReGeneraTing Agents mimic heparan sulfate in regenerative medicine: from concept to curing patients. *Glycoconjugate J* **2017,** *34* (3), 325-338.
[440.] G. S. Schultz; J. M. Davidson; R. S. Kirsner; P. Bornstein; I. M. Herman, Dynamic reciprocity in the wound microenvironment. *Wound Repair and Regeneration* **2011,** *19* (2), 134-148.
[441.] V. Barbier-Chassefiere; S. Garcia-Filipe; X. L. Yue; M. E. Kerros; E. Petit; P. Kern; J. L. Saffar; D. Papy-Garcia; J. P. Caruelle; D. Barritault, Matrix therapy in regenerative medicine, a new approach to chronic wound healing. *J Biomed Mater Res A* **2009,** *90* (3), 641-7.
[442.] A. Meddahi; F. Blanquaert; J. L. Saffar; M. L. Colombier; J. P. Caruelle; J. Josefonvicz; D. Barritault, New Approaches to Tissue Regeneration and Repair. *Pathology - Research and Practice* **1994,** *190* (9-10), 923-928.
[443.] A. Meddahi; J. P. Caruelle; L. Gold; Y. Rosso; D. Barritault, New concepts in tissue repair: skin as an example. *Diabetes Metabolism* **1996,** *22* (4), 274-278.
[444.] D. Barritault; P. Desgranges; A. Meddahi-Pellé; J.-M. Denoix; J.-L. Saffar, RGTA®-based matrix therapy – A new branch of regenerative medicine in locomotion. *Joint Bone Spine* **2017,** *84* (3), 283-292.
[445.] Y. Ikeda; S. Charef; M. O. Ouidja; V. Barbier-Chassefiere; F. Sineriz; A. Duchesnay; H. Narasimprakash; I. Martelly; P. Kern; D. Barritault; E. Petit; D. Papy-Garcia, Synthesis and biological activities of a library of glycosaminoglycans mimetic oligosaccharides. *Biomaterials* **2011,** *32* (3), 769-76.
[446.] V. Rouet; Y. Hamma-Kourbali; E. Petit; P. Panagopoulou; P. Katsoris; D. Barritault; J.-P. Caruelle; J. Courty, A Synthetic Glycosaminoglycan Mimetic Binds Vascular Endothelial Growth Factor and Modulates Angiogenesis. **2005,** *280* (38), 32792-32800.
[447.] V. Rouet; A. Meddahi-Pelle; H. Q. Miao; I. Vlodavsky; J. P. Caruelle; D. Barritault, Heparin-like synthetic polymers, named RGTAs, mimic biological effects of heparin in vitro. *J Biomed Mater Res A* **2006,** *78* (4), 792-7.
[448.] J. van Neck; B. Tuk; D. Barritault; M. Tong, Heparan Sulfate Proteoglycan Mimetics Promote Tissue Regeneration: An Overview. In *Tissue Regeneration - From Basic Biology to Clinical Application*, 2012.
[449.] S. Charef; M. Tulliez; L. Esmilaire; J. Courty; D. Papy-Garcia, Toxicological evaluation of RGTA OTR4120, a heparan sulfate mimetic. *Food and Chemical Toxicology* **2010,** *48* (7), 1965-1968.
[450.] C.-M. Timaru; C. Stefan; D. A. Iliescu; A. De Simone; M. Batras, Matrix regenerative therapy. *Rom J Ophthalmol* **2017,** *61* (1), 2-10.
[451.] S. Garcia-Filipe; V. Barbier-Chassefiere; C. Alexakis; E. Huet; D. Ledoux; M. E. Kerros; E. Petit; D. Barritault; J. P. Caruelle; P. Kern, RGTA OTR4120, a heparan sulfate mimetic, is a possible long-term active agent to heal burned skin. *J Biomed Mater Res A* **2007,** *80* (1), 75-84.
[452.] M. Tong; B. Tuk; I. M. Hekking; M. Vermeij; D. Barritault; J. W. van Neck, Stimulated neovascularization, inflammation resolution and collagen maturation in healing rat cutaneous wounds by a heparan sulfate glycosaminoglycan mimetic, OTR4120. *Wound Repair Regen* **2009,** *17* (6), 840-52.

[453.] M. Tong; M. M. Zbinden; I. J. Hekking; M. Vermeij; D. Barritault; J. W. van Neck, RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds. *Wound Repair Regen* **2008,** *16* (2), 294-9.
[454.] M. Tong; B. Tuk; I. M. Hekking; M. M. Pleumeekers; M. B. Boldewijn; S. E. Hovius; J. W. van Neck, Heparan sulfate glycosaminoglycan mimetic improves pressure ulcer healing in a rat model of cutaneous ischemia-reperfusion injury. *Wound Repair Regen* **2011,** *19* (4), 505-14.
[455.] M. Tong; B. Tuk; P. Shang; I. M. Hekking; E. M. Fijneman; M. Guijt; S. E. Hovius; J. W. van Neck, Diabetes-impaired wound healing is improved by matrix therapy with heparan sulfate glycosaminoglycan mimetic OTR4120 in rats. *Diabetes* **2012,** *61* (10), 2633-41.
[456.] D. Logeart-Avramoglou; J. Jozefonvicz, Carboxymethyl benzylamide sulfonate dextrans (CMDBS), A family of biospecific polymers endowed with numerous biological properties: a review. *Journal of Biomedical Materials Research* **1999,** *48* (4), 578-590.
[457.] T. H. Nguyen; S. H. Kim; C. G. Decker; D. Y. Wong; J. A. Loo; H. D. Maynard, A heparin-mimicking polymer conjugate stabilizes basic fibroblast growth factor. *Nat Chem* **2013,** *5* (3), 221-7.
[458.] A. van der Smissen; V. Hintze; D. Scharnweber; S. Moeller; M. Schnabelrauch; A. Majok; J. C. Simon; U. Anderegg, Growth promoting substrates for human dermal fibroblasts provided by artificial extracellular matrices composed of collagen I and sulfated glycosaminoglycans. *Biomaterials* **2011,** *32* (34), 8938-8946.
[459.] S. Rother; S. Samsonov; T. Hofmann; J. Blaszkiewicz; S. Köhling; S. Moeller; M. Schnabelrauch; J. Rademann; S. Kalkhof; M. von Bergen; M. T. Pisabarro; D. Scharnweber; V. Hintze, Structural and functional insights into the interaction of sulfated glycosaminoglycans with tissue inhibitor of metalloproteinase-3 – A possible regulatory role on extracellular matrix homeostasis. *Acta Biomaterialia* **2016,** *46*, 143-154.
[460.] G. Sawicki; Y. Marcoux; K. Sarkhosh; E. E. Tredget; A. Ghahary, Interaction of keratinocytes and fibroblasts modulates the expression of matrix metalloproteinases-2 and -9 and their inhibitors. *Molecular and Cellular Biochemistry* **2005,** *269*, 209-216.
[461.] M. M. Fuster; L. Wang, Endothelial heparan sulfate in angiogenesis. *Prog Mol Biol Transl Sci* **2010,** *93*, 179-2012.
[462.] R. V. Iozzo; J. D. San Antonio, Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. *Journal of Clinical Investigation* **2001,** *108* (3), 349-355.
[463.] R. D. Rosenberg; L. M. S. Fritze; J. J. Castellot; M. J. Karnovsky, Heparin-like molecules as regulators of atherogenesis. *Nouv. Rev. Fr. Hematol.* **1984,** *26*, 225-260.
[464.] S. E. Stringer, The role of heparan sulphate proteoglycans in angiogenesis. *Biochem Soc Trans* **2006,** *34*, 451-453.
[465.] X. M. R. van Wijk; T. H. van Kuppevelt, Heparan sulfate in angiogenesis: a target for therapy. *Angiogenesis* **2014,** *17* (3), 443-462.
[466.] J. Folkman, How is blood vessel growth regulated in normal and neoplastic tissue. *Cancer Res* **1986,** *46*, 467-473.

[467.] S. Matou; S. Colliec-Jouault; I. Galy-Fauroux; J. Ratiskol; C. Sinquin; J. Guezennec; A. M. Fischer; D. Helley, Effect of an oversulfated exopolysaccharide on angiogenesis induced by fibroblast growth factor-2 or vascular endothelial growth factor in vitro. *Biochem Pharmacol* **2005,** *69* (5), 751-759.
[468.] S. A. Mousa; X. Feng; J. Xie; Y. M. Du; Y. Hua; H. He; L. O'Connor; R. J. Linhardt, Synthetic oligosaccharide stimulates and stabilizes angiogenesis: structure-function relationships and potential mechanisms. *Journal of Cardiovascular Pharmacology* **2006,** *48* (2), 6-13.
[469.] G. Han; X. Xia; Z. Pan; Y. Lin; L. Li; Y. Jiao; C. Zhou; S. Ding, Different influence of sulfated chitosan with different sulfonic acid group sites on HUVECs behaviors. *Journal of Biomaterials Science, Polymer Edition* **2020**.
[470.] D. K. Lim; R. G. Wylie; R. Langer; D. S. Kohane, Selective binding of C-6 OH sulfated hyaluronic acid to the angiogenic isoform of VEGF(165). *Biomaterials* **2016,** *77*, 130-138.
[471.] N. Ferrara, Vascular endothelial growth factor and age-related macular degeneration: from basic

science to therapy. *Nature Medicine* **2010,** *16* (10), 1107-1111.
[472.] Y. Li; W. Wang; Y. Zhang; X. Wang; X. Gao; Z. Yuan; Y. Li, Chitosan sulfate inhibits angiogenesis via blocking the VEGF/VEGFR2 pathway and suppresses tumor growth in vivo. *Biomater Sci-Uk* **2019,** *7*, 1584-1597.
[473.] R. G. Azizkhan; J. C. Azizkhan; B. R. Zetter; J. Folkman, Mast cell heparin stimulates migration of capillary endothelial cells in vitro. *Journal of Experimental Medicine* **1980,** *152* (4), 931-944.
[474.] D. A. Kessler; R. S. Langer; N. A. Pless; J. Folkman, Mast cells and tumor angiogenesis. *International Journal of Cander* **1976,** *18* (5), 703-709.
[475.] J. Folkman; R. Langer; R. Linhardt; C. Haudenschild; S. Taylor, Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* **1983,** *221* (4612), 719-725.
[476.] M. Presta; P. Oreste; G. Zoppetti; M. Belleri; E. Tanghetti; D. Leali; C. Urbinati; A. Bugatti; R. Ronca; S. Nicoli; E. Moroni; H. Stabile; M. Camozzi; G. A. Hernandez; S. Mitola; P. Dell'Era; M. Rusnati; D. Ribatti, Antiangiogenic activity of semisynthetic biotechnological heparins: low-molecular-weight-sulfated Escherichia coli K5 polysaccharide derivatives as fibroblast growth factor antagonists. *Arterioscler Thromb Vasc Biol* **2005,** *25* (1), 71-6.
[477.] H. S. Adhikari; P. N. Yadav, Anticancer activity of chitosan, chitosan derivatives, and their mechanism of action. *International Journal of Biomaterials* **2018,** *2018*, 2952085.
[478.] J. Murata; I. Saiki; T. Makabe; Y. Tsuta; S. Tokura; I. Azuma, Inhibition of tumor-induced angiogenesis by sulfatad chitin derivatives. *Cancer Research* **1991,** *51*, 22-26.
[479.] B. Casu; M. Guerrini; S. Guglieri; A. Naggi; M. Perez; G. Torri; G. Cassinelli; D. Ribatii; P. Carminati; G. Giannini; S. Penco; C. Pisano; M. Belleri; M. Rusnati; M.

Presta, Undersulfated and glycol-split heparins endowed with antiangiogenic activity. *Journal of Medicinal Chemistry* **2004,** *47* (4), 838-848.
[480.] H. H. A. M. Hassan, Chemistry and biology of heparin mimetics that bind to fibroblast growth factors. *Mini-Reviews in Medicinal Chemistry* **2007,** *7*, 1206-1235.
[481.] M. Demir; O. Iqbal; D. A. Hoppensteadt; P. Piccolo; S. Ahmad; C. L. Schultz; R. J. Linhardt; J. Fareed, Anticoagulant and antiprotease profiles of a novel natural heparinomimetic mannopentaose phosphate sulfate (PI-88). *Clinical and Applied Thrombosis/Hemostasis* **2001,** *7* (2), 131-140.
[482.] X.-J. Liang; L. Yuan; J. Hu; H.-H. Yu; T. Li; S.-F. Lin; S.-B. Tang, Phosphomannopentaose sulfate (PI-88) suppresses angiogenesis by downregulating heparanase and vascular endothelial growth factor in an oxygen-induced retinal neovascularization animal model. *Molecular Vision* **2012,** *18*, 1649-1657.
[483.] C. R. Parish; W. B. Cowden Preparation and use of sulfated oligosaccharides. US 6143730, 2000.
[484.] K. Takahashi ; K. Tanabe; M. Ohnuki; M. Narita; T. Ichisaka; K. Tomoda; S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **2007,** *131*, 861-872.
[485.] K. Takahashi; S. Yamanaka, Induction of pluripotent stem Ccells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **2006,** *126*, 663-676.
[486.] J. Yu; M. A. Vodyanik; K. Smuga-Otto; J. Antosiewicz-Bourget; J. L. Frane; S. Tian; J. Nie; G. A. Jonsdottir; V. Ruotti; R. Stewart; I. I. Slukvin; J. A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells. *Science* **2007,** *318* (5858), 1917-1920.
[487.] C. E. Johnson; B. E. Crawford; M. Stavridis; G. Ten Dam; A. L. Wat; G. Rushton; C. M. Ward; V. Wilson; T. H. van Kuppevelt; J. D. Esko; A. Smith; J. T. Gallagher; C. L. Merry, Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-enhanced green fluorescent protein-expressing neural progenitor cells. *Stem Cells* **2007,** *25* (8), 1913-23.
[488.] D. C. Kraushaar; S. Rai; E. Condac; A. Nairn; S. Zhang; Y. Yamaguchi; K. Moremen; S. Dalton; L. Wang, Heparan sulfate facilitates FGF and BMP signaling to drive mesoderm differentiation of mouse embryonic stem cells. *J Biol Chem* **2012,** *287* (27), 22691-700.
[489.] F. Lanner; K. L. Lee; M. Sohl; K. Holmborn; H. Yang; J. Wilbertz; L. Poellinger; J. Rossant; F. Farnebo, Heparan sulfation-dependent fibroblast growth factor signaling maintains embryonic stem cells primed for differentiation in a heterogeneous state. *Stem Cells* **2010,** *28* (2), 191-200.
[490.] N. Sasaki; K. Okishio; K. Ui-Tei; K. Saigo; A. Kinoshita-Toyoda; H. Toyoda; T. Nishimura; Y. Suda; M. Hayasaka; K. Hanaoka; S. Hitoshi; K. Ikenaka; S. Nishihara, Heparan sulfate regulates self-renewal and pluripotency of embryonic stem cells. *J Biol Chem* **2008,** *283* (6), 3594-606.
[491.] L. Weinberger; M. Ayyash; N. Novershtern; J. H. Hanna, Dynamic stem cell states: naive to primed pluripotency in rodents and humans. *Nat Rev Mol Cell Biol* **2016,** *17* (3), 155-69.

[492.] L. Gasimli; A. M. Hickey; B. Yang; G. Li; M. dela Rosa; A. V. Nairn; M. J. Kulik; J. S. Dordick; K. W. Moremen; S. Dalton; R. J. Linhardt, Changes in glycosaminoglycan structure on differentiation of human embryonic stem cells towards mesoderm and endoderm lineages. *Biochim Biophys Acta* **2014,** *1840* (6), 1993-2003.
[493.] T. Mikami; H. Kitagawa, Sulfated glycosaminoglycans: their distinct roles in stem cell biology. *Glycoconj J* **2017,** *34* (6), 725-735.
[494.] D. *e. a.* Soares da Costa, Sulfation of Glycosaminoglycans and Its Implications in Human Health and Disorders. *Annu Rev Biomed Eng* **2017,** *19*, 1-26.
[495.] C. C. Rider, Heparin/heparan sulphate binding in the TGF-β cytokine superfamily. *Biochemical Society Transactions* **2006,** *34* (3), 458-460.
[496.] T. Izumikawa; B. Sato; H. Kitagawa, Chondroitin sulfate is indispensable for pluripotency and differentiation of mouse embryonic stem cells. *Sci Rep-Uk* **2014,** *4* (3701).
[497.] M. E. Levenstein; T. E. Ludwig; R. H. Xu; R. A. Llanas; K. VanDenHeuvel-Kramer; D. Manning; J. A. Thomson, Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* **2006,** *24* (3), 568-74.
[498.] X. Lin; G. Wei; Z. Shi; L. Dryer; J. D. Esko; D. E. Wells; M. M. Matzuk, Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice. *Developmental Biology* **2000,** *224* (2), 299-311.
[499.] A. V. Nairn; A. Kinoshita-Toyoda; H. Toyoda; K. Harris; S. Dalton; M. Kulik; M. J. Pierce; T. Toida; K. W. Moremen; R. J. Linhardt, Glycomics of proteoglycan biosynthesis in murine embryonic stem cell differentiation. *Journal of Proteome Research* **2007,** *6*, 4374-4387.
[500.] I. Klimanskaya; Y. Chung; L. Meisner; J. Johnson; M. D. West; R. Lanza, Human embryonic stem cells derived without feeder cells. *The Lancet* **2005,** *365* (9471), 1636-1641.
[501.] C. Xu; E. Rosler; J. Jiang; J. S. Lebkowski; J. D. Gold; C. O'Sullivan; K. Delavan-Boorsma; M. Mok; A. Bronstein; M. K. Carpenter, Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells* **2005,** *23* (3), 315-23.
[502.] R. H. Xu; R. M. Peck; D. S. Li; X. Feng; T. Ludwig; J. A. Thomson, Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* **2005,** *2* (3), 185-90.
[503.] C. E. Pickford; R. J. Holley; G. Rushton; M. P. Stavridis; C. M. Ward; C. L. Merry, Specific glycosaminoglycans modulate neural specification of mouse embryonic stem cells. *Stem Cells* **2011,** *29* (4), 629-40.
[504.] M. Forsberg; K. Holmborn; S. Kundu; A. Dagalv; L. Kjellen; K. Forsberg-Nilsson, Undersulfation of heparan sulfate restricts differentiation potential of mouse embryonic stem cells. *J Biol Chem* **2012,** *287* (14), 10853-62.
[505.] T. Miura; N. Yuasa; H. Ota; M. Habu; M. Kawano; F. Nakayama; S. Nishihara, Highly sulfated hyaluronic acid maintains human induced pluripotent stem cells under feeder-free and bFGF-free conditions. *Biochem Biophys Res Commun* **2019,** *518* (3), 506-512.

[506.] F. Haasters; W. C. Prall; D. Anz; C. Bourquin; C. Pautke; S. Endres; W. Mutschler; D. Docheva; M. Schieker, Morphological and immunocytochemical characteristics indicate the yield of early progenitors and represent a quality control for human mesenchymal stem cell culturing. *J Anat* **2009,** *214* (5), 759-67.
[507.] M. Zenobi-Wong; G. Palazzolo; R. Mhanna; J. Becher; S. Moller; M. Schnabelrauch Sulfated alginate hydrogels for cell culture and therapy. Apr 10, 2018, 2018.
[508.] P. Chopra; M. T. Logun; E. M. White; W. Lu; J. Locklin; L. Karumbaiah; G. J. Boons, Fully Synthetic Heparan Sulfate-Based Neural Tissue Construct That Maintains the Undifferentiated State of Neural Stem Cells. *ACS Chem Biol* **2019,** *14* (9), 1921-1929.
[509.] M. L. Huang; R. A. Smith; G. W. Trieger; K. Godula, Glycocalyx remodeling with proteoglycan mimetics promotes neural specification in embryonic stem cells. *J Am Chem Soc* **2014,** *136* (30), 10565-8.
[510.] F. Di Giacomo; D. Lewandowski; E. Cabannes; V. Nancy-Portebois; M. Petitou; S. Fichelson; P. H. Romeo, Heparan sulfate mimetics can efficiently mobilize long-term hematopoietic stem cells. *Haematologica* **2012,** *97* (4), 491-9.
[511.] M. Askari; S. Bonakdar; M. H. Anbouhi; H. Shahsavarani; S. Kargozar; V. Khalaj; M. A. Shokrgozar, Sustained release of TGF-beta1 via genetically-modified cells induces the chondrogenic differentiation of mesenchymal stem cells encapsulated in alginate sulfate hydrogels. *J Mater Sci Mater Med* **2018,** *30* (1), 7.
[512.] Y. Hazeri; S. Irani; M. Zandi; M. Pezeshki-Modaress, Polyvinyl alcohol/sulfated alginate nanofibers induced the neuronal differentiation of human bone marrow stem cells. *Int J Biol Macromol* **2020,** *147*, 946-953.
[513.] K. Ding; Y. Wang; H. Wang; L. Yuan; M. Tan; X. Shi; Z. Lyu; Y. Liu; H. Chen, 6-O-sulfated chitosan promoting the neural differentiation of mouse embryonic stem cells. *ACS Appl Mater Interfaces* **2014,** *6* (22), 20043-50.
[514.] G. P. Huang; R. Menezes; R. Vincent; W. Hammond; L. Rizio; G. Collins; T. L. Arinzeh, Gelatin Scaffolds Containing Partially Sulfated Cellulose Promote Mesenchymal Stem Cell Chondrogenesis. *Tissue Eng Part A* **2017,** *23* (17-18), 1011-1021.
[515.] G. Portocarrero Huang; A. Molina; N. Tran; G. Collins; T. L. Arinzeh, Investigating cellulose derived glycosaminoglycan mimetic scaffolds for cartilage tissue engineering applications. *J Tissue Eng Regen M* **2018,** *12* (1), e592-e603.
[516.] G. Frescaline; T. Bouderlique; M. B. Huynh; D. Papy-Garcia; J. Courty; P. Albanese, Glycosaminoglycans mimetics potentiate the clonogenicity, proliferation, migration and differentiation properties of rat mesenchymal stem cells. *Stem Cell Res* **2012,** *8* (2), 180-92.
[517.] F. Blanquaert; D. Barritault; J. P. Caruelle, Effects of heparanlike polymers associated with growth factors on osteoblast proliferation and phenotype expression. *Journal of Biomedical Materials Research* **1999,** *44*, 63-72.
[518.] G. H. Raguénès; A. Peres; R. Ruimy; P. Pignet; R. Christen; M. Loaec; H. Rougeaux; G. Barbier; J. G. Guezennec, Alteromonas infernus sp. nov., a new

polysaccharide-producing bacterium isolated from a deep-sea hydrothermal vent. *Journal of Applied Microbiology* **1997,** *82* (4), 422-430.
[519.] P. Albanese; D. Caruelle; G. Frescaline; J. Delbe; L. Petit-Cocault; E. Huet; N. Charnaux; G. Uzan; D. Papy-Garcia; J. Courty, Glycosaminoglycan mimetics-induced mobilization of hematopoietic progenitors and stem cells into mouse peripheral blood: structure/function insights. *Exp Hematol* **2009,** *37* (9), 1072-83.
[520.] A. Pulsipher; M. E. Griffin; S. E. Stone; J. M. Brown; L. C. Hsieh-Wilson, Directing neuronal signaling through cell-surface glycan engineering. *J Am Chem Soc* **2014,** *136* (19), 6794-7.
[521.] Q. Liu; Z. Lyu; Y. Yu; Z. A. Zhao; S. Hu; L. Yuan; G. Chen; H. Chen, Synthetic Glycopolymers for Highly Efficient Differentiation of Embryonic Stem Cells into Neurons: Lipo- or Not? *ACS Appl Mater Interfaces* **2017,** *9* (13), 11518-11527.
[522.] A. Pulsipher; M. E. Griffin; S. E. Stone; L. C. Hsieh-Wilson, Long-lived engineering of glycans to direct stem cell fate. *Angew Chem Int Ed Engl* **2015,** *54* (5), 1466-70.