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GLYCOSAMINOGLYCAN AND PROTEOGLYCAN BIOTHERAPEUTICS IN ARTICULAR CARTILAGE PROTECTION AND REPAIR STRATEGIES:
Novel approaches to viscosupplementation in orthobiologics

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Abstract

The aim of this study was to review developments in glycosaminoglycan and proteoglycan research relevant to cartilage repair biology and in particular the treatment of osteoarthritis. Glycosaminoglycans decorate a diverse range of extracellular matrix and cell associated proteoglycans conveying structural organization and physico-chemical properties to tissues. They play key roles mediating cellular interactions with bioactive growth factors, cytokines and morphogenetic proteins, and structural fibrillar collagens, cell interactive and extracellular matrix proteoglycans and glycoproteins which define tissue function. Proteoglycan degradation detrimentally affects tissue functional properties. Therapeutic strategies have been developed to counter these degenerative changes. Neo-proteoglycans prepared from chondroitin sulfate or hyaluronan and hyaluronan or collagen-binding peptides emulate the interactive, water imbibing, weight bearing and surface lubricative properties of native proteoglycans. Many neo-proteoglycans outperform native proteoglycans in terms of water imbibition, matrix stabilization and resistance to proteolytic degradation. The biospecificity of recombinant proteoglycans however provide precise attachment to native target molecules. Visco-supplements augmented with growth factors/therapeutic cells, hyaluronan and lubricin (orthobiologicals) have the capacity to lubricate and protect cartilage, control inflammation and promote cartilage repair and regeneration of early cartilage lesions and may represent a more effective therapeutic approach to the treatment of mild to moderate OA and deserve further study.

1. Introduction

Articular cartilage's functional properties as a shock absorbing weight bearing tissue stem from its structure and the intermolecular interactions between its constituent fibrillar (type I and II collagen) and lattice forming collagens (type VI collagen), cell and matrix proteoglycans (PGs) (biglycan, decorin, fibromodulin, lumican, perlecan and aggrecan) [1-6] and structural and cell attachment glycoproteins (COMP, PRELP, fibronectin, laminin, link protein) [7]. These cartilage component are assembled into a functional dynamic composite (**Figure 1**). Confocal immunolocalizations of some of these key extracellular matrix (ECM) components are presented later in this review.

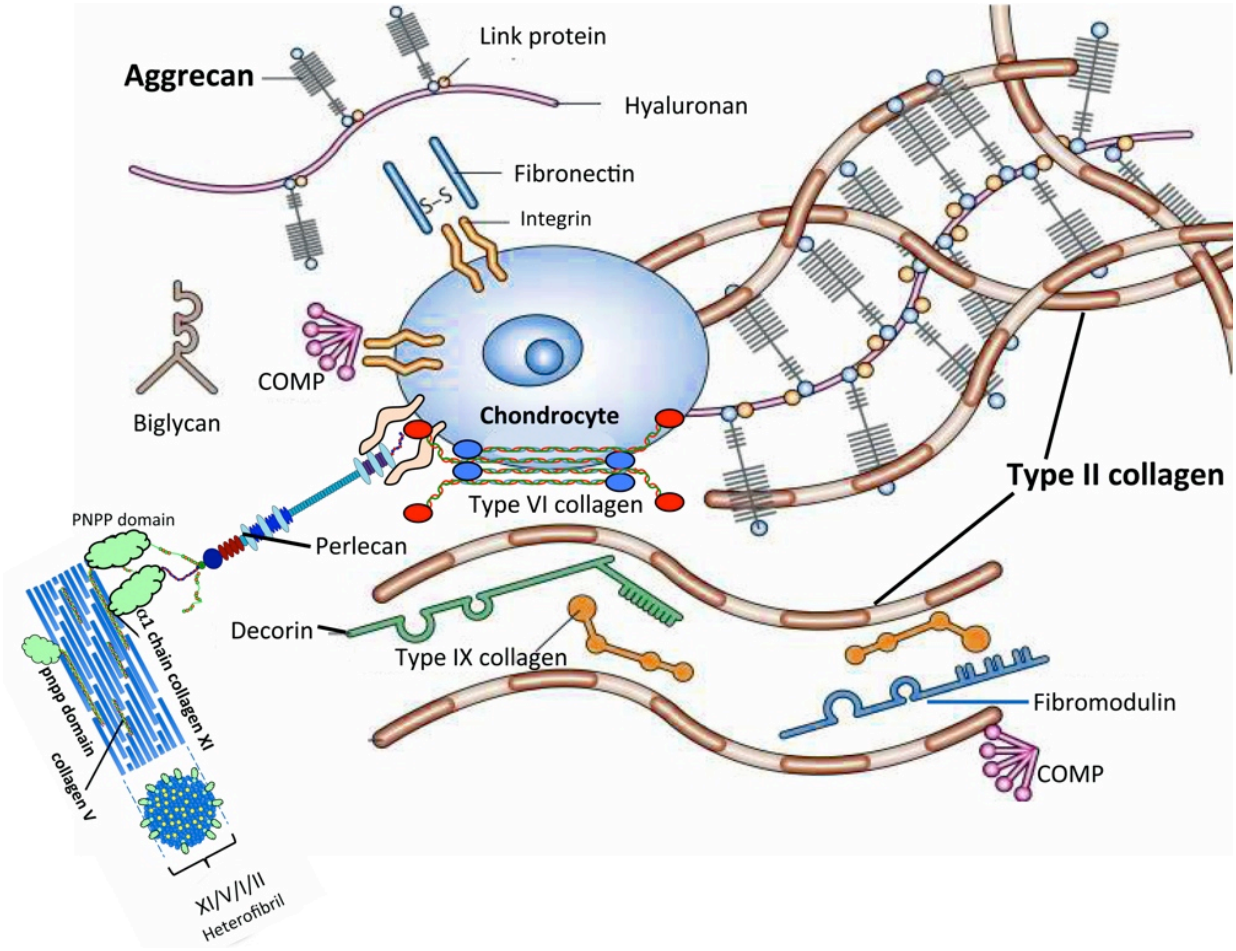

Figure 1. Schematic of articular cartilage depicting the chondrocyte and the collagen, proteoglycan and associated matrix components synthesized and assembled to provide dynamic weight bearing and visco-elastic properties. Figure modified from [8] with permission [doi:10.1038/ncprheum0216]; copyright 2006 Springer-Nature Publishers.

Synovial fluid has roles in the nutrition of the articular chondrocytes by diffusive processes and in the lubrication of the cartilage surface which protects it from surface abrasion particularly in weight bearing areas not protected by the meniscal fibrocartilages interposed between the femur and tibia. Hyaluronan (HA) forms massive mega Dalton sized aggregate structures with aggrecan stabilised by link protein. These space-filling structures are entrapped within the collagenous fibrillar networks in cartilage. HA-aggrecan aggregates have impressive water regain properties and these generate hydrostatic pressure within the tissue which equips cartilage with hydrodynamic weight bearing and visco-elastic properties. HA is also a key component of the synovial fluid which bathes the articular cartilage surface.

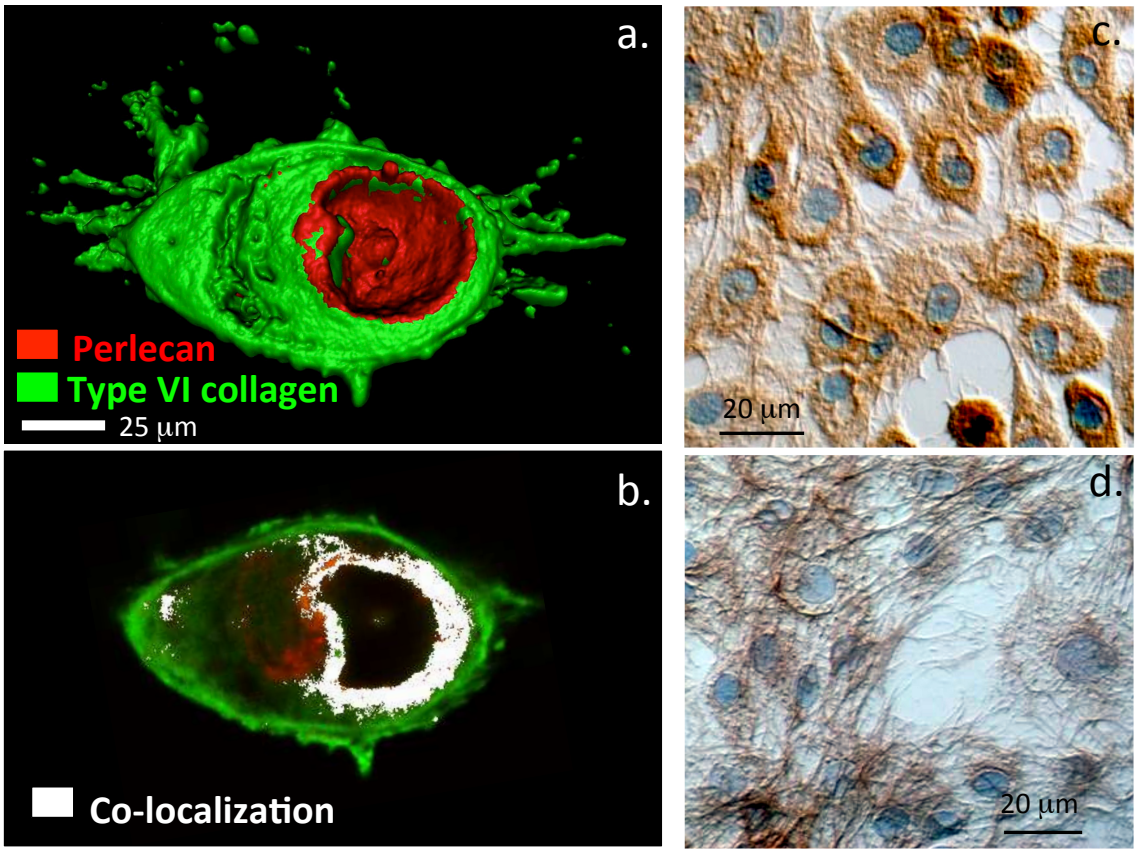

Figure 2. Surface rendered 3D image of a chondron containing a chondrocyte in a fluorescent confocal image. The chondron is composed oft he lattice forming type VI collagen (a), perlecan surrounds the chondrocyte pericellularly and interacts with type VI collagen as highlighted in the co-localisation mask (b). The perlecan C-terminus interacts with α2β1 integrin expressed on the chondrocyte surface. Chondrocyte monolayers expressing perlecan in culture (c) and type XI collagen which is present as fine fibrillar material. Perlecan interacts with type XI collagen and this forms a cell-ECM interconnection which facilitates communication and acts as a bio-sensor for the chondrocyte to perceive its biomechanical micro-environment, allowing it to respond to changes in

the ECM orchestrating the replenishment of deficient components and facilitating homeostasis of ECM composition and tissue function. Figure 2a courtesy of Dr AJ Hayes, University of Cardiff, UK Copyright AJ Hayes 2012 . Figure 2b reproduced with permission[9] [doi: 10.22203/eCM]2018 under the terms of licence CC-BY-SA; 2016 with permission of the copyright holders. Fig 2c,d: Reproduced with permission from[10] [doi: 10.1007/s10735-019-09823-1]; 2019, Nature Springer Publishers under terms of license 459010123588.

PGs and glycosaminoglycans (GAGs) have been the subject of intensive investigation in connective tissue biology for over ten decades. In more recent times a major breakthrough came from the appreciation that PGs/GAGs were not merely supportive scaffolding components of the ECM. In addition to their structural roles, they are also dynamic, responsive, regulatory elements of the ECM and participate in cell-cell and cell-ECM communication, the regulation of cellular metabolism, cell proliferation and differentiation - duties that make them essential for normal tissue function and homeostasis[11] (**Figure 2**). This has led to the realization that PGs/GAGs could potentially be used in a therapeutic mode to promote repair of tissue defects[12, 13-18] and the re-attainment of functional properties in tissues that had undergone degradative changes due to disease [15, 17, 19, 20, 21, 22]. Articular cartilage has an inherently low capacity for self-repair and degeneration of cartilage in diseases such as osteoarthritis (OA) are painful debilitating conditions leading to considerable interest in repair biology for the development of therapies to prevent disease progression to end stage OA [23]. Articular cartilage that has undergone osteoarthritic changes is a particularly challenging tissue to repair and one in which major efforts have been devoted world-wide for over five decades but with little success. OA imposes a large and rapidly increasing global disease burden that is challenging global health-care systems. Disease progression in OA, produces increasing pain and impaired joint function leading to the need for total knee joint replacement in end-stage debilitating OA. Data from the American Academy of Surgeons (AAOS) Annual Report in 2018 indicated OA resulted in ~160,000 total joint replacements in England and Wales and 492,000 in the USA in 2017. The World Bank has calculated from United Nations Population data collected from 1960 to 2017 a consistent world-wide trend in ageing populations predicting a dramatic global increase in

individuals aged ≥ 65 year in the next two decades (https://data.worldbank.org). Elderly OA prone patients will therefore represent a more significant proportion of the world population over the coming decades [24]. The prevalence of knee OA has doubled since the mid 20th century and it is predicted to become a leading global musculoskeletal condition by 2050 [25].

Identification of OA as a global disorder affecting all joint tissues has partially explained the lack of success of therapeutic approaches which have focused specifically on joint-specific articular cartilage repair. Of all of the many therapeutic approaches that have been examined, objectively, only one compound has achieved any success in terms of a recovery of joint tissue structure and function, and stimulation of resident cell populations to replenish joint tissues. This compound is hyaluronan (hyaluronic acid; HA), which is now available in many different formulations that have been used in broad and diverse applications with other therapeutic agents to promote joint functional properties. International guidelines by the Food and Drug Administration (FDA) and European Medicines Agency (EMA)-recommend that the management of knee OA should utilise combined non-pharmacological and pharmacological therapeutic interventions[26, 27]. In order for such therapeutic formulations to be effective, ideally, they should be applied to joints in the early pre-symptomatic stages of cartilage damage, which if left untreated have a high likelihood of progressing to an advanced OA phenotype. It should be stressed that the development of a preventive strategy combining the natural beneficial effects of compounds which maintain the lubrication of the weight bearing surfaces of joints with therapeutic compounds which stimulate cartilage repair (orthobiologicals) is a new treatment proposal we outline in this review and its merits should be given due consideration. It is the weight bearing regions of joints that initially develop lesions during the degeneration of knee-joint components and subsequent development of OA so it is logical that these regions should be the focus of any prospective therapeutic interventions. Visco-supplementation is a useful effective therapeutic

procedure that is simple to perform. In this review we provide evidence that visco-supplements could be further augmented with growth factors (or PRP), neo-PGs such as mLUB15, a lubricin biomimetic and therapeutic stem cells to further improve the effectiveness of visco-supplementation raising it from a maintenance modality to one with positive impact on the repair of degenerate joint tissues. While all components of this proposed multifunctional bioactive visco-supplement have proven effectiveness individually no such combination therapy has yet been examined for the treatment of OA but is certainly worthy of consideration and has considerable merit. A further important aspect of such a treatment is that for it to have optimal success it should target the earliest stages of OA lesion development to prevent progression to an advanced degenerative stage and is an important preventative aspect of such a therapeutic approach. Treatment of joints with well-established advanced OA lesions, have been singularly unsuccessful. The most appropriate therapeutic window to target to ensure successful treatment is clearly the earliest stages of OA. However, despite advances in imaging methodology, routine identification and selection of patients with early OA lesions is challenging and herein lies a major difficulty when attempting to undertake successful therapeutic intervention. However imaging of knee joint tissues is continually improving and will greatly aid in patient selection in the future when such improved methodologies become more widely accessible.

In articular cartilage, PGs play important and diverse multi-functional roles in the ECM and cell-associated environment[2, 28-32, 33, 34]. Not only do they mechanically stabilize the tissue, but they facilitate cell-matrix communication, acting as mechano-sensors that transmit sensory regulatory cues to the resident cell populations which allow them to sense and respond to ECM alterations and orchestrate replacement of deficient components in order to maintain tissue homeostasis and undertake intrinsic repair processes. These functional

properties are due to the PG core proteins and their attached GAG side chains. This review provides technical information on recombinant and synthetic neo-PGs and their prospective use in biomimetic procedures in repair biology [35].

1.1 Evolution of GAG mediator/proteoglycan multifunctional effector molecules.

GAGs decorate the core proteins of PGs which provide additional functional attributes to tissues through their particular modular core protein design[36]. GAG evolution over 500 million years of vertebrate and invertebrate evolution [37] has selected for GAGs equipped with molecular recognition and information transfer properties. These GAGs act as cellular mediators in the glycocalyx surrounding all cells and control responses to growth factors, cytokines and morphogens at the cellular boundary [38, 39, 40]. Thus GAGs and PGs evolved which have regulatory properties over downstream cell-signalling pathways and gene expression networks essential for physiological life processes. The reason GAGs and PGs have existed in a minimally altered form throughout evolution up to the present day despite a requirement for a considerable investment by the cells in the many genes encoding the multiple biosynthetic enzymes (eg heparan sulfate (HS) requires 20+ enzymes) required for GAGs and PG core proteins testifies to the importance of GAGs in cellular survival and the functional attributes they provide to tissues. Attempts are being made to better understand the glycocode of GAGs in order to better determine their specific contributions in tissue development and ECM remodeling since this information may be of potential application in repair biology [13, 14, 39-42, 43, 44].

1.2 Challenges of articular cartilage repair.

Inspiration for the development of biomaterials that interface with cartilage has been derived from the cartilage ECM which is abundant in both PGs and hyaluronan (HA). PGs,

comprised of core proteins decorated with GAG side-chains convey structural and functional properties to the ECM [36]. Advances in our understanding of their biological interactions have demonstrated key aspects that need to be designed into cartilage-interfacing biomaterials. Tissue engineers have designed synthetic and semi-synthetic biopolymers for use as structural, chemical and biological replacements for native PGs [45-47]. These are referred to as neo-PGs since they serve as functional and therapeutic replacements for natural PGs currently unavailable for tissue engineering studies. Although limitations exist in neo-PGs in terms of their cell signalling capability and biocompatibility, they nevertheless display promise as replacements for natural PGs through their cell and protein binding properties [45]. This review covers recent developments in the development and application of GAG-based biomaterials in articular cartilage tissue engineering and also considers their roles in supporting functional tissue regeneration.

2. Articular cartilage proteoglycans.

Approximately 45 ECM and 10 cell-associated PGs have been categorised, twelve of these occur in articular cartilage [36, 46, 48, 49], schematics are shown depicting the complexities of their structural forms (**Figures 3-5**). The essential roles these PGs provide in life-processes has ensured their phylogenetic longevity throughout vertebrate and invertebrate evolution. Every cell has a surface glycocalyx containing GAG mediator molecules that control crucial signalling pathways involved in cellular regulation and development [50, 51]. Synthetic materials that mimic the multi-valency of this three-dimensional GAG microarchitecture may serve as important tools for deciphering and exploiting GAG regulatory properties in repair biology [51, 52]. Nano-scale biomatrices have been developed for studies of such glycocalyx interactions [51] and GAG microarrays have aided in the elucidation of GAG sulphation patterns required to drive such processes [53]. PGs act as ECM scaffolding molecules and stabilise tissues [5, 18, 30, 54]. GAG side chains in PGs are variably sulfated and

have interactive properties with growth factors, chemokines, cytokines, morphogenetic proteins and structural matrix components, which aid in the stabilization and development of tissues[13, 28, 39, 41, 43, 55].

2.1 Aggrecan structure and function.

In many PGs the GAG side chains are heterogeneous structures which are subject to spatial and temporal variation in specific tissue locations in tissue development. HS heterogeneity and fine structure is a finely controlled process in PGs such as perlecan [56] and is of considerable importance in the regulation of tissue development, while the chondroitin sulfate (CS) chains on aggrecan are less variable in structure [57]. The density of the GAGs on perlecan and aggrecan also widely differ with endothelial perlecan containing typically 3 HS chains attached to its N-terminal domain-1 while aggrecan has ~100 CS chains variably distributed in its CS1 and CS2 domains located towards its C terminus, and 25-30 keratan sulfate (KS) chains located at the N-terminus of the CS-rich region [29] (**Figure 3c**). Other members of the lectican PG family do not contain this KS-rich region [58]. The function of HS and CS differ, the fine structure of HS is critical in determining perlecan's binding properties with growth factors and other mediators which initiate cell signalling [6, 59]. A comparative study of CS and HS in the promotion of three-dimensional chondrogenesis of mesenchymal stem cells (MSCs) showed that CS-hydrogels of low mechanical stiffness provide a scaffold which promotes MSC-based cartilage tissue regeneration. CS was more potent at inducing chondrogenesis than HS [60]. The high density of the CS chains in aggrecan provide a high fixed charge density critical to the performance of aggrecan in water imbibition, tissue swelling and the generation of internal hydrostatic pressure in weight bearing tissues [61]. These hydrodynamic properties are described by the Gibbs-Donnan effect [62] and are a function of the high density of CS chains and their mutual repulsive effects on the aggrecan core protein and their associated counter-ions and how these partition in cartilaginous tissues

due to osmotically-driven forces. This partitioning effect provides an internal hydrostatic pressure which provides weight bearing properties to articular cartilage and intervertebral disc. Less is known of the specific contribution which the KS chains make to aggrecan's functional properties however like the CS chains they may also contribute to water imbibition to some extent however the need for two different GAGs on the aggrecan core protein is unclear (**Table 1**). The KS domain in aggrecan (KS rich region) is located between the G2 and CS rich domains, and is encoded by exon 11. However the amino acid sequence in this domain region varies among species and this has consequences on the relative KS contents of aggrecan in these species. Some of these species like rodents are frequently used in animal models of human disease however rodent aggrecan is devoid of a KS rich region and findings with these models therefore need to be carefully interpreted. The consensus sequence for attachment of KS in human aggrecan core protein is E-(E,K)-P-F-P-S or E-E-P-(S,F)-P-S [63, 64]. In humans and cows, aggrecan contains a segment of 4–23 such hexapeptide repeats where the KS chains are located, rats and other rodents lack this motif and do not contain KS [63]. The rodent aggrecan core protein is truncated in the KS rich region and rodent aggrecan does not contain a KS rich region equivalent to that found in human or bovine aggrecan. This does not appear detrimental to aggrecan turnover or aggrecan's functional properties in rodent cartilages thus it is uncertain what the role of the KS chains on the aggrecan core protein is.

Rodent aggrecan does contain small N- and O- linked KS chains in the G1, G2 and IGD and these have been suggested to potentiate aggrecanolysis by ADAMTS metalloproteases [65] which are important in the normal turnover of this PG in cartilage. ADAMTS-1-knockout mice do not exhibit abnormalities in aggrecan turnover in vitro or in vivo [66]. ADAMTS-5 is the major aggrecanolytic enzyme in mouse cartilage and has roles in skeletogenesis and in the development of OA [67]. However, using mice lacking ADAMTS-5 activity it has been possible to identify additional genes involved in the initiation of OA which are responsible for

cartilage destruction. These act independently of ADAMTS-5 in post traumatic models of OA in mice [68]. Aggrecan mimetic neo-PGs have been prepared using CS and the absence of KS in these molecules is not apparently detrimental to their performance.

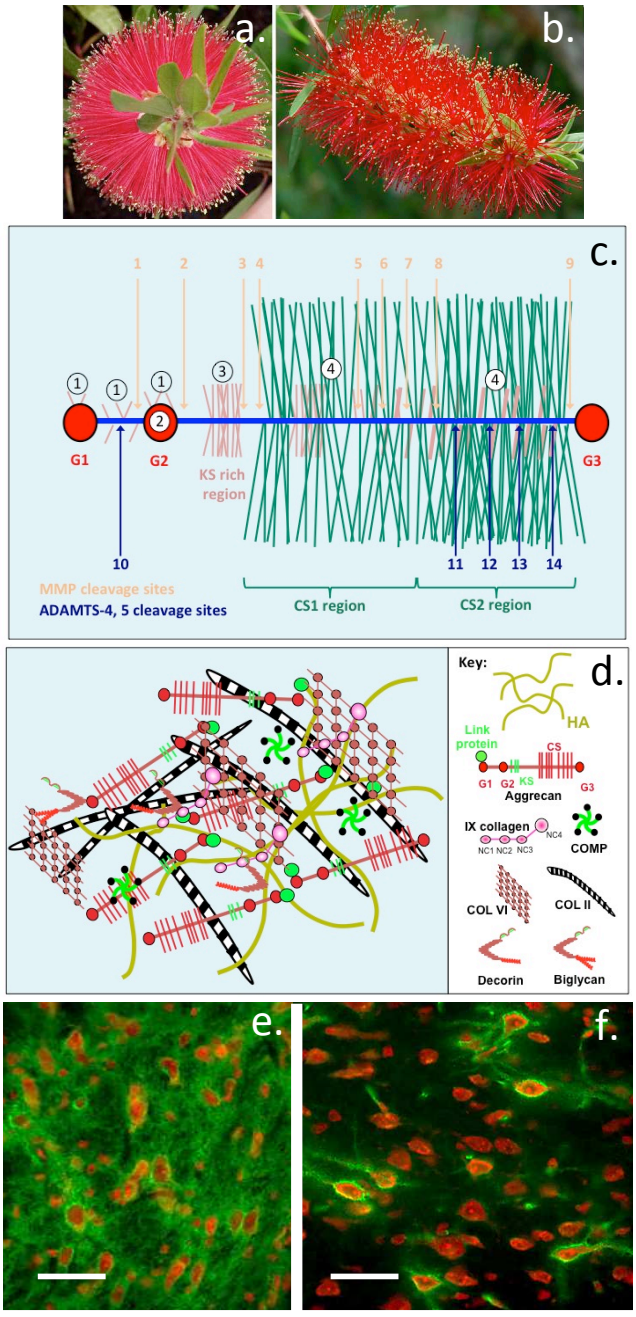

Figure 3. Composite figure depicting the spatial orientation of the petal distibution of the Australian bottlebrush *Callistomine rigidis (a, b)* which have been compared with the GAG chains oriented around and distributed along the aggrecan core protein (c). The aggrecan core protein contains three globular domains G1, G2, G3, KS rich region and CS1 and CS2 chains attached to a central core protein (c). The aggrecan core protein is susceptible to cleavage by MMPs and ADAMTS-4, 5 (aggrecanase-1, 2). These core protein cleavage sites are indicated as arrows 1-14. Despite the massive literature on aggrecan several aspects of its KS chains remains unresolved. These areas of interest in the G1, interglobular, G2, KS rich and CS1 and CS2 regions are indicated

with the labelled points (1-4) which are discussed further in the text. Schematic depiction of the aggrecan-HA-link protein ternary complex which is found entrapped within type II collagen networks in cartilaginous matrices and provides the hydrodynamic properties of weight bearing tissues (d). Matrix components which interact with aggrecans G3 domain include type VI collagen, cartilage oligomeric protein (COMP), decorin and biglycan which assemble extended networks between aggrecan macroaggregates and collagen networks in tissues (d). Similar protective perineural net structures assembled from the lectican proteoglycan family and HA also occur in the CNS/PNS. These so called perineuronal nets are visualized by immunolocalization of MAb 1B5 (+) epitope in rat brain (e) and pericellular 1B5(+) epitope expression by isolated neurons (f). Scale bars 100 μm. Fig 3e, f courtesy of Dr Anthony J. Hayes and Prof Bruce Caterson, Cardiff School of Biosciences, University of Cardiff, UK. Antibodies provided courtesy of Professor Bruce Caterson, Cardiff University, Cardiff, UK.

Table 1. How Do The KS Side Chains of Aggrecan Contribute To Its Structure and Function

(* For an explanation of features 1-4 see Figure 3c)

Feature*	Functional contributions
1 G1, G2 and IGD regions	The functions of the small *N*- and *O*- linked KS chains in the IGD, G1 and G2 domains of aggrecan are largely unknown. The IGD KS chains may potentiate and initiate cleavages in the aggrecan core protein by ADAMTS-4 and ADAMTS-5. KS chains in the G1 domain obscure a T cell epitope which otherwise makes the G1 a powerful arthritogen in auto-immune models of inflammatory arthritis.
2 G2 region	The G2 globular domain of aggrecan shares homology with its G1 domain but does not bind HA and the biological role of the G2 domain is unknown.
3 KS rich region	The function of the *O*-linked KS chains in the KS rich region of human aggrecan is not known. These are absent in murine aggrecan with no obvious detrimental effect on cartilage function and aggrecan turnover.
4 CS1 rich CS2 rich regions	KS chains are sparsely distributed in the CS2 region and are end-capped with L-Fucose and N-acetyl neuraminic acid. These KS chains are antigenically distinct from KS chains of the KS rich region. The KS chains in aggrecan isolated from non-weight-bearing nasal, laryngeal and tracheal cartilages do not contain such capping structures on KS thus their functions are unknown. In contrast the KS chains of aggrecan in amyotrophic lateral sclerosis (ALS) in brain tissues are heavily substituted with fucose and sialic acid and this may modulate ligand binding in the PGs these modified KS chains are attached to and may alter the turnover of these KS-PGs in brain tissues contributing to motor neuron impairment.

2.2 The cartilage proteoglycans.

Cartilage contains a number of large and small ECM and pericellular matrix (PCM) proteoglycans as well as transmembrane PGs attached to the chondrocyte cell surface. These have been recently reviewed [13, 15, 36, 69]. As already noted, aggrecan is the major cartilage PG responsible for the imbibition of water into cartilage which is the basis of cartilages ability to act as a weight bearing tissue (**Figure 3**), perlecan is also a prominent pericellular PG (**Figure**

4). A layer of chondrocytes at the cell surface of flattened morphology also synthesise a large 400 kDa CS-PG, versican as well as a 345 kDa mucin-like proteoglycan called lubricin (proteoglycan 4, PRG4) (**Figure 5d**). This was also previously known as surface zone protein or megakaryocyte stimulating factor. Versican interacts with HA via its globular (G1) HA binding domain localising HA at the surface of cartilage where along with lubricin it is responsible for the near frictionless articulatory properties of articular cartilage [70, 71]. The lubricin core protein has somatomedin B, heparin-binding and hemopexin domains and a mucin rich region which is heavily glycosylated with more than 168 *O*-linked glycan chains. Some of these mucin chains are also substituted with sialic acid [70]. Lubricin also contains a small CS chain thus should more correctly be considered a glycoprotein rather than a PG [71]. Some lubricins do not contain this CS chain [72]. Lubricin is discussed more fully later in this review.

Cartilage also contains two large basement membrane HS-PGs, perlecan (**Figure 4a**) and agrin (**Figure 5d**) and two families of cell-associated HS-PGs, the glypicans and syndecans [30, 33, 73]. Perlecan is a prominent modular, multifunctional pericellular PG which promotes chondrogenesis, matrix stabilisation, chondrocyte differentiation and growth factor signalling and has an extensive repertoire of interactive ligands (**Figure 4**). Perlecan has a 467 kDa core protein which contains 3 GAG chains in domain I. In endothelial cells perlecan contains three HS chains, whereas in chondrocyte perlecan up to two of these HS chains are replaced by CS. The CS chains of chondrocyte perlecan contain 4, 6-disulfated motifs which direct collagen fibrillogenesis [2, 4-6, 74].

Agrin is a 400 kDa HS-PG of basement membranes which interacts with low-density lipoprotein receptor-related protein-4 (LRP4) and α-dystroglycan in chondrogenic signalling

networks supporting chondrocyte differentiation and the upregulation of SOX9 and its transcriptional targets, COL2A1 and ACAN [75] (**Figure 5e**). Agrin-induced chondrocyte differentiation does not induce hypertrophy. LRP4 interacts with WNTs and BMPs [76] to regulate chondrocyte differentiation. Agrin has not been evaluated in cartilage repair strategies. Cartilage also contains several members of the small leucine rich proteoglycan (SLRP) family including the CS or DS-substituted decorin and biglycan and the KS substituted lumican, fibromodulin and keratocan[33, 77].

The SLRPs are horse-shoe shaped proteins which have a central region containing multiple leucine rich repeat (LRR) domains which are interactive with a wide range of proteins, another characteristic of the SLRPs are N-and C- terminal disulphide-stabilised globular domains. Decorin and biglycan contain one or two O-linked GAG chains attached to their N-termini (**Figure 5b, c**). Lumican, fibromodulin and keratocan have 3-4 N-linked KS chains located within their LRR regions. The SLRPs bind to fibrillar collagens and regulate the fibrillogenesis process. The SLRPs regulate tissue organization, cellular proliferation, adhesion, and responses to growth factors and cytokines [77] in inflammation [16], cell growth, tissue morphogenesis and innate immunity [21, 78]. Biglycan acts as a pathogen associated molecular pattern (PAMP)-like ligand interacting with toll-like receptor-2 and -4 (TLR2 and TLR4) [19, 78] and also interact with BMP/TGF-β to modulate fibrosis [79] and cell differentiation [21, 78]. While biglycan can interact with extracellular fibrillar collagens [80] and pericellular type VI collagen, its major interactive areas actually lie in cell mediated regulatory processes, whereas decorin has more prominent roles in collagen fibrillogenesis. With cartilage degeneration in the development of OA the SLRPs become progressively fragmented and their functional properties are lost from this tissue [1, 81]. The SLRPs are discussed further later in this review in the context of cartilage repair strategies.

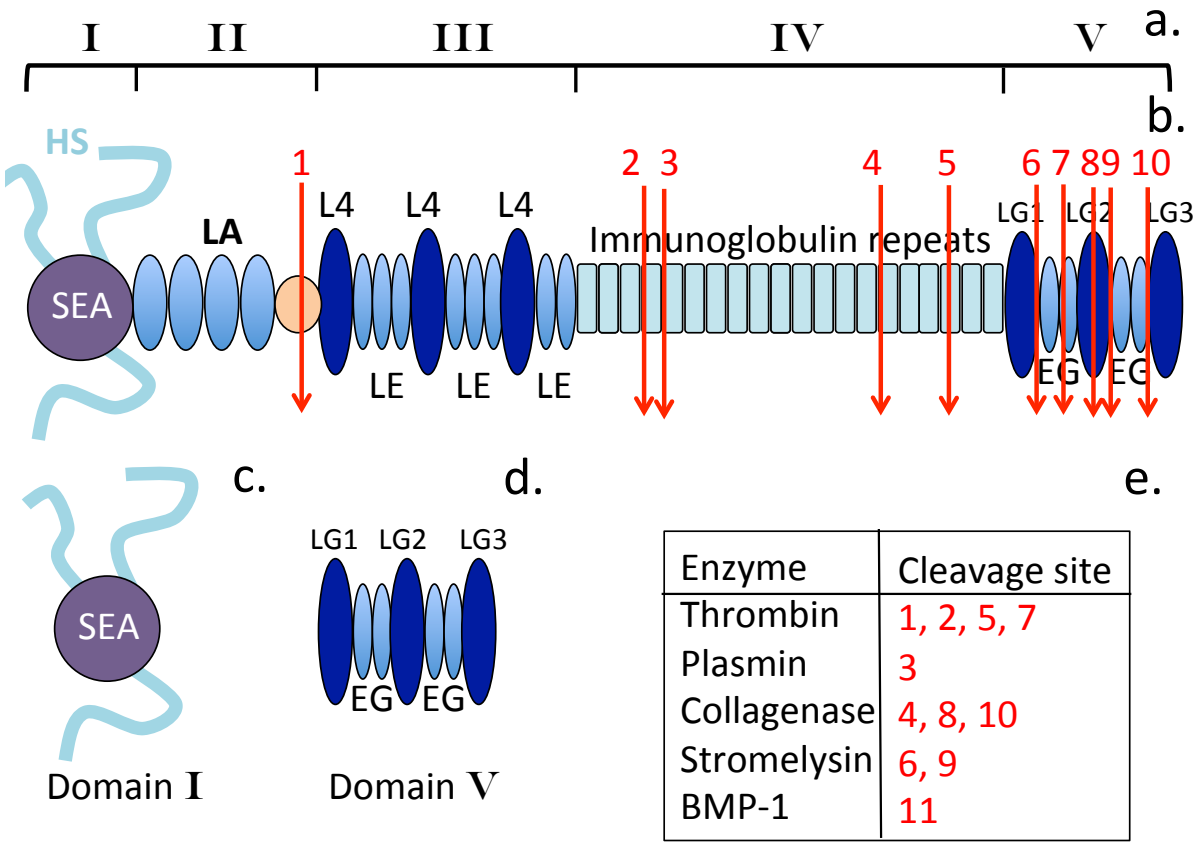

Figure 4. Schematic representation of the modular structural features of the heparan sulfate proteoglycan, perlecan showing its five domains (a) and HS attachment region and some of the known protease cleavage sites (b, e). Diagrams of recombinant domain-I (c) and domain V are also shown (d).

A further small PG found in cartilage is bikunun (**Figure 5a**). Bikunin, is an ancient Kunitz BPTI-like serine protease inhibitor of the inter-α-trypsin inhibitor (ITI) superfamily and pleiotropic cell regulatory PG. Bikunin contains one CS chain and is unique among the PGs in that this CS chain does not merely decorate the PG core protein but it also acts as an intramolecular linkage module for the attachment of a number of heavy chain (HC) proteins in ITI which are transferred to HA in particular cellular contexts leading to stabilization of the HA [82] up to six HCs can be attached to the CS chain of bikunin/ITI with tissue development and pathology [83]. This transfer of the HCs to HA occurs by a process of trans-esterification catalysed by the enzyme TSG-6 [84] and apparently stabilizes the HA countering its depolymerisation by free radicals released by inflammatory cells during OA and rheumatoid arthritis (RA)[84]. Such condensed HA in growth plate cartilage appears important in the

cartilage to bone transition during endochondral ossification[3]. Chondrocytes synthesize bikunin, its affinity for HA may localize bikunin in the surface regions of cartilage where HA is localized[85]. A number of catabolic proteases produced by leucocytes and mast cells released at sites of inflammation (leucocyte elastase, cathepsin G, chymase, tryptase) can digest lubricin [85, 86]but are inhibited by ITI thus bikunin/ITI has a protective role to play at the cartilage surface.

2.3 GAG heterogeneity and complexity confer inherent difficulties in their analysis.

The structural complexity and poly-dispersity of GAGs confounds their sequencing and structural analysis. Routine analysis of the GAG chains in PGs typically involves compositional and disaccharide analysis, mapping of 4-8 oligosaccharide segments and occasionally domain analysis, however extensive sequencing is not normally undertaken. Sequencing of GAGs is not a facile process and most laboratories do not have the expertise available to routinely undertake such procedures, however it is critical that such information should be known if the binding properties of native GAG chains are to be replicated [87, 88]. Embedded sulphation motifs such as CS-D and CS-E internally in the CS chains have growth factor interactive properties, the surrounding regions of the CS chain serve a carrier function for these biological motifs [89]. Such motifs can be relatively minor components thus their presence is not readily detected by conventional compositional analyses. Glycan sequencing methodology is steadily improving and it is now possible to sequence small GAG samples, however this still cannot be considered a routine procedure. Technological advances in mass spectroscopy are also improving such determinations [55, 88, 90, 91]

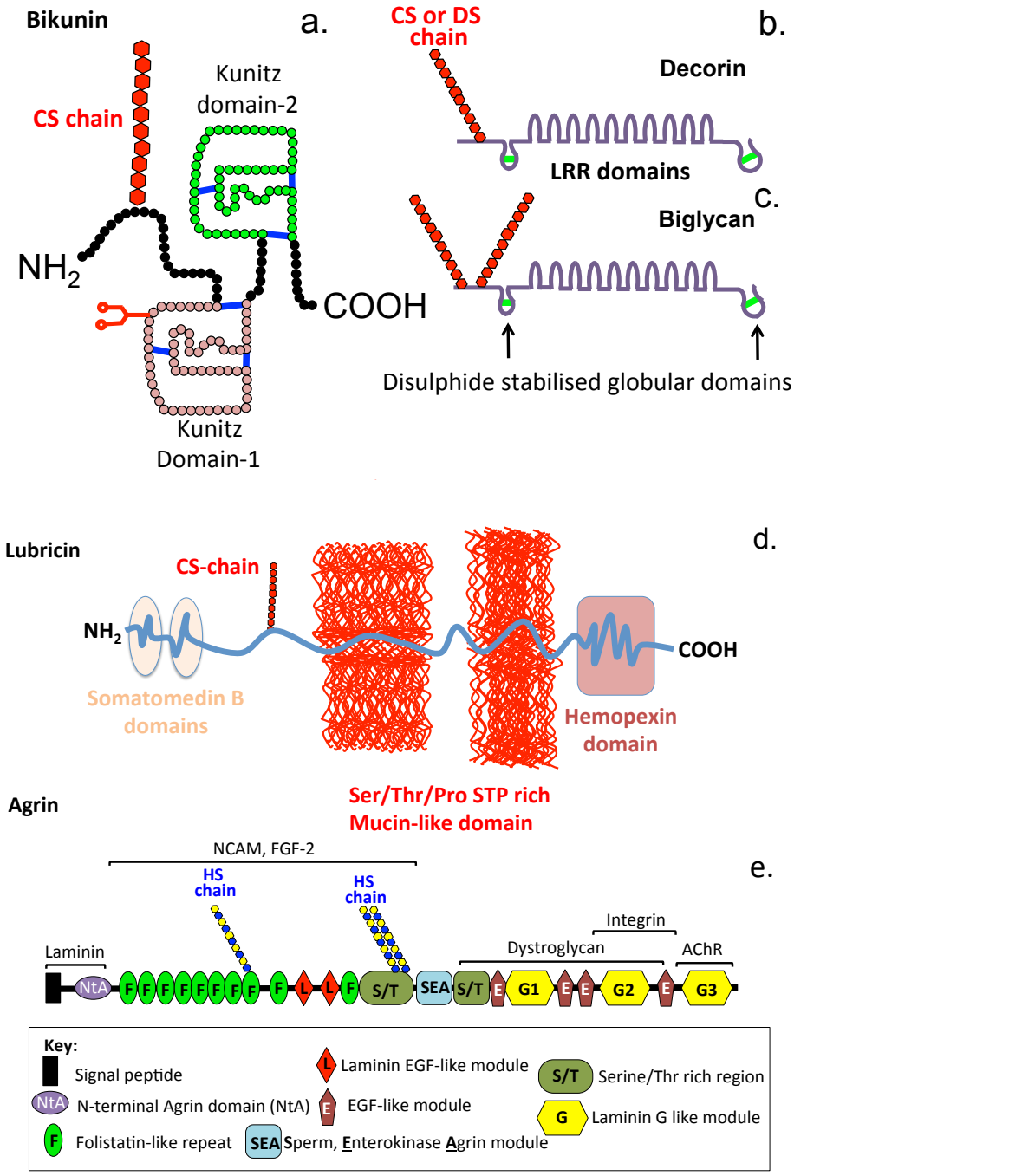

Figure 5. Schematic representation of the structure of bikunin, the simplest PG which is produced by chondrocytes and shows an affinity for HA thus it becomes localized at the cartilage surface (a) and the small leucine repeat PGs decorin (b) and biglycan (c). Lubricin (PRG4) is a mucin-like glycoprotein which provides boundary lubricative properties at the surface of articular cartilage (d). Agrin is HSPG synthesized by articular chondrocytes (e). Figure reproduced from [13] with permission Biochem J. Copyright Biochem J. 2018.

2.4. Functional organization of articular cartilage

The schematic organization of the major cartilage PGs and collagens (**Figure 1**), and chondrocyte PCM depicting type VI and XI collagen and perlecan in the pericellular

environment around a chondrocyte (**Figure 2**) demonstrates the complex inter-relationships between these components. Confocal imaging of whole thickness bovine articular cartilage further shows the distribution of aggrecan and perlecan throughout articular cartilage (**Figure 6**). Monoclonal antibodies (MAbs) to the core proteins of these PGs and MAb 4C3 to a CS sulphation motif co-localised these PGs throughout the articular cartilage (**Figure 6a, b**). Use of a white overlay clearly delineates the pericellular colocalisation of these components (**Figure 6b**). Type I Collagen is immunolocalised to the surface regions of cartilage where it provides resistance to tensional shear loads (**Figure 7a**) whereas type II collagen is immunolocalised throughout the full depth of the cartilage (**Figure 7b**). Type II collagen and supramolecular aggregates of aggrecan and HA, stabilized through link protein, make up the bulk of the tissue (**Figure 7b**), endowing it with its unique hydration properties to resist compressive loading. Aggrecan, a hyalectin containing CS and KS GAG chains, is abundant in cartilage and, like type II collagen, is a primary chondrogenic marker (**Figure 6a**). Perlecan, a modular, multi-functional heparan sulfate (HS)/CS PG is also a marker of early chondrogenesis in cartilage rudiments [3, 4] and a stem cell niche component (**Figure 6b**). The CS GAG chains of aggrecan carry unique sulphation motif epitopes, which facilitate interaction with growth factors and cytokines [6, 31, 92]. Versican is more typical of tensional fibrocartilaginous tissues such as meniscus, tendon or annulus fibrosus but can also be expressed by some chondrocytes with a flattened morphology in the cartilage surface regions (**Figure 8a**). Fluorescent colocalisation of aggrecan (identified by MAb 6B4) and perlecan (MAb 1948) with CS sulphation motifs recognized by MAb 4C3 demonstrated that both PGs are substituted with 4C3 and have overlapping distributions in the pericellular chondrocyte environment further illustrated via the white overlay mask depicted (**Figure 6b**). Versican, a CS hyalectin typical of tensional fibrous/fibrocartilaginous tissues such as meniscus, tendon or annulus fibrosus, was also expressed in superficial articular cartilage; however it was not associated with the 4C3 CS motif (**Figure 8a**) [93]. Versican and aggrecan both form ternary

complexes with HA and link protein in articular cartilage. Versican may have specific roles to play in the localization of HA at the articular surface [93]. Aggrecan, however, has a more widespread distribution, like perlecan, throughout the cartilage matrix which experiences compressive loading. Both of these PGs have biomechanical attributes which allow the cartilage to withstand compression. Perlecan is pericellularly distributed around chondrocytes along with type VI collagen providing compliancy to the rigid type VI collagen lattice. Perlecan compliant properties are considered cyto-protective in tensional and weight bearing tissues [2, 9, 94]. Lubricin (proteoglycan-4, PRG4) has a distinctive localisation at the articular surface, hence is also commonly referred to as surface zone proteoglycan (SZP) (**Figure 8b**). Lubricin interacts with HA, cartilage oligomeric matrix protein (COMP) and fibronectin, and [95] contributes to the lubrication and protection of the articular surface from mechanical damage and chondrocyte apoptosis [34, 96] thus protecting against the development of OA (**Figure 8b**). Mechanical damage to articular cartilage modulates lubricin biosynthesis [97]. In a meniscectomy model of OA in sheep, lubricin levels are significantly down-regulated [98]. In mice lacking PRG4 a loss of articular cartilage structure, stiffness and boundary lubrication severely depressed the articulatory properties of murine knee joints [99]. The above studies show a direct inter-relationship between lubricin, boundary lubrication, and cell survival suggesting that supplementation of synovial fluid or a visco-supplement with HA and lubricin could prevent further cartilage deterioration in OA and provide conditions conducive to intrinsic cartilage repair [100]. Gene therapy which upregulates PRG4 expression [101] and its beneficial autocrine effects on synoviocyte metabolism [102] is consistent with the cartilage protection afforded by recombinant PRG4 in a porcine meniscectomy model of OA [103]. Biglycan, a small leucine rich proteoglycan (SLRP) with two CS/DS GAG chains, also has a prominent pericellular immunolocalization pattern in articular cartilage (**Figure 9a**) while decorin, has a single CS or DS chain and is distributed more prominently in the inter-territorial matrix (**Figure 9b**). Decorin has important roles in the regulation of fibrillar

collagens [104], its incorporation into bioscaffolds promotes attachment of endothelial cells [105], however neither recombinant decorin [106] or biglycan [107] have so far been used specifically in articular cartilage repair strategies. Biglycan shows promise in bone repair strategies with BMP-2 and BMP-4 [107, 108] while decorin is implicated in the activation of growth factor and cytokine regulatory pathways in chondrogenic differentiation relevant to cartilage remodelling [109].

HA, the only GAG member which occurs devoid of a PG core protein, is non sulfated and has a relatively simple structure composed of the repeating disaccharide D-glucuronic acid (GlcA) glycosidically linked to the amino sugar N-acetyl glucosamine (GlcNAc). This simplicity in structural design hides the complexity of HA's cell regulatory properties [110]. HA evolved as a molecule which avoided immune detection and has been referred to as a stealth molecule [111] with material properties which provide hydration to tissues conducive to cellular survival and cellular migration in tissue development [112]. Cross-linked HA has been developed as a drug delivery vehicle and various formulations of HA have been used to deliver therapeutic stem cell preparations [113, 114]. Besides its application to combat OA and preserve joint function, HA preparations have also found widespread application in ophthalmic surgery, and embryo implantation procedures [115]. HA has also been widely used as a delivery vehicle in ophthalmic, nasal, pulmonary, parenteral, implant and gene transfer procedures [115] and in the development of smart new-generation multi-lamellar wound dressings (Hyalomatrix(®)[116]. HA has also been used as a nano-particle delivery vehicle for drugs, RNA, DNA and growth factors [117-119]. Nano-particles coated in HA have anti-inflammatory and ROS scavenging properties [120]. The free radical scavenging and anti-oxidant properties of HA have also been applied to new generation smart wound dressings [22, 54, 121]. HA is discussed more fully later in this review.

The ability of native HA to convey cell regulatory properties is doubly surprising given its lack of sulphation. The CS sulphation motifs have information and cell regulatory properties similar to other GAGs [13, 39, 48, 122, 123]. HA has important progenitor cell regulatory properties operative in embryonic and foetal development and is a common component of stem cell niches. It also has functional roles to play in the formation of macromolecular assemblies with aggrecan in mature cartilaginous tissues and with the lectican PG family in perineuronal nets [13, 32, 58, 124]. HA also supports the lubrication of articular cartilage surfaces in synovial joints [125]. Many PGs have interactive properties with HA that are important to tissue function and this is a trait that has been built into many neo-PGs by the incorporation of HA-binding peptides (HABPs) [45, 126]. HABPs are prominent components of the neo-PGs, mAGC and mLUB15 which are analogue forms of aggrecan and PRG4 and these make critical contributions to the water regain and tissue lubrication properties of cartilage. These traits are discussed in detail later in this review.

3. Application of proteoglycans in cartilage tissue engineering

Current attempts to regenerate articular cartilage aim to produce biological and functional neo-cartilage with an articular surface similar to that of the native tissue [127]. However a cartilage-centric approach may be short sighted and is liable to be unsuccessful in terms of the function of the whole joint unless the global health of all joint tissues are successfully addressed. Despite the major focus in OA being on the articular cartilage, meniscal degeneration is also highly prevalent, with pathological changes in menisci preceding those of articular cartilage [128, 129, 130]. Meniscal degeneration results in the generation of degradative proteases which contribute significantly to the total degradative enzyme pool in the joint synovial fluid. Many of these degenerative changes in joint tissues

are also reproduced in animal models of OA, allowing specific testing of particular parameters or components [131]. In-vitro approaches specifically examining features of meniscal pathobiology have also demonstrated focal tissue changes in response to stimulation by IL-1 and TNFα [129, 130, 132]. Articular chondrocytes are also responsive to these inflammatory mediators but to a lesser extent [129]. Just as PGs are now appreciated to be multi-functional proteins with additional ECM stabilising and space-filling supportive roles, functional roles for visco-supplement polymers other than in boundary lubrication and joint articulation also need to be considered [133, 134]. High molecular weight HA is an efficient visco-supplement but in combination with PRG4 improved boundary lubrication has been demonstrated [133]. PRG4 like HA also has anti-inflammatory [133] and cell directive properties. High molecular weight HA has chondro-protective properties through its ability to inhibit degradative MMP production by chondrocytes stimulated with inflammatory mediators [135] and also modulate synoviocytes within the joint [102]. Alkyl HA derivatives are also effective at inhibiting MMP production and activation [136].

PRG4 localises HA at the articular cartilage surface and promotes boundary lubrication but also inhibits attachment of immune cells which could lead to local inflammatory conditions causing depolymerisation of HA and impaired joint lubrication [96, 100, 103, 137, 138]. Thus novel formulations of visco-supplements incorporating HA and PRG4 are worthy of further evaluation and may have a global joint protective effect in OA. The synergism of PRG4 and HA in joint lubrication [100, 139], chondro-protective efficacy of PRG4 in a model of OA [103] and in the regulation of inflammation/innate immunity in the joint [96, 138, 140], has already been established. There is a great need for such globally-effective preventative therapeutic interventions to prevent the formation of more extensive deep cartilage defects that are extremely difficult to treat clinically[133]. A multi-functional visco-supplement approach to the treatment of OA is a novel strategy which deserves further

evaluation in the experimental systems described above and *in vivo* [134]. Supplementation of HA with platelet rich plasma (PRP) is an early attempt to improve the therapeutic properties of such formulations with growth factors and other biofactors which promote articular cartilage regeneration (**Table 4**).

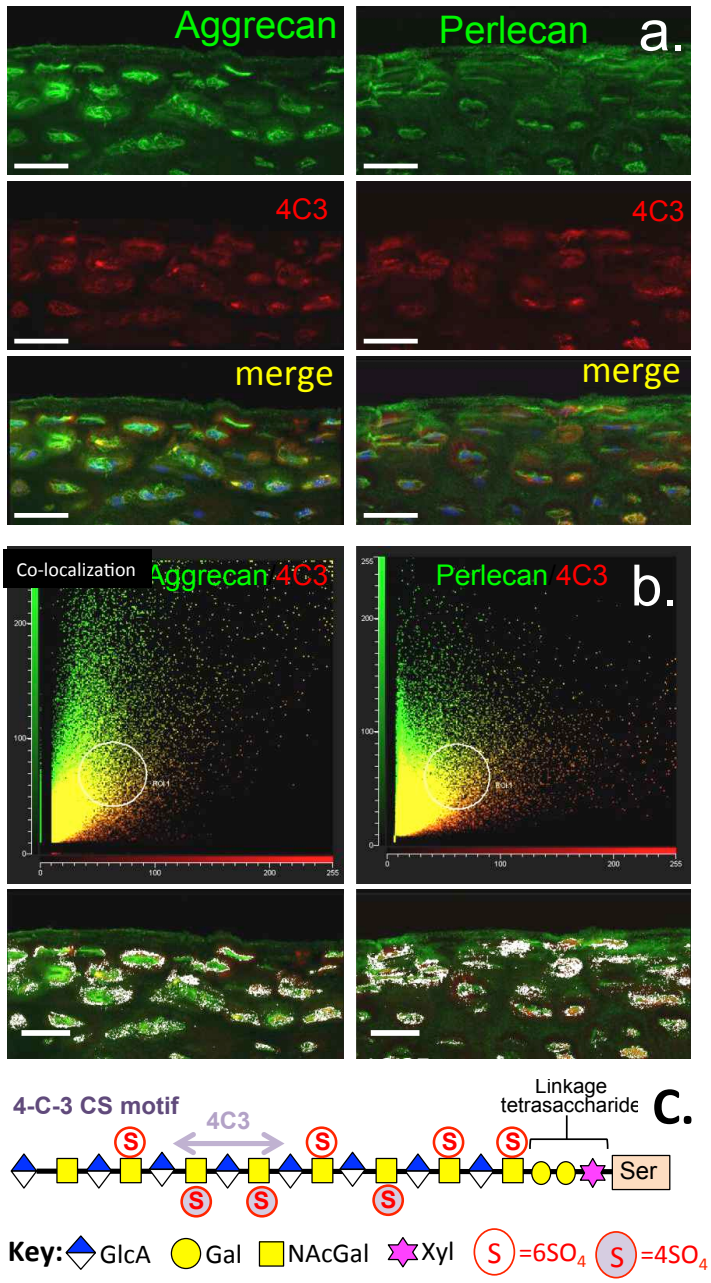

Figure 6. Confocal co-localizations in vertical bovine articular cartilage sections using monoclonal antibody (MAb) 4C3 and proteoglycan core protein antibodies comparing chondroitin sulfate (CS) sulphation motif epitopes in aggrecan and perlecan. (a) Confocal images depict immunolocalizations of aggrecan and perlecan core proteins (green) and native CS motifs detected using MAb 4C3 (red). (b) Cytofluorograms showing the frequency distributions of the fluorescent intensities of the green and red fluorochrome channels. Co-localized pixels, are also depicted below this using a white overlay mask on the underlying cartilage images. A diagram of the 4C3 CS epitope is depicted (c). Scale bar = 25 μm. Fig 6a,b reproduced (adapted) from [141] [doi 10.1042/BCJ20180283]; 2008, with permission of SAGE Publishers.

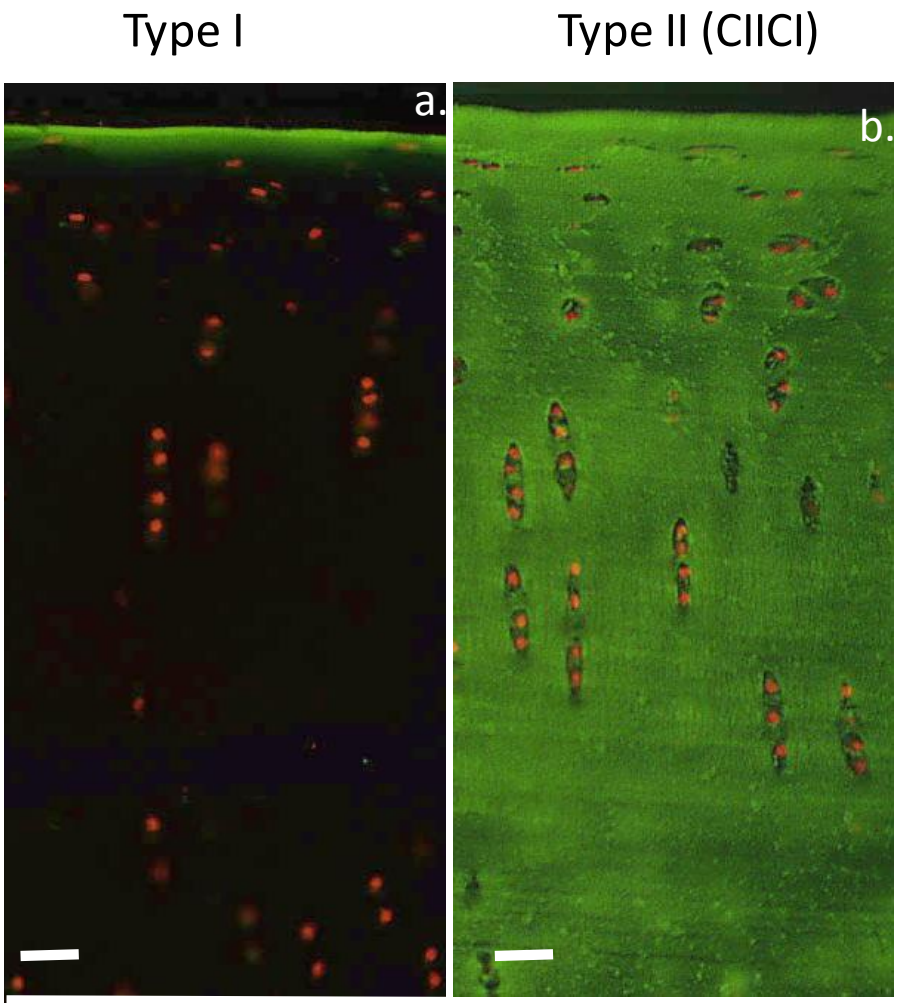

Figure 7. Fluorescent immunolocalizations of type I collagen (a) and type II collagen in full thickness bovine articular cartilage (b). Fig 7a, b courtesy of Dr. Anthony J Hayes, Cardiff School of Biosciences, Cardiff University, UK. Copyright AJ Hayes 2012. Scale bars represent 50μm.

3.1 Recombinant proteoglycans.

The production of recombinant PGs which faithfully reproduce the native PG and GAG structure is a technically demanding task [47]. A number of recombinant PGs have been successfully produced [46, 142]. Reproducing GAG fine structures attached to recombinant core proteins to recapitulate native GAG structure is a challenging exercise. Production of recombinant, large PGs such as aggrecan and perlecan have their own technical difficulties associated with their massive molecular dimensions and glycosylation characteristics. In the case of aggrecan, its high degree of GAG substitution represents around 90% of its mass and is of functional importance in water imbibition, space-filling and hydrodynamic properties

which equip tissues with their unique weight bearing properties [30, 46]. The stability of recombinant large PGs is also relevant to any prospective therapeutic application and it may be more expedient to use specific modules of the large PG in specific applications rather than the intact PG. These individual modules are also simpler to synthesize and may have better stability properties. Recombinant aggrecan has been prepared in a number of studies [reviewed in [46] however the GAG side chains of these recombinant proteins have not been a major focus in many of these studies and it is not clear to what extent the extensive GAG substitution of native aggrecan has been reproduced. A major focus of many research groups has therefore been to produce aggrecan mimetic analogue molecules to overcome these difficulties. Neo-aggrecans are discussed fully later in this review.

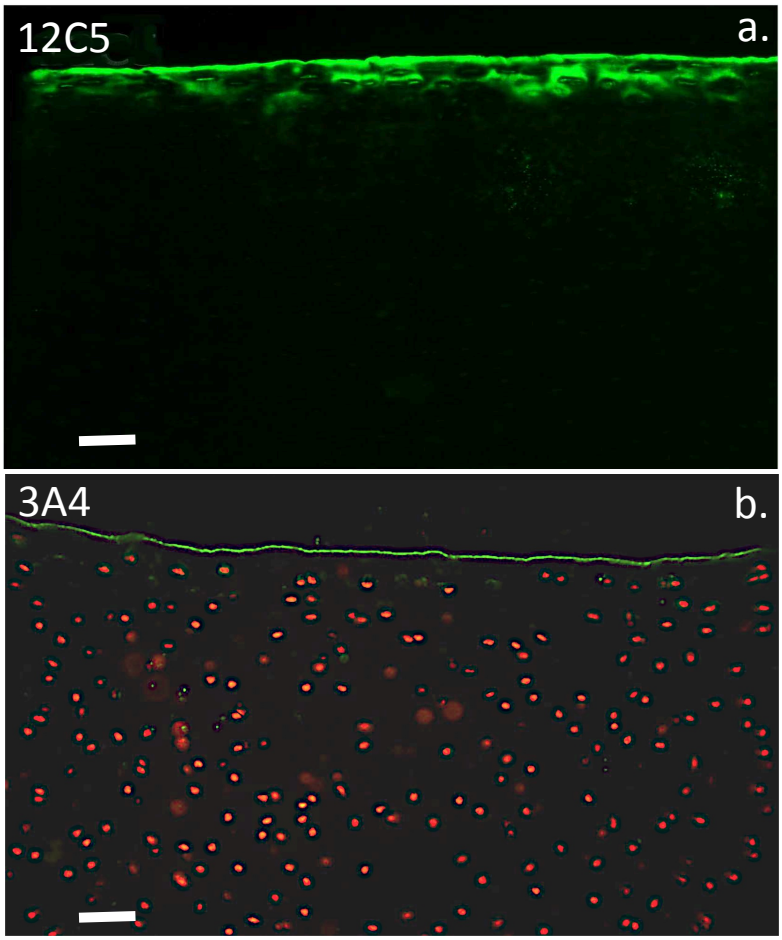

Figure 8. Surface localisation of versican using MAb 12C5 to the HA binding region of versican (Iowa Hybridoma Bank, USA)(a) and lubricin (PRG4) using MAb 3A4 (b). Fig 8a reproduced (adapted) from [141] [doi 10.1369/jhc.7A7320.2007]; 2008, SAGE Publishers. Figure 8b courtesy of Prof Bruce Caterson and Dr Anthony

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The human *HSPG2* gene on chromosome 1 covers 115,000 base pairs. The transcript contains 97 exons and is 14,327 base pairs in length. Recombinant approaches with perlecan have produced individual domains or clusters of modules [143-147] or perlecan sequence-containing peptides [148]. Endorepellin is an 85-kDa recombinant fragment of perlecan domain V. Perlecan domain V contains four EGF-like repeats and three laminin-like globular domains (LG1–3). Two EGF-like repeats separate the LG2 and LG3 domains. Perlecan contains five modular domains (**Figure 4a, b**) and is the largest PG which has been fully cloned and sequenced with a core protein of 467 kDa [149], displaying homology to structural glycoproteins with known functional properties in matrix assembly and stabilization, angiogenesis, chondrogenesis and in cellular attachment [6] (**Figure 4**). Rather than producing the perlecan core protein in its entirety the major focus in repair biology has been to concentrate on production of particular domains of perlecan for specific applications in repair biology (**Figure 4c, d, ; Table 2**).

Delivery of growth factors to tissues is hindered by their relative instability, the GAG side chains of PGs like aggrecan and perlecan which the growth factors bind to stabilizes and protects the growth factors from degradation in-situ. PGs in the PCM and ECM thus provide a stabilised local growth factor reservoir that can be accessed by the resident cells to undertake tissue homeostasis or for tissue remodelling during a wound healing response. GAGs also regulate growth factor-receptor interactions at the cell surface. The development of neo-aggrecan nanoparticles as growth factor delivery platforms mimics the growth factor binding and stabilization afforded by native aggrecan with growth factors retaining activity for more than three weeks and CS-based neo-aggrecans are as effective as native aggrecan [117]. CS bioscaffolds have also been developed which provide instructive cues to progenitor cells and have been applied in a number of tissue repair applications, reviewed in [47, 150]. Perlecan

domain I has been conjugated to HA hydrogels and used to promote chondrogenesis through delivery of BMP-2 [151]. BMP-2 delivery by perlecan domain I also promotes osteogenesis [146] and cartilage repair in a murine model of OA [152]. Soluble perlecan domain-I increases VEGF-165 receptor phosphorylation by bone marrow endothelial cells [153]. Recombinant perlecan domain-I can also be used for FGF-2 delivery [91]. All five domains of perlecan have been recombinantly produced and these fragments have been shown to retain the interactive properties of the native molecule (Table 2). Most of the tissue repair strategies which have been developed with these fragments have focussed on perlecan domain-I and domain-V. As already mentioned, perlecan domain-I has been used to promote chondrogenesis and osteogenesis [146, 151, 152, 154, 155]. Pre-clinical studies show that domain V of perlecan contains modules which interact with α2β1 integrin and disrupt tube formation by endothelial cells inhibiting angiogenesis [156]. Domain V also inhibits amyloid-β Induced activation of α2β1 integrin-mediated neurotoxicity [157] and inhibits amyloid-β induced brain endothelial cell toxicity restoring angiogenic functions [158]. Domain V shows promise in the treatment of stroke victims [159], in the treatment of post-ischemic cerebral angiogenesis [160] and in the treatment of vascular dementia [161].

Recombinant PGs show much promise, however they are not without limitations. These include expense of production at a commercial level, and the high fidelity reproduction of native GAG side chain fine structures. Some GAGs require the biosynthetic machinery only found in mammalian cells for GAG biosynthesis and thus the non-mammalian expression systems often used in recombinant protein technology are unsuitable for their production. These are impediments to the routine cost-effective production and use of recombinant PGs in tissue engineering. It is implicit in any design plan that in order to reproduce the precise GAG side chain microstructure of a native PG consistently then its GAG side chain sequence and distribution pattern along the PG core protein must have been

fully determined. A number of polymers act as structural, chemical and biological PG mimetics, or neo-PGs. Given the expense and greater technical requirements in their production, recombinant PGs or modular domains of these PGs are less likely to be used in a generic manner but may be useful to target specific areas of tissue repair while neo-PGs do not specifically target specific physiological events or disease processes but are cheaper and easier to produce.

3.2 Biomimetic neo-PGs.

Neo-PG core structures attached to scaffolds have their own intrinsic limitations based on potential toxic side effects of the associated scaffold components [162]. Some nano-materials can display unexpected redox regulatory properties and detrimental oxidative effects on mitochondria [163], nevertheless neo-PGs and GAGs in bio-scaffolds can provide positive attributes in tissue engineering aiding in cellular proliferation and migration, acting as anchoring modules for the scaffold to tissue components and for cells to the scaffold. GAGs incorporated into bioscaffolds can thus sequester bioactive signaling molecules, minerals, growth factors and cytokines in a similar way to natural PGs [13]. Despite their limitations, the ease of preparation and versatile structural modification of neo-PGs that are possible makes these components flexible alternatives to natural and recombinant PGs for the preparation of tissue engineering scaffolds.

3.3 GAG-biomaterials versus native PGs.

Many GAG bio-scaffolds have been developed and shown to have utility in a diverse range of tissue repair strategies [47] and these have provided invaluable insights as to how specific GAGs direct cellular behaviour to effect connective tissue repair[22], An important aspect of PGs which is not reproduced to the same degree in artificial scaffolds decorated with GAGs is the bio-integration of these therapeutic agents in tissue repair settings. The functional properties of PGs is due not only to the fine structure and density of their attached

GAG side chains but also to functional domains within the PG core protein which also have an extensive range of interactive properties with many ECM components [6, 31]. These interactions not only stabilize the ECM but also serve to bio-integrate the PG with the tissue repair zone [142].

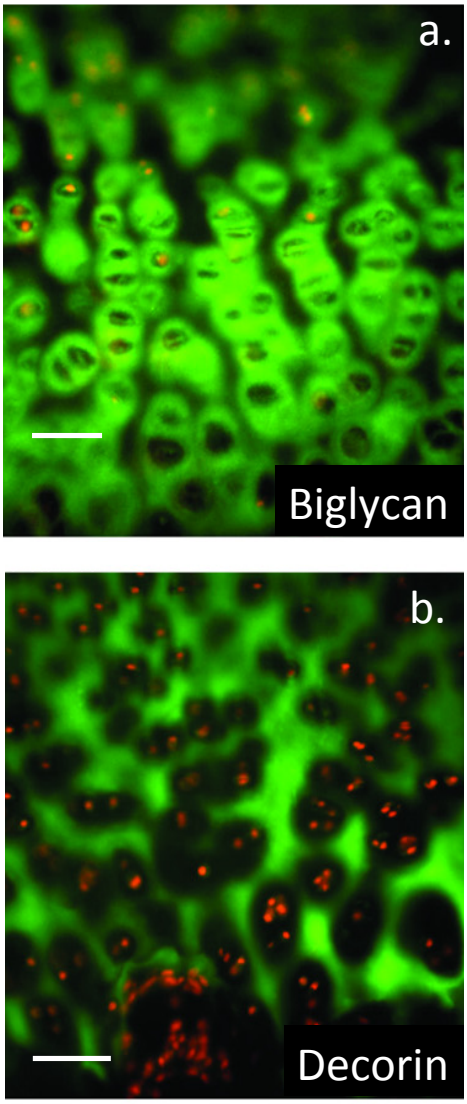

Figure 9. Immunofluorescent localisations of biglycan (a) and decorin (b) in vertically sectioned bovine articular cartilage showing biglycan distributed as a pericellular component while decorin is more prominent in the inter-territorial matrix. Nuclei were counter-stained red with propidium iodide. Scale bar 25 mm. Fig 9a, b reproduced (adapted) from [164] [doi: 10.1016/j.ymeth.2008.01.011], 2008, Elsevier Publishers under terms of license 4587040979458.

Table 2. Features of Recombinant Perlecan Domains

Domain	Domain features	Ref
I	Contains GAG attachment sites for 3 HS or CS/HS chains	[146, 147]

II	Contains LDL-like receptor	[145]
III	RGDS containing domain	[143]
IV	Immunoglobulin repeats	[148, 165, 166]
	Bioactive, domain IV peptides	
V	Endorepellin anti angiogenic protein, 85 kDa domain V fragment	[144, 167, 168]

Therapeutic Applications of Recombinant Domains of Perlecan

Domain	Application	Ref
I	(i) delivery of recombinant human BMP-2 for bone regeneration	[146, 152, 154]
	(ii) mediates cartilage repair through BMP2 delivery in a murine model of early OA	[152]
	(iii) enhanced VEGF165 activity/receptor phosphorylation in human bone marrow endothelial cells	[153]
	(iv) domain I-conjugated, HA hydrogel, chondrogenic differentiation via BMP-2	[151]
	(v) electrospun collagen/gelatin fibers coated with domain I increases GF binding	[169]
	(vi) chondrogenic differentiation on perlecan domain-I/Coll II/BMP-2 matrices.	[155]
	(vii) human perlecan domain I recombinant HSPG with 20-kDa GAG chains	[170]
	(viii) human perlecan domains I & II synthesized by baculovirus-infected insect cells	[171]
II	(i) characterization of recombinant domain II	[172]
	(ii) human perlecan domains I and II synthesized by baculovirus-infected insect cells	[171]
III	(i) high-affinity nidogen-1 interactions with immunoglobulin-like domain 3 of perlecan	[173]
	(ii) cell-adhesive properties of three recombinant peptide fragments of domain III	[174]
	(iii) Ab mapping/tissue localization of globular cysteine-rich regions of domain III.	[175]
	(iv) characterisation of rh domain III-3 containing a globular domain EGF-like motif	[145]
IV	(i) electrospun PCL-domain IV peptide scaffolds, 3D pharmacokinetic cancer model	[176]
	(ii) stimulation of salivary gland cell assembly in-vitro by domain IV peptide	[177]
	(iii) novel domain IV peptide supporting cell adhesion, spreading and FAK activation	[148]
	(iv) chondrogenic activity of perlecan mapped to N-terminal domain I	[147]
	(v) rh domain IV-nidogen, laminin-nidogen, fibronectin, fibulin-2 and heparin binding.	[165]
V	(i) domain V-silk biomaterial modulates endothelial cell-platelet interaction	[178]
	(ii) vascular proteoglycan	[179]
	(iii) inhibition of amyloid-β activation of α2β1 Integrin-mediated neurotoxicity	[158]
	(iv) treatment of vascular dementia	[161]
	(v) treatment of post-ischemic cerebral angiogenesis	[160]
	(vi) neuroprotective agent giving functional improvement in photothrombotic stroke	[180]
	(vii) inhibits amyloid-β induced brain endothelial cell toxicity restoring angiogenesis	[157]
	(viii) stroke therapy	[159]
	(ix) induction of VEGF in brain endothelial cells integrin α5β1 and ERK signaling	[144]
	(x) upregulated in human brain arteriovenous malformation mediating VEGF effects	[181]
	(xi) modulates astrogliosis in-vitro and focal cerebral ischemia mediated through multiple receptors and increased nerve growth factor release	[182]
	(xii) neuroprotective and proangiogenic following ischemic stroke	[156]
	(xiii) endorepellin peptide anti-angiogenic module of domain V angiostatic module, antagonises α2β1 integrin and VEGFR2 interactions	[168]
	(xiv) inhibits α2 integrin-mediated amyloid-β neurotoxicity	[183]
	domain V β1 integrin-mediated cell adhesion, HS, nidogen and fibulin-2 binding	[184]

3.3.1 The stability of native proteoglycans in tissues.

Perlecan and the lectican PG family, and aggrecan in particular, are highly susceptible to proteolytic degradation in tissues (**Figure 2c, Figure 3a, Table 3**). A large number of cleavage sites have also been determined for perlecan [185] but the catabolism of this PG has not been as extensively investigated as that of aggrecan. Cleavage sites on the aggrecan core protein have been determined for ADAMTS-4 and ADAMTS-5, the so called aggrecanases,

and ADAMTS-1 and other ADAMTS family members as well as a number of matrix metalloproteases (MMPs) [186] (**Figure 3c**). Several serine proteases (tryptase, chymase, leucocyte elastase, cathepsin G) released when mast and other immune cells degranulate at sites of inflammation are also capable of degrading aggrecan directly [187] or by activating metalloprotease degradative enzymes [188]. The neutral protease calpain-m [189], and the lysosomal cysteine proteases cathepsin B, D, L, K, S [190] can also degrade aggrecan.

Table 3 MMP & ADAMTS-4, 5 cleavage sites in the aggrecan core protein

Protease cleavage sites	
MMP Cleavages	**ADAMTS-4,5 cleavages**
1. $PEN_{360\ 361}FFG$	10. $EGE_{373\ 374}ARG$
2. $VEE_{698\ 699}WIV$	11. $ELE_{1545\ 1546}GRG$
3. $VGD_{952\ 953}LSG$	12. $EEE_{1714\ 1715}GLG$
4. $VED_{1028\ 1029}SGL$	13. $AQE_{1819\ 1820}AGE$
5. $VEE_{1295\ 1295}ISG$	14. $SQE_{1871\ 1872}LGQ$
6. $VEE_{1332\ 1333}ISG$	
7. $VED_{1409\ 1410}LSR$	
8. $AED_{1470\ 1471}LSG$	
9. $PAE_{2166\ 2167}THL$	

3.3.2 The biological half-life and function of aggrecan in tissues.

In adult articular cartilage the chondrocytes have an inherently low metabolic activity and undergo cell division infrequently. Synthesis of ECM components is also low but sufficient to replenish degraded ECM components and maintain the tissue in a state of functional homeostasis. The majority of cartilage matrix molecules are present in this tissue for many years, aggrecan, the major cartilage PG has a half-life close to 25 years [191], while type II collagen has a half-life of >100 years [192]. The half-life of high buoyant density A1D1 aggregatable aggrecan from the IVD isolated by CsCl isopycnic density gradient ultracentrifugation is estimated to be 5.5 years whereas low buoyant density A1D6 IVD PGs

(decorin, biglycan) have a half-life of 21.5 years [193] and IVD collagens have a half-life of between 95 and 215 years [194].

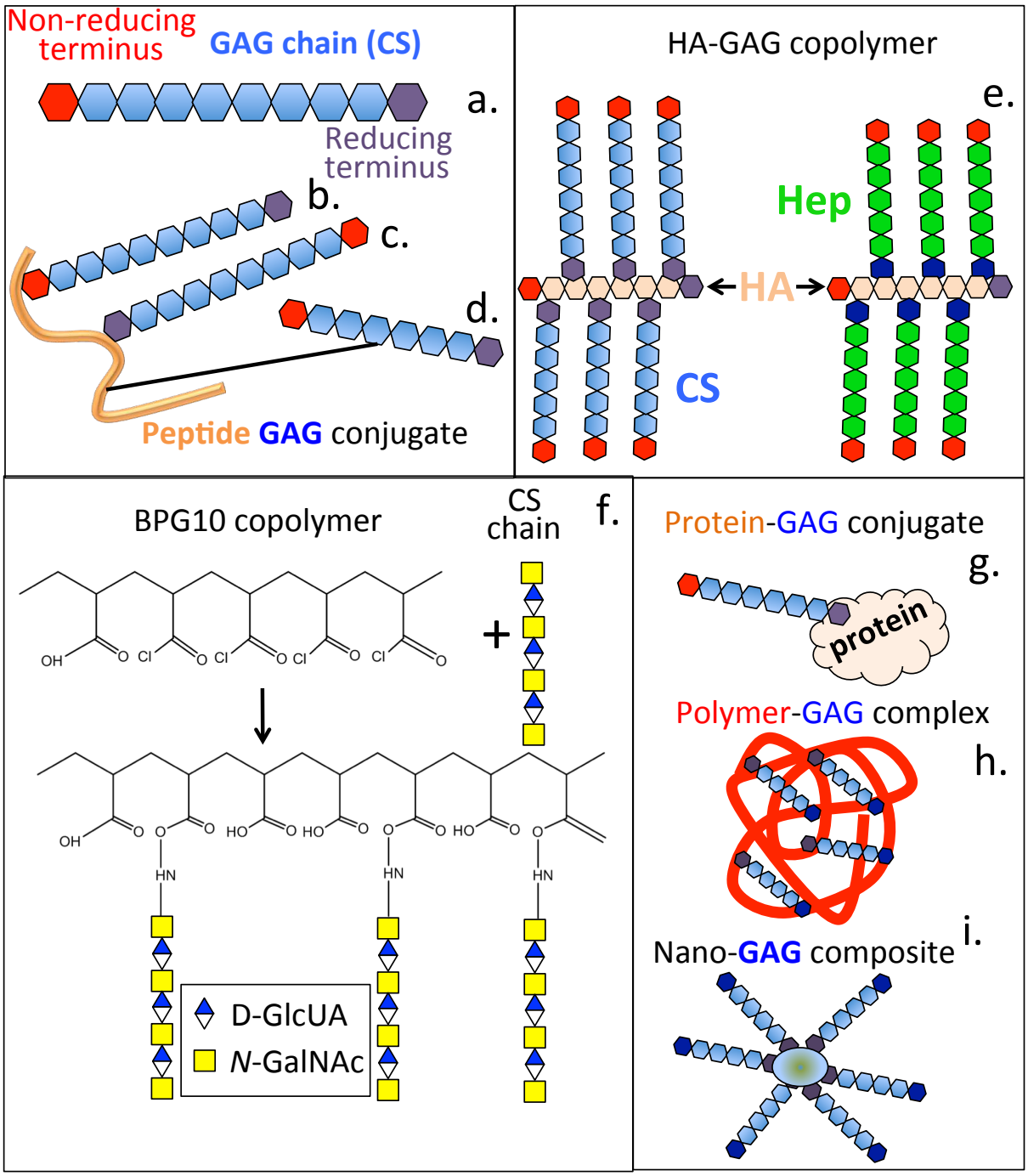

Figure 10. Summary of the different forms of GAG polymers which have been assembled for tissue engineering applications. Schematic of CS chains (a) linked to a peptide backbone via their non-reducing (b) or reducing termini (c) or by intra-chain attachment (d) in a prospective neo-proteoglycan. Biomimetic peptidoglycan neo-aggrecan and neo-lubricin (e) GAG derivatised hyaluronan co-polymer (e) BPG10 co-polymer aggrecan mimetic (f) protein-GAG conjugates (g), polymer GAG complexes (h) Nano-composite (i) which have have all been used as proteoglycan mimetics.

Aggrecan in mature cartilage is heavily substituted with ~100 CS and ~25-50 KS chains representing ~90% of the mass of aggrecan (**Figure 3c**) [29]. KS is concentrated in a KS-rich region adjacent to the CS1 and CS2 regions in all species but rodent which have truncated core proteins devoid of this KS-rich region [195]. Small KS chains are however found in the G1 and G2 globular domains of aggrecan and in the interglobular domain (IGD) in all species [196]. The role of these KS-rich domain GAG chains is not known, however KS chains in the G1, G2 have been shown to suppress a T cell-mediated response when G1 is used as an arthritogen in an inflammatory OA model [197]. IGD KS potentiates aggrecanolysis within the IGD by aggrecanases [198] (Fig 3c). No deleterious effects have been observed in the performance or turnover of aggrecan in murine cartilage, there would therefore appear to be no absolute requirement for KS in aggrecan or in a neo-aggrecan mimetic. The other members of the lectican PG family do not contain KS. Aggrecan forms massive link protein stabilized ternary structures with HA in cartilage and with tenascin-R in neuro-protective PNNs in the CNS/PNS (Fig 3d-f). Monoclonal antibody 1-B-5 detects an unsulfated stub epitope on the CS chains of aggrecan and has been used to immunolocalise PNN structures in neural tissues, other members of the lectican PG family which have also been found in PNNs would also be identified by this antibody (**Figure 3e, f**). PNNs are mesh-like structures, composed of a hierarchical assembly of ECM molecules which encapsulate neurons and regulate their plasticity however much has still to be learnt of such interactions in the PNN and how they regulate neurons [199]. Although the basic components in PNNs are similar, they are not identical in all regions of the brain and the precise composition of their components varies in different regions of the brain [200]. The high localization of GAGs in PNNs is neuro-protective, chelating bioreactive molecules such as free Fe^{2+} and Fe^{3+} ions protecting the neuron from oxidative stress through free radical generation in ferrous and ferric ion catalyzed reactions [201]. Brain tissue is rich in

polyunsaturated fatty acids and susceptible to lipid peroxidation. Fe2+/Fe3+ are important initiators of free radical generation in such peroxidation reactions [202].

A number of strategies have been used to prepare bio-scaffolds containing neo-aggrecan mimetic molecules. Three dimensional scaffolds containing GAG mimetic nanofibres mimic the biochemical and mechanical properties of native cartilage [203]. A number of scaffolds supplemented with CS have been used to promote MSC differentiation into osteogenic, chondrogenic and neurogenic cell lineages to improve cartilage and neural repair (reviewed in [47]).

3.3.3 Rationale for the use of CS but not KS chains in aggrecan mimetic neo-PGs.

When the structure of native aggrecan is examined in detail, the rationale for only using CS in the design of a neo-aggrecan becomes apparent. Despite the intensive investigation of aggrecan's structure and function for over five decades, there are still gaps in our knowledge of this molecule. The water binding properties of aggrecan stem from the high negative fixed charge density provided by its CS side chains and their associated counter-ions and how these partition in cartilage as described by the Gibbs Donnan Equilibrium. In Figure 1c and Table 1 we have identified unresolved aspects of aggrecan's structure mainly involving its KS chains. This information provides a rational basis for the use of CS but not KS in neo-PG design. Rodent aggrecan is deficient in KS chains with no obvious impediment in the articulation of rodent joints.

3.3.4 Production of biomimetic neo-aggrecan.

Several strategies have been used to prepare aggrecan mimetics (**Figure 10a-i**). One method uses a polyacryloyl chloride backbone to attach CS chains. In this BPG10 aggrecan

mimetic, 22 kDa CS chains are attached via reaction of a terminal primary amine on the CS with acyl chloride groups on the polymer backbone (**Figure 10j**) with unreacted acyl chloride hydrolysed to carboxyl groups contributing to the negative charge density of this polymer [204]. BPG10 is an aggrecan mimetic of dissimilar molecular dimensions to native aggrecan, but its CS side chains have a similar bristle-like arrangement around the core structure to native aggrecan and these display a similar high fixed negative charge density (**Figure 3a, b**). BPG10 has impressive water regain characteristics, displaying 50% greater water regain on a weight basis to native aggrecan or CS chains in isolation. Moreover, the backbone in BPG10 is not susceptible to proteolytic degradation thus the biological half-life of this neo-aggrecan is superior to that of native aggrecan. AFM confirms the bottle-brush type distributions of the CS chains (**Figure 3 a, b**) around the polyacryloyl chloride backbone and the relative size of this neo-aggrecan which is significantly smaller than that of native aggrecan (**Figure 10j**).

In another approach a CS backbone has been subjected to periodate oxidation to introduce reactive aldehyde groups in glucuronic acid residues of the CS chain and these were reacted with an HA binding peptide (GAHWQFNALTVRGGGC)[205] (**Figure 11a**). This peptidoglycan aggrecan mimetic (mAGC) containing CS chains with attached HA-binding peptides replicated the HA-binding properties of native aggrecan ensuring that GAG localization was maintained within the scaffold [205]. Furthermore, localization of mAGC in the scaffold also stimulated synthesis of type II collagen. AFM demonstrated that the attached peptide chains in mAGC assumed bottle-brush-type orientations on the CS-core structure similar to the CS chain arrangements in native aggrecan core protein [204]. mAGC had superior water regain properties to that of native aggrecan and mAGC constructs had superior compressive strength (78% increase), The catabolism of mAGC constructs by MMP-13 and ADAMTS-5 [206, 207] was also reduced compared to in animal models of OA [206, 208]. A peptidoglycan lubricin-mimetic, mLUB15 has also been prepared by similar methodology to

that of mAGC using HA binding peptide (GAHWQFNALTVRGGGC) and a type II collagen binding peptide (WYRGRL) attached to the CS backbone [209] (**Figure 11b**).

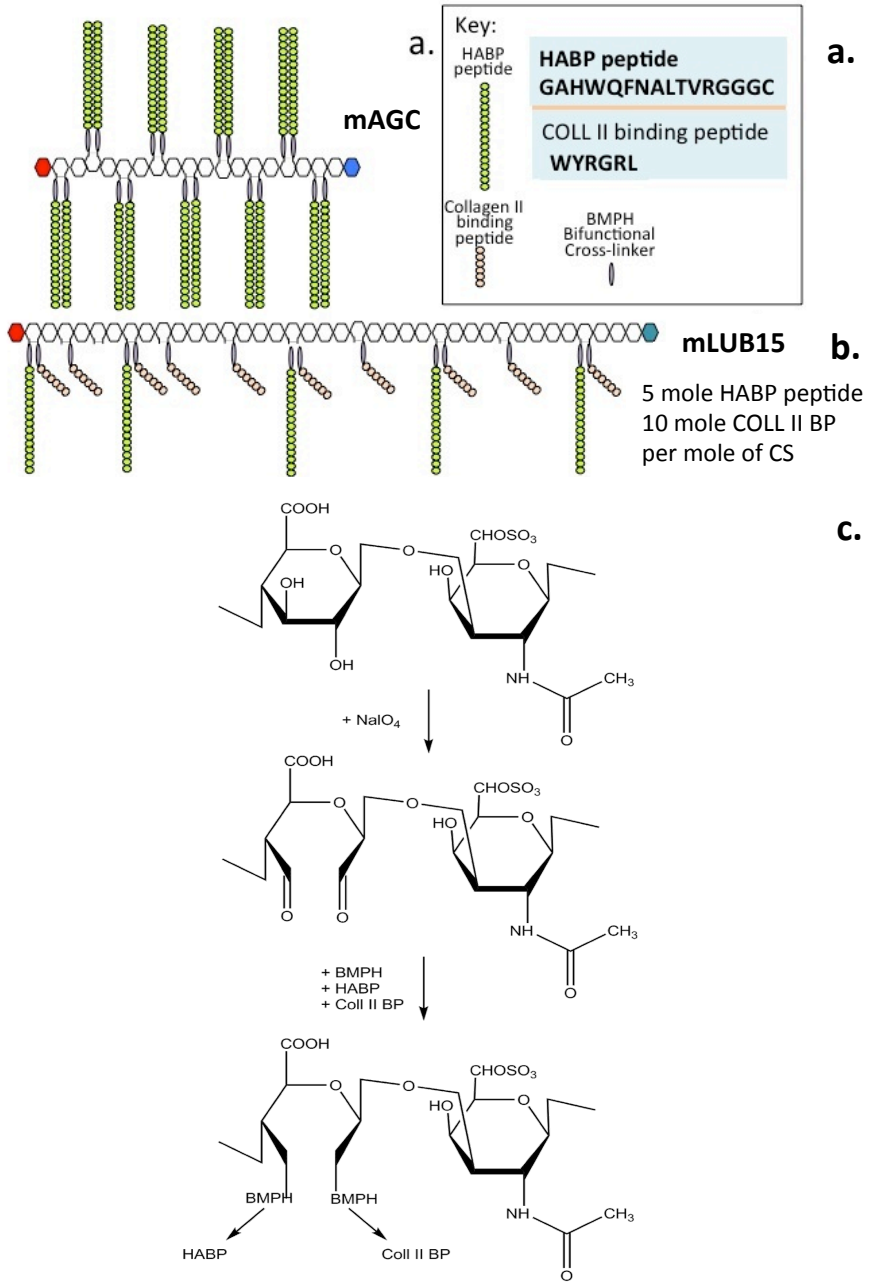

Figure 11. Aggrecan, **mAGC** (a) and lubricin, **mLUB15** (b) biomimetic neo-proteoglycans. The native structure of lubricin is shown in (c). See Fig 2 for the native structural organization of aggrecan. Reaction steps in the production of peptidoglycan mimetic neo-PGs (d). Periodate oxidation of GlcA in CS produces reactive aldehyde groups (21 aldehyde groups per CS chain). HA binding peptide **GAHWQFNALTVRGGGC** or type II Collagen binding peptide **WYRGRL** are attached to the reactive aldehydes using the heterobifunctional crosslinker N-[b-maleimidopropionic acid] hydrazide (Trifluoroacetic salt) (BMPH). In **mAGC** only the HA binding peptide is attached to the CS chain (a). This product has a molecular weight of 40 kDa. Production of the lubricin biomimetic **mLUB 15** involves attachment of HA binding peptide and the type II collagen binding peptide (b). 5 mole HABP and 10 mole type II binding peptide are attached per mole of CS.

mLUB15 prevented pathological age-related effects on bovine vitreous, and enzymatically-induced degradation [210]. A further GAG copolymer mimetic aggrecan has been developed using HA as a support backbone to which CS or heparin chains were attached using a hydrazide bi-functional reagent [211] (**Figure 10i**). In yet another approach, collagen has been used as a backbone structure and CS chains attached by reaction of the CS chains with the e-amino groups of lysine and hydroxylysine residues on the collagen by reductive amination using cyanoborohydride to produce a collaggrecan aggrecan mimetic [212] (**Figure 12**). Lee et al [213] used ring-opening metathesis polymerization (ROMP) methodology to attach CS chains to microarray and surface plasmon resonance platforms for the assessment of CS mediated interactions with growth factors and binding proteins. This methodology could also be used to produce alternative forms of aggrecan-mimetic molecules [213].

3,3,5 Features of peptidoglycan neo-aggrecan and lubricin mimetic PGs.

In the production of mAGC, 16 amino acid HA-binding peptides (GAHWQFNALTVRGGGC) are attached to the CS backbone (**Figure 11a**) using periodate oxidation to produce reactive aldehyde groups in the CS chain (21 aldehyde groups per CS chain) and these are reacted with HA-binding peptide using the hetero-bifunctional cross-linker N-[b-maleimidopropionic acid] hydrazide to attach ten HA-binding peptides per CS chain. Periodate ($NaIO_4$) oxidation cleaves between the C2 and C3 vicinal diols in GlcA to form reactive aldehydes to attach CS to peptides or some other primary amine using a bi-functional reagent. Ring opening by periodate oxidation also forms highly flexible 'hinges' in an otherwise inflexible CS chain altering its physical properties and is permissive of the exploration of a more extensive range of conformational orientations than the native CS chain [214].

CS is a linear chain of 100-200 β1-3 and β1-4 linked D-GlcA residues attached to D-GalNAc. In order to accommodate the bulky space-filling sulfate groups, CS adopts a helical

structure [215]. X-ray fibre diffraction studies show that like HA, CS adopts a left-handed 3 fold helix with sulfate groups pointing outwards towards the periphery of the helix where they interact with a surrounding cation and a water molecule [216]. The swapping of Na+ for Ca2+ changes the pitch of the helix from a 3 fold to a more compact 2 fold form. Opening of the GlcA ring structure by periodate oxidation introduces two aldehyde functionalities which are reactive with bi-functional reagents and these can be used to attach peptides to the CS backbone. AFM confirmed that the attached peptides adopted a bristle-like distribution in a bottle-brush type arrangement on the CS core and they thus explored all available spatial orientations which would be expected to optimize the space-filling and interactive properties of the peptidoglycans [45]. mLUB15 is a biomimetic peptidoglycan lubricin prepared using HA binding and type II collagen binding peptides [209] . This neo-lubricin has a dissimilar structure to that of native lubricin (**Figure 11b**) but its surface lubricative properties in articular cartilage provided efficient joint lubrication and it also had an increased resistance to proteolysis compared to native lubricin. The attached interactive binding peptides on mLUB15 equip it with interactive properties with synovial fluid components which promote boundary lubrication[217]. mLUB15 displayed synergistic properties with fibronectin [218], its interactive properties provided by the HA and type II collagen binding peptides ensure consistent mLUB15 localisation in squeeze films at the articular surface even under high pressure conditions.

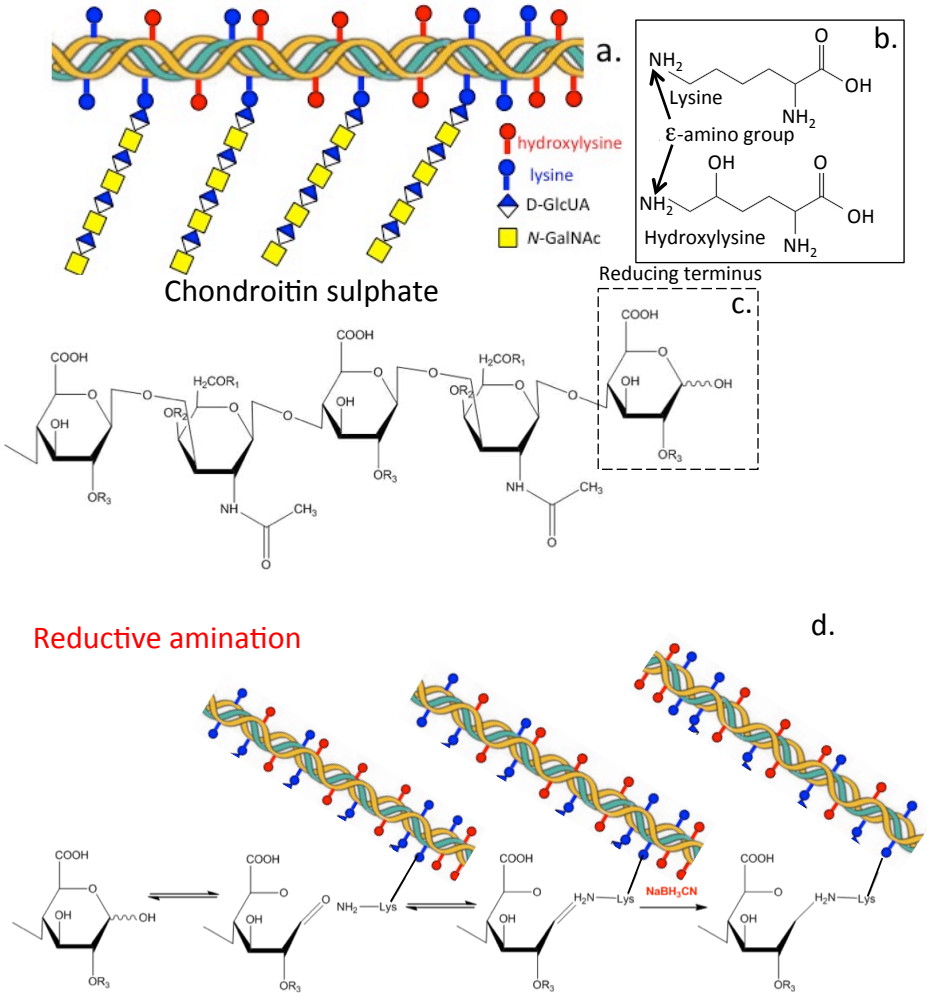

Figure 12. Preparation of the neo-PG collaggrecan (a) by attachment of CS chains to the epsilon amino groups of lysine or hydroxylysine (ε-Lys, ε-Hydroxyls) (b) on a collagen chain through the CS reducing terminus (c) using reductive amination and sodium cyano borohydride ($NaBH_3CN$) (d). Ring opening of the reducing terminal glucuronic acid residue produces a tautomeric reactive aldehyde which reacts with the e-amino Lys as shown by reaction with the reducing agent $NaBH_3CN$ in a reductive amination step.

3.3.6 Tissue protective properties of neo-PGs.

While the specific design features of neo-PGs may make them resistant to degradation by proteases, the high charge density provided by their GAG components may provide an added bonus by enhancing the properties of naturally occurring protease inhibitory proteins within tissue repair environments. Binding of GAGs to members of the plasma Serpin protease inhibitor family can significantly enhance their inhibitory properties [219]. GAGs enhance the inhibition of some of the coagulation cascade proteases including thrombin, factor Xa and plasmin [220]. Charge density appears to be an important requirement for

protease inhibitory activity [221]. Cell surface GAGs have also been reported to enhance the inhibitory properties of AT-III and tissue factor protease inhibitor displayed against VIIa [222]. GAGs also modulate the activity and activation of the lysosomal exopeptidase tripeptidyl-peptidase-I thus may affect the processing of peptide hormones and growth factors in-situ and tissue repair [122]. The protective effect reported for some neo-PGs over ECM components could potentially therefore, be exerted by effects on resident protease inhibitory proteins in tissues [22]. Interactions between GAGs and TIMP-3 suggest that this may improve TIMP-3 inhibitory properties against MMPs and be protective in repair environments [55, 223]. TIMP-3 is the only TIMP which has interactive properties with ECM components which localise it in tissues. High molecular weight and cross-linked HA preparations such as hylan either down-regulate the production of cartilage degrading MMPs or actively inhibit these enzymes in tissues thus they have beneficial chondroprotective properties [135]. These points are covered further in Table 4.

3.3.7 Application of nano-delivery systems employing stimulatory peptides, GAGs and MSCs in cartilage repair strategies.

As already mentioned, CS has been applied in conventional scaffold systems for the stimulation and differentiation of MSCs, promotion of chondrogenesis and application in cartilage engineering (reviewed in [47]). CS (or heparin) nano-delivery systems and MSC or chondroprogenitor stimulatory peptides are also promising strategies in articular cartilage repair (**Figure 8f**). Enzymatic treatments to release these bioactive peptides from the scaffold, increases their bio-availability, representing a novel methodological advance [224, 225]. Encapsulation of MSCs within collagen microparticles induces chondrogenic differentiation and the cartilaginous matrix elaborated within these micro-particles can be readily bio-integrated into cartilage defects [226]. Triple helical collagen mimetic GPC(GPP)5-GFOGER-

(GPP)5GPC-NH_2 and fibronectin RGD cell attachment peptides incorporated into degradable and non-degradable polyethylene glycol gels have been examined for their ability to promote cartilage repair through the promotion of chondrogenic differentiation of MSCs. Interestingly, the collagen mimetic peptide provided superior results in cartilage repair than the fibronectin peptide [224, 227]. Pre-chondrogenic cells cultured on bioactive self-assembled peptide nano-fibres undergo elevated growth and chondrogenic differentiation thus this substrate is appropriate for further assessment in cartilage regeneration strategies [228].

HA derivatised with CS (or heparin) using hydrazide bifunctional reagents and reductive amination [229] is a highly flexible methodology in terms of the density of CS (or heparin) which can be achieved and is also suitable for the generation of nanoparticles. CS-HA and heparin-HA conjugates both promote cartilage repair, however heparin-HA is more effective as an FGF-2 delivery system. Aggrecan-mimetic GAG nanoparticles stabilize and optimize FGF-2 delivery for repair applications [117]. Cellulose based-CS mimetic scaffolds also display positive traits in cultures of MSCs in articular cartilage repair [230]. Recent advances with artificially sulfated polysaccharides to improve cell growth and differentiation and drug delivery also show promise in tissue engineering applications [231]. Biphasic semi-interpenetrating polymer network hydrogels impregnated with CS nanoparticles, (Zein nanoparticles, ~150nm) interspersed in a calcium cross-linked alginate-PVA blend, have an interconnected porous microstructure conducive to cartilage repair. Primary chondrocytes loaded into these hydrogels exhibit high expression levels of sox9, aggrecan and type II collagen but low expression of type I collagen and this hydrogel is considered a useful mimetic for repair of irregularly shaped cartilage defects [118]. NP cells cultured on laminin peptides and surface conjugated α3 integrin receptor peptides P4 and P678, and the α2, α5, α6, β1 integrin recognizing peptide AG10 on polyacrylamide matrices showed elevated

expression of aggrecan, N-cadherin, and types I and II collagen. Integration of the neo-tissue with existing NP tissue suggested that this methodology promoted NP cell vitality conducive to repair or regeneration of the IVD [232]. RGDS functionalized *Streptococcal* collagen-like (SCL) mimetic hydrogels have been developed for MSC stimulation to promote articular cartilage repair [225]. An SCL protein modified with GAG-binding peptides was processed into a hydrogel format containing a RGDS peptide which could be released by treatment of the hydrogel with MMP-7. MSCs cultured in this hydrogel expressed a 3.9 fold increase in *COLAII,* 7.6 fold increase in *Acan* and 5.6 fold increase in *Sox9*, and significantly elevated levels of aggrecan and type II collagen synthesis at the protein level. Temporal activation of embedded stimulatory biomimetic peptides in hydrogels therefore represents a particularly innovative approach to cartilage repair. Recent studies have also demonstrated the usefulness of aldehyde-modified ECM proteins for targeted adherence to biological tissue surfaces. Aldehyde-modified lubricin displays enhanced binding to PRG4-depleted articular cartilage and has been used for cartilage resurfacing and is a convenient method for overcoming loss of lubrication during the early stages of OA [137]. Collagen fibrils have also been embedded in a network of photo-cross-linked acrylated HA, CS, or sulfated HA (sHA) 3D hydrogels [233]. Endothelial cell proliferation was significantly increased in sHA gels compared to CS-derivatised or non-derivatised HA hydrogels. Sulphation of HA increases the hydrogels growth factor binding capacity, and reduce its susceptibility to degradation by hyaluronidases [114]. Sulfated HA hydrogels are also suitable as carriers for hMSCs, promote chondrogenesis, inhibit hypertrophy and have been used in intra-articular injections to delay/reverse OA changes in joint tissues [114]. sHA micro-particles have been prepared for the controlled delivery of growth factors including BMP-2 and TGF-β1 [119, 234]. HA micro-particles containing heparin have also been used for the delivery of BMP-2 [235]. An HABP-PEG-collagen-binding peptide co-polymer (HABP2-8-arm PEG-COLBP) has been used to immobilise HA in cartilage and to improve surface lubrication to protect the articular surface

from abrasive damage [236]. Quartz crystal microbalance - isothermal calorimetry has demonstrated this polymer effectively reduced the progression of post-traumatic OA [236]. High molecular weight HA also protects articular cartilage by inhibiting aggrecanase expression by the resident chondrocyte cell populations [135].

4. Protection of the articular cartilage surface from abrasion and preservation of its lubricative properties

The surface of articular cartilage is a key component of the articulating joint structure and is the region where articular lesions first appear in OA arising from surface abrasions which occur due to overloading of this surface tissue (**Figure 13**). These lesions continue to develop with the progression of OA eventually leading to full depth defects down to the calcified cartilage and bone by a process known as eburnation. OA represents a large and rapidly increasing global disease burden challenging health-care systems worldwide. Disease progression in OA results in increasing pain and loss of joint function, it is at early symptomatic stages that OA treatment is more likely to be successful. Treatment of end stage OA lesions have so far met with little success despite a concerted global anti-arthritic program spanning five decades. With changing global ageing trends OA prone individuals >65 years of age will be prominently represented in the general population. This will result in OA becoming a leading musculoskeletal condition by 2040 and in the USA it is estimated that 78.4 million individuals will suffer from OA [Arthritis Foundation. Arthritis By the Numbers / Book of Trusted Facts & Figures. 2018; v2; 4100.17.10445 arthritis.org]. The incidence of OA has already doubled since the mid 20th century [25].

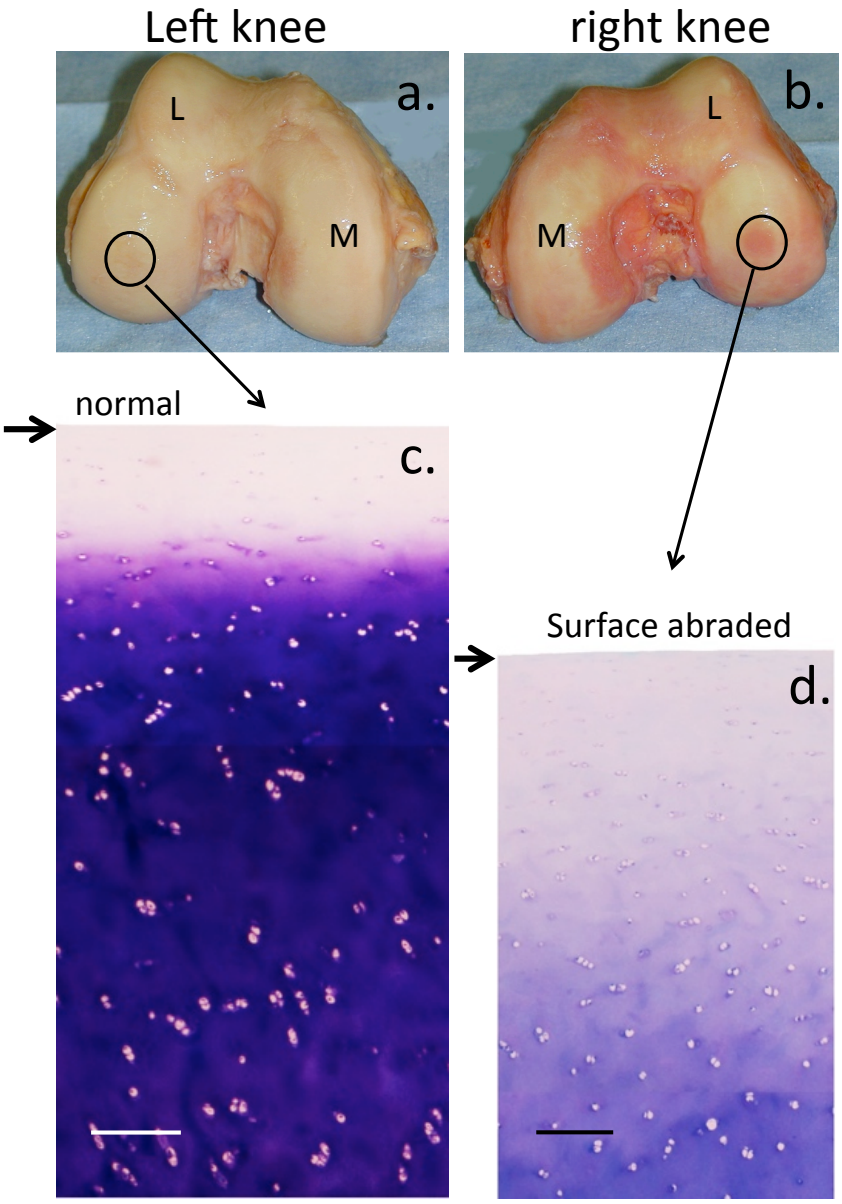

Figure 13. Morphological features of 55 year old male donor knees (a, b).Toluidine blue stained vertical sections of weight bearing regions of femoral condyle articular cartilage normal (c) and mildly abraded (d) from the areas depicted in (a) and (b) showing less intense staining and reduced cartilage thickness in the abraded specimen. Arrows depict the articular surface, M, medial; L, lateral compartment. The left knee is non-degenerate while the right knee shows very early stages of OA with areas of surface damage in the weight bearing region of the condyle. Clinically this is the type of cartilage recommended for treatment using viscosupplements, without such a preventative approach the indicated abraded areas will expand into full depth lesions severely impacting on joint function.

As already shown, images of bovine articular cartilage demonstrate that type I collagen, versican and lubricin/PRG4 are prominent components of the articular surface (**Figure 7-9**). Type-I collagen counters the tensional shear forces generated during joint articulation. Versican, is an HA binding PG and may contribute to the retention of HA at the articular

surface. Lubricin/PRG4 is a mucin glycoprotein/PG produced by surface zone synoviocyte-like cells of a flattened morphology and is deposited as a fine deposit at the articular surface. Lubricin as its name indicates has roles in the lubrication of the surface of articular cartilage. The major space-filling hydrodynamic PG of articular cartilage is aggrecan which is distributed throughout this tissue. Aggrecan equips cartilage with its ability to withstand compressive load. A schematic already presented shows the organization of these articular components (**Figure 1**). Type VI and XI collagen have also been shown to provide cell-pericellular interconnections which may contribute to cell ECM communication (**Figure 2**). HA conveys many beneficial properties to the structural integrity and function of synovial joints. Through its interaction with aggrecan, it serves as a hydrodynamic space filler in articular cartilage protecting resident chondrocyte cell populations from the significant weight bearing and shear forces they experience in the joint [115]. HA is also a major component of the synovial fluid that bathes and lubricates the articular joint surfaces along with lubricin ensuring smooth low frictional movement during normal day to day joint articulation. A comparison of the boundary lubrication properties of two batches of HA, including high molecular weight Healon® failed to provide as efficient joint lubrication as lubricin [237]. A synthetic lubricin neo-PG, mLUB also provided efficient lubrication of the surface of cartilage and acted in a protective capacity preventing the generation of surface lesions typical of those that occur in the development of OA [209].

4.1 Synovial fluid HA.

The average half-life of synovial fluid high molecular weight HA of 6 x 10^6 Da is 13.2h in rabbits while a smaller HA of molecular weight 9 x 10^4 Da had a half-life of 10.2 h. Steady state values for synovial HA in rabbits and sheep are reported to be 0.5-1 day [238]. Native synovial HA is a thixotropic non-Newtonian viscoelastic solution whose viscosity is shear dependent [239-245]. At high shear the viscosity of HA gels may drop by as much as

~10^3 times and it then obeys as a Newtonian fluid [240]. The elasticity of HA increases with increasing molecular weight and concentration of the HA molecular network. These rheological properties of HA are important in the lubrication of joints [239]. Commercial preparations of HA of variable sizes are available (**Table 5**). These differ in their rheological characteristics in-vitro and the properties they convey to synovial fluid and joint-lubrication in-vivo [241-245].

Synovial fluid HA in normal healthy knees has a molecular weight of 7 x 10^6 Daltons whereas HA in the OA or RA knee HA has a molecular weight of 4.8-5.0 x 10^6 Daltons [246]. HA in the OA knee has a broad polydisperse size distribution with a weight average molecular weight of 1.2-4.5 x 10^6 whereas the HA from a normal joint has a weight average molecular weight of 1.6-10.9 x 10^6 Da [115, 247, 248]. The concentration of HA in the OA or RA knee (1.09-1.20 g/l) is lower than in the normal healthy knee (1.45-3.12 g/ml) and is a smaller size range [246, 247, 249] thus the rationale for the use of HA in visco-supplementation procedures is to replenish these deficient HA levels to recover normal knee-joint function.

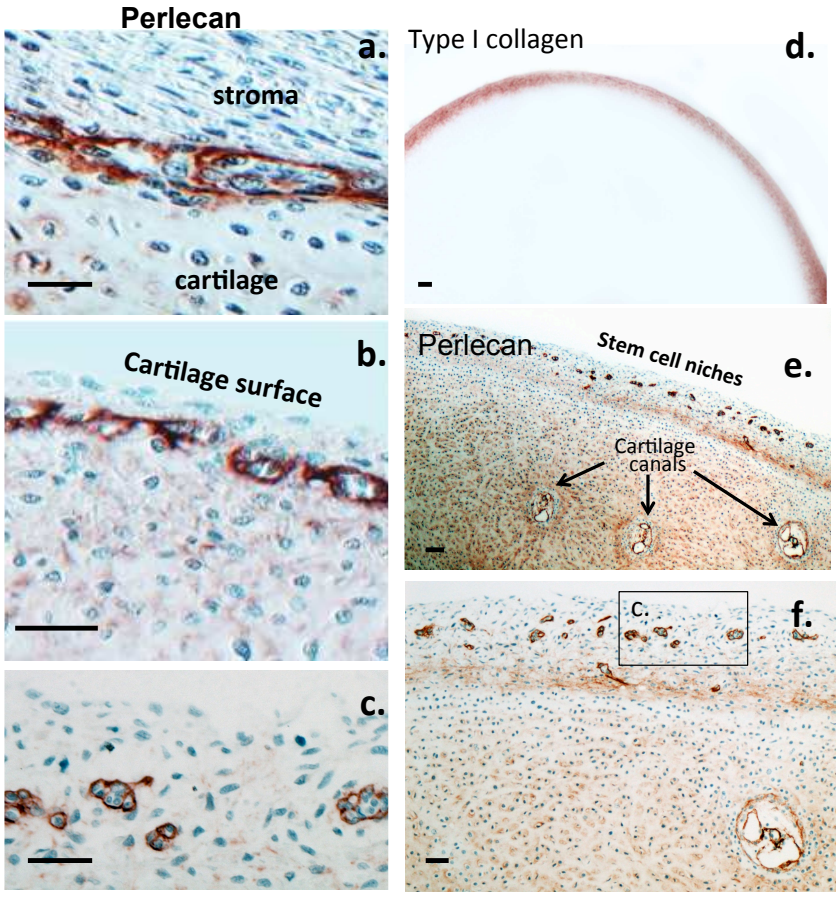

Figure 14. Stem cell niches delineated at their margins by the immunolocalization of perlecan in the surface regions of second trimester human foetal knee (a, b) and hip (d, e, f) articular cartilage using anti-perlecan domain IV antibody A7L6. The boxed region in e is provided at higher magnification in segment f. Type I collagen is also a prominent component of human foetal articular cartilages, type I collagen is immunolocalised in a foetal hip joint in photosegment c. Scale bars 100 μm. Images reproduced from [250] [doi: 10.24966/SRDT-2060/100009] with permission. Images copyright J. Melrose 2014.

4.2 HA and visco-supplementation.

A number of purified HA preparations have been developed for visco-supplementation (Table 4). HA preparations, can be broadly categorized into (i) HAs (unmodified HA) and (ii) cross-linked HAs (Hylans). Standard HAs are low to medium molecular weight HA fractions extracted from rooster combs or bovine vitreous[251, 252]. Hylans are chemically cross-linked high molecular weight, HA derivatives. Hylan G-F 20 (Synvisc® , Genzyme), consists of two hylan polymers, hylan A and hylan B[253]. Hylan A extracted from rooster combs is pre-treated with formaldehyde resulting in cross-link formation between hydroxyl groups on the HA and amino/imino group of proteins between 2 and 8 native HA chains are thus cross-linked in hylan preparations producing an HA product of ~ 6 MDa [253, 254]. The average

protein content of hylan A is 0.4–0.8% [255]. Hylan B is formed by further cross-linking of hylan A using divinyl sulfone [251] to form a visc-oelastic gel [253, 254] with ~20% divinyl sulfone crosslinks. NASHA (non-animal stabilized HA) was subsequently developed to provide a high molecular weight cross-link stabilized HA preparation from a bacterial ferment to avoid any immunological sensitivity issues which have been reported for animal sourced HAs [256]. NASHA are new-generation HAs with improved biophysical properties compared to unmodified HA. NASHA injections deliver an increased density of HA and display a high resistance to intra-articular degradation increasing its intra-articular residency time and clinical effect. NASHA was designed to deliver a single injection of high density intraarticular HA sufficient for it to be an effective visco-supplement. Despite the small extent of molecular cross-linking in NASHA, the stabilization provided has a major effect on the rheologic properties of NASHA, and is a key aspect of its functional properties as a lubricant and shock-absorber within the knee joint. NASHA has greater elasticity and viscosity, compared to existing commercial HA preparations, and outperforms endogenous HA synthesized by young, healthy joints. Q-Med AB originally developed Durolane® for ophthalmic applications but it also had application as a visco-supplement [257]. Q-Med AB manufactures all of the formulations of NASHAs including Durolane® and Restylane ®. NASHA is stabilized using 1, 4-butanediol diglycidyl ether, this cross-linking reagent reacts with the hydroxyl groups of the repeat disaccharide unit. Cross-linking conditions are carefully controlled to ensure that only ~0.5-1.0% of the residues are modified. This represents ~ 1 residue in every 100 disaccharide units linking two native HA molecules together and while it changes the physical properties of the HA forming a 3D network the majority of the functional groups are unmodified and available to undertake interactions which normally occur with the native HA molecules. Each HA gel microbead thus formed is effectively a single HA molecule however it has a massively increased molecular weight increased by a factor of around ten thousand billion ie 10^{13} [256]. This size imparts important

beneficial therapeutic properties to NASHA in terms of its rheological and visco-elastic performance as a visco-supplement and increases its residence time in the intra-articular space. Furthermore, NASHA is less susceptible to depolymerisation by free radicals which further increases its biological half-life in the knee-joint. Treatment with NASHA is by a single injection of 60mg [209] which ensures a rapid response to pain alleviation in the OA knee and further advantages in its clinical management over protocols which require multiple (up to five) injections of HA to produce the same pain alleviation effect.

Table 4. HAs used for visco-supplementation procedures for the treatment of OA

Mw (Da)	HA	Manufacturer	Ref
0.5-1 x 10^6	Hyalgan® Suplasyn® Fermathron®	Fidia Pharma USA Inc Rubio Laboratories MegaChem, Berlin GmbH	[243, 258-260]
0.8-1.5 x 10^6	Go-on Matrix	Rottapharm|Madaus, Monza, Italy	[261]
1.1 X 10^6	Gelsyn-3	IBSA Farmaceutici Italy	[262]
1-1.8 x 10^6	Ostenil® Orthovisc® Viscoseal®	TRKB Chememdica, UK DePuys Synthes TRKB Chememdica, UK	[243, 258, 259]
1.5-2.0 x 10^6	HyalOne®/Halubrix® Gel-one® cross-linked HA	Zimmer-Biomet Rottapharm|Madaus, Monza, Italy	[263] [244]
1.0-2.9 x 10^6	Monovisc®	DePuy Synthes Mitek Sports Medicine	[243]
2.4-3.6 x 10^6	Euflexxa® Synocrom Forte	Ferring Pharmaceuticals	[259, 264]
6 x 10^6	Synvisc (Hylan G-F 20)® Synvic One (Hylan G-F 20)®	Sanofi (USA)	[259, 265]
6.2-11.7 x 10^6	Supartz® Supartz FX®	BioVentus , USA	[243, 258, 259, 266]
HMW but weight not provided	Hymovis, HYADD 4 High molecular weight viscoelastic HA.	Fidia Pharma USA Inc	[267]
2.5-3 x 10^6	Viscoplus_Matrix®, Viscoplus_Gel®,	Biomedical B Baumann Group	[241]
10 x 10^{12} Da	NASHA HAs Durolane®	Bioventus (USA)	[242, 256, 268, 269]

4.3 HA for reconstructive surgery and cosmetic applications.

Several HA formulations have been developed specifically for applications in cosmetic [270] and ophthalmic corrective surgical applications (**Table 5**). HA ophthalmic visco-elastic devices are used in vitro-retinal, anterior segment and glaucoma surgery, and corneal transplantation procedures in the eye and on its surface to prevent dehydration and to

promote wound healing [271]. Dermal filling with cross-linked HA such as Princess® Filler is a viable treatment option for the correction of various soft tissue defects of the face resulting facial lipoatrophy, morphological asymmetry of the face, or debilitating depressed scars [272]. The space-filling and hydrating properties of HA preparations [273] are important attributes in cosmetic and restorative applications[270, 274]. The longer residence time due to improved stability of high molecular weight cross-linked HAs such as Restyllane are also important performance indicators. Non-stabilised HA has a relatively short half-life in tissues and is susceptible to depolymerisation by free radicals generated during inflammation. The resultant small molecular weight oligosaccharide products have angiogenic properties and also stimulate many cell types leading to synthesisis and activatation of MMPs leading to unwanted ECM remodeling. Some of the HAs used in cosmetic and reconstructive surgery have similar properties to the HAs used for visco-supplementation of synovial joints and are made by the same manufacturer eg Restyllane® and Durolane® NASHA. Many HA formulations for dermal injection have additives such as sorbitol or mannitol anti-oxidants and local anaesthetics (lidocaine) to ease extended injections.

Table 5. FDA Approved Hyaluronan Dermal Fillers for Cosmetic and Reconstructive Procedures

HA Product	Manufacturer	Application
1. Revanesse Versa 2. Revanesse Versa + Lidocaine	Prollenium Medical Tech Inc,Aurora,USA.	mid to deep dermal injection to correct facial wrinkles/folds [275]
1. Restylane Lyft 2. Restylane Refyne, 3. Restylane Defyne	Q-Med AB, Uppsala, Sweden	Dermal implantation for aesthetic use in the hands mid-deep dermal injection for aesthetic correction of moderate to severe facial wrinkles and folds in adults >21 years old [276]
1. Juvederm Volbella XC 2. Juvederm Vollure XC 3. Juvederm Violift® 4. Juvederm Voluma®	Allergan Inc, Dublin, Ireland.	dermal injection, correction of facial wrinkles and folds licenced in USA for lip injection/lip augmentation and correction of perioral rhytids in adults >21 years old [277]. cross-linked 90% LMW HA and 10% HMW HA giving better filling qualities than HMW HA fillers [278].
Princess® Dermal filler	Croma-Pharma GmbH,	Treatment of facial lipoatrophy, morphological asymmetry, or debilitating scars [272].
Cohesive polydensified matrix HA volumizer (CPM-HA)	Belotero-Balance, Geneva, Suisse	Cheek augmentation, aesthetic improvement of nasolabial folds [279] and Etched-In Fine Facial Lines [280]. Augmentation with lidocaine, epinephrine.
1. Stylage S cross-linked HA 2. Stylage Hydromax 3. Stylage L, Stylage XL **4. Stylage Special Lips**	Vivacy Labs, France	(i) Cross-linked HA inter penetrating network gel+ lidocaine + Mannitol; for the correction of superficial lines and wrinkles [281]. (ii) transdermal injection/rehydration of dehydrated skin [282]. (iii) treatment superficial wrinkles, crow's feet, frown lines, fine cheek perioral wrinkles, oral commissures, dark eye circles [282]. (iv) dermal injection of very deep wrinkles/naso-labial folds (v) dermal/subcutaneous deep injection to treat facial volume defects, restore facial contours, augment cheekbone area [282]. (iv) superficial/mid dermal injection, augment lip volume
Surgiderm facial fillers	Allergan	HMW cross-linked HA hydrogel dermal filler.

Surgiderm® 24XP, Surgiderm 30XP.	Irvine, CA, USA	Surgiderm 24XP used to treat medium to deep lines/wrinkles Surgiderm 30XP treats mid-deep dermis [242, 283].

4.4 The efficacy of high or low molecular weight HA in OA therapeutics.

The size of HA in the external environment may influence the synthesis of HA by resident synovial fibroblasts in the knee joint [284-286] with HA sized 0.5 x 10^6 Da stimulating endogenous HA synthesis but not higher Mw HA [287]. HAs within the molecular weight range of 0.5 -1.0 x 10^6 Da partially restore rheological properties of synovial fluids and synovial fibroblast metabolism in animal models [284]. High molecular weight HA of 2 x 10^6 Da inhibits arachidonic acid release by synoviocytes in-vitro but lower molecular weight forms of HA do not. High molecular weight HA displays anti-inflammatory properties which coupled with its inhibition of arachidonic acid release contribute to a beneficial clinical and pain relief OA treatment profile [288] and it provides greater beneficial effects on PG synthesis and in the maintenance of visco-elastic joint lubrication [289, 290]. A comprehensive review [291] of MEDLINE, EMBASE, and PUBMED databases uncovered 2,782 articles on HAs used in OA treatment. Of these, six major categories were identified to describe the beneficial effects afforded by treatment with high molecular weight HA preparations. These were (i) chondroprotection [135, 241, 285, 289, 292, 293, 294, 295-297, 298-300] (ii) promotion of PG/GAG biosynthesis [287, 295, 297, 301, 302, 303], (iii) anti-inflammatory effects [285, 290, 293, 295, 303, 304], (iv) mechanical effects [296, 299, 302, 305], (v) effects on subchondral bone [294, 306]and (vi) analgesic properties [300, 307].

The development of high molecular weight HAs such as Hylan G-F and Durolane represent significant technological advances in terms of their in-vitro rheological properties which translate in-vivo into improved visco-elastic, chondroprotective weight bearing properties and pain alleviation in the treatment of OA. These forms of HA also have longer residence times in the knee-joint due to their resistance to de-polymerisation by free radicals

during the inflammatory conditions which occur in the OA joint thus they are active for prolonged periods protecting the cartilage surface from further damage. Therapeutic visco-supplementation procedures developed for Durolane and the Hylans use single injections, a significant improvement over multiple injection protocols with associated adverse effects at injection sites.

4.5 Augmented Visco-supplement HA formulations for the treatment of OA.

HA has been used in combination with steroids, NSAIDs, anti-oxidants, DMOADs, anaesthetics, and GAG additives in order to improve the clinical performance of these formulations in the treatment of OA. These are summarized in Table 6.

Table 6. Augmented HA Viscosupplement Formulations used for the Treatment of OA

HA	Mw (kDa)	Additive	Property	Ref
HA solution	500-730	Carprofen	NSAID	[308]
		Triamcinolone acetonide	Cortocosteroid	[309]
		Dexamethasone	Cortocosteroid	
		Prednisolone	Cortocosteroid	
HA hydrogel	n/a	Dexamethasone	Cortocosteroid	[310]
Adant (Suprahyal)	500-1000	Tenoxicam	NSAID	[311]
Variofill (Adoderm, Langenfeld, Gmbh)	HMWCR	Diclofenac	NSAID	[312]
		Sodium clodronate	DMOAD	[313]
		Triamcinolone acetonide	Cortocosteroid	
		Ropivacaine HCl	Anaesthetic	
Hydros, Hydros-TA	HMW	Triamcinolone acetonide	Cortocosteroid	[314]
Cingal	1900	Triamcinolone acetonide	Cortocosteroid	[315]
Hylan GF-20	HMWCR	Triamcinolone acetonide	Cortocosteroid	[316]
HA solution	800	Mannitol	Anti-oxidant	[317]
Go-on matrix	n/a	Sorbitol	Anti-oxidant	[261]
Go-on	800	Mannitol	Anti-oxidant	[318]
Hanox-M	1000	Mannitol	Anti-oxidant	[319]
Hanox-M-XL	n/a	Mannitol	Anti-oxidant	[318]
Hyal G-F	1000	Mannitol	Anti-oxidant	[298]
Synolis V-A	2000	Sorbitol	Anti-oxidant	
Ostenil Plus	1500	Mannitol	Anti-oxidant	[320]
Happyvix	1500	Mannitol	Anti-oxidant	[320]
Happycross	1500	Mannitol	Anti-oxidant	[320]
Synolis V-A	2000	Sorbitol	Anti-oxidant	[320]
HA 4AR conjugate	2200	4 aminoresorcinol	Anti-oxidant	[321]
Ostenil	1000-2000	L-glutathione	Anti-oxidant	[322]
Arthrum HCS	2800	CS	GAG	[320]
Surgical Syonium	2800	CS	GAG	[320]
HA-sCT conjugate	200	Salmon calcitonin	DMOAD	[323]
HA ADAMTS inhibitor	n/a	ADAMTS inhibitor	DMOAD	[324]
HA hydrogel	60-120	Doxycycline	DMOAD	[296]
Hanox	1500	Cortivazol	Cortocosteroid	[325]
		Triamcinolone hexacetonide	Cortocosteroid	
Hanox M-XL	n/a	Mannitol + lidocaine clorhydrate	Anti-oxidant + local anaesthetic	

n/a not available, HMW , high molecular weight; HMWCR, high molecular weight cross-linked.

4.6 Versatility of conjugated HA as a delivery vehicle for therapeutic compounds.

HA is an extremely versatile carrier molecule which has been conjugated with drugs and bioactive compounds in hydrogel, micelle, nano-particle and liposome formulations for drug delivery with the amphiphilic properties of the HA adding to the delivery process. The targeting of tumour cells has been intensely investigated [326, 327, 328] and some particularly innovative HA targeting systems have been developed [329] and improved methods of delivery of compounds to previously impenetrable deep target sites deep in tumor masses [330]. The ability of HA to target CD44 overexpressing tumor cells is a useful trait for the specific delivery of therapeutic compounds [328, 331] and has also been exploited in some novel imaging modalities for tumor cells [332]. Such approaches can also be utilized to target cells other than tumor cells, a thermo-responsive HA nanogel drug delivery system which targets macrophages has been developed [333]. The versatile properties of HA as a delivery vehicle for specific cell targeting and improved cellular uptake of compounds represents another facet of the biology of HA separate from its roles as a visco-supplement. The examples outlined in Tables 7 and 8 clearly demonstrate the versatility of HA as a delivery vehicle. A number of glyco-polymers with appropriate rheological properties and low cytotoxity have also been evaluated with a view to application in visco-supplementation procedures [245, 334].

Table 7 Conjugation of HA with Bioactive Compounds as a Therapeutic Delivery Vehicle

Attached compound	Conjugate form	Application	Ref
Methotrexate	Coated magnetic polydopamine nanoparticles	Multimodal Imaging-Guided Multistage Targeted Chemo-Photothermal Therapy	[335]
	Optimized HA-methotrexate conjugates	OA therapy	[336]
Steroids	Steroid grafted on to HA	Antioxidant delivery	[337]
EGF	HA-EGF conjugate	Chronic wound healing	[338]
		Skin wound healing and regeneration	[339]
Anti-tumor necrosis factor-α	antitumor necrosis factor-α HA conjugate	early healing effects in a rat burn model	[340]
Epigallocatechin-3-O-Gallate	HA-Epigallocatechin-3-O-Gallate Conjugates injectable hydrogel	Free Radical Scavenging	[341]
Sonic hedgehog	Sonic Hedgehog-HA conjugates	Wound healing	[342]
	Multivalent Sonic Hedgehog conjugate	Diabetic Wound Healing	[343]
Catechin	hyaluronic acid-green tea catechin	Targeted intracellular protein	[344]

	nanogels	delivery	
Quercetin	HA- quercetin conjugated micelles	A tumor cell-targeted prodrug	[345]
Opiods	HA-opioid conjugate	Controlled delivery treatment for pain alleviation applications	[346]

Table 8. The versatility of HA delivery systems for anti-cancer drugs

Drug	Conjugate form	Application	Ref
Adriamycin	Adriamycin pro-drug HA micelles	targeted drug delivery with enhanced antitumor efficacy	[347]
Tocopherol succinate	Redox-responsive disulphide stabilized HA -tocopherol succinate micelles	Melanoma	[348]
Mertansine Toxin	HA-Shelled Disulfide-Cross-Linked Nano polymersomes	Ultrahigh-Efficiency Reactive Encapsulation CD44-Targeted Delivery	[349]
curcumin and alendronate	Multifunctional redox-responsive and CD44 receptor targeting polymer-drug nanomedicine	targeted drug delivery with enhanced antitumor efficacy	[350]
Iridium(III) Anticancer Drugs	Reduction- and pH-Sensitive Hyaluronan Nanoparticles	targeted drug delivery with enhanced antitumor efficacy	[351]
Nimesulide	Nimesulide-HA conjugate	Treatment of CD44-overexpressing HT-29 colorectal cancer	[352]
5-fluorouracil	Thermosensitive chitosan hydrogel containing 5-fluorouracil nanoparticles	Targeted controlled delivery of 5-fluorouracil Transcorneal tumours	[353]
Gold nanoclustered HA	Gold-Nanoclustered HA Nano-Assemblies	Photothermally Maneuvered Photodynamic Tumor Ablation	[354]
HA oligosaccharide Ca Phosphate	hybrid HA oligosaccharide-Ca phosphate nano crystals (Chrysalis)	Dual pH/redox responsive and CD44 receptor targeting	[355]
Death Receptor-5 Antibody Conjugate	HA conjugate	Targeted Treatment of Liver Metastasis	[356]
Lanthanum HA-Pt	Chemotherapeutic HA-Pt	Chemotherapeutic Agent HA-Pt to Track In Vivo Distribution of HA-Pt	[357]
Silybin	HA-glycyrrhetinic acid micelles	Liver targeted hepato protection	[358]
trastuzumab.	HA-tyramine sustained release hydrogels	HA-tyramine sustained release hydrogels for trastuzumab.	[359]
Doxorubicin	Reversibly crosslinked hyaluronic acid nanoparticles	doxorubicin delivery to drug resistant CD44+ human breast tumors	[360]
	multiwalled HA-carbon nanotubes	targeted intracellular delivery of doxorubicin into cancer cells	[361]
	GE11 peptide modified reduction-responsive HA nanoparticles	High efficacy doxorubicin for breast carcinoma	[362]
Doxorubicin-glycyrrhetenic acid	Doxorubicin self-assembled HA nano-particles conjugated with glycyrrhetinic acid	targeted drug delivery with enhanced antitumor efficacy	[363]
camptothecin-doxorubicin	Combinatorial HA conjugate	Synergistic camptothecin-doxorubicin antitumor dual HA conjugates	[364]
Doxorubicin-cisplatin	HA Doxorubicin-cisplatin conjugates	Cellular uptake and internalization of Doxorubicin-cisplatin	[365]
gemcitabine and doxorubicin	sequential delivery of gemcitabine/doxorubicin	treatment of triple negative breast cancer cells	[366]
Docetaxel	Docetaxel-HA conjugate	CD44-targeted docetaxel conjugate for cancer cells and cancer stem-cells	[367]
Camptothecin	bifunctional HA camptothecin prodrug	targeted drug delivery with enhanced antitumor efficacy	[368]
Gemcitabine	HA-coated liposomes	active targeting of gemcitabine to tumor cells	[326]
	redox sensitive vitamin E succinate paclitaxel-HA nanoparticles	targeted drug delivery with enhanced antitumor efficacy	[369]
	paclitaxel C-6 hexanediamine-modified-HA-	targeted drug delivery system	[370]
	HA oligomer-HPMA paclitaxel copolymer	HA oligomer-HPMA copolymer conjugated paclitaxel targeted to	[371]

		CD44-overexpressing ovarian tumors	
Paclitaxel	amphiphilic HA-deoxycholic acid conjugated paclitaxel	Micellar targeted intracellular delivery of paclitaxel	[372]
	HA-paclitaxel conjugate	targeting of human squamous cell carcinomas of the head and neck	[373]
	HA- paclitaxel conjugated micelles	Intracellular antitumor targeting	[374]
	redox-responsive polymeric paclitaxel HA conjugate	Intracellular antitumor drug delivery	
	HA nano particle directed cytosolic paclitaxel prodrug delivery	HA-paclitaxel prodrugs for direct cytosolic delivery and enhanced antitumor activity	[375] [376]
	multi-functional tLyP-1-hyaluronic acid-paclitaxel conjugate	Broad anti-cancer treatment	[377]
	paclitaxel-glycyrrhetinic acid-graft-HA conjugate synergistic targeting	Improved anti-tumor activity and safety delivery of paclitaxel	[378]
siRNA	hydrophobized HA-spermine conjugates	tumor-targeted delivery for efficient receptor-mediated siRNA delivery	[379]
Cisplatin	HA-cisplatin conjugate	Intralymphatic chemotherapy	[380]
	HA-Cisplatin Nanoparticles	Lung Cancer	[381]
	Fibrin gels loaded with cisplatin and cisplatin-hyaluronate complexes	subcutaneous human melanoma treatment system	[382]

5. Therapeutic Properties of Platelet Rich Plasma

The reparative properties of platelet rich plasma (PRP) are controversial and based on its ability to provide supraphysiologic levels of essential growth factors which act as a regenerative stimulus promoting reparative processes in tissues which have an intrinsically low healing capacity [383] The alpha-granules of platelets contain platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), platelet factor interleukin-1 (IL-1), platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF) all of which have the ability to promote tissue repair [384].

PDGF stimulates blood vessel formation along with PDAF and VEGF from existing vessel networks and thus contributes to tissue repair processes. PDGF also stimulates cellular proliferation and migration of fibroblasts, osteoblasts, tenocytes, vascular smooth muscle cells. Platelet-derived IL-1 stimulates cytokine production of vascular smooth muscle cells, indicating that it may regulate cytokine networks during tissue repair processes [385]. TGF-β is a multifunctional cytokine of the TGF-β superfamily with the ability to promote synthesis of

major ECM components such as PGs and collagen by many cell types thus contributing to tissue repair and regeneration. Overexpression of TGF-β however can also lead to fibrosis [386] and tumour development [387], and it has crucial roles to play in the regulation of stem and T-cell differentiation [388]. PRP has anti-inflammatory, chondroprotective and potential regenerative properties stimulated when TGF-β, PDGF, EGF, VEGF, FGF, and IGF are released upon de-granulation. These growth factors stimulate cartilage regeneration [389] and inhibit the actions of inflammatory mediators such as IL-1β [390]. PRG4 also inhibits adhesion of inflammatory cells to the articular surface reducing cartilage inflammation that can potentially lead to de-polymerisation of HA and loss of its lubricative properties and further degradative changes in the articular cartilage.

Pilot studies on the clinical efficacy of intra-articular PRP in the treatment of knee OA demonstrated clinical improvement in terms of self-reported pain and functional capacity with little or no adverse effects [391]. A systematic review, which included data from six randomised controlled trials comparing the effectiveness of PRP with other intra-articular injections, exercise or analgesia for a minimum of 6 months PRP produced statistically significant improvements in WOMAC scores for up to 12 months [392, 393]. Adverse events with PRP treatment were not significantly increased in comparison with other knee OA treatments consistent with earlier studies using PRP as an intervention in knee OA [393-395]. Studies with intra-articular HA injections with PRP have indicated that younger patients with mild OA benefitted more than older patients with established OA [396]. The PRP formulation PRGF-Endoret has been shown to provide superior pain relief than HA [397]. Randomized controlled clinical trials in 2015 and 2017 however failed to show that PRP gave superior clinical benefit than HA in the treatment of OA [398]. AAOS guidelines do not currently recommend for or against the use of PRP for knee OA. However a randomized controlled

clinical trial conducted in 2016 showed HA and PRP-HA injections had promise as a therapeutic combination for the treatment of knee OA [399].

5.1 PRP as a therapeutic treatment for mild to moderate knee OA: the added benefit of combined HA-PRP treatment.

While PRP and HA are both used as individual therapeutic treatments for mild to moderate OA, when used in combination**,** a synergistic effect has been reported with HA inducing the release of growth factors from PRP [400]. These promote anabolic responses that improve cartilage regeneration [401] and joint function [402]. Inclusion of a cellular matrix along with PRP-HA intra-articular injections is reported to further improve long term beneficial effects in over 50% of patients avoiding surgery in 80% of cases at 4 year follow up [403]. Randomised controlled clinical trials comparing, PRP, HA and PRP+HA for the treatment of mild-moderate OA confirmed these beneficial aspects of using the combined treatment [399, 404] although PRP did not offer superior visco-supplementation to HA [394]. A Meta-analysis of randomized controlled trial data confirmed the efficacy and safety of PRP injections for the treatment of knee OA [405]. American College of Rheumatology (ACR) and Osteoarthritis Research Society International (OARSI) guidelines on the use of PRP and HA intra-articular injections for the treatment of knee OA (Kjellgren-Lawrence grade II-III) state that accurate phenotyping and selection of patients should be mandatory in future randomized controlled trials [26].

As already stated, the therapeutic properties of PRP are based on the presence of PDGF, TGF-β, VEGF, IGF-I, PF4, EGF, HGF and SDF-1α stored in the platelet a-granules [406]. The potential for variable results in therapeutic applications using such a complex biological mixture is to be expected, which may explain some of the inconsistent results reported for PRP treatment. The standardization of procedures for the use of PRP and

selection criteria for subjects who will maximally benefit from the procedure will hopefully minimize this biological spread. Although PRP is reported to provide pain relief and improved joint function its mechanism of action is appears not to involve effects due to enhancement of MSC-mediated hyaline cartilage formation. PRP added to infrapatellar fat pad-adipose stem cells and bone marrow MSCs cultured in high density pellet cultures did not enhance cellular proliferation or matrix production [407].

6. The promise of MSCs for the treatment of OA

Intra-articular MSCs and chondroprogenitor cells have yielded promising results in cartilage repair in animal OA models and in preclinical studies on the treatment of knee OA. Chondroprogenitor cells transfected to produce elevated TGF-β levels are currently under phase III evaluation in clinical trials for the treatment of grade II and III Kjellgren and Lawrence OA patients.

6.1 Animal models of OA which demonstrate the utility of MSCs.

Repair and regeneration of the knee joint meniscal fibrocartilages of the knee has been examined by intra-articular injection of MSCs from adipose tissue, bone marrow, synovium, or meniscus alone or in combination with implantable or injectable scaffolds [408]. Fibrochondrocytes, chondrocytes, and transfected myoblasts [409] seeded into various combinations of bioscaffolds have also been examined for their abilities to carry out meniscal repair and regeneration [408]. In-vitro and in-vivo preclinical and laboratory based studies using allogeneic cells for cartilage repair have been just as successful as studies employing autologous cells [410, 411]. Implantation or injection of cell-seeded scaffolds has increased tissue regeneration and led to better structural organization of the repair tissue compared to the use of a scaffold alone. Intra-articular administration of MSCs in a caprine model of OA resulted in a pronounced regeneration of the medial meniscus however degeneration of the articular cartilage, osteophytic remodeling, and subchondral sclerosis were also reduced with MSC

treatment compared to vehicle alone [412]. Intra-articular injections of MSCs in sheep OA models retards the progression of OA and promotes repair of 60mm full thickness cartilage defects [413] and stimulates cartilage regeneration in a sheep OA model induced by complete resection of the anterior cruciate ligament and medial meniscus [414]. MSCs administered with Hyaff-11, (Hyaff-11® is a 3D fibrous membranous scaffold material composed of a benzyl ester of HA [415]) in a sheep meniscectomy OA model resulted in a reduction of inflammation in cartilage, meniscus , and synovium and produced a reversal of fibrotic and hypertrophic tissue changes which normally occur in this model without MSC administration. Cartilage degeneration was also prevented and regeneration of the excised meniscus was prominent [416]. The knee joint menisci protect other knee joint tissues from degenerative changes thus repair of the menisci is important for the global health and function of the knee-joint[130, 417]. .

MSCs are present in multiple niches in the knee-joint in subchondral bone, articular cartilage, meniscus, synovial fluid, synovium and the adipose fat pad. Developmental studies demonstrate that MSCs have chondrogenic roles during embryogenesis and diarthrodial joint development [250], and also suggest that synovium-derived MSCs have migratory properties homing to sites of cartilage damage where they attempt to undertake cartilage repair in adulthood [418]. Injection of MSCs with HA in a rabbit OA model promoted the migration of MSCs to cartilage. It has been proposed that priming of progenitor cells with HA modulates cell homing and favours their attachment to and integration with articular cartilage, a feature conducive to cartilage repair processes in the treatment of OA [419]. A sheep OA model has also demonstrated that intra-articular injection of allogeneic adipose derived MSCs combined with HA efficiently blocked OA progression and promoted cartilage regeneration [420]. Furthermore, intra-articular injection of allogeneic MSCs with HA in an in-vivo anterior cruciate ligament transection rabbit model of OA, the joint surface showed less

cartilage loss and surface abrasion after MSC injection compared to tissues receiving HA injection alone thus the MSCs apparently reduced the progression and severity of OA [421].

Chondrogenic priming of bone marrow stromal MSCs in the laboratory is reported to improve their anabolic ECM gene expression profiles and the development of their ability to undertake cartilaginous repair within cartilage defects in a full thickness cartilage defect model in sheep [422]. Further studies have shown that chondrogenic pre-conditioning of MSCs enhanced cartilage regeneration through epigenetic methylation modifications of *Nanog* and *Oct4* in committed chondrogenic cell populations suitable for the treatment of OA cartilage lesions [423]. FGF-2 and FGF-18 have also been used to pre-condition bone marrow derived stromal MSCs promoting cellular proliferation for expansion of MSC numbers and guiding them to appropriate chondrogenic repair phenotypes in-vitro [424, 425]. This suggests that this step could improve their cartilage repair capabilities if FGF-2/FGF-18 and MSCs were co-administered intra-articularly. This possibility has not been considered as a potential mechanism whereby MSC cell proliferation could be enhanced in-situ and the cartilage repair properties of undifferentiated stem cells could be guided to a reparative phenotype. If MSC differentiation could be manipulated as suggested by the findings of in-vitro experiments conducted with these components [425] this would be expected to be beneficial in the treatment of OA.

6.2 Human pre-clinical studies on MSCs.

Adult MSC-based tissue engineering is a promising technology for the development of a transplantable cartilage replacement to improve joint function [8]. Delivery of MSCs to the OA affected knee could be undertaken by intra-articular injection or by graft of an engineered construct seeded with MSCs. Thus a 3D construct with mechanical properties appropriate for

the weight-bearing function of the joint can be designed for specific applications to support the use of MSCs for regenerative procedures.

An innovative strategy has been developed using hyperbranched multi-acrylated poly(ethylene glycol) macromers (HP-PEGs) and thiolated hyaluronic acid (HA-SH) as an injectable stem cell delivery and retention platform to deliver encapsulated adipose-derived stem cells (ADSCs) to treat diabetic wounds in a diabetic murine animal model. This strategy yielded enhanced wound healing of diabetic wounds which have a notoriously poor repair capability similar to OA lesions in articular cartilage. This polymer has a proven ability to maintain stem cell stemness and viability and ability to secrete trophic repair factors conducive to tissue regeneration. Thus ADSCs maintained their regenerative ability when delivered into wound sites using this hydrogel. Such an approach resulted in remarkable enhancement of diabetic wound healing, including inhibition of inflammation, enhanced angiogenesis and re-epithelialization. This approach represents a significant advance in the healing of one of the most serious problematic clinical wounds and could be adapted to an orthobiological visco-supplementation format for the treatment of OA[426].

Another innovative approach is the use of resident MSCs harvested from OA knee arthroscopic flushing fluids recovered from initial clinical knee examinations. These cells were incorporated into HP-PEG/HA-SH hydrogels and delivered into a rat full thickness cartilage defect model and induced repair after an 8 week repair period. This study demonstrated the repair capability of knee-joint MSCs harvested in such a manner and the effectiveness of the HP-PEG/HA-SH hydrogel delivery system for cartilage repair warranting further studies in an orthobiological cartilage repair format[427].

A further approach for the delivery of therapeutic cells for cartilage repair is the use of bioadhesives to deliver these cells to the defect site. Pullulan has been used to successfully attach MSCs to fibrillated cartilage regions dramatically improving their retention in eroded articular cartilage lesions where the MSCs promote cartilage repair. Pullulan is a biocompatible and effective cytoadhesive material for tissue engraftment of MSCs. Prolonged exposure to pullulan had no negative impact on the phenotype, viability and differentiation potential of the cells [428] with MSCs maintaining a stable phenotype in terms of metabolic activity, proliferation and differentiation. Further bioadhesives which have strong wet-adhesive properties have been developed to avoid suturing in abdominal and heart surgery should also be of application in this area of bioadhesive mediated cell delivery for cartilage repair.

6.3 Manipulation of the MSC phenotype using bioscaffolds.

An interesting approach inspired by the cell directive capability of GAGs of PGs has been developed using acellular implants that can become colonized by resident stem cell populations in tissues [429]. Thus bioactive signals and scaffolds can actively recruit endogenous stem cells which potentially have the capacity to repair OA lesions. As proof of principle the authors showed that endogenous stem cells actively homed to defect sites and could be used to regenerate the surface cartilage in a rabbit model [430]. Attempts have been made to model the chemotactic responses of different joint stem cell populations in order to understand how they can be induced to undertake chondrogenesis and cartilage repair [431]. HA has cell interactive properties and is known to promote cell migration. This has led to the development of HA binding scaffolds which may provide an environment conducive to cartilage repair processes [432]. Nano-fibres have also been developed which stimulate chondrogenesis and cartilage repair [433]. Furthermore, CS- bioscaffolds have been shown to direct stem cell differentiation in-situ and is of potential exploitation in improved cartilage

repair strategies [47]. Transplantation of MSCs seeded in such biomatrices represents a promising strategy [434] given the high proliferative capacity of MSCs and their potential to differentiate into cartilage-producing cells [435] however further work is required before this will become a modality suitable for clinical evaluation [436]. Besides articular chondrocytes, cells from the synovial membrane, fat pad, and meniscus all have variable abilities to form cartilage [437]. However in order to obtain sufficient cell numbers for preclinical evaluation these cells need expansion in-vitro under conditions which direct the cells to a chondrogenic phenotype. A co-culture approach of MSCs with cartilaginous cells may represent a simpler approach [410, 438] for the expansion of chondrogenic cell numbers. A phase I/II clinical study has shown that intra-articular injection of expanded autologous bone marrow MSCs is a safe procedure for the treatment of moderate to severe knee OA [439] research is on-going to perfect this technique [440].

6.4 Human clinical trials with intra-articular MSCs and chondroprogenitor cells.

Clinical trials using intra-synovial injection of MSCs for the treatment of advanced OA of the knee were started in 2013 [441] and are ongoing in 10 European Centres using patient-derived adipose stem cells (https://cordis.europa.eu/result/rcn/193066_en.html European Union 2018, adipoa2-z-fold-brochure-jpg.jpg). In 2017, INVOSSA™ (Kolon TissueGene) TGF-β transfected cells were approved for the treatment of knee OA in South Korea [442]. This allogeneic cell therapy utilised chondrocytes transduced with a retrovirus overexpressing TGF-β which is irradiated to prevent any risks of insertional mutagenesis. These genetically modified cells expressed TGF-β at therapeutic levels to promote cartilage repair. On Nov 21st 2018, an FDA approved Phase III placebo controlled double blind trial was initiated in the USA using INVOSSA™(TG-C) allogeneic primary chondrocytes transduced to express TGF-β1 for the treatment of Kjellgren and Lawrence grade II and III

OA [https://www.prnewswire.com/news-releases/kolon-tissuegene-doses-first-patient-in-us-phase-iii-clinical-trial-300754257.html]. Phase II trials with this product already conducted yielded encouraging results in terms of pain relief and improvements in clinical indices of knee-joint function [443]. This phase III trial using INVOSSA™(TG-C) has 1020 patients enrolled and will be conducted in 60 clinical centres throughout the USA. Although expert formulated guidelines indicate that visco-supplementation is only appropriate for cases of mild OA, [26, 444] it is conceivable that stem cells or engineered chondrocytes could be delivered in a visco-supplement to treat more advanced stages of OA - localising the administered cells at the articular surface in an effort to promote focal cartilage regeneration and recapitulating the early stages of rudiment and diarthrodial cartilage development [43, 44, 250] (**Figure 8**). Such a proposal has considerable merit and is worthy of future investigation. Furthermore, a recent review of the use of GAG copolymer hydrogels and bioscaffolds has demonstrated their ability to direct stem cell differentiation and promote anabolic repair processes [47]. Such processes could potentially be incorporated into a visco-supplement preparation to promote cartilage re-surfacing and chondrogenic repair. Several novel options therefore exist in the development of new generation visco-supplement therapeutics which certainly are worthy of further examination.

7. Future Research

Pre-clinical and animal model based studies have demonstrated that neo-aggrecan has useful water imbibing properties and is more resistant to proteolytic degradation than native aggrecan. Recombinant domain-I and domain V of perlecan also have useful properties in BMP-2 and FGF-2 delivery applicable to cartilage and bone regeneration and anti-angiogenic properties of potential application in neurovascular therapeutic applications respectively. Continued development of these neo-PGs will bring them a further step closer to clinical application. Neo-lubricin also has impressive properties as a visco-supplement and has

proposed in this review for prospective inclusion as a component to augment HA visco-supplement formulations to improve their performance in an orthobiological approach to the treatment of knee OA. PRP and MSCs also have important biological properties which warrant their inclusion in such multifunctional orthobiological formulations. It will be interesting in future studies to observe whether protocols are developed using such a therapeutic approach.

7.1 The critical importance of patient selection in visco-supplementation procedures.

In introductory comments to this review we indicated the importance of appropriate patient selection to select individuals affected by mild to moderate OA who would optimally benefit from these procedures. This has been re-iterated by guidelines on the use of PRP in the treatment of OA released by the ACR and OARSI mandated that future clinical trials with PRP should follow strict patient selection guidelines with the procedure only being offered to Kjellgren and Lawrence grade II and III OA patients since these were the patients which would optimally benefit from this procedure [445]. These comments were made in response to inconsistent reports of the efficacy of PRP in the treatment of knee OA possibly due to inappropriate patient selection. Similarly, despite numerous studies clearly showing the beneficial effects of HA injections for pain reduction and recovery of joint function reports still persisted questioning the efficacy and utility of intra-articular injection of HA visco-supplementation procedures possibly fuelled by inconsistent findings from clinical trials not conducted in a standard format [446]. Furthermore, in 2013 AAOS clinical practice guidelines for the treatment of knee OA did not recommend using HA for patients with symptomatic knee OA and these guidelines also stated that there was inconclusive evidence to recommend for or against the use of intra-articular corticosteroids to treat knee OA [447]. Robust debate continued in this area fuelled by the inconsistent findings of some studies until in 2018 EUROVISC issued guidelines on how clinical trials should be conducted on HA

viscosupplementation procedures [448]. Furthermore, in July 2018, a key opinion leader panel discussion by ten leading expert physicians made two major recommendations. 1. "Visco-supplementation with the use of hyaluronic acid injection is a treatment option for knee OA and can provide lubrication and elastic shock absorption, leading to potential pain relief, improved function, and reduced stiffness". 2.The panel also concluded that "visco-supplementation with HA injections represented a viable, cost-effective, and safe alternative for the treatment of knee OA" [449].

7.2 Orthobiologics: an emerging, promising, therapeutic modality

As we have outlined earlier, HA, has established outstanding credentials as an agent for the improvement of articular lubrication and the maintenance of the visco-elastic properties of synovial fluid which provides weight bearing and pain alleviation in OA. A number of pharmacologic compounds developed over the last five decades have also usefully treated some degenerative features of OA articular cartilage. These compounds include corticosteroids, NSAIDs, DMAODs and anti-oxidants. Each of these have intrinsic beneficial properties in their own right in the treatment of OA but in isolation they are incapable of alleviating all OA symptomatology. A recent approach has been to use some of these compounds to augment HA viscosupplements in order to improve their therapeutic performance in the treatment of OA. Addition of these compounds is generally acknowledged to improve the therapeutic properties of HA viscosupplements compared to HA alone. PRP has also been shown to act synergistically with HA in viscosupplements improving the efficacy of HA formulations in the treatment of OA. MSCs have also yielded promising results in animal models and in preclinical studies in the treatment of knee OA and are currently under evaluation in clinical trials for the treatment of grade II and III Kjellgren and Lawrence OA patients. mLUB15, a mimetic neo-lubricin also has useful properties in

cartilage lubrication and surface protection and could usefully augment HA viscosupplement formulations to improve their therapeutic performance.

Recent findings in clinical studies on the therapeutic use of visco-supplementation with HA for the treatment of OA have yielded promising but heterogenous findings of variable quality. With so many formulations of HA available commercially for intra-articular injection, variable weight ranges, different concentrations of HA formulations and non-uniform clinical trial formats this was a somewhat predictable outcome. Thus there is a need for more robust studies to determine the place of visco-supplementation in the management of knee OA but sufficient positive findings to warrant continuing such studies. To this end EUROVISC recently issued guidelines on how clinical trials should be conducted on the use of HA for visco-supplementation [448]. One option to improve the performance of visco-supplementation was to augment such visco-supplement formulations with growth factors, neoPG biomimetics and therapeutic cells to promote maintenance of the lubricative properties of articulating joints, control inflammation and provide an environment conducive to proliferation of resident cell populations for the replenishment of matrix components and repair of cartilage defects aided by the therapeutic cells provided in the augmented visco-supplement formulation. A comparison of the efficacy of intra-articular corticosteroids with HA for knee OA showed each were equally effective at pain relief over 1-4 weeks after injection while HA had a superior effect over 5-13 weeks [259]. Augmentation of triamcinolone in HA visco-supplements also improved WOMAC (The Western Ontario and McMaster Universities Osteoarthritis Index) and VAS (Visual analog scale for pain) scores after 1 week compared with HA alone [316]. Recent trials comparing triamcinolone with HA found HA to be more effective at pain reduction and it also increased range of joint motion [66]. Combined with the outstanding performance of high molecular weight HAs in the lubrication

of articular cartilage these added biological properties further establish HA as an important component of visco-supplement formulations.

7.3 The outstanding performance of high molecular weight HA as a visco-supplement.

Cross-linked high molecular weight HA preparations such as Hylan G-F 20 [450] and Durolane NASHAs [269] and similar HA products [451] with their higher molecular weights, improved visco-elastic properties, improved residence times and high injection concentrations which facilitate single injection protocols have clearly provided an improvement in the clinical treatment provided by visco-supplementation. A recent comparison of the efficacy of corticosteroids, visco-supplementation, PRPs, and MSCs for improved joint function has shown that each have positive attributes [452]. Corticosteroid injections are recommended as a first-line treatment for OA patients resistant to other non-surgical treatments such as NSAID medication, physical therapy, weight loss, and an acceptable amount of activity modification. However corticosteroid injections are not recommended for prolonged usage. Intra-articular injections of HA are recommended to replace the deficient levels of endogenous HA in OA knees and to allow the recovery of efficient joint articulation and associated pain-relief due to the improved weight-bearing provided by the visco-elastic properties of the injected HA. PRP has anti-inflammatory, chondroprotective and regenerative properties due to TGF-β, PDGF, EGF, VEGF, HGF, FGF and IGF which are released upon de-granulation when PRP is injected as a supplement in HA intra-articular injections. These growth factors stimulate cartilage regeneration [389] and inhibit the actions of inflammatory mediators such as IL-1β [390]. It may well be that a combined biotherapeutic approach such as that proposed in a multifunctional orthobiological may well provide an effective new approach to the treatment of OA. This option is certainly worthy of further evaluation in future research.

8. Conclusions

1) By understanding how the core proteins and GAG side chains of native PGs contribute to their functional properties in tissues it has been possible to identify critical features which need to be built in to neo-PG design to emulate these features.
2) While native PGs such as aggrecan rely on macro-aggregate formations to convey biophysical properties to tissues, it has been possible through innovative design to prepare more robust neo-aggrecans of smaller dimensions and with improved water retention and matrix stabilizing properties. These neo-aggrecans are less susceptible to proteolytic degradation in-situ thus their biological half-lives are increased relative to the native PGs making them amenable to many applications in repair medicine.
3) Retention of biopolymers within bio-scaffold frameworks, or in the squeeze-film during boundary lubrication, are also important considerations in particular applications and interactive peptides have been included in neo-PG design to convey these interactive retentive properties. The interactive properties of these molecules with other endogenous ECM components within the superficial regions of articular cartilage further support their retention at the tissue's surface.
4) Development of many combinations of GAG copolymers, GAG peptidoglycans, nano-delivery systems, bioactive visco-supplements testify to the diverse innovative applications possible.
5) Recombinant PGs have specific targeting properties which neo-PGs cannot match, thus they will also continue to be of application in specific repair strategies..
6) Viscosupplementation is a valuable procedure in the treatment of mild to moderate knee OA. Many strategies and formulations with HA have been developed in a procedure known as orthobiological visco-supplementation. These are multifunctional formulations which can also contain PRP, MSCs neo-PGs such as mLUB15 and growth factors/anti-oxidants.

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