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Reproducing the biomechanical environment of the chondrocyte for cartilage tissue engineering

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Abstract

It is well known that the biomechanical and tribological performance of articular cartilage is inextricably linked to its extracellular matrix structure and zonal heterogeneity. Furthermore, it is understood that the presence of native extracellular matrix components such as collagen II and aggrecan promote healthy homeostasis in the resident chondrocytes. What is less frequently discussed is how chondrocyte metabolism is related to the extracellular mechanical environment, at both the macro and micro scale. The chondrocyte is in immediate contact with the pericellular matrix of the chondron, which acts as a mechanocoupler, transmitting external applied loads from the ECM to the chondrocyte. Therefore, components of the pericellular matrix also play essential roles in chondrocyte mechanotransduction and metabolism. Recreating the biomechanical environment through tuning material properties of a scaffold and/or the use of external cyclic loading can induce biosynthetic responses in chondrocytes. Decellularised scaffolds which retain the native tissue macro and micro-structure also represent an effective means of recapitulating such an environment. The use of such techniques in tissue engineering applications can ensure the regeneration of skeletally mature articular cartilage with appropriate biomechanical and tribological properties to restore joint function. Despite the pivotal role in graft maturation and performance, biomechanical and tribological properties of such interventions is often underrepresented. This review outlines the role of biomechanics in relation to native cartilage performance and chondrocyte metabolism, and how application of this theory can enhance the future development and successful translation of biomechanically relevant tissue engineering interventions.

Impact statement

Physiological cartilage function is a key criterion in the success of a cartilage tissue engineering solution. The in-situ performance is dependent on the initial scaffold design as well as ECM deposition by endogenous or exogenous cells. Both biological and biomechanical stimuli serve as a key regulators of cartilage homeostasis and maturation of the resulting tissue engineered graft. An improved understanding of the influence of biomechanics on cellular function and consideration of the final biomechanical and tribological performance will help in the successful development and translation of tissue engineered grafts to restore natural joint function post-cartilage trauma or osteoarthritic degeneration, delaying the requirement for prosthetic intervention.

Introduction

Articular cartilage is a highly specialised connective tissue found lining the end of bones in synovial joints, where it provides a low friction and low wear surface for joint articulation, permitting tissue longevity over tens of millions of loading cycles over a lifetime (1). It also serves to withstand and redistribute the compressive, tensile and shear forces imposed during joint articulation (2). These functions of cartilage derive from its unique extracellular matrix (ECM) structure and composition. However, the tissue is susceptible to disruption through age-related degeneration and acute trauma, accompanied by poor healing due to the avascular nature of articular cartilage (3, 4). Inflammation mediates further cartilage degradation (5), beginning a positive feedback loop resulting in significant pain, immobility and emotional distress for patients who develop osteoarthritis (OA) (6).

Total knee arthroplasty in younger patients with active lifestyles can lead to high failure rates (7, 8), therefore restorative techniques such as mosaicplasty, osteochondral allo/autograft transfer, microfracture and autologous chondrocyte implantation (ACI) are more commonly used in first line treatment. Despite pioneering the use of cell therapy for cartilage repair (9-12), there are concerns surrounding the limited biomechanical function prior to regeneration with ACI, as well as cell leakage from the site of implantation (13) and fibrocartilage formation (14-16).

Cartilage tissue engineering has the potential to address these limitations and allows the possibility of tailoring the biological, biomechanical and tribological properties of a scaffold to provide a biocompatible environment that is most suitable for delivery with therapeutic cells or to support endogenous cell repopulation. However, the role of biomechanics in the development and performance of these interventions currently is underrepresented in the literature. Here we will outline the complex biomechanical and tribological requirements of articular cartilage and detail the importance of considering these in the design of scaffolds for cartilage repair. We propose that recapitulation of the chondrocyte micromechanical environment and cartilage biomechanical function will result in improved outcomes for patients undergoing ACI and future tissue engineering interventions.

Zonal structure and biomechanical function of articular cartilage

Articular cartilage is described as a biphasic material due to the presence of both fluid and solid extracellular matrix (ECM) phases (17). Interstitial fluid makes up approximately 80% (w/w) of the wet weight of the tissue. The solid ECM phase is represented most prevalently by collagen (10-20%) and proteoglycans (5-10%) (18). The tissue is anisotropic, and can be divided into three principal zones, the superficial, middle and deep zones (Figure 1). The heterogeneity is present in both density and organisation of the collagen fibres and proteoglycan aggregates, as well as in the fluid and cellular composition (Table 1).

This heterogeneic structure of cartilage is intrinsically linked to its function. In the superficial zone, the highly aligned collagen fibres provide a smooth, congruous surface for movement of the knee joint as well as a resistance to the shear and tensile stresses of articulation (19). The tensile stiffness of this zone has been measured to be 5-fold higher than that of the deep zone of cartilage (20). However, in the deep zone, the perpendicular fibre alignment provides a means of integration between the cartilage ECM and the underlying subchondral bone (20, 21). Both functions are a result of the ability of collagen to resist tensile forces in the direction of fibre alignment. This property is also evident in the middle zone, where the alignment and tensile resistance of collagen acts to provide shear-resistance, upon deformation in the direction of shear (4). The stiffness of collagen in tension is not matched in compression, a phenomenon referred to as tension-compression non-linearity (33).

Proteoglycans and glycosaminoglycans (GAGs) play an important role in the load-bearing capacity of articular cartilage. The density of proteoglycans varies throughout the zones (Table 1). Aggrecan is tightly packed between the collagen fibres, and the close proximity of the negatively charged GAG side chains creates a fixed charge density in the ECM (25, 34). In order to maintain electroneutrality, there is a cation influx which creates a swelling pressure known as Donnan osmotic pressure (26). This swelling pressure may have a contribution of 50-60% towards the total tissue stiffness (27, 28). On compression, the fixed charge density becomes greater, and hence the permeability decreases, and the osmotic pressure increases. This pressurised interstitial fluid can support up to 90% of the

compressive load (30), reducing the stress on the solid ECM matrix. Due to this contribution of the bulk and physicochemical properties of proteoglycans to the compressive stiffness of articular cartilage, their differential distribution leads to anisotropic compressive properties throughout the tissue. The compressive modulus is lowest in the superficial zone and increases with proteoglycan content into the deep zone of the tissue (25, 27).

Interstitial fluid support is also important in the low friction and low wear function of cartilage (2), being critical to the mechanism of biphasic lubrication (35). Upon loading the interstitial fluid is pressurised and bears most of the load, contributing to low friction at the articulating surfaces (23). Further lubrication mechanisms also exist, such as boundary and fluid film lubrication, which also enable low friction articulation (Reviewed in: (23, 36, 37)).

These biphasic and zone dependent mechanical properties are essential in healthy articular cartilage function and are important to recapitulate in cartilage tissue engineering intervention to restore native joint function.

Biomechanical microenvironment of the chondrocyte

The ECM components provide a scaffold for the resident chondrocyte cells. As described in Table 1, chondrocyte morphology and properties vary throughout articular cartilage, a result of the differential experience of mechanical force, which is accompanied by a zone dependent metabolic profile with higher GAG production from middle and deep zone chondrocytes (38). From a cartilage tissue engineering perspective, harnessing and maximising the chondrocytes healthy metabolic activity is an imperative. Such anabolic and catabolic activities of chondrocytes are regulated by both the biological and biomechanical stimuli experienced at a macro (tissue) and micro (chondron) level.

As well as the depth dependent ECM structure, there are also concentric structural differences in the tissue structure surrounding the cell. The interterritorial region is the largest of three matrix regions and describes the region often referred to as the ECM and makes up the greatest proportion of the tissue structure (39). Closer to the cell, there is a zone of fine collagen fibrils which form a mesh surrounding the cell called the territorial

matrix. It is this layer which is hypothesised to contribute towards the cells ability to withstand deformation (40). The innermost zone is the PCM which is a 2 – 4 μM zone composed of biomolecules such as; collagen VI, collagen IX, perlecan, decorin, biglycan, aggrecan and hyaluronic acid (HA) (41) (Figure 2). The PCM is hypothesised to function as a supportive niche for healthy chondrocyte growth, demonstrated by studies showing that isolated chondrons grow larger in pellet culture compared to chondrocytes alone (42).

As the immediate buffer between the chondrocyte and the ECM, the PCM is hypothesised to both protect and amplify loads experienced by the chondrocyte during loading cycles. This effect is zone dependent, with protection offered in the superficial zone and amplification deeper in the tissue (43). The chondrocyte has been well documented as a mechanosensitive cell (44, 45) and it is understood that interaction with the surrounding ECM influences chondrocyte cell signalling pathways and therefore cartilage homeostasis (Figure 3). The response of the cell to mechanical loads is dependent on the magnitude and frequency of the load, as well as the strain rate and nature (static or dynamic) of the applied load. Strain below 20%, and stress below 18 MPa in vivo are considered physiological (46). The PCM is thought to play a pivotal role in translating these macro-mechanical forces experienced on a tissue scale to the chondrocyte, resulting in changes to cell biosynthesis. This process is known as mechanotransduction and is an essential concept in cartilage homeostasis (47).

The chondrocyte is anchored to the PCM through surface integrins and collagen VI. Collagen VI is a PCM hallmark biomolecule and has also been shown to tether the chondrocyte to territorial and ECM molecules such as collagen II and hyaluronan via biglycans and decorin (48). This connection is hypothesised to facilitate mechanotransduction, and the presence of PCM components has been shown to promote chondrocyte responsiveness to static and cyclic loading (49).

The cell surface receptors present on the chondrocyte such as discoidin domain-containing receptor (DDR)-2 (50) and Cluster of Differentiation (CD)-44 (51) allow direct or indirect interaction with ECM molecules such as collagen II and hyaluronic acid respectively, facilitating a continuous connection with the extracellular environment.

Deformation of the tissue can be detected by cell primary cilia, which protrude from the chondrocyte into the PCM and act as principal mechanosensors (52). Transgenic mice studies have shown that without cilia, chondrocytes produce cartilage with significantly reduced mechanical properties (53). Cilia act as an intermediary between the intracellular golgi apparatus and the ECM (54), and under compression release adenosine triphosphate (ATP) allowing for the activation of ATP gated Ca^{2+} channels (55, 56). The Ca^{2+} influx activates the transcription of chondrogenic genes (53). Deformation is also experienced at a cellular level, which activates stretch activated cation channels and membrane hyperpolarisation. This mechanism has been associated with the activation of anti-inflammatory interleukin (IL)-4, and the subsequent inhibition of matrix metalloproteinase (MMP)-3 as well as increased aggrecan production (44, 57, 58).

Studies have also shown that the laminar and non-pulsatile shear stress applied by fluid flow can also activate chondrocyte signalling through increasing ATP and prostaglandin efflux. Upon interaction of these molecules with cell surface receptors, intracellular protein kinase A (PKA) cell signalling cascades result in the production of lubricin from the superficial zone (59). P38 mitogen-activating protein kinase (MAPK) is also activated through fluid flow shear, stimulating Cbp/p300-interacting transactivator (CITED)-2 mediated inhibition of MMP activity (60). Interstitial fluid pressure has also been shown to activate the mechanosensitive transient receptor potential vanilloid sub type 4 (TRPV4) Ca^{2+} channel (45). Activation of this channel with small molecule treatment in the absence of mechanical loading has been shown to increase aggrecan deposition (45).

The anabolic response of the chondrocyte is partially governed by the mechanical stimuli it receives from the ECM and PCM. In order to maximise the regenerative response of a tissue engineering intervention, consideration of the extracellular environment and how it will translate stimulation to the cell is an important design consideration.

Functional and metabolic consequences of cartilage damage

Thus far, we have provided an overview of the intricacies of articular cartilage structure and function, as well as the delicately balanced homeostatic condition. Maintenance of this balance favours cartilage longevity, however, age related changes or mechanical insult

can skew this balance towards inappropriate catabolism, beginning a vicious cycle of degradation and loss of function.

Upon mechanical insult, resident chondrocytes secrete interleukin-1-β (61). This triggers a cascade of pro-inflammatory events culminating in the upregulation of MMP expression and the subsequent degradation of damaged cartilage (62). At this point chondrocytes begin to express transforming growth factor (TGF)-β to stimulate both chondrogenesis and regeneration of the damaged joint (63). As articular cartilage is an avascular tissue, chondrocytes have a low turnover rate in terms of ECM secretion. Therefore, if the damage is greater than the chondrocytes natural capacity to regenerate, the damage will persist, triggering a vicious loop of damage, inflammation and degradation. Furthermore, the resident chondrocytes can undergo phenotypic switch towards a degradative phenotype, characterised by aberrant expression of catabolic enzymes such as MMP-13 (64). Studies have suggested that upon damage to cartilage, collagen II exposure is detected by DDR2 domains on the chondrocyte surface triggering an up regulation of Runx-2, which drives chondrocytes into a hypertrophic state, characterised by MMP-13 expression (65, 66).

Focal cartilage lesions disrupt the tissues integrity, and the size of the defect affects the outcome of this initial damage. There is a threshold known as a critical defect size, which dictates a defect size which is capable of resolution, above this, however, there are progressive degenerative changes. Focal cartilage defects have been shown to result in increased stress at the rim of the defect, resulting in hyper-physiological loads experienced by the cartilage and chondrocytes in this area (67). Such damage and/or degeneration is also causative in changes to the bulk mechanical environment such as a reduction of the compressive stiffness (68, 69), and both shear and elastic moduli (70, 71). Through osteoarthritic mechanisms, cartilage proteoglycans can become depleted, affecting the interstitial fluid pressurisation and biphasic lubrication mechanism (72).

There are also alterations to the micromechanical environment of cartilage, specifically in the PCM mechanical properties. Chondrons isolated from human OA cartilage exhibited 40% lower Young's moduli and a 2-3 times higher permeability compared to chondrons isolated from healthy samples (12, 47). These changes in bulk and micro-mechanical

properties increase the exposure of chondrocytes to hyper-physiological loads (73). Such loads are applied dynamically and acutely, as in an injury setting, and can cause resident cell death and the promotion of inflammation which triggers increased catabolic activities (74, 75).

There are several mechanisms by which these loads can trigger catabolic signalling pathways which are illustrated in Figure 4. Disruption to the cytoskeleton from mechanical trauma is detected by integrin receptors, activating MAPK signalling, and catabolic gene expression of MMPs and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Damaged collagen exposes intracellular domains to DDR2 receptors, activating MMP-13 expression (76). Hyperphysiological strain (> 50%) can also increase membrane tension activating the transport of Ca^{2+} ions, via Piezo channels into the cell, culminating in chondrocyte cell death through Caspase activity (77). Strains of 20% have also been shown to decrease primary cilium length, which activates Indian hedgehog (Ihh) mediated chondrocyte hypertrophy (78, 79).

Finally, loads of 20 MPa have been used in loading regimes as models of hyperphysiological loading. Such magnitudes of stress can stimulate intracellular reactive oxygen species (ROS) signalling, followed by a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) positive feedback loop and expression of hypoxia induced factor (HIF) -2α and MMPs (80). It is therefore of paramount importance that any intervention aiming to treat cartilage lesions, acts to restore biomechanical function at a bulk and micromechanical level to protect chondrocytes, breaking the cycle of catabolic cell signalling to restore a healthy cell phenotype.

It is clear that mechanotransduction is important for cartilage homeostasis and should be considered to deliver successful tissue engineering repair therapies.

Cartilage Tissue Engineering

Despite pioneering the field of cell therapy for tissue repair (81) and reports of long-term success in generating hyaline-like cartilage, ACI in its simplest form disregards many of the elements that contribute to the functional re-engineering of a specialised connective tissue such as articular cartilage. This often results in sub-optimal mechanical properties

(82), requiring long post-operative rehabilitation times (10-21 months) until the tissue is of suitable biomechanical strength (14).

Chondrocytes are very sensitive to changes in their microenvironment. Therefore the removal from this niche and expansion in 2-D tissue culture conditions can cause de-differentiation of chondrocytes (83), characterised by an increased collagen I expression which favours fibrocartilage repair, resulting in a repair tissue with inferior tribological and biomechanical properties (84).

Fortunately, de-differentiation is not only preventable but reversible, through the use of a 3-D scaffold it is possible to re-differentiate chondrocytes back to a chondrogenic phenotype (85). Cartilage tissue engineering offers the possibility of generating such 3-D scaffolds which can accurately represent the biomechanical and tribological behaviour of native articular cartilage.

Table 2 gives an overview of the range of tribological and biomechanical parameters that have been observed in native cartilage and provide a range of values to aim for in the fabrication of tissue engineered cartilage constructs. However, such properties are merely an example of bulk properties observed, and can vary greatly depending on species, location, zone of cartilage as well as the test set up. It is also important to emphasise that bulk biomechanical properties are only part of the solution to developing functional cartilage tissue engineering interventions. The in-situ scaffold performance is also highly dependent on graft tribological properties, the depth dependent structure and mechanical properties, as well as the stability of the graft in the site of implantation. Determination of such parameters requires more physiologically relevant testing methodologies (Reviewed in: (86)).

The following sections will focus on four aspects of reproducing the biomechanical environment of the chondrocyte: 1) the use of external biomechanical stimuli, 2) the rational design of mechanically relevant scaffolds, 3) engineering chondron structure and 4) the use of decellularised cartilage scaffolds.

Mechanical stimuli for functional tissue engineering

Mechanical stimuli have been shown to prevent de-differentiation. Through tuning scaffold mechanical properties Li et al. (96) demonstrated that chondrocytes cultured in a stiffer hydrogel matrix (29.9 kPa versus 17.1 kPa and 3.8 kPa) were more likely to maintain a chondrogenic phenotype, characterised by increased collagen II and aggrecan expression as well as GAG secretion. Chondrocytes respond to these regions of higher moduli with increased aggrecan deposition. Interestingly, blocking mechanosensation with the small molecule blebbistatin reverses this effect (97). Research in this area gave rise to the term functional tissue engineering (98) which encompasses the use of mechanical stimuli into the existing paradigm (99), recognising their role in the development of mature tissue engineered grafts (Table 3).

Compressive stimuli are a staple in the maintenance of balanced metabolic activity in chondrocytes and hence the structural integrity of articular cartilage (111). Moderate exercise regimes that load cartilage within its physiological limit improve GAG content and reduce the risk of developing OA (112). Conversely, once afflicted with OA, joint distraction has been shown to reduce disease progression. However, this is suspected to be a result of decreasing hyperphysiological loading and OA mediators such as MMP-13 (113).

Dynamic in vitro loading regimes which aim to mimic in vivo loading, at frequencies of 0.1 – 1 Hz have been shown to increase proteoglycan (114) and cartilage oligomeric matrix protein (COMP) synthesis (115) in cartilage explant models. Kisiday et al. (116) utilised this paradigm in a self-assembling peptide hydrogel model. Through application of a dynamic compression loading regime (2.5% strain and 1.0 Hz frequency), an increased GAG accumulation and accompanied increase in stiffness and equilibrium modulus of the scaffold was observed against the free swelling control (116). Additionally, as shown in Table 3, a combination of compression and shear can enhance biosynthesis over either stimulus alone. This response to loading can be enhanced through supplementation with growth factors such as TGF-β and Insulin growth factor (IGF) -1, as well as through emulation of the hypoxic environment which naturally occurs in articular cartilage (102). However, loading regimes that are either static (109, 110), high velocity impact (106) or

high strain (106, 108) are shown to have negative effects on ECM deposition and cell viability.

Another key challenge in cartilage tissue engineering is the production of an ECM which represents skeletally mature tissue. Luo et al. (117) state that many tissue engineered scaffolds more commonly represent immature cartilage tissue, characterised by a lack of concentric and depth dependent structure which ultimately impacts their mechanical performance (118) and the load experienced by the cells. Middendorf et al. (119, 120) have also indicated that inconsistent aggrecan deposition and distribution can result in construct buckling, and cell death as a result of high local strains. The importance of the presence of a skeletally mature tissue on chondrocyte metabolism has also been shown in bovine cartilage models. When subject to cyclic stress (1 – 5 MPa), chondrocytes within immature cartilage (4 – 8 weeks old) were much more susceptible to injury compared with chondrocytes embedded in mature cartilage (1.5 – 2 years old). The authors observed less intense collagen VI staining as well as the presence of flattened chondrocytes in the immature samples, indicating a role of the skeletally mature structure as well as the PCM in the protection of chondrocytes from injury inducing mechanical loads. Although beyond the scope of this review Khan et al. (121) have shown that it is possible to biochemically induce the maturation of immature cartilage, defined by maturation dependent changes in stiffness, collagen alignment and the presence of distinct concentric territorial, interterritorial and pericellular domains, through the use of FGF-2 and TGF-β.

The knowledge of how loading regimes can affect chondrocyte metabolism and construct maturation, allows for the use of tailored regimes to produce constructs with more appropriate biomechanical and tribological properties. For example, Mauck et al. (122) achieved a 6-fold increase in the equilibrium aggregate modulus (from 0.015 to 0.1 MPa) when loading bovine chondrocytes within an agarose hydrogel constructs, utilising an intermittent (1 hour on/off) loading cycle at 1Hz for 28-days. This demonstrates the importance of utilising a pause in compressive dynamic loading regimes to enhance de novo cartilage synthesis (123). Furthermore, using static hydrostatic pressure (10 MPa) alone, Elder et al. (124) improved the aggregate modulus of bovine chondrocyte seeded agarose constructs up to 0.248 MPa.

Whilst the majority of research focuses on compression, loading regimes have been investigated which can enhance the tensile properties of constructs, through improvements to the ultimate tensile strength (UTS). Lee et al. (125) demonstrated, using a tension stimulation device to impart 15% tensile strain for 1 hour a day over to course of day 10-14 during a 28-day culture cycle, that it is possible to achieve an ultimate tensile strength of 3.3 MPa when used in conjunction with TGF-β and lysyl oxidase-like protein 2 with copper sulphate and hydroxylysine (LOXL2). This value was 3.3 times higher than the untreated control value. Furthermore, loading regimes can also encourage improvements in the tribological properties of cartilage. Through application of a combination of dynamic compression and sliding motion, Bian et al. (126) engineered an agarose hydrogel scaffold seeded with chondrocytes to yield an equilibrium friction coefficient of ~ 0.08, which was significantly lower than the constructs loading under compression alone.

Engineering biomechanically functional constructs

In recognition of the need for a biomechanically functional environment as well as structural support for chondrocytes, the ACI technique has evolved into matrix assisted chondrocyte implantation (MACI). This is referred to as third generation ACI, whereby chondrocytes (0.5-1 x 10^6 cells/cm^2) are seeded into a collagen I/III matrix prior to implantation (127). In a large cohort (827 patients) improved results with MACI versus ACI were shown (128). Furthermore, from a clinical perspective MACI offers an implantation time 20 minutes faster than ACI (129) and an accelerated rehabilitation programme of just 6-weeks has been reported (130).

Despite promising indications, there are still key limitations in the mechanical strength of the repair material. Compression testing of MACI repaired cartilage only achieved 15% of the aggregate modulus of native tissue in a canine model (131). Furthermore, an equine model demonstrated no difference in mechanical properties in repair tissue from an empty defect versus a MACI implant treated defect (132). This is common in tissue engineered grafts due to the lower concentrations and different distributions of both collagen and aggrecan, resulting in local tissue weakness and construct buckling (120). Interestingly, Griffin et al. (133) report aggregate moduli up to 70% of the native tissue.

However, they suspect this is due to the use of a long-term implant and a large animal model.

Beyond this, there is a 4th generation of MACI which encompasses more innovative approaches such as the implantation of allogeneic mesenchymal stem cells (MSCs) with intact whole chondrons. The key benefit being the retained presence of the PCM, which has been shown to cause greater production of proteoglycans and collagen II (42).

The use of synthetic materials offers the possibility of finely tuning mechanical properties of a scaffold. McCullen et al. (134) used electrospinning to create a scaffold with the same zonal fibre orientation as articular cartilage using electrospun poly(ε-caprolactone) (PCL). They achieved an increased tensile modulus compared to articular cartilage and a comparable compressive modulus at 10% strain (134). The use of aligned fibres was found to improve the resistance to tension and damage arising from shear when tested in a multi-axial testing rig (135). Using a polyethylene terephthalate (PET) scaffold, another group achieved compressive moduli values of 2.8 MPa at 15% strain with compressive culture with chondrocytes over an 84-day period, which was only slightly higher than reported native value of 1.8 MPa (136). Whilst only one paper demonstrates any tribological analysis and neither of these papers details in vivo performance data, both show potential strategies of replicating the native mechanical properties of cartilage with a biocompatible material.

Despite this potential replication of biomechanical properties, there are several problems frequently observed with synthetic biomaterial scaffolds for tissue engineering applications. Many lack natural binding motifs and hydration, are hydrophobic and often fail to precisely recapitulate the bioarchitecture and biphasic properties found in cartilage which the chondrocytes are sensitive to.

The biocompatibility of synthetic scaffolds can be improved through hybrid fabrication methods. Owida et al. (137) incorporated polylactic acid (PLA) nanofibre mesh within hyaluronic acid (HA) hydrogels. The fibre alignment matched native cartilage and as a result chondrocytes exhibited zone dependent morphology and distribution, and the repair tissue had higher GAG content in the deeper zones accompanied by a higher

compressive modulus (137). Nguyen et al. (138) used poly(ethylene glycol) (PEG)-based hydrogels with incorporated chondroitin sulphate in the top and middle zones, and PEG:HA in the deep zones. MSCs cultured within the matrices deposited collagen II in the superficial zone and collagen X and proteoglycan in the deep zone, resulting in respective compressive moduli of 0.5 and 1.7 MPa (138) which were consistent with their literature reference for native cartilage (139).

Despite a simplified cartilage structure, Wang et al. (140) fabricated a tri-composite osteochondral scaffold from poly(D,L-lactic acid-co-trimethylene carbonate) (P-(DLLA-TMC), with TGF-β loaded collagen I hydrogel in the cartilage layer. The scaffold had a compressive strength of 0.12 MPa, which the authors report approaches the range of native human cartilage (0.4 – 1.0 MPa (141)), however there was again no native control in the study. Interestingly, they demonstrated that the addition of TGF-β enhanced MSC chondrogenesis and GAG deposition (140).

Means et al. (142) developed a 'double network hydrogel' scaffold composed of a poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) network and poly(N-isopropylacrylamide-co-acrylamide) (P(NIPAAm-co-AAm)). The scaffold exhibited a low coefficient of friction, a compressive modulus greater than 1 MPa, a compressive strength of 25 MPa and demonstrated a similar creep response profile to native cartilage. The group showed cytocompatibility compared to the tissue culture plastic control but are yet to investigate whether cells can be successfully encapsulated. However, this study demonstrates the potential of successfully recapitulating multiple biomechanical and tribological properties for restoration of cartilage function.

Engineering the Chondron

The presence of PCM has been shown to be beneficial for chondrocytes in terms of mechanocoupling and in promoting the deposition of cartilage matrix molecules (143). One approach to engineer constructs containing chondrons, is using scaffold free cartilage constructs, using the technique of self-assembly of chondrocytes within non-adherent agarose wells (144). Jeon et al. (143) also have demonstrated that culturing chondrocytes

for 2-weeks prior to further treatment with compression and saw an increase in collagen VI production and the formation of chondron structures within the agarose gels.

It has been shown that scaffold morphology has an influence over the formation of chondron structures. Using a hyaluronic acid polymer (HYAFF11), Fraser et al. (145) demonstrated that bovine chondrocytes seeded onto a non-woven scaffold formed chondron-like collagen VI structures in the neotissue, whereas those onto a sponge scaffold lacked distinct chondron formation.

Stoddart et al. (123) have shown that in response to compressive loading with pauses, distinct chondron-like structures are formed which integrate with the surrounding agarose hydrogel. Steward et al. (146) demonstrated chondrogenesis of MSCs seeded into agarose hydrogels and found that MSCs produced a denser and stiffer PCM in the presence of a stiffer hydrogel. Furthermore, gene expression analysis of chondrocytes cultured in a 3D collagen sponge demonstrated higher expression of the PCM component matrilin in response to cyclic loading (147).

Few studies have investigated the production of PCM biomolecules or structures through mechanical loading regimes. This may be a result of multiple studies demonstrating PCM production in tissue engineered scaffold without the requirement for loading (143, 148). Interestingly, the aforementioned study by Jeon et al. (143) also observed that following the 2-week preculture period which allowed for the production of PCM structure in their construct, increased the cellular response to mechanical loading. This suggests that recapitulating the PCM biomechanical niche may be key to successful tissue engineering, and also elucidates a potential strategy to optimise pre-implantation loading regimes for tissue engineered constructs.

Overall, there appears a disparity in the literature between research advocating the benefits of an intact PCM in cartilage tissue engineering and the rational design of scaffolds with chondron like structure or with a structure primed for the formation of such structures (123).

Decellularised cartilage tissue scaffolds

So far, this review has indicated a variety of strategies for engineering functional cartilage constructs. One approach which encompasses all of these elements (native composition, biomechanical properties and depth-dependent/concentric ECM structure) is decellularisation, a process that can remove cells from a range of human and xenogeneic biological tissues whilst preserving the macro and micro-structure of the native tissue, as well as the ECM composition (149), producing a fully functional regenerative tissue scaffold. These tissues have been shown to induce favourable cell responses such as chemotaxis, differentiation and induce tissue regeneration (Reviewed in: (150)). This technique has been applied successfully to generate decellularised cartilage and osteochondral scaffolds (151-153). These scaffolds have taken many forms summarised below in Table 4.

The biological response to decellularised cartilage has been extensively shown, using animal models. Li et al. (157) report significant improvement in macroscopic ICRS and histological O'Driscoll scores, accompanied by integration of the scaffold with the surrounding tissue and a lack of collagen I expression in the neocartilage. Xue et al. (161) showed similar repair after 6-months in vivo implantation of acellular cartilage sheets seeded with BMSC's. Histology showed good integration at the cartilage interface and lack of fibrosis in the repair tissue, and mechanical testing saw Youngs moduli and maximum compressive strength of regenerated cartilage approaching that of native cartilage. Due to the retention of native ECM structure, as well as chondrogenic growth factors such as TGF-β, IGF-1 and BMP-2, these scaffolds have been shown to direct chondrogenesis (162).

A key observation in the performance of decellularised grafts is the improved histological and functional outcomes when seeded with cells versus scaffold alone controls. This is likely due to the immobility of chondrocytes and the low porosity of cartilage, resulting in low endogenous cell migration into the scaffold. Furthermore, cartilage is an avascular tissue with low cell density (4), therefore acellular grafts without either large porosity or biomolecular homing signals are unlikely to undergo mass endogenous cell repopulation.

The structural composition of decellularised cartilage, in terms of collagen alignment and composition has been shown to match that of the native tissue (117). However, initially post-decellularisation there is a reduction in GAG content, which translates in increases in deformation (152) and decreases in Youngs moduli under compression compared to native cartilage (157). Despite the reduction from native values, instances where the compressive modulus is measured, values above 1 MPa are reported (163). However, several studies show the recovery of both GAG content and mechanical properties post-recellularisation. Li et al. (157) report that decellularised scaffolds seeded with autologous chondrocytes recover the native Youngs modulus and increase DNA and GAG content with respect to the native controls after 8-weeks of in vivo implantation in a rabbit defect model. Using decellularised cartilage microfilaments, Kang et al. (164) created a lyophilised ECM-derived scaffold, showed a similar recovery up to 83% of the stiffness of native cartilage after 6-months in vivo implantation.

A recent study by Luo et al. (117), exemplifies many of the strategies suggested in this review. Mature and immature cartilage was decellularised and seeded with fat pad derived stem cells for 4-weeks. The regenerated tissue mimicked the zonal collagen arrangement of the surrounding decellularised scaffold, and displayed depth dependent compressive moduli in the mature tissue but not the immature tissue (117). This study provides an example of how scaffolds designed to accurately recreate tissue structure can influence cell behaviour towards appropriate neo-tissue formation with depth dependent mechanical properties.

Conclusion

This review has aimed to emphasise how the complex heterogeneic structure and anisotropic properties of cartilage governs tissue biomechanical and tribological function in vivo, and how these functions are much more complex than simply an ability to withstand compression.

As well as permitting healthy joint function, appropriate biomechanical properties are essential for healthy cellular function. As the intermediate material between applied loads and the cells, the ECM and PCM directly influence how the chondrocyte experiences the

load and the response (47). Harnessing these concepts in the scaffold design, alongside application of the correct mechanical and biological cues should yield an environment primed for graft maturation and the deposition of functional cartilage to restore joint function.

Decellularised cartilage tissues offer an ideal scaffold for matrix assisted chondrocyte implantation, due to the retention of much of the skeletally mature cartilage macro/microstructure and biochemical cues to promote healthy homeostasis. The provision of an immediate restoration of functional performance may allow for faster rehabilitation times for patients and improve long term graft performance (165).

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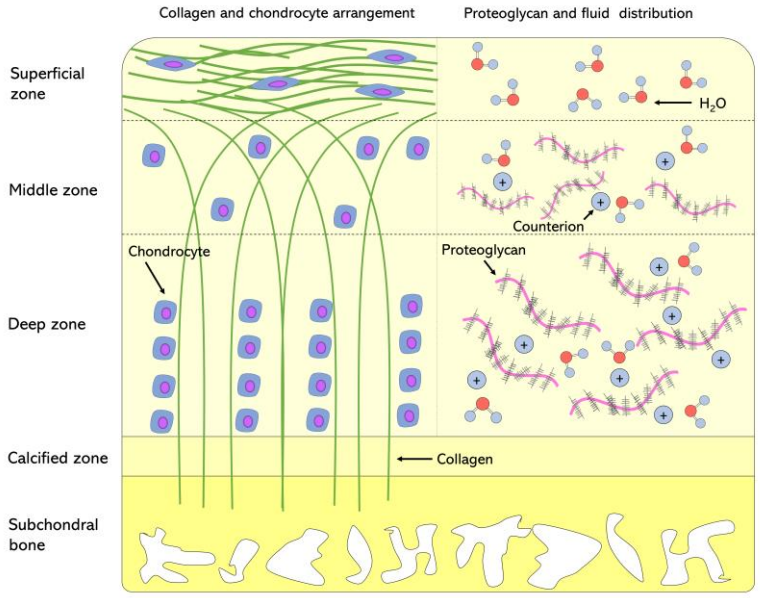

Figure 1: Arrangement of key extracellular matrix components and chondrocyte distribution. Left: collagen fibre structure and zone dependent chondrocyte morphology and arrangement. Right: zonal distribution of proteoglycans, associated counterions and water molecules.

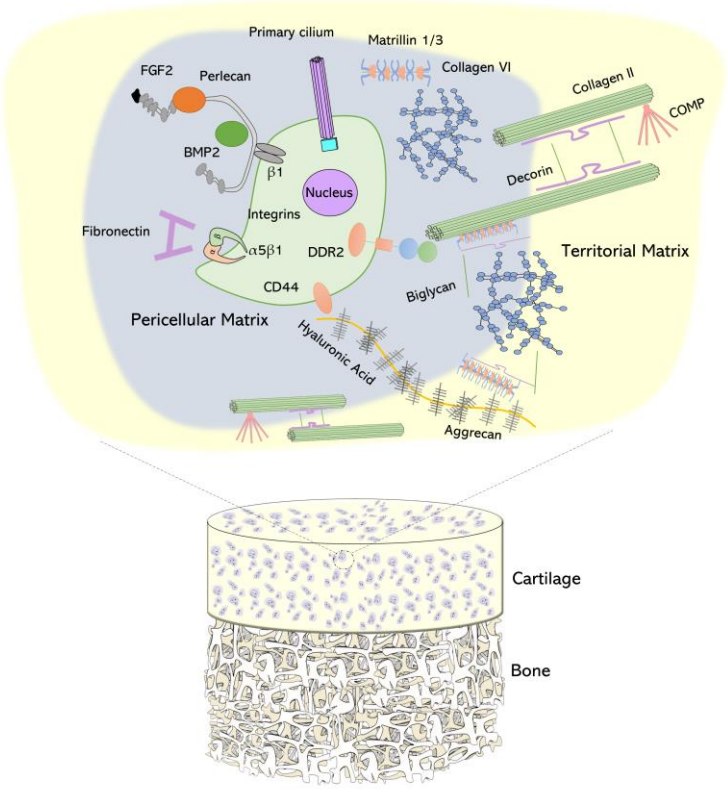

Figure 2: Pericellular matrix arrangement in relation to the osteochondral unit. Magnified view demonstrates the pericellular matrix and territorial matrix components. Collagen II fibrils, linked by decorin/biglycan, from the extracellular matrix can interact directly with chondrocytes via discoidin domain-containing receptor (DDR)-2 receptors on the surface. CD44 also links the chondrocyte to hyaluronic acid which in turn can associate with collagen VI and II via matrillin 1/3 and biglycans. Cell surface integrins also permit interaction with fibronectin and perlecan, which itself acts as a biomolecule repository of fibroblast growth factor (FGF)-2 and bone morphogenetic protein (BMP)-2. Primary cilia are also located on the cell surface membrane which deform in response to mechanical loading.

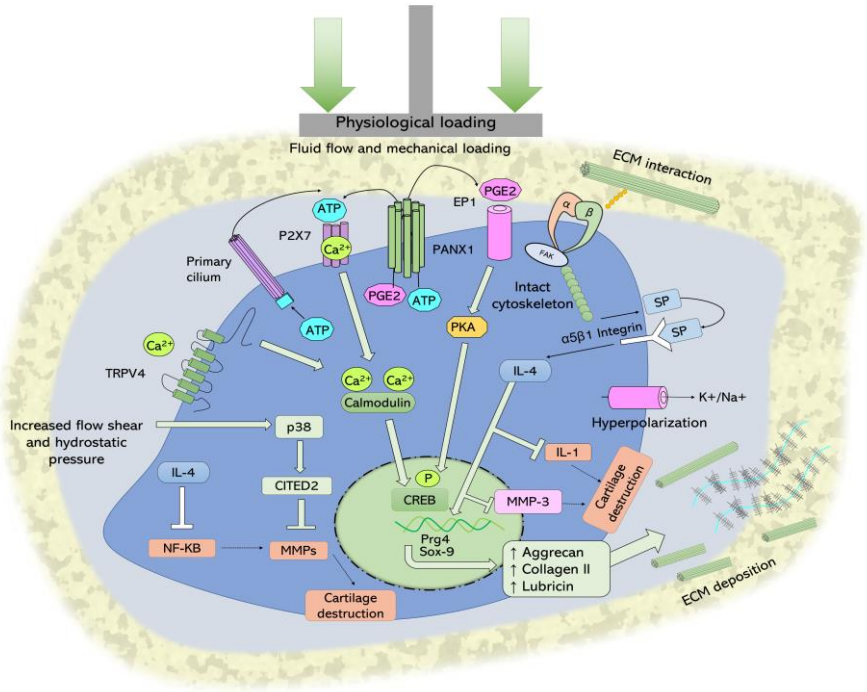

Figure 3: Chondrocyte cell signalling response to physiological mechanical loads. Mechanical stimuli cause interaction of ECM with cell surface integrins, allowing membrane hyperpolarisation through cation efflux and interaction with the intact cytoskeleton. The result is substrate P (SP) secretion which interacts with NK1 channel to activate intracellular IL-4 activity, increasing anabolic aggrecan production and inhibiting IL-1 and expression of matrix metalloproteinase (MMP)-3. Fluid flow promotes efflux of prostaglandin (PG)-E2 and ATP through pannexin 1 (PANX1) channel. Extracellular ATP can interact with purinergic type 2X receptor (P2X) 7 channels to increase intracellular [Ca^{2+}]. PGE2 interacts with EP1 G-protein coupled receptor, to activate protein kinase A (PKA) which in turn phosphorylates cAMP response element-binding protein (CREB) on serine-133, allowing for transcription of Pgr4 and lubricin production. Mechanosensitive transient receptor potential vanilloid sub type (TRPV)-4 receptors respond to osmotic pressure to mediate Ca_{2+} influx, which interact with many downstream signalling pathways, such as calmodulin, CREB to increase transcription of anabolic genes such as sex determining region Y-box (Sox)-9, p38 mitogen-activated protein kinase (MAPK) to activate Cbp/p300-interacting transactivator (CITED)-2 which can inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated MMP production and cartilage destruction.

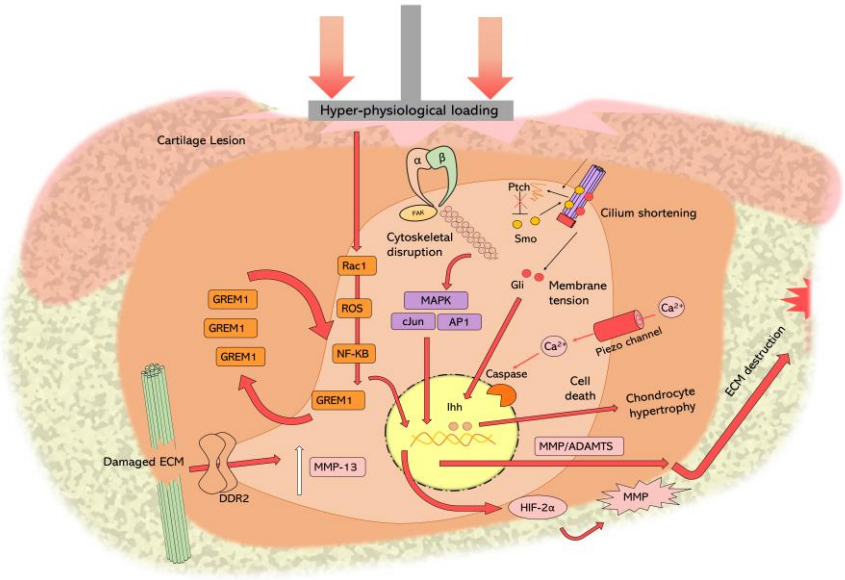

Figure 4: Chondrocyte cell signalling response to hyper-physiological loads. Upon exposure to hyper-physiological loads, integrins interact with the disrupted cytoskeleton, activating MAPK signalling, and catabolic gene expression of MMPs and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). This membrane tension activates the transport of Ca_{2+} ions, via Piezo channels, into the cell which activates Caspase activity, resulting in chondrocyte cell death. High loads decrease primary cilium length through histone deacetylase (HDAC)-6 causing release of Ptch, relieving inhibition of Smo which can activate Gil to express Indian hedgehog (Ihh) and downstream ADAMTS5 and chondrocyte hypertrophy. Rac1 is activated in response to high mechanical loads, which stimulates ROS mediated activation of NF-κβ, triggering a positive feedback loop with Gremlin (GREM)-1, further increasing NF-κβ expression of Hypoxia induced factor (HIF)-2α. High loading also causes damage to the ECM surrounding the chondrocyte. Damaged collagen exposes intracellular domains to DDR2 receptors, activating MMP-13 expression.

Table 1: Anisotropic distribution and function of ECM components, interstitial fluid and chondrocytes in chondrocyte biological and biomechanical/tribological function. SZ = superficial zone, MZ = middle zone, DZ = deep zone, GAGs = glycosaminoglycans, PG = proteoglycans, FCD = fixed charge density.

	Zonal Anisotropy	Function	Role
Collagen	**SZ:** High density of **c**ollagen II fibres align in direction of articulation (19) **MZ:** Lower density, random alignment. Associated with PG. **DZ**: Perpendicular alignment with subchondral bone (20, 21).	High tensile strength in direction of stress, due to inter/intramolecular crosslinking of collagen fibres (19).	**Tensile strength: :** Congruous surface for articulation. Resist tensile stress of articulation. Collagen-PG mesh with low porosity (22). Resistance to shear (4). Solid matrix tensile strength to resist fluid pressurisation (23). Integrates cartilage with subchondral bone
PG/GAGs	**SZ:** Low concentration. PGR4 acts as boundary lubricant (24). **MZ:** Tightly packed mesh with collagen fibres (22). **MZ/DZ:** Highest	Negatively charged GAGs create high FCD (25). Cation influx creates high osmotic potential (Donnan Osmotic potential (26). Loading increases FCD.	**Physicochemical/compressive strength:** Higher concentration in MZ/DZ associated with higher compressive moduli. Donnan osmotic pressure can contribute 50-60% to tissue stiffness (27, 28).

	concentration of PGs. **DZ:** Highest concentration of GAGs (25).		
Fluid	**SZ:** Highest (80%) fluid content. **MZ:** Decreases in composition with depth. **DZ:** Lowest composition (65%) (29).	Retained by Collagen-PG mesh. Low permeability of matrix allows for interstitial fluid pressurisation (4), which can bear compressive load.	**Biphasic lubrication:** On loading, interstitial fluid pressure can support 90% of compressive load (30), reducing solid-solid contact. Greatest in **SZ,** lowest in **DZ** (31).
Chondrocytes	**SZ:** Fibroblastic morphology. Highest density. **MZ:** Lower cell density, randomly arranged. **DZ:** Round morphology, align in columnar fashion to subchondral bone (4).	Overall cell density = 2.4 x 10^6 cells/cm^2 (32). Produce ECM, PCM in development and response to injury. Sensitive to environment.	**Cartilage homeostasis:** Respond to mechanical (and biochemical) stimuli to govern tissue anabolic/catabolic balance).

Table 2: Bulk biomechanical and tribological properties of human and animal articular cartilage obtained through defined testing regimes.

Mechanical Behaviour	Value	Test	Reference
Compressive moduli			
Youngs Modulus (Compressive)	0.41 to 0.85 MPa	Unconfined compression	(87, 88)
Aggregate Modulus	0.1 to 2.0 MPa	Confined compression/Indentation	(88, 89)
Tensile strength			
Tensile Strength	2 to 8 MPa	Constant strain rate testing	(90)
Young's Modulus (Tensile)	5 to 25 MPa	Constant strain rate testing	(88, 90)
Tribology			
Friction Coefficient (Cartilage-Cartilage)	0.014 to 0.030	Pin-on-plate	(91, 92)
Shear			
Equilibrium Shear Modulus	0.2 to 0.37 MPa	Biaxial shear test/Indentation/ Shear relaxation	(93-95)
Permeability (*k*)	1.2 to 6.2 (x 10^{-16}) m^4/N.s	Confined compression	(88)

Table 3: Chondrocyte metabolic response to various loading regimes for cartilage tissue engineering constructs seeded with chondrocytes or MSCs.

Load regime	Set up	Frequency	Strain/ Amplitude	Time	Results
Physiological					
Biaxial	Biaxial loading bioreactor (100)	1 Hz	30% compression 1 mm shear	1h/day, 28-days	↑Anabolic mRNA; ↑Col II deposition; ↓Col I
-	Biaxial pin-on ball bioreactor (101)	1 Hz	0.4mm compression 25˚ shear	1h/day. 15-days.	↑Anabolic mRNA vs uniaxial loading
-	Biaxial loading; hypoxic (102)	0.5 Hz	10-20% (0.4 mm) compression 25˚ shear	1h/day, 4-weeks	↑ Aggrecan content; ↑ Col II/ACAN; Stable phenotype w/ hypoxic
Dynamic	Stepper motor with loading pins (103)	0.3 Hz	5 -20% Intermittent (I) and continuous (C)	(I) 1hr on/off x6/day. (C) continuous. 7-days	I: ↑ Anabolic + MMP; C: ↑Aggrecan, ↓MMP expression
-	Compression with 10 ng/ml TGF-β (104)	1 Hz	10%	3 h/day, 1 hour on/off, 5d/week. 5-weeks	↑Equilibrium aggregate modulus vs other stimuli alone

Hydro-Static	Hydrostatic bags (105)	1 Hz	1, 5 and 10 MPa	4h/day, 1 or 4 days	4 days and 10 mPa ↑ Aggrecan + Collagen mRNA
Non-physiological					
High velocity impact load	Custom indentation (106)	0.1%/Sec 80%/Sec	40%	1 test	↓Cell viability at Day 0, 4 and 7 post-injury
Repeated overloading	Custom indentation (107)	0.5 Hz	40% (1 MPa)	3h/day. 7 days.	↓Respiratory activity compared with lower load
High strain rate	Unconfined compression (108)	0.1-1/sec	18 – 24 MPa	5 minutes	↓Biosynthesis ↓Compressive stiffness
Static load	Custom compression chambers (109)	Static	50% compression	24 hours	↓Proteoglycan + collagen II synthesis
-	Unconfined spring loaded compression (110)	Static	84 kPA	24 hours	↓GAG + DNA synthesis

Table 4: Summary of methods for producing decellularised/acellular cartilage scaffolds from both human and xenogeneic cartilage sources.

Approach	Description	Decellularisation?	Recellularisation?
Devitalised cartilage (154)	Dehydration in ethanol, lyophilisation, freeze-thaw in liquid nitrogen	Cell fragments remained	Seeded chondrocytes retained viability *in vitro*
Cartilage ECM-derived matrix (155)	Pulverised, centrifuged, Triton X-100 in hypotonic, nuclease, lyophilised and crosslinked to form a scaffold. Seeded with MSCs and implanted into mice	No cells present on histology	Cell viability maintained; neocartilage contained GAGs/collagen II
Acellular cartilaginous matrix (156)	Freeze dried, Triton X-100, DNase and RNAase. Seeded with adipose stem cells (ASCs)	-	Chondrogenesis; Collagen II deposition; high cell density and an inhomogeneous cell distribution
Decellularised osteochondral scaffold (153)	Freeze/thaw, waterpik, hypotonic, hypertonic, 0.1% SDS, nuclease	DNA below 50 ng/mg threshold; Reduced GAGs	-
Laser modified decellularised cartilage (157)	2% SDS, deionised water, DNAase-I and PBS washes. Laser micro-drilled using 8-24ms pulse duration to	DNA below 50 ng/mg; Reduced GAG	Improved cell adhesion; Post-recellularisation recovery of Youngs Moduli, GAG content; Significant improvement in macroscopic and

	create pores		histological scores
Chondrofix osteochondral allograft plugs (158, 159)	Remove lipids and bone marrow elements, soaked in methylene blue under light to inactivate viruses	-	72% failure and 19.6% clinical survivorship; Age predictive of failure (average 35 years); acute graft delamination
Acellular cartilage sheets (160)	Chondrocytes cultured to form cell sheets, decellularised using 1% SDS.	DNA reduced below 50 ng/mg threshold	Rabbit defect model showed substantial remodelling.
Acellular cartilage sheets (161)	1% SDS and lyophilised. Seeded with BMSCs	-	Cartilage-like repair; satisfactory interface healing after 6-months; significantly higher Young's moduli