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Sulfated polysaccharide-based scaffolds for orthopaedic tissue engineering

Abstract

Given their native-like biological properties, high growth factor retention capacity and porous nature, sulfated-polysaccharide-based scaffolds hold great promise for a number of tissue engineering applications. Specifically, as they mimic important properties of tissues such as bone and cartilage they are ideal for orthopaedic tissue engineering. Their biomimicry properties encompass important cell-binding motifs, native-like mechanical properties, designated sites for bone mineralisation and strong growth factor binding and signaling capacity. Even so, scientists in the field have just recently begun to utilise them as building blocks for tissue engineering scaffolds. Most of these efforts have so far been directed towards in vitro studies, and for these reasons the clinical gap is still substantial. With this review paper, we have tried to highlight some of the important chemical, physical and biological features of sulfated-polysaccharides in relation to their chondrogenic and osteogenic inducing capacity. Additionally, their usage in various in vivo model systems is discussed. The clinical studies reviewed herein paint a promising picture heralding a brave new world for orthopaedic tissue engineering.

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1 Sulfated polysaccharide-based scaffolds for orthopaedic tissue engineering

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23

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25 Abstract

26 Given their native-like biological properties, high growth factor retention capacity and porous nature, sulfated-
27 polysaccharide-based scaffolds have shown great promise in a number of tissue engineering applications.
28 Specifically, as they mimic important properties of tissues such as bone and cartilage they are ideal for orthopaedic
29 tissue engineering. Their biomimicry properties encompass important cell-binding motifs, native-like mechanical
30 properties, designated sites for bone mineralization and strong growth factor binding and signalling capacity. Even

1 so, scientists in the field have just recently begun to utilise them as building blocks for tissue engineering scaffolds.
2 Most of these efforts have so far been directed towards *in vitro* studies, and for these reasons the clinical gap is
3 still substantial. This review paper highlights some of the important chemical, physical and biological features of
4 sulfated-polysaccharides in relation to their chondrogenic and osteogenic inducing capacity. Additionally, their
5 usage in various *in vivo* model systems is discussed. The clinical studies reviewed herein paint a promising picture
6 heralding a brave new world for orthopaedic tissue engineering.

7 **1. Introduction**

8 Orthopaedic diseases are the second largest contributor to disability worldwide and are expected to grow
9 rapidly in the foreseeable future due to the aging population.[1] They include debilitating diseases such as
10 osteoarthritis, tendinopathies, osteoporosis, as well as skeletal and joint fractures.[2, 3] The current approaches
11 for addressing this grand challenge rely on various prosthetic, allograft and autograft-based strategies. Even
12 though the prosthetic-based interventions have shown exciting results in recent years, they still face major
13 shortcomings such as suboptimal long-term outcomes, the need for revision surgeries and risk of infection.[4]
14 Allograft and autograft strategies on the other hand impose their own limitations including the possibility of
15 disease transmission, insufficient autologous resources, rejection of allograft tissue and potential need for
16 immunosuppression therapies.[5] To overcome these hurdles a great variety of tissue engineering approaches have
17 been proposed over the years (Figure 1).[3, 6]

18 The grand goal of tissue engineering is to generate artificial tissues with the capacity to bring normality back
19 to dysfunctional tissues by replacing them with more functional ones.[4] The tissue engineering paradigm involves
20 scaffolds combined with potent cell sources and suitable biochemical signals [7], which together can promote the
21 formation of new organs and tissues.[8] Ideally, these scaffolds emulate key physical and molecular features of
22 the native extracellular matrix (ECM) in order to facilitate cell attachment, proliferation and differentiation and
23 ultimately new tissue growth (Figure 1).[9] The key in this regard is to provide the cells with a native-like milieu
24 with the capacity to guide them into tissue specific phenotypes.[10-13] Generally speaking, bioactivity is included
25 into scaffolds by using: i) insoluble signals, such as bio-ceramics and carbon-based nanocues [14], ii) introducing
26 growth factors and other biological moieties into the scaffold matrix [15], or ii) by incorporating cell adhesion
27 and differentiation promoting oligopeptides (such as the cell binding RGD peptide [16, 17]).

28 While all of these methods have shown promise in the synthesis of bioactive scaffolds, they still face certain
29 limitations in the clinic. For instance, i) some insoluble signals such as carbon-based nanomaterials can cause a
30 foreign body response that can facilitate tissue fibrosis [18, 19], ii) growth factors often face issues such as loss
31 of bioactivity, low tissue penetration and dosage-dependent toxicity [20] and iii) many of the bioactive
32 oligopeptides do not facilitate the needed intracellular signalling pathways for optimum tissue generation; even
33 though a number of proteins (such as fibronectin[21, 22], collagen[23], osteopontin,[24] vitronectin[25] and
34 fibrinogen[26]) stimulate much more robust intracellular signalling than bioactive oligopeptides[27-30] they are
35 limited by either foreign body responses from the host or in some cases high cost and low scalability. For these
36 reasons, native-like and abundant biopolymers with inherent bioactivity have attracted much attention in
37 biomaterials science. In particular, sulfated polysaccharides are by now widely recognized for their ability to bind
38 to important cell receptors to facilitate cell adhesion, proliferation and differentiation.[31, 32] They can also bind

1 to and signal a number of important growth factors such as fibroblast, vascular endothelial and bone
2 morphogenetic protein growth factors for controlled growth factor release; and they can improve growth factor
3 bioavailability by protecting them against proteinase degradation.[31, 33-36]

4 In simple terms, sulfated polysaccharides can be classified under three distinct categories including i)
5 sulfated GAGs, ii) marine sulfated glycans and iii) chemically sulfated polysaccharides. While the first two
6 categories are inherently sulfated polysaccharides, the third one consists of non-sulfated polysaccharides that are
7 chemically modified with various sulfating agents. Regardless, the bioactivity of sulfated polysaccharides depends
8 on factors such as degree of sulfation and sulfation pattern.[34, 37] For instance, hyaluronic acid (HA)/collagen
9 type I matrices were shown to inhibit differentiation and resorption of osteoclasts, mainly relying on degree of
10 sulfation of HA.[38] To this end, highly sulfated HA was capable of improving bone regeneration in *in vitro* and
11 *in vivo* models.[39-41] In other studies, an intimate link between sulfation pattern and chondrogenesis has been
12 proposed.[42] For example, it was shown that chondroitin sulfate (CS) rich in 4,6-O-disulfated disaccharides, had
13 a higher potential to upregulate the expression of important chondrogenic biomarkers when compared to other CS
14 derivatives containing either 4- or 6-O-sulfated disaccharides.[42]

15 Accordingly, sulfated polysaccharides have been rapidly picked up by scientists in the field in order to
16 manufacture more bioactive scaffolds that can facilitate better skeletal tissue regeneration.[43-53] These scaffolds
17 were made via various fabrication methods such as casting, electrospinning and 3D printing from either
18 individually sulfated polysaccharides or in combination with other biopolymers. Generally speaking, the scaffolds
19 have been used in two different ways to assist osteogenesis or chondrogenesis: i) in conjugation with growth
20 factors to facilitate differentiation of cells via sustained release of growth factors, or ii) in the absence of any
21 growth factors by solely relying on intermolecular interactions with important cell-membrane receptors.[54, 55]

22 This paper reviews the most recent progress in sulfated polysaccharide-based scaffolds for skeletal tissue
23 engineering, with particular focus on bone and cartilage tissue engineering. Specifically, three different groups of
24 sulfated polysaccharides, sulfated GAGs, marine sulfated glycans and chemically sulfated polysaccharides, and
25 their usage as building blocks in orthopaedic scaffolds are reviewed; since these polysaccharides present the most
26 promising avenues in this field. This review also highlights the ability of these scaffolds to direct progenitor cells
27 into either chondrogenic or osteogenic differentiation. Finally, application of these scaffolds in various preclinical
28 studies related to mending bone and cartilage defects along with more complex osteochondral lesions are
29 reviewed, as such studies are of utmost importance for bridging the current gap between the laboratory and the
30 clinic.

31 **2. Naturally Sulfated Polysaccharides**

32 Sulfated polysaccharides can be derived from the ECM of animal tissues in the form of sulfated GAGs or
33 from plants such as marine algae in the form of alginate, carrageenan, fucoidan and ulvan (Figure 2). The sulfate
34 groups in the abovementioned biopolymers can also be chemically conjugated to the sugar backbones of non-
35 sulfated molecules such as HA, chitosan, alginate and cellulose. Along these lines, this section is divided into
36 three subsections dealing with sulfated GAGs and polysaccharides derived from natural sources as well as sulfated
37 polysaccharides that are custom-made in the laboratory. Notably, the wide variety of sulfated polysaccharides
38 reviewed can display differing bioactivity depending on the sulfate position and degree.

1 **2.1 Glycosaminoglycans (GAGs)**

2 Sulfated GAGs are present in the ECM, cellular membrane and intracellularly within eukaryotes (Figure 2).
3 They therefore, play an essential role in modulating extracellular and intracellular interactions. In simple terms,
4 GAGs can be defined as negatively charged heteropolysaccharides, whose disaccharide units are made from
5 repeating disaccharide units consisting of either an amino sugar (glucosamine or galactosamine) or a uronic acid
6 (iduronic or glucuronic acid). Based on their disaccharide composition, they are grouped into four different
7 families including heparin/heparan sulfate, chondroitin/dermatan sulfate, keratan sulfate and HA. While heparin,
8 heparan, chondroitin, dermatan and keratan sulfate are sulfated and post-translationally synthesised via attachment
9 to a core protein, HA is non-sulfated and synthesised at the cell surface without a protein core. Importantly, GAGs
10 can differ significantly from one another in terms of bioactivity and structural complexity depending on their
11 specific biosynthesis pathway and source of derivation.[56]

12 *Heparin and Heparan Sulfate*

13 Heparin is a highly sulfated GAG only produced by connective tissue mast cells that exclusively decorates
14 the protein core of serglycin. [57] In contrast, heparan sulfates (HS) decorate intracellular, ECM and cell surface
15 proteoglycans and are produced by almost all cell types. Specifically, they participate in a wide range of biological
16 events including cell proliferation and differentiation, immune responses, as well as angiogenesis.[58-61] Both
17 heparin and HS are composed of repeating disaccharide units of either iduronic or glucuronic acid and
18 glucosamine units but with less iduronic acid and less overall sulfation in HS compared to heparin. Importantly
19 HS does not contain sulfation at the C3 position and does not possess anti-coagulant activity.[62-64] They also
20 interact with a variety of proteins, including heparin-binding growth factors, which together with their cell
21 signalling role, make them ideal choices for scaffolding materials.[60]

22 Heparin has been widely explored in tissue engineering, owing to its ease of supply, especially in the clinical
23 as an anticoagulant. It is also often used as an analogue of HS.[65-67] Heparin and HS bind to a range of proteins
24 via electrostatic interactions that are controlled by its three-dimensional structure, anionic nature and sulfation
25 patterns. Heparin is known to enhance the osteogenic potential and bioavailability of bone morphogenetic protein-
26 2 (BMP-2) through its binding, stabilization and presentation to cells.[68-70] Indeed, in a study by Hettiaratchi *et*
27 *al.* [71] it was shown that methacrylated heparin microparticles could bind high quantities of BMP-2, vascular
28 endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), which in turn could stimulate alkaline
29 phosphatase (ALP) activity in skeletal myoblasts (C2C12) and increase the cell division rate. Notably, such
30 heparin microparticles typically demonstrate better presentation of growth factors in comparison to gelatin
31 microparticles and soluble heparin; something which has been speculated to arise from heparin's higher charge
32 density.[71] Similarly, PLGA microspheres when functionalised with both heparin and BMP-2, could
33 significantly up regulate MG-63 osteosarcoma cell differentiation as seen through the enhanced expression of
34 osteocalcin (OCN) and osteopontin (OPN), whilst simultaneously increasing both ALP activity and deposition of
35 important bone minerals.[72]

36 However, heparin's anticoagulant capacity can hinder bone regeneration through antithrombin III activation,
37 which can prevent the accumulation of various tissue regenerative growth factors and cytokines in the defected
38 bone region. Thus, the lesser negatively charged HS could be a more useful bioactive supplement. To this end,
39 Bramono *et al.* [73] compared the osteogenic potential of heparin and HS from various sources; as regulators of

1 BMP-2 activity, and found that heparin could up regulate osteogenic differentiation of C2C12 cells (induced by
2 BMP-2) in the short term, however they did not observe any significant BMP-2 stimulated bone matrix
3 mineralisation after 14 days. Interestingly, HS delivered BMP-2 in a prolonged and controlled manner, at more
4 physiologically relevant concentrations whilst retaining its osteogenic activity when compared to heparin. This
5 was thought to be associated with the higher growth factor binding and signaling capacity of HS compared to
6 heparin which enables the more efficient presentation of osteogenic ligands to their cell associated receptors.[74]
7 HS has also been shown to regulate other growth factors in the transforming growth factor beta (TGF- β)
8 superfamily. For instance, Chen *et al.* [75] demonstrated that, in the presence of TGF- β 3, HS induced
9 chondrogenic differentiation of human MSCs whilst activating important TGF- β related signaling pathways.
10 Similarly, heparin in combination with a self-assembling peptide (RAD 16-I) could drive adipose-derived stem
11 cells (ADSCs) into the chondrogenic lineage as evidenced by collagen type II up regulation; a phenomenon that
12 was speculated to arise from heparin's affinity towards VEGF.[76] More recently, a biphasic silk fibroin
13 biomaterial incorporating heparin was reported to increase growth factor retention and thereby preventing the
14 undesired initial burst-like release that is so common in many traditional scaffolds.[77] Interestingly, the addition
15 and controlled release of TGF- β 2 and GDF5 (growth differentiation factor 5) into the scaffold up-regulated
16 chondrogenic markers, including SOX9, aggrecan and collagen type III (Figure 3).

17 In summary, several studies have demonstrated the versatility of heparin and HS to efficiently deliver and
18 preserve the function of important chondrogenic and osteogenic growth factors. As mentioned, the prominent
19 anticoagulant capacity of heparin can diminish the accumulation of growth factors and cytokines in a bone defect
20 site and subsequently hinder tissue regeneration. HS, the less sulfated heparin analogue, on the other hand holds
21 promise as an alternate delivery vehicle without such undesirable side effects. In this regard, HS has already
22 showed promise at permitting sequestration and controlled local delivery of growth factors resulting in an
23 improved bone and cartilage matrix production. Overall, HS and heparin-based biomaterials have shown immense
24 promise in multiple branches of tissue engineering including but not limited to growth factor and cytokine delivery
25 vehicle for bone and cartilage tissue regeneration.

26

27 *Chondroitin Sulfate*

28 Chondroitin sulfate (CS) is the most abundant GAG found in vertebrate and invertebrate ECM and decorates
29 intracellular, ECM and cell surface proteoglycans. It is a linear polysaccharide composed of repeating disaccharide
30 units of glucuronic acid and galactosamine that can be sulfated at carbons 2 on the glucuronic acid, and 4 and/or
31 6 on the galactosamine, which provide heterogeneity in structure.[78] Aggrecan is the major CS proteoglycan in
32 cartilage that binds to HA to form aggregate structures that have a high water retention capacity and provide the
33 hydrodynamic weight bearing properties of cartilage.[79] CS has been shown to stimulate the synthesis of HA,
34 aggrecan, glucosamine and collagen II, as well as preventing chondrocyte apoptosis and cartilage degradation by
35 inhibiting ECM degrading enzymes. Accordingly, CS has been greatly utilized for repairing cartilage as well as
36 assisting stem cells to undergo chondrogenic differentiation.[80] For a more in-depth analysis of the influence of
37 CS hydrogels on stem cell fate the reader is referred to a comprehensive review published recently by Farrugia *et*
38 *al.* [81]

1 A number of recent studies have harnessed the abovementioned biomimicry properties of CS in cartilage
2 tissue engineering with exciting outcomes. For instance, a study by Levett *et al.* [82] aimed to enhance
3 chondrocyte behaviour in gelatin methacrylate-based (GelMA) hydrogels by incorporating GAGs including HA
4 methacrylate (HAMA) and CS methacrylate (CSMA) into the hydrogels; both separately and together.
5 Interestingly, they found that the integration of HAMA enhanced chondrocyte re-differentiation and improved
6 matrix distribution, whereas CSMA showed marginal improvements over both the GelMA control and
7 GelMA/HAMA/CSMA triple composite. This means that HAMA positively influences bioactivity and the
8 mechano-physiological properties of GelMA hydrogels when compared with CSMA. Although, HA provides the
9 biochemical cues for chondrogenesis, it was shown that the inclusion of CS in the HA hydrogels can upregulate
10 mRNA expression of chondrogenic markers, while decreasing expression of the hypertrophic markers that are
11 normally associated with HA hydrogels.[84] Additionally, incorporation of CS into HA hydrogels led to an
12 increase in GAG accumulation both *in vitro* and *in vivo*. Similar results were observed by Costantini *et al.* [85]
13 during bioprinting of bone marrow derived hMSCs in a composite matrix containing GelMA, HAMA and CS
14 amino ethyl methacrylate (CSAEMA). In the absence of HAMA, the ratio of collagen II/collagen I and collagen
15 II/collagen X increased suggesting neocartilage formation, whereas differentiation towards hypertrophic cartilage
16 was observed with HAMA alone. This may be due to the stiffness increase from 59 kPa (GelMA/CS) to 100 kPa
17 (GelMA/CS/HAMA), as MSC differentiation is sensitive to interface stiffness.[86, 87] In summary, the chemical
18 composition, network density and stiffness of the 3D microenvironment in combination play an important part in
19 determining the chondrogenic potential of MSCs, with CS showing the most promising cartilage regenerative
20 capacity.

21 CS has also been employed together with other biopolymers such as polyethylene glycol (PEG), chitosan,
22 and alginate to constitute bioactive scaffolds for cartilage tissue engineering.[46, 88-93] In a noteworthy example,
23 with the aim of evaluating the effect of CS sulfation degree on its interaction with positively charged growth
24 factors, researchers made two different types of scaffolds composed of poly(ethylene glycol)-diacrylate (PEG-
25 DA) with either CS or desulfated CS.[89] *In vitro* experiments demonstrated that the release of a positively
26 charged model protein, histone, from hydrogels containing desulfated CS resulted in an increased histone release
27 when compared to a hydrogel containing normal CS, indicating that sulfation plays an essential part in modulating
28 protein interactions with GAG hydrogels, and thereby also the growth factor release profile. Interestingly, in
29 chondrogenic medium, MSCs in hydrogels containing desulfated CS had significantly higher expression of
30 collagen II and aggrecan at day 21, compared to PEG control scaffolds or CS containing scaffolds. This was
31 speculated to arise from the augmented TGF- β 1 pull-down from culture media caused by the presence of CS in
32 the hydrogels.

33 In another study, a biomaterial composed of chitosan and CS was utilized in engineering cartilage
34 tissue.[46] The *in vitro* results with a pre-chondrocyte cell line (ATDC5) showed that chitosan/CS induced a more
35 collagen II/collagen I ratio (a characteristic of hyaline cartilage formation) after 21 days, when compared to
36 pristine chitosan. Furthermore, the collagen X expression in chitosan/CS showed an increase after 21 days
37 compared to pristine chitosan scaffolds, indicating that these scaffolds can drive ATDC5 cells into a hypertrophic
38 state. CS has also been employed in conjugation with alginate to establish porous scaffolds for chondrogenesis of
39 hMSCs.[93] After 14 days, it was shown that under chondrogenic conditions total collagen and GAG contents
40 were higher in cells seeded onto CS-containing scaffolds as compared to the CS-free ones.

1 Apart from cartilage tissue engineering, CS has been utilized to promote osteoblast adhesion for engineering
2 of bone tissue.[94] In this respect, Vandrovcová *et al.* [95] coated PLGA with collagen I with and without CS and
3 showed that CS improved both the osteoconductivity and osteoinductivity of the (osteoblastic) MG-63 cell line,
4 observed through the increased proliferation and upregulation of osteocalcin, as compared to pristine collagen I
5 coatings. Similarly, titanium implants have also been coated with CS/collagen[96] or CS,[97] as sulfated GAGs
6 are known to bind calcium and calcium phosphates such as hydroxyapatite [98]. The former compared three forms
7 of CS (4-sulfated CS (CS A); 6-sulfated CS (CS C) and dermatan sulfate (CS B)), and found that both CS A and
8 CS B stimulated local osteoblast adhesion. We also note, that the study by Dudeck *et al.* [97] demonstrated a
9 synergistic effect between CS and hormone replacement therapy in an osteoporotic rat model, and thus indicates
10 that CS scaffolds could open new therapies for osteoporosis.

11 In summary, CS has been used in conjunction with biopolymers to form more functional composite
12 biomaterials that can facilitate both chondrogenesis and osteogenesis. When used with cartilage forming cells, it
13 has been seen that the inclusion of CS increases the expression of collagen II, while facilitating a more hyaline-
14 like cartilage formation, as a result of enhanced binding with growth factors and integrin-mediated cell-matrix
15 interactions the CS structure, and specifically the location of the sulfates on the CS backbone, directly influences
16 its ability to bind to cells and direct their differentiation. Therefore, CS holds great promise for skeletal tissue
17 engineering since it can both have an impact on chondrogenesis and bind to important components of the hard
18 phase of bone; all because of its many sulfate groups.

19 **2.2 Marine sulfated Glycans**

20 Over 70% of the earth's surface is inundated by oceanic environments, rich in biodiversity. Among these
21 marine organisms lies algae and seaweed that are abundant with bioactive compounds of use in the field of
22 biomedicine owing to their numerous health benefits stemming from their anti-inflammatory, anti-cancer,
23 anticoagulant and immunomodulatory properties.[88, 99, 100] Although seasonal disparities can influence their
24 overall composition,[101] their sustainable cultivation is not constrained by climate as with various terrestrial
25 plant species. Notably, some of these algae are also made up of monosaccharides joined by glycosidic bonds
26 (Figure 2) that resemble GAGs and they can promote protein binding and cell growth without giving rise to
27 immunogenicity. As with other GAG-like polymers, the bioactivity of sulfated marine sugars depends on their
28 composition, molecular weight, degree and location of sulfate groups. The three most prevalent marine-based
29 sulfated polysaccharides currently used in biomedicine are carrageenan, fucoidan and ulvan, derived from red,
30 brown and green algae, respectively.

31 *Carrageenan*

32 In simple terms, Carrageenans (CARs) can be described as linear and water-soluble anionic-sulfated
33 polysaccharides. They are derived from red algae of the class *Rhodophyceae* and identified based on their
34 disaccharide sulfation. They have previously been successfully exploited in cartilage and bone tissue engineering
35 applications, owing to their thermoreversible gelling behaviour in the presence of non-toxic cations, as well as
36 their ability to facilitate bone apatite formation.[102-110]. As a noteworthy example, Popa *et al.* [102]
37 demonstrated that kappa (κ) - CAR hydrogels were capable of supporting the proliferation and chondrogenic
38 differentiation of encapsulated ADSCs. Following 21 days of culture they also observed a raise in hydrogel storage

1 modulus and viscoelastic properties possibly related to the ECM deposition from the cells. Additionally, following
2 compression, hydrogel's mechanical properties were observed to be in the range of native human cartilage. In
3 another study, Oliveira *et al.* [111] investigated how variations in the primary structure of CARs can influence
4 bone mineralisation. They compared the osteogenic properties of three different CAR sugar backbones, kappa (κ),
5 iota (ι), and lambda (λ), within a chitosan/polycaprolactone (PCL)-based scaffold. In this respect, it was shown
6 that bone apatite formation varies significantly between different CAR species. Specifically, of the three CARs
7 employed, the ι -variant demonstrated significantly higher biomineralization, possibly due to an increased affinity
8 for various bioactive compounds from the osteogenic media as a result of higher sulfur, oxygen and nitrogen
9 content within its sugar-like backbone. In a similar vein, the osteogenic capacity of a composite containing ι -
10 CAR/chitosan/gelatin was recently explored.[112] Here, the researchers found that the inclusion of gelatin with
11 its native RGD peptides and chitosan with its favorable cationic and osteogenic properties,[113] into the CAR
12 hydrogel network, promoted the osteogenic differentiation of ADSCs. Notably, they found that the inclusion of a
13 10 wt % ι -CARs substantially increased the ALP activity of encapsulated cells in comparison to the composites
14 containing 0, 5 and 15 wt % of ι -CAR. Correspondingly, an osteogenic-specific histology assay suggested that the
15 5 and 10 wt % ι -CAR-based composites caused higher mineral deposits following a 28-day *in vitro* study than the
16 other groups. In another recent investigation, κ -CAR was blended into biodegradable polyesters to consummate
17 a biocompatible scaffold for bone tissue engineering.[50] Interestingly, the authors found that like the other studies
18 reviewed herein the presence of κ -CAR could facilitate the formation of nanosized apatite crystals when compared
19 to pure polyesters, which instead gave rise to non-native-like and larger microsized crystals. Of interest, the
20 introduction of κ -CAR in the polyester material also enabled tailored degradability. In a related study, Liang *et*
21 *al.* [51] found that the expression of genes specific to cartilage (SOX9, collagen II and aggrecan) were up regulated
22 with increasing CARs concentrations within chitosan, when compared to pristine chitosan. They also showed that
23 CARs promoted cellular responses such as adhesion, viability and proliferation in the composite hydrogel. These
24 benefits were attributed to the chemical similarities between CARs and CS, which is widely recognized for its
25 chondrogenic capacity.

26 The thermoreversible and thixotropic gelling behaviour of κ -CAR under physiological conditions also
27 makes them suitable to be used as injectable hydrogels in cartilage tissue engineering, as evidenced by a recent
28 study by Rocha *et al.*[114] Specifically, in this study, it was found that ADSC-laden κ -CAR hydrogels cultured
29 in TGF- β 1 supplemented growth media did not induce chondrogenic differentiation, though when used with
30 chondrogenic medium, the cells developed a spherical, chondrogenic-like phenotype. Likewise,
31 immunohistochemical analysis revealed increased collagen II deposition following the integration of TGF- β 1 in
32 the κ -CAR hydrogels under chondrogenic conditions, suggesting the production of cartilage-specific
33 proteoglycans. Interestingly, the heated gelling conditions did not elicit thermal stress on encapsulated hASCs
34 following live-dead staining, justifying their potential future use as *in situ* forming hydrogels in cartilage tissue
35 engineering.

36 *Fucoidan*

37 Fucoidan is a sulfated polysaccharide stemmed from the cell-wall matrix of brown seaweed. It contains a
38 significant amount of L-fucose and sulfate ester groups which varies based on the source species.[100] The species
39 that is most frequently used in the field, is - *Fucus vesiculosus* - which typically gives rise to Fucoidan consisting

1 of 1,2- α -fucose, with its sulfate groups primarily located at C4 position.[115] Interestingly, fucoidan has been
2 demonstrated to interact with transforming growth factor (TGF)- β_1 , which was speculated to be associated with
3 its heparin-like chemical structure,[116] and like the CARs, fucoidan can also facilitate bone-like apatite
4 formation.[117] Specifically, it was demonstrated that the addition of fucoidan promoted osteocalcin and ALP
5 production whilst supporting human bone marrow stromal cell (hBMSC) growth. The increase in ALP was
6 indicative of initial osteogenic differentiation, which happened after a rapid cell division, a stage in osteogenic
7 differentiation of stromal cells in culture. Interestingly, they also found that fucoidan could more than double the
8 compressive strength of the scaffolds from 191 ± 5 KPa to 414 ± 3 MPa, something that could come to use later,
9 due to the intimate link between cartilage/bone formation and biomaterial stiffness.[118] In another study,
10 Puvanewary *et al.* [49] developed a porous fucoidan scaffold to influence bone mineralisation and apatite
11 formation. These scaffolds promoted hBMSC attachment, proliferation and differentiation. Though the lengthy
12 process of mineralisation was not significant, upregulation of collagen I under osteogenic conditions demonstrated
13 osteogenesis within the fucoidan composite. Additionally, Runt-related transcription factor-2 (RUNX2) and
14 osteonectin (ON) were significantly upregulated compared to the chitosan only hydrogel.

15 Owing to the TGF- β -binding properties of fucoidan, it was also exploited for cartilage tissue engineering
16 applications. For instance, Karunanithi *et al.* [119] studied the chondrogenesis of encapsulated hMSCs within a
17 fucoidan-alginate composite. The results revealed that hMSCs cultured in chondrogenic medium supplemented
18 with fucoidan expressed a higher level of chondrogenic markers (including tenascin-C, SOX9, collagen II,
19 aggrecan and cartilage oligomeric matrix protein). In addition, the cultures expressed a significantly lower level
20 of hypertrophy markers (including Col X and Runx2), when compared to alginate hydrogels. Furthermore, cells
21 encapsulated in the fucoidan-alginate hydrogel produced a higher GAG content at day 21 when compared to
22 alginate hydrogels, which is a widely recognized indicator of mature chondrocyte phenotype. Thus fucoidan may
23 enhance the chondrogenic differentiation of stem cells owing to its affinity to multiple growth factors, such as
24 TGF- β_1 . Likewise, cell condensation – a hallmark for chondrogenic differentiation - were observed in this study,
25 which puts further emphasis on the promise that Fucoidan holds in cartilage tissue engineering.

26 *Ulvan*

27 Ulvan, a lightly branched anionic-sulfated polysaccharide, is derived from the cell wall of green algae; and
28 consist of sulfated rhamnose, iduronic and glucuronic acids.[120] The ulvan sugar share a chemical similarity
29 with GAGs, due to its glucuronic acid and sulfate groups.[88, 121] As with the previously investigated marine
30 glycans, ulvan has been employed in conjugation with chitosan to generate osteogenic coatings for titanium
31 implants. To this end, coatings seeded with 7F2 osteoblasts showed complete confluency after 6 days; something
32 significantly different as compared to cells seeded on pure ulvan or pure chitosan. From this point-of-view
33 ulvan/chitosan composite induced the attachment and proliferation of 7F2 osteoblasts while maintaining the cell
34 morphology and viability. In a related study by Dash *et al.* [122] ulvan was used for bone tissue engineering
35 applications. Purposely, the group introduced methacrylate groups to the ulvan backbone to further increase the
36 physiological stability of the hydrogel through UV-crosslinking. Hydrogels were incubated with ALP at varying
37 concentrations to gauge mineralisation capacity, as mineralisation is known to promote bioactivity through the
38 formation of chemical bonds with surrounding bone tissue after implantation. The lowest methacrylated-ulvan

1 group, saw the highest concentration of ALP resulting in pre-osteoblast cells differentiating towards an osteogenic
2 lineage, as interpreted from increased ALP activity and a reduction in cell proliferation.

3 Overall, these naturally sulfated marine glycans have seen limited use thus far in orthopaedic tissue
4 engineering applications. Since they're known to have chemical compositions that mimic several ECM-based
5 GAGs and proteoglycans there's no doubt they could be used to drive the R&D engine of the next-generation of
6 biomaterials for orthopaedic tissue engineering. Especially, their strong affinity towards a wide range of tissue
7 regenerative growth factors makes them ideal growth factor delivery vehicles, which in turn further improve their
8 tissue regeneration capacity. Additionally, their high abundance and sustainability along with reduced
9 immunogenicity strongly advocates their promise in the broader field of tissue engineering.

10 **2.3 Chemically sulfated**

11 The biological features of sulfated polysaccharides from mammalian and plant-based sources are vast. In
12 fact, their bioactivity is a function of molecular weight, sugar-backbone variant and sulfate degree[123] However,
13 naturally-derived polysaccharides typically give rise to batch-to-batch variations, which further hinders the
14 reproducibility of their ensuing biophysical properties.[124, 125] As a result, in an effort to produce sulfated
15 polysaccharides with more specific and controllable functional properties, researchers have started to chemically
16 manipulate non-sulfated polysaccharides such as HA, chitosan, alginates and cellulose, with either sulfate groups
17 or sulfate-containing biomolecules. Controlled chemical sulfation of these polysaccharides can be achieved
18 through various surface immobilisation strategies including chemical binding[126] and electrostatic
19 assembly.[127] Modifying or combining these polysaccharides with sulfate-containing moieties could exploit
20 their native chondrogenic or osteoblastic potential whilst prolonging growth factor delivery to promote
21 proliferation and differentiation of tissue specific stem cells, as well as circumventing shortcomings such as
22 hypertrophy or rapid enzymatic scaffold degradation.[128]

23 *Hyaluronic acid (HA)*

24 HA is a naturally occurring GAG, that has been widely utilised in tissue engineering as it possesses cell
25 surface receptors such as CD44 that enable cell binding,[129] and is immunoneutral at the same time.[130] Indeed,
26 the CD44-based cell binding receptor has been utilised and shown to increase chondrogenesis.[82, 131] Various,
27 groups have also studied the effect of modifying the HA with sulfate groups, to enable sustained growth factor
28 delivery through improved growth factor binding. For instance, Xu *et al.* [132] investigated the effect of decorating
29 HA with heparin. It was seen that when MSCs were seeded onto a HA-heparin hydrogel with BMP-2 present,
30 there was significant upregulation of mRNA and key chondrogenic genes including collagen II, SOX9 and
31 aggrecan, as compared to pristine HA. These improvements can be attributed to the heparin subgroups that contain
32 sulfate groups, which were seen to have a higher binding capacity for BMP-2. Importantly, a sustained release
33 profile over 13 days was observed, compared to pristine HA which displayed an initial burst release profile.

34 In a similar vein, Jha *et al.* [133] chemically modified HA with HS-bearing perlecan domain I perlecan, a
35 recombinantly produced proteoglycan. Here, the HA-perlecan hydrogel exhibited the ability to bind significantly
36 more BMP-2 as compared to HA alone and promoted chondrogenesis. Likewise, Srinivasan *et al.* [134]
37 chemically modified HA with HS and demonstrated a targeted and controlled delivery of BMP-2 for cartilage
38 tissue engineering. For bone tissue engineering HA-based hydrogels have been chemically modified with heparin

1 for BMP-2 delivery *in vitro* and *in vivo*. [135] In this study a rapid burst release of BMP-2 in non-heparin hydrogels
2 was observed, with sustained release only seen in heparin containing hydrogels, which in turn maintained the
3 osteogenic potential of BMP-2 over 28 days. Another study by Hintze *et al.* [136] compared HA, sulfated HA and
4 CS hydrogels, and found that, native HA, low sulfated HA and CS showed low affinity for all TGF- β isoforms.
5 Specifically, the highly-sulfated HA had the greatest affinity for TGF- β 1 and TGF- β 2 but not TGF- β 3. [137]

6 Overall, HA has proven to be a favorable material for various tissue engineering applications as it contains
7 the important CD44 receptor and is capable of binding to important tissue regenerative growth factors. Some
8 studies in the field also suggest that by decorating HA with sulfated materials such as heparin, perlecan and CS,
9 it is possible to significantly increase its affinity towards important growth factors for skeletal tissue engineering
10 as well as delaying their release in a controlled manner.

11 *Chitosan*

12 Chitosan is a non-sulfated, linear polysaccharide with a semi-crystalline and biodegradable nature. It's
13 typically derived from chitin extracted from insects, crustaceans and fungi (Figure 2). Chitosan is known to have
14 intrinsic antimicrobial properties against fungi and bacteria. [138] The molecular weight of chitosan varies from
15 300 – 1000 kD and it is comprised of glucosamine and N-acetyl glucosamine linked by β (1–4) glycosidic bonds.
16 Notably, chitosan behaves as a polycation under acidic conditions, and thus is capable of forming hydrogels in
17 the presence of polyanions and polyelectrolytes. Additionally, the degradability of chitosan directly relates to its
18 degree of crystallinity and can thus be tailored to correspond to the targeted tissue. [139]

19 To even further improve the already impressive biological properties of chitosan, tissue engineers have
20 recently tried to modify its polymeric backbone with sulfate groups. For instance, Cao *et al.* [140] transformed
21 chitosan into 2-N, 6-O-sulfated chitosan (2,6SCS); and demonstrated that this particular sulfated chitosan is useful
22 for sustained and dose-dependent BMP-2 delivery among many sulfated variants. [140] In a follow-up study they
23 made a comparison between BMP-2-gelatin (G)-based scaffolds, BMP-2 loaded 2,6SCS chitosan nanoparticles
24 (BMP-2/NPs) incorporated into these gelatin scaffolds (BMP-2/S-NP/G) and a BMP2-2,6SCS-G composite. To
25 this end, the authors found that the BMP-2/S-NP/G variant could significantly prolong the growth factor release
26 and up-regulate *in vitro* ALP activity as compared to the other variants (Figure 4); something which was thought
27 to be associated with the synergistic action of released BMP-2 and the unique material properties of 2,6SCS
28 sulfated nanoparticles. [141] Interestingly, the addition of nano-particles also had an impact on the mechanical
29 properties of the scaffold, thereby significantly prolonging its degradation time, to create an optimal condition for
30 balancing scaffold removal with the deposition of fresh bone tissue. Building on these results, a recent approach
31 by Pan *et al.* [142] demonstrated that 2,6SCS can also be used to improve the angiogenic and osteogenic capacity
32 of BMP-2, confirmed both on a protein and genetic level. In another recent study, Cao *et al.* used 2,6SCS in
33 combination with poly(lactide-co-glycolide) (PLGA), to manufacture a composite scaffold (S-PLGA). Here they
34 demonstrated that the BMP-2 binding efficiency within the PLGA scaffold could increase almost 10-fold in the
35 presence of 2,6SCS. The release profiles of BMP-2 were 30% slower in S-PLGA scaffolds as compared to pristine
36 PLGA. In the same study, BMSC cells showed an elongated and spindle-shaped morphology when interacting
37 with the hydrophilic surface of S-PLGA. Additionally, these cells were seen to circumvent Noggin inhibition, a
38 BMP antagonist that binds extracellular BMP-2, which in turn inhibits important receptor interactions ultimately
39 leading to reduced osteogenic capacity. Modification of the chitosan backbone with arginine yields a water-

1 soluble molecule that is able to interact efficiently within the biological environment in contrast to the acid soluble
2 starting material. Sulfate modification of this molecule has been achieved at the 2N as well as C2, C3 and C6
3 positions on the chitosan backbone.[143, 144] These sulfated derivatives bind and signal members of the fibroblast
4 growth factor family replicating the activities of HS. While chitosan-arginine has been reported to induce
5 osteogenesis in primary chondroblasts without exposure to osteogenic medium, sulfated chitosan-arginine could
6 facilitate chondrogenesis instead.[143] These data demonstrate how subtle changes in sulfation affect cell
7 phenotype and can direct stem cell differentiation.

8 In summary, the high abundance of chitosan in nature along with its favorable biocompatible and
9 biodegradable properties makes it an attractive biomaterial for skeletal tissue engineering. The modification of
10 chitosan with sulfate groups can further improve the already amazing bioactivity of this material. Indeed, the
11 controlled introduction of sulfate groups onto chitosan's backbone can expand its use as a potential coagulator
12 and a growth factor delivery vehicle.[142] Interestingly, the cationic nature of chitosan enables negative GAGs
13 and proteoglycans to easily be incorporated into such scaffolds to promote better tissue regeneration. What's more,
14 sulfated chitosan is in many ways structurally similar to GAGs, and thus share many of the same biological
15 properties; as its capable of modulating both cell morphology and function – two important hallmarks of cell
16 proliferation and differentiation.[145, 146] Overall, these exciting biomaterial properties of chitosan justify its
17 continued usage as a novel biomaterial in orthopaedic tissue engineering applications.

18 *Alginate*

19 Alginate is a sustainable polysaccharide extracted from brown algae (Pheoophyceae) and less frequently
20 from gram-negative bacteria (Azotobacter and Pseudomonas sp.). Alginates are linear-anionic polymers with
21 favorable biocompatibility for various tissue engineering applications (Figure 2).[147, 148] Notably, alginate has
22 the capacity to form ionic hydrogel networks through chelation with divalent cations, such as Ca^{2+} , broadening its
23 use towards drug delivery[149]. Additionally, due to the innate adhesive and tailorable shear thinning viscoelastic
24 properties of alginate it has found widespread use in bioprinting applications.[150-152] As with other plant-based
25 hydrogels, alginate does not natively support cell adhesion and has been described as a “blank slate” by many
26 engineers in the field.[153] Even still, alginate can be customised through sulfation and peptide modifications to
27 control the phenotypes of encapsulated osteoblasts,[154] chondrocytes[155] and hMSCs.[156]

28 Alginate sulfation based on sulfur trioxide (SO_3) [157] and sulfuric acid[158] treatments have been widely
29 used over the years. To this end, some studies have demonstrated that such sulfated alginates can retain growth
30 factors and promote chondrogenesis through various cellular signaling pathways;[159] and for these reasons they
31 are considered as heparin analogues (Figure 5). Along these lines, Mhanna *et al.* [160] employed an SO_3 /pyridine
32 method of alginate sulfation for cartilage tissue engineering. In this study, the formation of ionic networks was
33 restricted to a degree of sulfation of 0.8 (per monosaccharide unit), as higher degrees of sulfation (2.6) did not
34 facilitate hydrogel formation, possibly due to strong electrostatic forces and/or steric effects between adjacent
35 polymers. Interestingly, they found that sulfation maintained the proliferative capacity as well as phenotype of
36 encapsulated chondrocytes, in contrast to previous studies showing initial dedifferentiation in a non-sulfated
37 hydrogel microenvironments.[161-163] The introduced sulfate groups also influenced Ras homolog gene family
38 member A (RhoA) activity, which is known to be associated with chondrocyte proliferation and

1 differentiation[164]; though the expression of collagen I and collagen II as well as proteoglycan synthesis was not
2 significantly impacted.

3 Thus, sulfated alginate-based scaffolds are promising alternatives to mammalian derived GAGs due to their
4 biocompatibility, low immunogenicity, protein retention capacity and the great variety of readily implementable
5 gelling and functionalisation strategies that can improve their bioactivity. Their extensive and continued use will
6 definitely empower researchers with the knowledge to effectively understand the regulatory role of sulfated-
7 alginate in extracellular and intracellular interaction, something, which hopefully will lead to their more frequent
8 use in skeletal tissue engineering in the foreseeable future.

9 *Cellulose*

10 Cellulose is the most abundant natural polysaccharide available in the world.[165, 166] Its chemical
11 structure consists of unsubstituted, linear glucose homosaccharide with six available hydroxyl groups.
12 Intriguingly, it has been seldom used in tissue engineering, potentially due to difficulties in hydrogel assembly
13 caused by solubility inadequacies.[167] The sulfation of cellulose can improve solubility, through the disruption
14 of intermolecular hydrogen-bonds[168] to potentially broaden its applicability towards various tissue engineering
15 applications.[169]

16 One study by Huang *et al.* [170] explored the use of sulfated cellulose scaffolds for cartilage tissue
17 engineering. Initially MSC induction media was spiked with a fully sulfated form of sodium cellulose (NaCS)
18 leading to a significant upregulation of collagen II and aggrecan. In the same study, NaCS was combined with
19 gelatin to develop scaffolds through electrospinning. Interestingly, the scaffolds with the lowest concentration
20 (0.1%) of NaCS added to induction media resulted in the highest production of collagen II both on a protein and
21 genetic level after 56 days of culture. Additionally, cells on the 1% and 5% NaCS/Gelatin-based scaffold showed
22 low collagen X production, suggesting higher NaCS may result in a reduced propensity towards hypertrophy.
23 These higher sulfate concentrations may have an inhibitory effect on chondrogenesis because of irreversible
24 growth factor-biomaterial bindings, which in turn can comprise the release and delivery of TGF- β 3 to the targeted
25 cells.[171] The same group took this a step further and introduced partially sulfated cellulose (pSC) into gelatin
26 hydrogels instead, and discovered an enhanced expression of chondrogenic markers (collagen II/collagen I ratio,
27 aggrecan and SOX9) upon increasing pSC concentration in the scaffolds, indicating the potential of pSC as a
28 scaffold for cartilage tissue engineering.[172]

29 For these reasons, cellulose sulfate is an interesting carrier for growth factor delivery in cartilage tissue
30 engineering and could have broader uses in the foreseeable future due to its abundance, sustainability and reduced
31 immunogenicity. Specifically, the backbone sulfation of cellulose allows for precise control over the sulfation
32 pattern and sulfation degree, and thereby enables the biological properties of such scaffolds to be fine-tuned in a
33 customizable manner. The range of available chemical modifications can also pave the way for tuneable
34 mechanical and pharmaceutical properties, and could thereby potentially enable an even greater variety of
35 biomaterials. [173] [174]

36 **3. Tissue engineering**

1 While sulfated polysaccharides have been shown to successfully act as delivery vehicles for growth factors
2 in an *in vitro* environment, their ability to elicit this response in an *in vivo* model needs to be evaluated as well.
3 Indeed, many tissue engineering approaches have shown significant benefits in *in vitro* studies yet when they
4 progress to animals models they show some limitations.[28] Understanding, whether the successful *in vitro*
5 strategies also show promise in an *in vivo* setting, is therefore critical to successfully translate tissue engineering
6 strategies from the laboratory and into the clinic. This section, highlights recent advances in translating the hard
7 tissue regenerative potential of scaffolds made from sulfated polysaccharides in various animal models both alone,
8 in combination with various growth factor or with other biopolymers.

9 **3.1 Bone**

10 The number of people at risk of bone fractures has grown steadily in most parts of the world due to the
11 ageing population. In 2015 around 160 million people worldwide experienced a bone fracture; a number that is
12 expected to double to 320 million by the end of 2040.[175] Traditional clinical therapies for mending bone
13 fractures rely on various forms of casts to fixate the broken fracture to enable the native bone to heal itself on its
14 own terms, however, native bone displays a restrictive regenerative capacity, that is haunted by a number of
15 challenges including non-anatomical reduction of the fracture, a-vascular necrosis, as well as non-union and mal-
16 union fracture healing.[176] These issues are more prevalent in older people and will thus grow steadily in the
17 near future as the median lifetime is expected to increase significantly in the coming decades. Autologous bone
18 grafts are commonly utilized to promote osteoconduction and osteoinduction in bone defects to avoid the
19 abovementioned scenarios. While these grafts have shown some promise for healing bone defects, they require
20 multiple invasive surgeries and are hindered by low availability and donor site morbidity associated with
21 relocating native bone tissue from the patient's own bone and into the defect site.[177] Allografts on the other
22 hand are limited as a consequence of lack of available donor tissues and unwanted foreign body responses; and
23 bone implants in some cases do not facilitate sufficient bone healing and therefore revisions surgeries are common
24 with this methodology.[178]

25 For these reasons, a number of bone tissue engineering strategies have emerged to address this critical
26 challenge by delivering the promise of a better method to mend bone defects.[179] As such, these approaches rely
27 on developing synthetic bone tissues by combing 3D biomaterials with stem cells either exogenously or by
28 recruiting them from native bone-tissue in a post-implantation scenario. The 3D biomaterials have the potential
29 to drive stem cells into bone-like cells that under the right conditions can form mature tissues either in the
30 laboratory or within the body depending on which one of the abovementioned strategies has been employed
31 (Figure 1). However, many of the tissue engineered scaffolds explored to date have not reached this full potential
32 and in many cases fall short of the performance of autografts.[180] A number of studies, including those by Wang
33 *et al.* [177] and Lee *et al.* [15] suggest that such results could be related to the uncontrolled release of growth
34 factors that collaterally interfere with untargeted cells. As sulfated polysaccharides can bind and regulate the
35 signalling of a number of important growth factors they are likely to be essential components of next-generation
36 biomaterials for bone tissue engineering.

37 Indeed, sulfated polysaccharides are considered one of the most important biological and mechanical
38 components of the native ECM of hard tissues.[181] They have therefore in recent years emerged as new and

1 promising building blocks for bone tissue engineering scaffolds.[182] Heparin is one of the most widely employed
2 sulfated polysaccharides in this respect, due to its ability to capture, stabilize and present growth factors to bone
3 progenitor cells in a controllable manner. For instance, Yang *et al.* [183] developed heparin-conjugated fibrin
4 scaffolds for orthotopic *in vivo* models to control the release of BMP-2 in order to prolong the bioactivity of ALP.
5 This prolonged activity ultimately translated itself into significant improvements in bone mineralization when
6 compared with pristine fibrin scaffolds. Notably, by using heparin, they were able to obtain a similar amount of
7 new tissue formation with lower concentrations of BMP-2 than previously reported in the literature.[184]
8 However, some studies have reported that exogenous heparin under certain circumstances reduces the bioactivity
9 of osteogenic biomolecules and can thus compromise the bone healing process, by inhibiting the binding of BMP-
10 2 to the BMP receptor. What's more, the potent anticoagulant activity of heparin is, by many in the field, thought
11 to be counterproductive for bone growth.[185]

12 To address these issues, sulfated chitosan, has been used as an alternative due to its good biocompatibility
13 and similar growth factor binding ability as heparin without the abovementioned native biological issues
14 associated with heparin.[186] In this direction, Zhou *et al.* [187] synthesized BMP-2 loaded chitosan with varying
15 degrees of sulfation and compared their responses *in vivo*. These *in vivo* results revealed that the most sulfated
16 chitosan-based scaffold was the best promoter of BMP-2 bioactivity, and could even surpass the bone regeneration
17 capacity of heparin-based scaffolds. Similarly, Bai *et al.* [188] and Lü *et al.* [189] developed a self-healing,
18 biocompatible and injectable dual cross-linked CS-based hydrogels for *in vivo* delivery of BMP-4. This hydrogel
19 was crosslinked through both diels-alder (DA) and acylhydrazone bonds; and the authors used these bonding
20 schemes to fine-tune various hydrogel properties such as rigidity and degradation. Through this sophisticated
21 crosslinking scheme they were also able to manufacture a superior hydrogel, which could prevent excessive
22 hydrogel swelling *in vivo*; and thereby prevent poor stem cell differentiation and tissue regeneration.[190] In both
23 instances, histology staining's demonstrated new bone formation in the BMP-4 loaded hydrogel samples after 12
24 weeks, with controls primarily stimulating fibrous tissue growth. Additionally, initial sproutings of blood vessels
25 were observed. In another noteworthy study, Kim *et al.* [181] evaluated the inclusion of UV-crosslinked
26 methacrylated CS (MeCS) in PEGDA hydrogels at various concentrations in terms of their bone regenerative
27 properties within the body (Figure 6). Specifically, these scaffolds were implanted in critical sized calvarial defects
28 (4mm diameter) in six-week-old female mice (n = 4) for up-to eight weeks. Interestingly, scaffolds containing the
29 highest concentration of CS induced the most effective bone formation evidenced by larger bone mineralization
30 density. This was speculated to arise from the ability of the sulfate groups within CS to bind to calcium ions and
31 facilitate the formation of fresh hydroxyapatite; one of the most important components of the mineral phase of
32 bone. Additionally, Hematoxylin, Eosin and Masson's trichrome staining's also showed significant improvements
33 in bone tissue formation with increasing CS concentration.

34 Although, a wide range of sulfated polysaccharides have been studied in the literature, these biomaterials
35 are seldom employed in clinical treatments due to the lack of more standardized clinical studies.[191] Indeed, a
36 number of important parameters such as the size of the bone defect, the place of the defect, the implanted cell
37 type, and implantation time needs to be considered to fully unravel the bone tissue engineering potential of such
38 scaffolds. Unfortunately, these parameters have not been studied enough to turn this promising strategy into a
39 clinical therapy which can benefit the many sufferers of bone disorders.[191] Consequently, more in-depth *in vivo*

1 studies are necessary to validate the efficiency of sulfated polysaccharides for bone tissue engineering, and to
2 identify the best combination to use in the clinic.

3 **3.2 Cartilage**

4 The primary cause of cartilage damage within the body is due to osteoarthritis (OA) in articular cartilage.
5 The clinical treatment for OA is currently suboptimal as the “state-of-the-art” surgical approaches are limited in
6 terms of their efficacy and high invasiveness. First stage interventions include arthroscopy, which involves the
7 flushing and removal of damaged cartilage and meniscus.[192] For more severe cases, the implantation of
8 autologous osteochondral graft (mosaicplasty) into the defect site and surgical drilling into the subchondral bone
9 (microfracturing) can be employed.[193] However, unfortunately both measures are controversial as they often
10 result in fibrous cartilage rather than native articular cartilage.[194] For the most severe cases, extremely invasive
11 and costly total knee replacements can be performed.[195] Notably, these measures are aimed at slowing the
12 impact of OA without actively regenerating native cartilage.

13 Recently, techniques such as stem cell therapy have been used to regenerate cartilage tissue, by injecting
14 regenerative cells into the damaged region.[43, 48, 174, 196] This technique is limited by low cell retention and
15 a low cell viability, caused by the shear-forces that cells experience when passing through the thin injection needle.
16 It also does not provide the cells with a 3D microenvironment to properly differentiate them into the required
17 tissues. The usage of hydrogels can provide a mechanical shield during the needle-injection phase and provide a
18 proper 3D microenvironment for guiding cells into the desired cell phenotypes in a post-injection scenario.
19 Especially, sulfated hydrogels hold great promise in this respect, since they display high affinity towards important
20 growth factors for cartilage regeneration; and in many ways resemble – CS - one of the most important
21 components of the native cartilage ECM. Indeed, such biopolymers have recently been used to develop scaffolds
22 with the capacity to deliver growth factors such as BMP-2 and TGF- β 3 in a sustainable manner to significantly
23 improve the cellular performance of chondrocytes.[75, 76] In another related study by Han *et al.* [197] a mussel
24 inspired CS-based hydrogel was created for enhanced adhesion between graft and native cartilage tissue (Figure
25 7). Specifically, the inclusion of CS promoted an upregulation of chondrogenic differentiation markers such as
26 aggrecan and collagen II. The scaffolds were also evaluated in a full thickness defects (diameter: 3.5 mm;
27 thickness: 5 mm) in the patella groves in the right legs of white rabbits (n = 8). Following a three-month
28 implantation period, the scaffolds showed significantly higher tissue formation in terms of Modified O’Driscoll
29 and International Cartilage Repair Society grading scores.

30 The abovementioned studies on using sulfated polysaccharides for cartilage regeneration clearly
31 demonstrate the great promise that they hold for the field of cartilage tissue engineering. Indeed, considering the
32 importance of cell therapy in treating acute cartilage injuries, sulfated polysaccharides can be ideal scaffolding
33 materials to support the chondrocytes temporarily until the implanted cells replace them by matrix components.
34 Collectively, the use of such scaffolds is expected to reduce chondrocyte leakage from the transplant site, facilitate
35 a more homogeneous chondrocyte distribution, and diminish graft hypertrophy.[198] Regardless, if these
36 scaffolds were to be used in cartilage tissue engineering, we would need to consider important parameters such as
37 lesion location and damage size, activity level and patient’s age. These parameters are by many in the field
38 considered the important parameters when it comes down to deciding which cartilage repair approaches to use

1 and evaluating the treatment.[199] Finally, the biomaterials utilised in *in vivo* cartilage tissue engineering need to
2 demonstrate appropriate biomechanical and biochemical cues without triggering immune responses. Therefore,
3 biomaterials and cell therapy techniques should also be compared to ‘gold standard’ techniques such as
4 microfracture and grafting in order to accurately gauge their efficacy *in vivo*. The continued investigations into
5 the usage of sulfated polysaccharides as growth factor delivery vehicles is also needed to fully elucidate their
6 potential as tissue engineering scaffolds for cartilage regeneration.

7 **3.3 Osteochondral**

8 Defects that impact both the articular cartilage and the underlying subchondral tissues are termed
9 osteochondral defects. Such lesions are caused by tissue degradation from aging, sports injuries or severe cases
10 of osteoarthritis. They typically result in joint instability, significant discomfort for the patient and loss of patient
11 mobility. Much like cartilage, osteochondral defects can be treated through microfracturing, allografting and
12 mosaicplasty, or even total knee replacements, however, all of these therapies unfortunately have similar issues
13 as those briefly mentioned in the previous section.[200] The abovementioned tissue engineering approaches could
14 remedy these shortcomings by recapitulating the highly hierarchal structure of osteochondral defects.

15 In this direction, Zhou *et al.* [201] recently combined silk fibroin with CS to develop a composite scaffold
16 that could mend osteochondral defects in a rabbit animal model. Indeed, this composite material produced greater
17 neo-tissue formation and improved structural restoration compared to the pristine silk scaffold at 6 and 12 weeks
18 as evident from an International Cartilage Repair Society histological analysis (Figure 8). Additionally, when
19 analysed *in vitro*, the composite scaffold was seen to maintain better chondrocyte morphology compared to the
20 silk scaffold alone, in combination with a higher expression of SOX9, collagen II, aggrecan and lower expression
21 of TNF- α 2 (an important inflammation marker) (Figure 8). In a similar vein, Liao *et al.* [202] implanted a
22 biomaterial composite consisting of methacrylated CS and poly(ethylene glycol) methyl ether- ϵ -caprolactone-
23 acryloyl chloride (MPEG-PCL-AC) incorporated with graphene oxide, into full-thickness osteochondral defects
24 (thickness: 3mm, diameter: 4mm, n = 27) in the hind limbs of rabbits. When combined with chondrocytes, the
25 scaffold was seen to improve chondrocyte morphology, integration, and subchondral bone formation. Notably,
26 this strategy could rapidly induce the formation of both new and thicker cartilage tissue as compared to a cell-free
27 scaffold.

28 In another study Feng *et al.* [52] conjugated sulfate groups onto the backbone of methacrylated hyaluronic
29 acid (MeHA) in order to deliver growth factors in a osteochondral rodent (n = 10) model in a controlled and
30 sustainable manner (Figure 9). Typically, HA is degraded rapidly by hyaluronidases *in vivo* and lacks high protein
31 binding affinity. They found that the introduction of sulfate groups reduced the degradation and deformation of
32 hydrogel scaffolds and promoted cartilage matrix deposition, as indicated by immunohistochemical stainings of
33 collagen II and CS, following 4 weeks *in vivo* studies. Additionally, the sulfated-HA in combination with hMSCs
34 was capable of attracting and retaining supplemented TGF- β 1, and thereby promoting chondrogenesis and
35 suppressing hypertrophy. Overall, the paper by Feng *et al.* [52] demonstrates that sulfated HA hydrogels enable
36 the generation of high quality neocartilage via intra-articular injection. Another noteworthy study used a heparin
37 immobilised polycaprolactone (PCL)/Pluronic F127 scaffold combined with TGF- β 2 and BMP-7 to facilitate even
38 more cartilage tissue formation as compared to PCL/Pluronic scaffolds alone. However, no significant histological

1 differences following implantation into large (diameter = 6mm, depth = 3mm) distal femur defects in rabbits (n =
2 12) was seen in this study.[203] Finally, Re'em *et al.* [204] recently created a bilayer scaffold with alginate-sulfate
3 incorporating both TGF- β 2 and BMP-4. This scaffold was subsequently implanted into subchondral defects
4 (diameter = 3mm, depth = 3mm) in the femur of rabbits. Encapsulated hMSC's were successfully differentiated
5 into both osteoblasts and chondrocytes at respective layers over 4 weeks, confirming the controlled release of the
6 growth factors. Additionally, the cartilage–bone interface formation remained the same in hMSC incorporated
7 scaffolds, indicating that native cells were able to migrate into the scaffolds and sense the biological cues spatially
8 present in there, and respond accordingly by differentiating to the appropriate cellular lineage.

9 History has shown that applying promising laboratory strategies to animal models is not always as
10 successful. Even a rudimentary understanding, through the use of pilot studies, of the *in vivo* efficacy of such
11 techniques can create a much more efficient process for producing novel, viable tissue engineering solutions. For
12 these reasons, sulfated-scaffolds for osteochondral tissue engineering are also beginning to be translated into *in*
13 *vivo* environments. Most often, these materials are used in composites to capitalise upon the benefits of multiple
14 materials and to develop the hierarchical scaffolding architecture needed for optimal osteochondral repair. To this
15 end, the effects of growth factor delivery and improved cellular performance observed in *in vitro* studies appear
16 to translate into *in vivo* outcomes. Additionally, the studies reviewed here indicate that sulfated polysaccharide do
17 not elicit any significant inflammatory responses when implanted *in vivo*, confirming that they indeed are suitable
18 biomaterials for osteochondral tissue engineering.

19 **Conclusion and future directions**

20 Tissue engineering has shown tremendous potential in several facets of biomedicine, particularly in skeletal
21 tissue engineering. With the ongoing development of novel sulfated biomaterials along with sophisticated *in vitro*
22 culturing systems tissue engineering will enhance our capacity to recapitulate bone and cartilage regeneration
23 through the sustained delivery of relevant growth factors. Overwhelmingly, the most commonly studied and
24 successful naturally sulfated biomaterials include CS and heparan sulfate and its analogues. The benefits that these
25 naturally sulfated ECM components provide can be chemically incorporated into non-sulfated biomaterials.
26 Specifically, HA and chitosan sulfation allows for the controlled binding and release of growth factors in a
27 localised environment. The use of composite materials in tissue engineering is omnipresent and can capitalise
28 upon the benefits of multiple materials. These four materials, CS, Hep/HS, HA and chitosan, can be easily utilised
29 in a composite system, where the scaffold can provide cells with controlled, prolonged and protected growth factor
30 delivery. Though, the translational capacity of animal-derived sulfated biomaterials is limited *in vivo* due to
31 immunogenicity, further exploration into plant-derived substrates could be a worthy endeavour. Intriguingly, as
32 these materials don't have specific enzymes for degradation their use could potentially extend growth factor
33 delivery beyond the body's native capacity. Many areas within the vibrant field of tissue engineering could readily
34 benefit from the utilization of sulfated biomaterials as a vehicle for providing growth factors to the target tissues
35 to elicit improved cellular performance both *in vitro* and *in vivo*.

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17

18 **Figures**

19

20 **Figure captions**

21

22 **Figure 1:** A schematic showing the core-principles behind tissue engineering.

23

24 **Figure 2:** Some of the most important sulfated polysaccharides reviewed herein have been
25 highlighted in this figure along with their derivation source and chemical structure.

26

27 **Figure 3: The growth factor retention capacity of heparin.** (a) Schematic showing the
28 design principle behind the biphasic silk fibroin scaffold used in the study. (b) The heparin
29 loading efficiency and its release profile from the scaffold is displayed here. (c) The sustained
30 release of TGF- β 2 and GDF5 is displayed here. Crosslinked heparin significantly delayed the
31 growth factor release. Modified from[77], with permission from Elsevier, Copyright 2018.

32 **Figure 4: The growth factor retention capacity of sulfated chitosan.** (a) Schematic
33 showing the design principle behind the S-NP incorporated gelatin scaffolds. (b) The size
34 distribution and scanning electron microscopy images (SEM) of the S-NP's are displayed here.
35 (c) The sustained release of BMP-2 from the scaffolds employed in this study is displayed here.
36 Modified from[141], with permission from Elsevier, Copyright 2014.

1 **Figure 5:** A schematic showing the growth factor bind properties of sulfated alginates and
2 their ability to promote chondrogenesis through important signalling pathways. Modified from
3 [159], with permission from MDPI, Copyright 2017.

4 **Figure 6: A chondroitin (CS)-based scaffold for bone tissue engineering.** (a) The
5 manufacturing of the PEGDA-MeCS hydrogel and its hydroxyapatite (HAP) formation
6 capacity is shown here. (b) The calcification and HAP formation of the cell-laden hydrogels
7 after 21 days are shown here through photographic images of the hydrogels at relevant time
8 points. (c) The bone regenerative capacity of the respective scaffolds incorporating different
9 concentrations of CS was quantified through Micro-CT analysis after 8 weeks of implantation.
10 (d) The bone area (BS/TS) and bone volume (BV/TV) were also calculated and are displayed
11 here. Adapted with permission from [181]. Copyright (2017), American Chemical Society.

12

13 **Figure 7: A tissue adhesive CS-based scaffold for cartilage tissue engineering.** (a) The
14 CS-based scaffold was made tissue adhesive by polymerizing dopamine (DA) and acrylamide
15 (AM) into it. (b) The tissue adhesive properties of the scaffold was mediated by the many
16 amino groups present on PDA and PAM. (c) The adhesion strength of the various manufactured
17 scaffolds towards porcine skin is shown here. (d) The cartilage regenerative potential was
18 highest for the PDA-CS-PAM hydrogel. (e) This was further validated by analysing the
19 Modified O' driscoll scoring for the implanted scaffolds after 3 months of implantation.
20 Adapted with permission from [197]. Copyright (2018). American Chemical Society.

21 **Figure 8: A Silk-CS-based scaffold for osteochondral tissue engineering.** (a) The
22 manufacturing process behind the Silk-CS scaffold is shown here. (b) The chondrogenic and
23 anti-inflammatory capacity of the Silk-CS was quantified from expression of relevant gene
24 markers. (c) Histological evaluation of the scaffolds after 12 weeks of implantation. H&E is
25 short for hematoxyling and eosin and SO for Safranin O. (D) The histological scores for
26 subchondral bone formation was evaluated after 6 and 12 weeks. Modified from [201], with
27 permission from Elsevier, Copyright 2017.

28

29 **Figure 9: A sulfated hyaluronic acid scaffold for osteochondral tissue engineering.** (a)
30 The manufacturing scheme behind the scaffolds are shown here, where LS-MeHA and HS-

1 MeHa are short for low sulfated and high sulfated methacrylated hyaluronic acid (HA),
2 respectively. (b) The TGF- β 1 retention capacity of the various scaffolds employed in the study
3 is shown here. (C) Histological staining of the respective hMSCs-laden scaffolds after 42 days
4 of implantation. Modified from [52], with permission from Elsevier, Copyright 2017.