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REVIEW ARTICLE

Glycosaminoglycan-Mediated Interactions in Articular, Auricular, Meniscal, and Nasal Cartilage

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Glycosaminoglycans (GAGs) are ubiquitous components in the cartilage extracellular matrix (ECM). Ultrastructural arrangement of ECM and GAG-mediated interactions with collagen are known to govern the mechanics in articular cartilage, but these interactions are less clear in other cartilage types. Therefore, this article reviews the current literature on ultrastructure of articular, auricular, meniscal, and nasal septal cartilage, seeking insight into GAG-mediated interactions influencing mechanics. Ultrastructural features of these cartilages are discussed to highlight differences between them. GAG-mediated interactions are reviewed under two categories: interactions with chondrocytes and interactions with other fibrillar macromolecules of the ECM. Moreover, efforts to replicate GAG-mediated interactions to improve mechanical integrity of tissue-engineered cartilage constructs are discussed. In conclusion, studies exploring cartilage specific GAGs are poorly represented in the literature, and the ultrastructure of nasal septal and auricular cartilage is less studied compared with articular and meniscal cartilages. Understanding the contribution of GAGs in cartilage mechanics at the ultrastructural level and translating that knowledge to engineered cartilage will facilitate improvement of cartilage tissue engineering approaches.

Keywords: chondrocytes, extracellular matrix, macromolecules, mechanobiology

Impact Statement

Reviewing the complex contributions of glycosaminoglycan in cartilage mechanics at the ultrastructural level will enable translation of this knowledge into engineered cartilage efforts and facilitate improvement of tissue engineering approaches.

Introduction

Cartilage is classified into three types, namely, hyaline cartilage, elastic cartilage, and fibrocartilage, primarily due to the differences in the fibrous components making up each type. These different cartilage types perform a variety of mechanical and protective functions in the human body, which, when affected by disease or trauma, can have

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debilitating results for the patient. There is an increasing need to develop options to replace or restore cartilage that is malfunctioning or missing due to injury, disease, or congenital disease. Tissue engineering (TE) is one approach that shows tremendous promise to mimic the heterogeneous nature of all cartilage types[1–4] and reduce the increasing burden of cartilage-related medical conditions. However, due to the complexity and heterogeneity of the cartilage structure, current TE approaches have yet to create a construct that produces a tissue with comparable functional quality to native cartilage. Challenges in manipulating and optimizing the cellular response in an *in vitro* environment and the inability to replicate ultrastructural features of the native tissue have been identified as key barriers to producing TE products with structural and functional integrity.[5–8]

The three types of cartilage vary both compositionally and structurally from one another. The basic building blocks of each type of cartilage are similar and include chondrocytes, collagen, elastin, proteoglycans (PGs), and glycosaminoglycans (GAGs). In addition, auricular and nasal cartilage are surrounded by a layer of connective tissue, called perichondrium.[9] The proportions and composition of these components differ according to the cartilage type, a difference which impacts their functional properties. The specific PGs and GAGs present in each type are not well investigated. Besides these basic compositional differences, the structural interactions of extracellular matrix (ECM) components according to anatomical location are also unknown. These variations can be subtle but are important for understanding the ultrastructure and tissue homeostasis of native cartilage. Furthermore, the precise ultrastructure that should be mimicked in the TE field is not well described for some cartilage types.

This article reviews literature related to four types of permanent (adult) cartilage: articular and nasal septal cartilage (hyaline), auricular cartilage (elastic), and meniscal cartilage (fibrocartilage). First, ultrastructure is discussed, followed by an exploration of reported interactions of GAGs with chondrocytes, collagen, and elastin. Efforts to replicate this cartilage ultrastructure in TE efforts for all cartilage types are described. Finally, a roadmap for further investigating GAG-mediated interactions in cartilage is outlined. Unless otherwise stated, the review is focused on human cartilage. Where human studies are missing and nonhuman data are available, this is reported for completeness.

Ultrastructure of Different Cartilage Types

Chondrocyte arrangement

The arrangement of chondrocytes in each type of cartilage depends on the anatomical location. Chondrocytes in articular cartilage of the knee show zonal variation in arrangement and morphology (Fig. 1A). The superficial layer of articular cartilage has elongated chondrocytes with parallel arrangement to the articulating surface. The middle-zone chondrocytes have a rounder morphology. The deep-zone chondrocytes are arranged as columnar clusters perpendicular to the articular surface.[13]

In meniscal cartilage, there are three different cell populations, namely, fibroblast-like cells, fibrochondrocytes, and fusiform cells, each located in a different region of the meniscus[12] (Fig. 1B). These cells are morphologically and phenotypically different from one another.[12] Fibroblast-like cells present in the outer one third of meniscal cartilage have oval fusiform shape with long extensions. In contrast, fibrochondrocytes are present in the inner region and have a round shape. Fusiform cells, present in the superficial zone, have flattened and fusiform morphology similar to fibroblast-like cells but without cell extensions.[12] This is also seen in rabbit meniscus.[12,14]

Nasal septal cartilage and auricular cartilage have three different zones (Fig. 1C).[15,16] The peripheral zone, which is adjacent to the perichondrium, has small flat cells oriented parallel to the cartilage surface. The intermediate zone has ovaloid cells where the axis runs more perpendicular to the cartilage surface. The central zone consists of cells which are more spherical and more or less arranged in columnar patterns perpendicular to the cartilage surface. The cell density decreases from the peripheral to central regions.[17] Comparatively, chondrocyte density in human nasal septal cartilage and auricular cartilage is similar; 390.76 ± 38.30 and 365.36 ± 74.36 cells/mm^2, respectively, while no significant differences are reported for chondrocyte size (area, mm^2).[10]

Collagen fiber arrangement

As with chondrocytes, the arrangement of collagen fibers in articular cartilage varies from the superficial layer to the deep zone (Fig. 2A). Collagen fibers in the superficial layer are arranged parallel to the articulating surface. The orientation of the collagen fibers in the middle zone is oblique, before anchoring perpendicular to the subchondral surface in the deep zone.[19] This zonal variation describes the different load bearing requirements of each zone. Collagen of the superficial layer is subjected to shear stress, whereas in the deep zone, they experience higher tangential normal stresses.[20] In addition, the amount of collagen in each zone differs (between 10% and 20% wet weight), with the highest amount found in the superficial zone.[20]

The arrangement of collagen fibers in meniscal cartilage also shows a zonal variation (Fig. 2B). Type I collagen fibers are oriented in circumferential and radial directions in the interior layers of the meniscus.[21] In contrast to the large amounts of collagen type I in outer regions of the meniscus (with only trace amounts, <1%, of types II and V), the inner region was reported to contain more collagen type II (60%) than type I (40%) in a study using porcine menisci.[22,23] The circumferentially arranged fibers of the meniscus contribute to a high tensile load bearing capability of the outer region,[22] and those arranged radially resist compressive stresses.[24]

Histology studies of human and bovine nasal septal cartilage have shown a higher density of collagen II in the peripheral regions in nasal septal cartilage compared with the central regions (Fig. 2C).[15,25] The fibers are aligned parallel at the surface next to the perichondrium and perpendicularly aligned fibers when moving down the surface.[26]

Collagen II in auricular cartilage is arranged together with a dense elastin fiber network surrounding the chondrocytes (Fig. 2D).[9,27] However, the zonal variation of collagen fibers in auricular cartilage are not well defined.

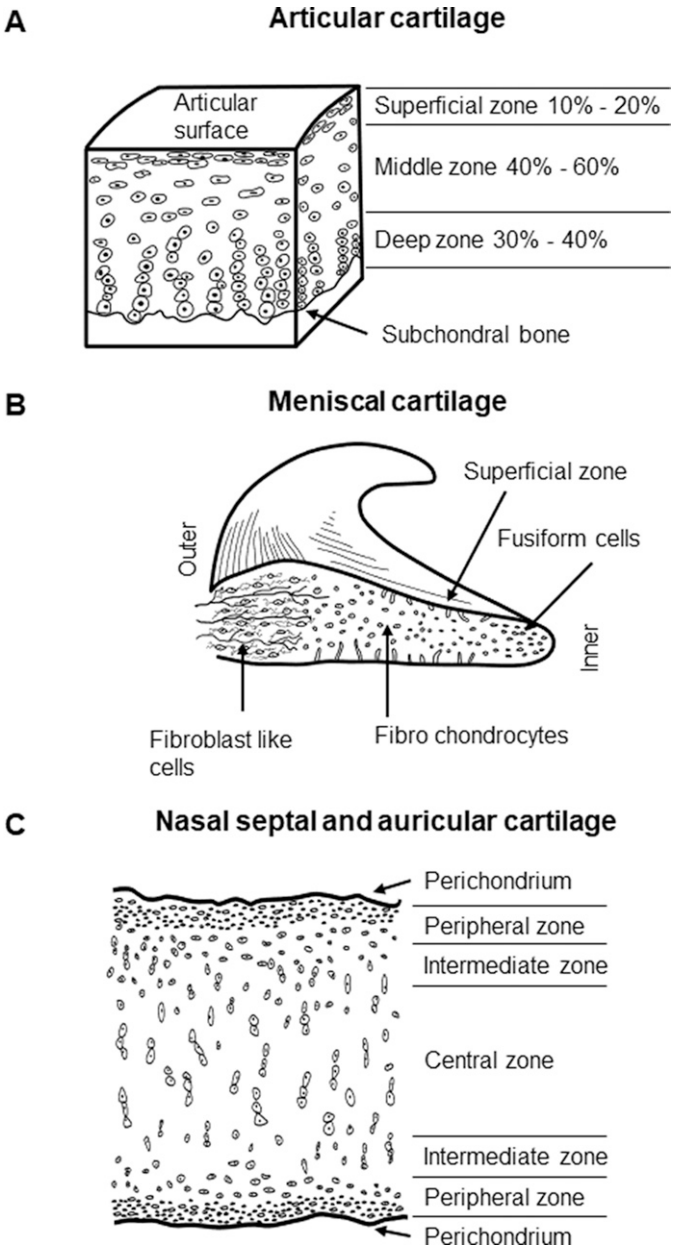


FIG. 1. Zonal arrangement of chondrocytes in different cartilage. **(A)** Articular cartilage: the superficial zone of articular cartilage has elongated chondrocytes aligned parallel to the articulating surface. Middle-zone chondrocytes have a rounder morphology. In the deep zone, chondrocytes are arranged as columnar clusters perpendicular to the subchondral bone surface. **(B)** Meniscal cartilage: Fibroblast-like cells present in the outer one-third of meniscal cartilage have oval fusiform shape with long extensions. Fibrochondrocytes are present in the middle region and have a round shape. Fusiform cells, present in the superficial zone, have flattened and fusiform morphology similar to fibroblast-like cells but without cell extensions. **(C)** Nasal septal cartilage and auricular cartilage: Peripheral zone consists of small, flat cells oriented parallel to the cartilage surface. Intermediate zone has ovaloid cells where their axis runs perpendicular to the cartilage surface. Central zone consists of cells which are spherical and arranged in columnar cluster perpendicular to the surface. There is no significant difference in chondrocyte density or size between nasal septal and auricular cartilage.[10] Adapted from **(A)** Buckwalter et al.[11] and **(B)** Makris et al.[12]

Elastin arrangement

Auricular cartilage has a high elastin content compared with other cartilage types, where it is only reported in trace amounts. Elastin is arranged as a 3D honeycomb around the auricular chondrocytes (Fig. 2D).[28] Interestingly, an organized elastin network at the superficial layer, arranged parallel to the cartilage surface, has been observed in kangaroo, bovine, and equine articular cartilage models.[29–31] The middle and deep zones of bovine articular cartilage have also reported an elastin microfibril arrangement around chondrocytes (pericellular matrix) and in the ECM.[31] Similar studies in human articular cartilage have not indicated the presence of elastin. Meniscal cartilage contains less than 1% elastin arranged at the meniscal surface and bridging the collagen fibers.[12,18]

In summary, the arrangement of zone-specific collagen in articular and meniscal cartilage is well understood, while such aspects of auricular and nasal cartilage are yet to be defined. However, understanding that the collective behavior of collagen and elastin is in concert with PGs and GAGs is required to provide an improved understanding of the specific tissue mechanics and mechanobiological function.

Evidence of the Importance of PGs and GAGs in the Mechanical Integrity of Cartilage through Interaction with Other ECM Molecules

PG and GAG ultrastructural arrangement

PGs are ubiquitous macromolecules found in ECM of all cartilage types. They compose a protein core to which GAG chains are covalently attached.[32] Some PGs are attached to hyaluronan through the glycoprotein known as link protein. Such PGs that are referred to as aggregating PGs and other PGs which do not connect to hyaluronan are known as

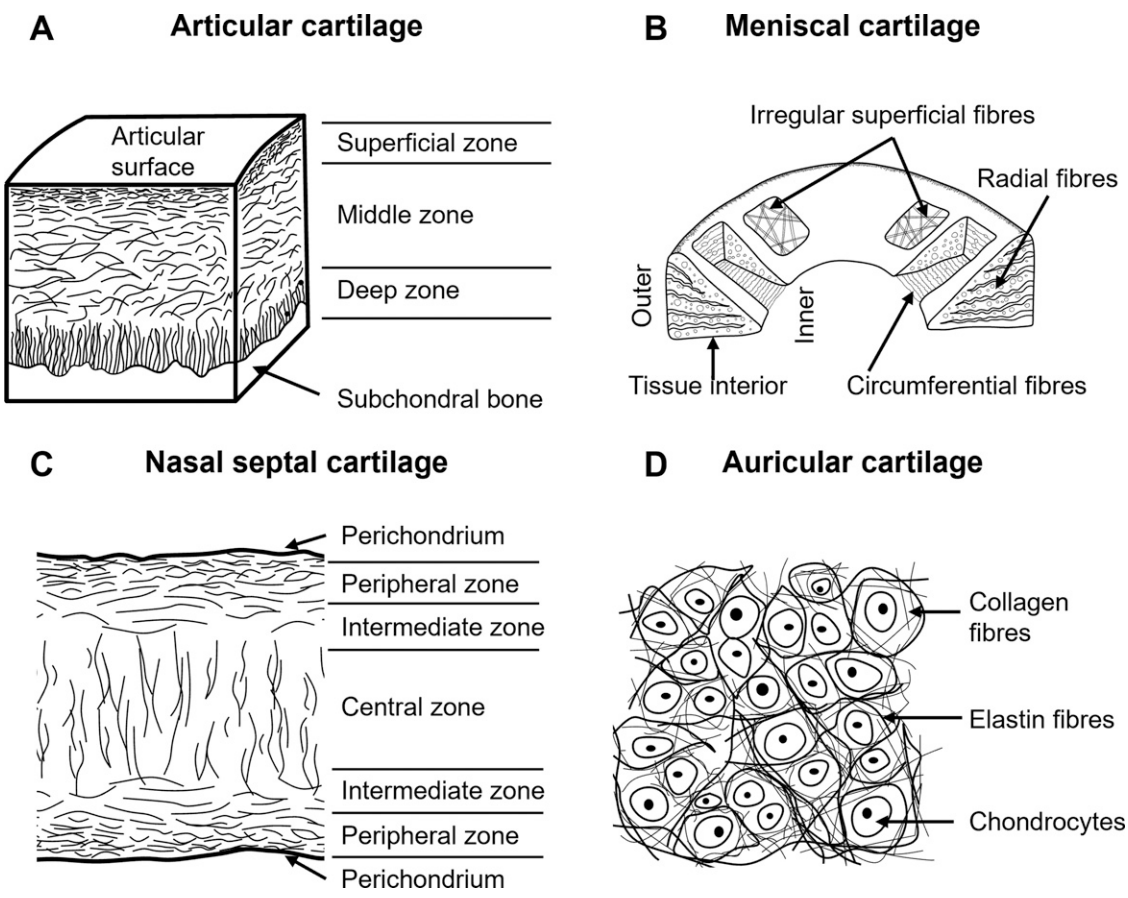


FIG. 2. Collagen fiber arrangements in different cartilage. **(A)** Articular cartilage: collagen fibers in the superficial zone are arranged parallel to the articulating surface enhancing the ability to withstand the tensile shear stress. Orientation of the collagen fibers is random in the middle zone and perpendicular to the articular surface in the deep zone. **(B)** Meniscal cartilage: Collagen fibers are oriented circumferentially in the interior zone of the meniscus. Radially orientated collagen fibers are also present in the tissue interior. Fibers at the superficial zone are arranged irregularly. **(C)** Nasal septal cartilage: Collagen fibers are aligned parallel next to the perichondrium and perpendicularly toward central region **(D)** Auricular cartilage: Collagen present in auricular cartilage is arranged together with a dense elastin network surrounding the chondrocytes. Elastin fibers are arranged in honeycomb-like 3D structures around the chondrocytes. Adapted from **(A)** Buckwalter et al.[11] and **(B)** Scotti et al.[18]

nonaggregating PGs.[28,33] GAGs are linear polysaccharides and are divided into four families as follows: (1) hyaluronan, HA, (2) keratan sulfate, KS, (3) chondroitin sulfate, CS/dermatan sulfate, DS, and (4) heparan sulfate, HS/heparin. Detailed reviews on these GAG types can be found in the following literature; hyaluronan,[34,35] keratan sulfate,[36] chondroitin sulfate,[37] and heparan sulfate.[38,39] In addition, Table 1 provides a list of interactive functions mediated by several prominent PGs reported in articular cartilage.

Mechanical integrity relies on homeostatic static synthesis and degradation of PGs and GAGs. Articular cartilage chondrocytes can synthesize 17,000 PG molecules per minute[49] while turnover rates can vary for different PGs from days to months and years depending on species.[49] The biosynthesis of sulfated GAGs is a nontemplate-driven multistep process that is initiated in the Golgi, through post-translational modification of the PG protein core.[50–55] For KS, CS/DS, and HS/heparin, initiation of biosynthesis is through coupling of a xylose residue to serine, beginning the formation of the linkage tetrasaccharide: xylose–galactose–galactose–glucuronic acid. Following the linkage tetrasaccharide, the repeating disaccharide chain is formed, galactose–*N*-acetyl glucosamine, glucuronic acid–*N*-acetyl galactosamine, or glucuronic acid–*N*-acetylglucosamine, for KS, CS/DS, or HS/heparin, respectively. Multiple enzymes are involved in the polymerization of the GAG chain, addition of sulfate groups at various positions, and epimerization, giving rise to the vast heterogeneity of this family of molecules. Hyaluronan (HA) is an unsulfated GAG, synthesized through linkage of repeating polysaccharide chains: glucuronic acid–*N*-acetylglucosamine. Unlike other GAGs, HA synthesis does not require attachment to a core protein to initiate polymerization and does not occur within the Golgi.[56]

Degradation of PGs can be attributed to enzymes specific for the protein core, for example, regarding aggrecan degradation can occur through aggrecanases and matrix metalloproteinases (MMPs), and similarly enzymes specific for GAG, including keratanase,[36] heparanase,[57] and hyaluronidase[58,59] for KS, HS, and HA/CS, resulting in both protein and GAG fragments.

The PG concentration in healthy mature articular cartilage shows a depth dependent gradient with a higher concentration in the deep zone compared with the superficial zone. More

Table 1. Specific Functions of Proteoglycans Found in Articular Cartilage

Proteoglycan	*Reported function in articular cartilage*
Aggrecan	Filling interfibrillar space.[40] Compressive stiffness.[41]
Biglycan	May have an involvement in cell–matrix interactions and binding to growth factors; however, exact function is unknown.[33,42]
Decorin	Binds to collagen II and has a role in regulating collagen fibril diameter. It also may have a role in stabilizing collagen meshwork and acting as an intermediary in binding growth factors.[41,40,42]
Fibromodulin	Binds to collagen I and II and may have a role in regulating the collagen fibril diameter.[43,40,44]
Laminin	Found to promote cell attachment in immortalized rat chondrocytes ex vivo, suggesting its involvement in cell attachment in the matrix.[45]
Perlecan	Protects cartilage ECM from degradation.[46] Chondrocyte attachment in the matrix.[45]
Syndecan III	Proliferative responses of articular cartilage chondrocytes facilitating cell–cell and cell–matrix interactions.[47,43]
Versican	Compressive stiffness.[28,48]

PGs are present in the deep zone of the tissue compared with superficial zone. In the pericellular matrix (PCM) surrounding the chondrocytes, the PG concentration is higher than in the ECM. These PGs in the PCM of articular cartilage are mostly aggrecan and other PGs such as perlecan, biglycan, and decorin.[41,60,61] Meniscal cartilage has a significantly lower PG content with PG-rich regions interspersed between collagen fibers. The PCM in porcine and bovine meniscal cartilage is also mainly composed of perlecan.[22,62–64] In bovine auricular cartilage PGs are colocalized with elastin fibers, mainly around chondrocytes.[65–67] Nasal septal cartilage has the highest GAG content and more uniform distribution compared with articular cartilage.[9,26,68]

Table 2 provides an overview of the PGs and GAGs reported in literature for the four cartilage types, including the most abundant types, the relative quantity, and the zones/regions they are located. There are no data available in the literature describing the PCM of nasal septal and auricular cartilage, nor its functional importance or relevance. This represents an opportunity for further study.

Interaction of PG and GAG with collagen

Besides their biological role in tissue development and tissue homeostasis, PGs and GAGs play a key role in the mechanical behavior of articular cartilage. Collagen fibrils are ~30–80 nm in diameter and ~100 nm apart and the PGs are dispersed in between. The negatively charged GAG chains attract ions creating an osmotic imbalance that attracts water and causes swelling of the cartilage.[28] The surrounding collagen meshwork physically restricts the swelling capacity, causing the PGs to be entrapped in the collagen meshwork (Fig. 3).[33,83,84] This creates an internal rigidity for the ECM to resist compressive loads.[28] The ECM and resulting water content give cartilage its biphasic properties. Upon loading these two phases are responsible for its time dependent poroviscoelastic behavior as the fluid flows out of the solid phase (ECM). While the fluid flows out, the ECM molecules slide relative to each other creating friction between the collagen fibers and PGs.[85–87] There is some evidence to suggest similar

Table 2. Ultrastructure Overview of PGs and GAGs Reported for Different Cartilage Types

	Articular cartilage	*Auricular cartilage*	*Meniscal cartilage*	*Nasal cartilage*
PGs present as reported in literature	Mostly aggrecan Perlecan, biglycan, decorin, laminin,[69] syndecan-3,[70] and versican[71]	Aggrecan[72,73] (rabbit, human) Laminin[74] Versican[75,76]	Mostly perlecan Also: aggrecan[77]	Aggrecan[78] Biglycan, decorin, and fibromodulin[33] (bovine) Biglycan, decorin[79] Laminin[80] Perlecan[45]
GAGs present as reported in literature	Hyaluronan, CS, DS, KS[49]	Hyaluronan, CS, KS (small and not stained densely, porcine)[81]	Hyaluronan, CS, DS[82]	Hyaluronan, CS, KS[78]
Zonal distribution of GAGs as reported in literature	GAGs have a depth dependent gradient, increasing toward the deep zone[20]	Most GAGs are located around the chondrocytes within the elastic fibers[67]	Not found in literature	Uniform distribution of GAG[25]
Mean absolute GAG content in bovine cartilage ± standard deviation (μg/mg dry weight)	169.1 ± 28.0[66]	173.1 ± 12.6[66]	28.9 ± 15.8[66]	427.2 ± 72.7[66]

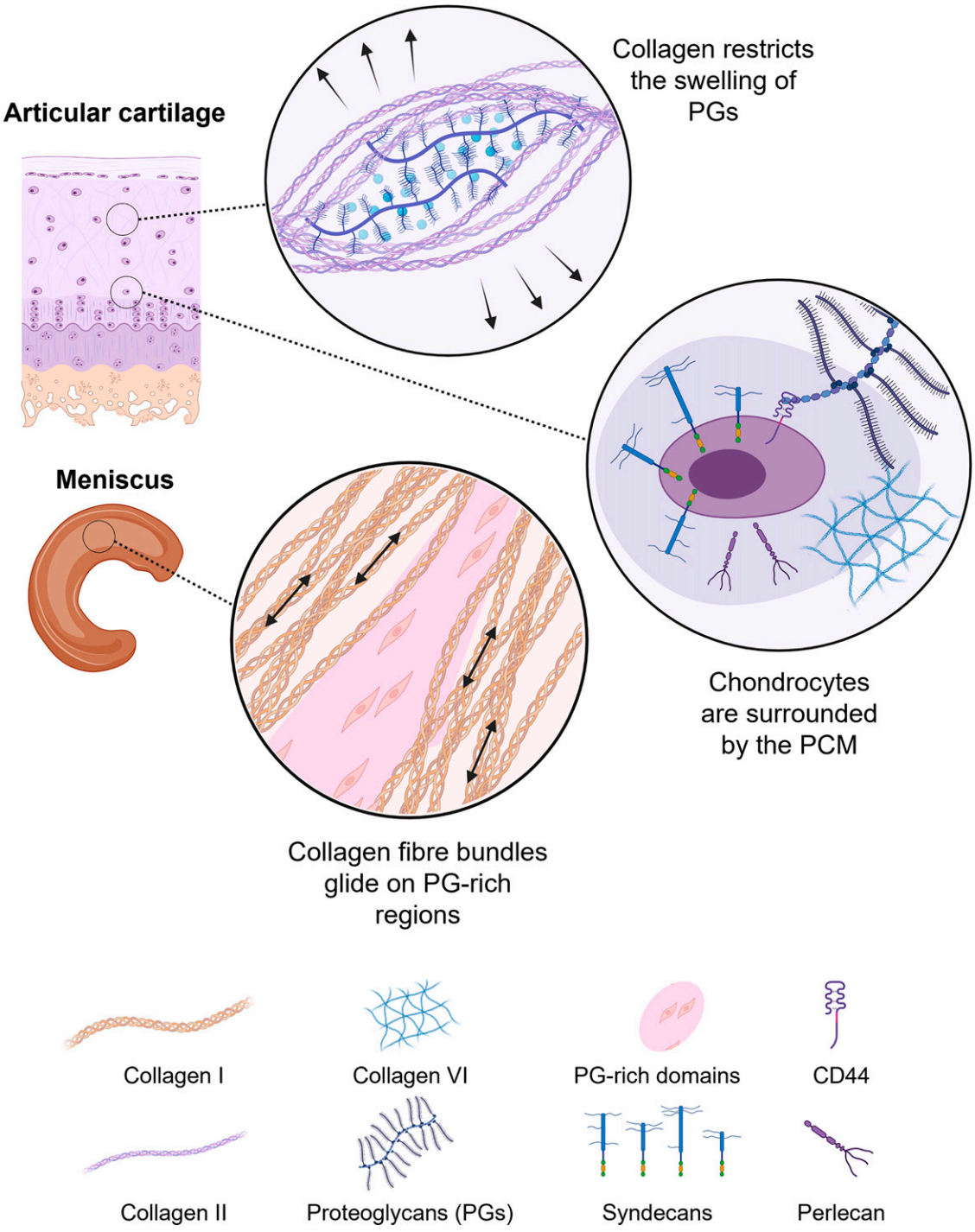


FIG. 3. Proteoglycan (PG)- and glycosaminoglycan (GAG)-mediated interaction with collagen and chondrocytes in articular and meniscal cartilage. The negatively charged GAG chains attract ions creating an osmotic imbalance that attracts water and causes swelling of the cartilage. The surrounding collagen meshwork physically restricts the swelling capacity, causing the PGs to be entrapped in the collagen meshwork. The pericellular matrix (PCM) of cartilage contains a high amount of perlecan and aggrecan and has important functions for cellular bifunctionality and mechanotransduction. The interactions for cell-PCM signal transductions are mediated by transmembrane receptors such as syndecans and CD44. PG-rich regions in the meniscus aid the gliding and realigning of the collagen bundles under loading and tensile forces. In addition, GAGs influence collagen fiber alignment and thickness in all cartilage types. Figure created with BioRender.com

hyperelastic behavior due to collagen and PG/GAG interactions in other cartilage types, although experimental data are scarce.[56,88]

In meniscal cartilage the interaction of PGs with collagen is also important for its mechanical behavior. The load-bearing properties have been attributed to the swelling

properties of the PGs, especially aggrecan which introduces a high charge density.[89] The concentration of aggrecan is highest in the inner zone of the meniscus, where compressive forces are largest. Aggrecan networks in sheep and bovine meniscus are reported to break up collagen I bundles.[90,91] This is thought to aid the gliding and realigning of the collagen bundles under loading and tensile forces (Fig. 3). Furthermore, the interspersed aggrecan areas between the collagen fibers protect the cells from high stresses in the tissue. Under tension, GAGs have been shown to have strain attenuating properties, therefore shielding the cells from these strains.[64,91,92]

In addition to promoting resistance to loads through physical entanglement with collagen, GAGs, specifically CS, have also been shown to influence their fibril formation and arrangement in all cartilage types. This could have an influence on tissue mechanics, as an increase in collagen fiber thickness and decrease in interfibril spacing have been linked to a decrease in mechanical properties.[93] The GAG chains of biglycan and decorin influence collagen fibrillogenesis by interacting with collagen II and collagen I fibers in articular and meniscal cartilage.[33,94,95] They control the lateral growth of collagen fibers and spacing between bundles through anionic repulsion of its GAG chains.[94,96] The presence of decorin leads to thick fibrils and lower interfibrillar spacing, whereas biglycan aids the formation of thinner fibers with more interfibrillar spacing.[97] Fibromodulin, a small leucine-rich PG, inhibits collagen fibrillogenesis through binding to sites on collagen fibrils in articular and meniscal cartilage. It hinders the addition of collagen monomers and delays fiber formation which results in thinner collagen fibers. In the porcine meniscus it is mostly found in the inner region where it inhibits growth of collagen fibers.[98] The inner region of the meniscus is subject to the largest compressive forces, which are resisted mainly by the high aggrecan content in that region. Therefore, control of collagen fiber alignment and thickness likely enables the tissue to support high compressive loads.

PGs (unspecified) and GAGs (CS, DS, KS) from nasal cartilage and auricular cartilage have been shown to bind electrostatically to collagen I and II as well. It is therefore likely that they also play a role in collagen fibril arrangement in these cartilage types, although literature on these cartilage subtypes is scarce and limited to nonhuman studies.[99–101]

Interaction of PG and GAG with elastin

The elastin fiber matrix of auricular cartilage contributes significantly to its mechanical stiffness.[67] Auricular cartilage has distinctly different viscoelastic behavior compared with articular cartilage. Articular cartilage shows a higher instantaneous modulus, attributed to a higher content of GAGs and no elastin present in bovine tissue.[67] The high amount of elastin in auricular cartilage however leads to a higher stiffness at equilibrium and compressive integrity, a more elastic response with elastin being the key contributor to the mechanical response. Despite similar GAG and water content, differences in stiffness are suggested to be due to the association of GAGs and elastin fibers.[67] In bovine and chicken aortas, elastin fibers have shown specific ultrastructural attachment (i.e., covalent or ionic binding) to the heparan sulfate PGs.[102,103] Similar interactions could be expected in auricular cartilage. It has also been reported that removal of positive binding charges on matrix GAGs may play a role in elastin fibrogenesis by preventing spontaneous aggregation of tropoelastin.[104] Further investigation of such interactions could be used to tune and evaluate tissue-engineered constructs and benchmark them against native tissues.

Interaction of PG and GAG with chondrocytes

The PCM in cartilage is rich in perlecan and aggrecan, protects chondrocytes from high strains, and has an important function in mechanotransduction.[105] The interactions for cell-PCM signal transductions are mediated by transmembrane receptors.[106,107] Syndecan is one of the most discussed nonintegrin receptors in articular cartilage.[108] Syndecans are a family of transmembrane heparan sulfate PGs which have four members: syndecan 1, 2, 3, and 4.[47,108] Syndecan 1 and 3 can be decorated with heparan, decorin, or chondroitin sulfate side chains, whereas syndecan 2 and 4 only comprise heparan sulfate side chains.[108] It is through these GAG chains that syndecan can interact with ECM molecules such as fibronectin, laminins, tenascin, and vitronectin. Syndecans also bind with growth factors such as fibroblast growth factor-2 (FGF-2), transforming growth factor-beta (TGF-β), bone morphogenetic protein-2 (BMP-2), morphogens, chemokines, and cytokines through their GAG chains. Known effects of these interactions are diverse and include functions such as regulating cartilage development and tissue remodeling and integrity.[43,47,109]

Perlecan is another PG that mediates ECM-chondrocyte attachments. Perlecan in bovine articular cartilage comprises heparan sulfate and chondroitin sulfate chains.[45] It can interact with cells either through GAG side chains or the core protein.[60] Perlecan is found in higher concentrations in the pericellular matrix of articular and meniscal cartilage compared to the other ECM regions.[61,110] The high concentration of perlecan, as well as aggrecan, in the PCM is crucial for physiological chondrocyte mechanotransduction as it shields the cells from deformation and high strains.[111–114]

In articular cartilage, the hyaluronan core where aggrecan and versican monomers are attached is known to bind with the cell membrane receptor, CD44, on chondrocytes. Since aggrecan and link protein only interact with hyaluronan extracellularly, this binding suggests a possible mechanosensitive mechanism for chondrocytes to receive signals from the PCM and ECM requiring a cellular response (e.g., biosynthesis/degradation activities).[115,116]

PG- and GAG-mediated interactions in articular cartilage are well established compared with other cartilage types. However, understanding the collective behavior of PGs and GAGs in concert with other ECM components is required to provide an improved understanding of the specific tissue mechanics and mechanobiological function.

Replicating GAG-Mediated Interactions in TE

TE as a means to replicate native tissue is showing great potential. Various attempts of combining scaffolds, cell types, and growth factors all have the same goal: to build functional tissue. However, it has proven challenging to reproduce tissue with structural and functional integrity. Several studies have attempted to harness the properties of PGs

and GAGs to replicate the mechanical behavior and ultrastructure to provide the right mechanical environment for cells to promote their biofunctionality and get functional replacements.

Replicating macromolecular interactions of GAGs with other ECM molecules

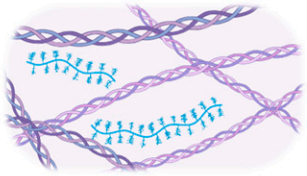
Various studies have developed GAG-based biomaterials to mimic the native ECM and utilize their biochemical cues, which have been shown to increase chondrogenesis (reviewed in Ref.[117]). However, hydrogels consisting of GAGs usually have weak mechanical properties. Therefore, they are often combined with synthetic polymers or other biomaterials to increase the mechanical properties. Often the desired mechanical properties match that of native cartilage. Therefore, mimicking this stiffness gradient to achieve full functional tissue with adequate mechanical integrity has become subject to numerous studies. Although in articular cartilage the depth-dependent gradient of stiffness correlates with the increase in PGs in deeper zones,[62,118,119] these studies have purely focused on biomaterial stiffness and have not taken GAGs into consideration to replicate the mechanics. Nevertheless, it has been shown that cells from human and bovine tissues express zone specific matrix deposition in stiffness gradient biomaterials, i.e., materials designed with different stiffnesses although the material depth, where the stiffnesses match that of native cartilage zones.[120,121] Zhu et al. added chondroitin sulfate (CS) as a biochemical cue in addition to a stiffness gradient, which enhanced cell proliferation, GAG production, and collagen deposition compared to a stiffness gradient alone. The addition of GAGs did not influence the mechanical properties of the constructs.[122]

Other studies have been trying to achieve the desired mechanical properties by replicating the biphasic properties of cartilage. They have harnessed the swelling properties of GAGs and combined them with fiber networks to replicate the collagen arrangement and to create high interstitial fluid pressure.[123–126] Biomaterials have been developed that combine GAG-based hydrogels such as HA, CS, or gellan gum, an anionic polysaccharide which is structurally similar to GAGs, with polymers such as polylactic acid (PLA), poly(ethylene glycol) (PEG), and poly(ethylene glycol) diacrylate (PEGDA). All reported stiffnesses are similar to native cartilage. Cao et al.[126] have created zonal-specific biphasic scaffold containing polycaprolactone (PCL) fibers in combination with gelatin methacryloyl (gelMA). Seeded mesenchymal stem cells (MSCs) and costal chondrocytes have shown increased chondrogenesis and zone-specific GAG and collagen production. Furthermore, they have reported Young's moduli of their scaffold similar to native articular cartilage. Despite aiming to replicate the interplay between PGs and collagen in cartilage, these studies have only reported the stiffness of their biomaterials. However, the Young's modulus does not give sufficient information due to biphasic nature and heterogeneous complexity of cartilage tissue which give it its poroviscoelastic properties. A more extensive mechanical evaluation has revealed comparable compressive and viscoelastic properties of woven PCL scaffolds combined with alginate and polyacrylamide (PAAm) hydrogels developed by Liao et al.[127] In a follow-up study, seeded MSCs have shown high cell viability.[128] More recently, Castilho et al.[129] have created a microfiber-reinforced hydrogel to replicate structural arrangement and mechanical properties of different zones in articular cartilage (Fig. 4A). They have used melt electrowriting of PCL to mimic the collagen structure and combined this fiber network with a gelMA hydrogel. Equine chondrocytes seeded in the scaffolds increased synthesis of GAGs and maintained their chondrogenic phenotype. Despite being able to create a similar collagen fiber structure, they have reported that native collagen spacing and fiber diameter are smaller than in the scaffold. The measured peak moduli of their scaffold did not match that of native cartilage; however, the viscoelastic behavior (relaxation behavior) was similar to native cartilage. This is a promising result as viscoelastic biomaterials have been shown to have favorable effects on cell proliferation, spreading, migration, and differentiation.[134–137]

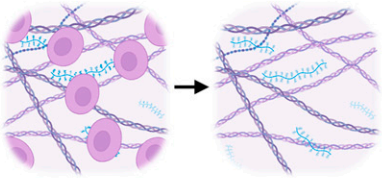
In meniscal cartilage, regions with a high PG content are arranged between collagen I bundles. These local areas with varying ECM composition have been replicated by Han et al. (Fig. 4B).[63] They developed a meniscal cartilage mimetic consisting of fibrochondrocytes seeded on electrospun PCL fibers to replicate a collagen I fiber network that was interspersed with MSC micropellets to mimic the PG-rich regions. Constructs with micropellets deposited more PGs and collagen II than constructs without micropellets, while the fibrochondrocytes deposited more collagen I in fiber-rich regions (compared with regions containing micropellets). Furthermore, the resulting construct showed similar heterogeneous microstrain profile than native meniscal cartilage. The cells in the PG-rich regions showed a distinct response to mechanical stimulation due to the attenuating properties of GAGs, similar to native cartilage.[63] These results highlight the importance of the right mechanical

FIG. 4. Tissue engineering (TE) approaches to recapitulate micromechanics of proteoglycans (PGs) and glycosaminoglycans (GAGs). **(A)** Polycaprolactone (PCL) fibers were used to replicate the collagen structure and filled with a gelatin methacryloyl (gelMA) hydrogel to increase the swelling properties and to mimic biphasic nature of cartilage. The resulting constructs showed comparable viscoelastic properties to native articular cartilage.[129] **(B)** Meniscal cartilage was replicated by PG-rich domains (*blue*) containing mesenchymal stem cells (MSCs) and fiber-rich domains (*red*) consisting of PCL electrospun fibers replicating the collagen I fibers in meniscus. The PG-rich domains showed attenuated strain profiles under tensile strain compared with the fiber-rich regions, which is thought to protect the cells.[63] **(C)**3D-printed ear construct from auricular cartilage decellularized cartilage tissue (dECM)-based bioinks.[131] **(D)** *Safranin-O* histology and SEM images of decellularized full thickness auricular cartilage. While the collagen and elastin network both stayed intact, there was a significant loss of GAG content, as well as mechanical properties.[130] **(E)** MSCs seeded in soft fibrin hydrogels surrounded by a stiff alginate hydrogel to replicate the PCM.[132] **(F)** Single chondrocytes were encapsulated in soft alginate hydrogels and seeded in stiff agarose construct to replicate the PCM.[133] Images used with permission.

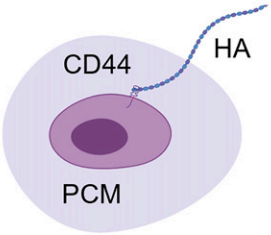
Replicating ECM interactions



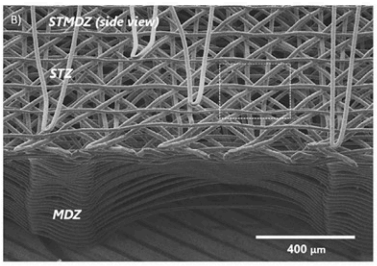
Decellularised tissue

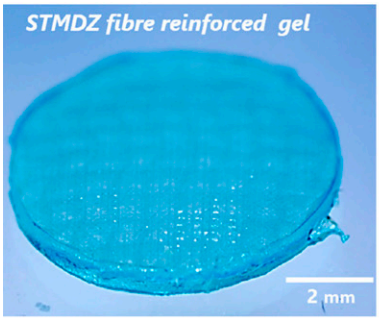


Replicating PCM

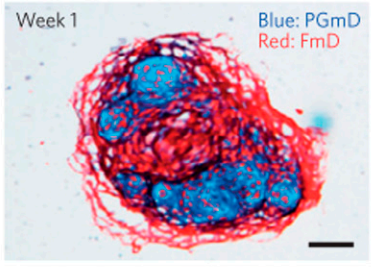


A)



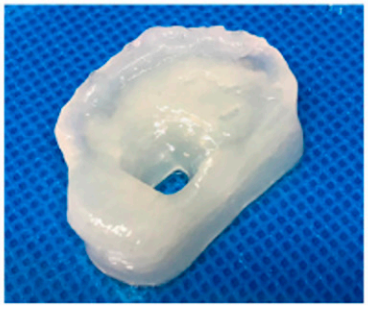


B)

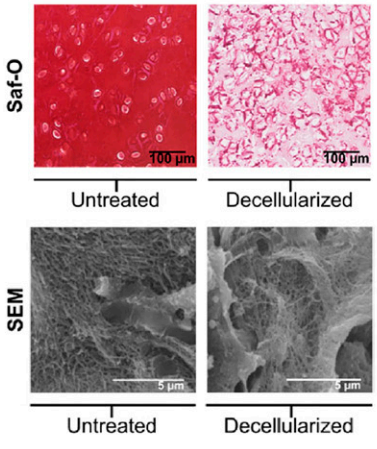


C)

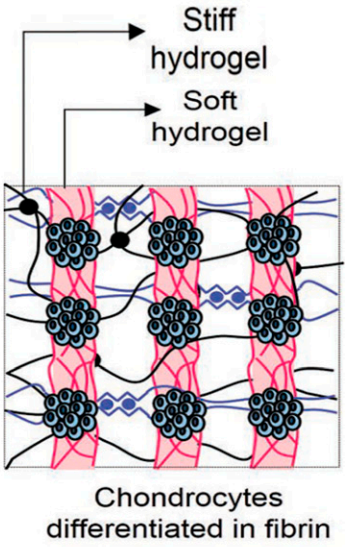
3D bioprinted ear construct



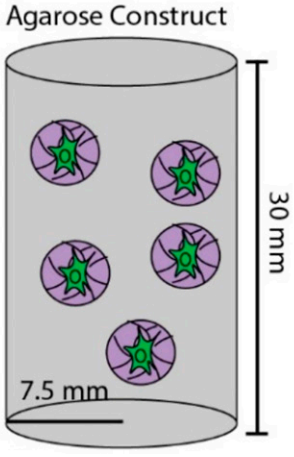
D)



E)



F)



environment for cells when trying to replicate native tissues. Having the importance of PGs in mind as well, Lopez et al.[138] replicated the role of GAGs in meniscus development through interaction with collagen fiber arrangement in collagen hydrogels. They enzymatically removed GAGs during maturation of the constructs and showed increased fiber alignment and organization leading to closer viscoelastic properties as those of native meniscus.

Even though structural and mechanical integrity is equally desirable for auricular and nasal cartilage reconstruction, the role of GAGs in the mechanical integrity to create biomaterials has not been taken into consideration to our knowledge.

Utilizing decellularized ECM to replicate ultrastructure

Another promising approach to replicate native ECM interactions of cartilage is utilizing biomaterials from decellularized cartilage tissue (dECM). Decellularization can be achieved through different methods such as physical (freeze–thaw, sonication), chemical (detergents, EDTA, hypotonic buffers), and biological (enzymes, digestion).[139,140] The aim is to remove any cells and antigens while retaining biochemical cues and mechanical integrity. Resulting dECM has been used in a slurry form in combination with a mold or 3D printing to create the shape of the outer ear.[140,141] Visscher et al.[131] have made printable bioinks derived from dECM of porcine auricular cartilage using methacrylation to form hydrogels (Fig. 4C).[131] However, despite maintaining cell viability, as well as GAG and collagen production, this approach does not preserve either the ultrastructure or architectural organization of the tissue. To counteract that, Bhamare et al.[142] have used decellularized goat auricular cartilage and molded into a patient-specific ear shape. However, using this approach, several studies have reported limited recellularization as a drawback.[142,143] Utomo et al.[130] were the first to decellularize full thickness auricular cartilage (Fig. 4D). They have reported a significant decrease in GAG content, while the native collagen and elastin content stayed intact. The removal of most GAGs was reflected in the measured mechanical properties of the decellularized scaffolds. The viscoelastic properties were significantly reduced in dECM scaffolds with lower GAG content. Different decellularization methods could be explored to achieve dECM with a high GAG content to retain the mechanical properties of the native tissue.

Replicating the PCM and interactions with the chondrocytes

Despite the importance of the PCM for cellular biofunctionality and mechanotransduction, there has been limited effort to replicate the PCM in TE. The focus is often placed on replicating the bulk stiffness and mechanical properties. However, the stiffness of the PCM is of several magnitudes lower compared with the ECM. Having a biomechanically functional environment for chondrocytes in mind, recent studies have been focusing on the PCM using different approaches. Chondrocytes do produce a PCM-like matrix in their surrounding when encapsulated in soft hydrogels (<10 kPa). They also retain their chondrogenic phenotype and do not dedifferentiate.[144,145] Studies used HA based hydrogels to achieve cell interaction with CD44. It has been shown that modification of HA hydrogels has an influence on chondrogenesis and binding to CD44.[146] However, these approaches still use homogeneous hydrogels and the bulk mechanics do not match that of native tissue. Using biomaterials with different mechanical properties can address that. De Melo et al.[132,147] have used two biomaterials with different stiffnesses to replicate native tissue (Fig. 4E). They have encapsulated MSCs in a soft fibrinogen hydrogel that replicates the PCM. These cell-hydrogel constructs were then printed in a PEG-alginate polymer mixture that had a higher stiffness. The MSCs showed increased cell viability and chondrogenic behavior compared with cells seeded in homogeneous hydrogels without a PCM. Frederikson et al.[133] used drop-based microfluidics to culture single chondrocytes in alginate microgels to closely mimic PCM environment embedded in high stiffness agarose hydrogels (Fig. 4F). They could show an increase in GAG content, as well as collagen VI, which is mainly found in the PCM.

These different promising approaches all try to harness the contributions of PGs and GAGs to the mechanical behavior of cartilage. The goals are to either provide a native environment for the cells or provide the cells with the right mechanical cues in order to produce the matrix that ultimately replicates native tissue.

Future Directions and Conclusion

This review discusses cartilage ultrastructure and GAG-mediated interactions of different cartilage types and attempts in literature to replicate these in TE. In the current literature, the ultrastructure of auricular cartilage and nasal septal cartilage is less defined compared with articular and meniscal cartilage. In addition, the specific GAG types present in each cartilage type have not been well-established in the literature. Therefore, identifying GAG types specific to each anatomical location is recommended. It will provide a benchmark for TE applications against native tissue.

Coupling 3D imaging techniques with mechanical testing approaches is suggested to understand the collective behavior of ECM macromolecules under dynamic loading.[148] Collective interactions of these tissue components can be imaged under different loading conditions and analyzed using image processing techniques to determine their deformation patterns.[63] Similarly, tissue engineered constructs should also be assessed and compared with native cartilage behavior, allowing tissue engineers to identify the features of the TE cartilage that require further development.

There is a need to combine efforts to unravel native tissue structure and related mechanical behavior while trying to replicate these features to get optimal outcomes in TE. Moreover, the field would benefit from understanding the contribution of GAGs in cartilage mechanics and translating that knowledge to engineered cartilage. Mechanical evaluation of TE constructs often lacks crucial details about how the construct is behaving at the microscale. This is necessary in order to produce functional tissue in the long term if the goal is to replicate the complex mechanical biphasic behavior of cartilage.

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Authorship Confirmation/Contribution Statement

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