Published in final edited form as:
J Med Biol Eng. 2013 February 1; 33(1): 1–16. doi:10.5405/jmbe.1254.

Multimodal evaluation of tissue-engineered cartilage

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Abstract

Tissue engineering (TE) has promise as a biological solution and a disease modifying treatment for arthritis. Although cartilage can be generated by TE, substantial inter- and intra-donor variability makes it impossible to guarantee optimal, reproducible results. TE cartilage must be able to perform the functions of native tissue, thus mechanical and biological properties approaching those of native cartilage are likely a pre-requisite for successful implantation. A quality-control assessment of these properties should be part of the implantation release criteria for TE cartilage. Release criteria should certify that selected tissue properties have reached certain target ranges, and should be predictive of the likelihood of success of an implant *in vivo*. Unfortunately, it is not currently known which properties are needed to establish release criteria, nor how close one has to be to the properties of native cartilage to achieve success. Achieving properties approaching those of native cartilage requires a clear understanding of the target properties and reproducible assessment methodology. Here, we review several main aspects of quality control as it applies to TE cartilage. This includes a look at known mechanical and biological properties of native cartilage, which should be the target in engineered tissues. We also present an overview of the state of the art of tissue assessment, focusing on native articular and TE cartilage. Finally, we review the arguments for developing and validating non-destructive testing methods for assessing TE products.

Keywords

Cartilage; Tissue engineering; Properties; Testing

1. Introduction

Articular cartilage is a composite of materials with disparate properties. The whole tissue is approximately 70 to 85% water by weight, with the remainder composed primarily of proteoglycans and collagen (see below). The distribution of proteoglycans and the structured collagen architecture suggest that cartilage is an inhomogeneous and anisotropic material. Uniquely, it is also avascular and aneural. It functions as a bearing surface in synovial joints such as hip and knee, where it provides low friction with excellent wear characteristics. It helps to distribute joint contact loads over a larger area than would be present if there was bone-to-bone contact, and it performs these functions for decades while loaded at several times body-weight.

Cartilage has limited ability to repair itself. Cartilage defects are a major health issue in countries with high life expectancies. In the USA, over 40 million people (15% of the population) suffer from arthritis, making it the leading cause of chronic disability [1]. That

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number is expected to exceed 60 million by 2020 [1]. Most current treatments target pain relief but do not modify the long-term, irreversible course of cartilage degeneration. Thus, the majority of these patients will eventually be candidates for prosthetic joint replacement. In 2006, there were 230,144 primary total hip replacement procedures and 516,435 primary total knee replacement procedures in the US alone, performed almost exclusively for an underlying osteoarthritis (OA) diagnosis [2]. Kurtz *et al.* project an increase to 572,000 primary total hip and almost 3.5 million total knee replacements annually by 2030 [3]. Joint prosthetics are costly, have a finite life-span, and are difficult to revise and replace. A living biological, rather than a prosthetic replacement of defective tissues would have definite advantages. Tissue engineering (TE) could provide such a repair and has promise as a biological solution and a disease modifying treatment for arthritis. TE cartilage must be able to perform the same functions as native tissue. This applies to chondrocyte- as well as to stem cell-based TE. Strategies for producing *in vitro* cultured cartilage tissue have been developed and could emerge into the clinic shortly [4]. An individualized autologous approach, *i.e.*, engineering tissue that is custom-designed and fabricated for each patient using his own cells, would be ideal. However, substantial donor-to-donor variability in the proliferation, differentiation potential, metabolic demands, and biosynthetic activity of isolated cells make it impossible to guarantee optimal, reproducible results in all cases [5,6]. Even within one donor, cell quality degrades when the cells are culture-expanded. Some of this variability can be reduced by growth factor treatment [7,8], and some can be mitigated by using robust scaffold material, but since, in the end, mechanical and biological properties approaching those of native cartilage are likely a pre-requisite for successful implantation, a quality-control assessment of these properties should be part of the release criteria for TE cartilage.

Currently, formal criteria for specifying that a specific TE construct is ready for implantation do not exist. The absence of release criteria is a significant gap in the translational route of cartilage TE from the *in vitro* to the *in vivo* domains. Release criteria should certify that selected tissue properties have reached certain target ranges, and should be predictive of the likelihood of success of an implant *in vivo*. A complete set of properties needed to establish release criteria is not known. Achieving properties approaching those of native cartilage requires a clear understanding of the target properties and reproducible assessment methodology [9-25].

In this paper, we examine several main aspects of quality control as it applies to TE cartilage. First, we review the known mechanical and biological properties of native cartilage in order to establish target values for these properties in engineered tissues. It has yet to be established what fraction of these target values represent the minimum requirements in mechanical or biochemical properties [26]. Second, we present an overview of the state of the art of tissue assessment, again with focus on native articular and TE cartilage. A third consideration in TE is that current methods for evaluating the tissue tend to be destructive, which negates clinical utility of the tissue after testing. Therefore, future research emphasis should be placed on developing and validating non-destructive testing methods for assessing TE products.

2. Biomechanical considerations

When placed in a joint, TE cartilage must be able to function under a wide range of loads and loading rates. Joint loads on the articular surface are a combination of compression and shear. Compressive loading is typically in the range of two to four times body weight during normal gait, but can be much higher in other activities [27-29]. These loads correspond to average peak compressive stresses in the range of about 3 – 11 MPa on the hip during for activities of daily living [30]. Loading rates have been estimated to be 6 – 25 MPa/s during

walking, 50 – 210 MPa/s during jogging, and 140 – 250 MPa/s during jumping. Loading rates of around 800 MPa/s are believed to cause subchondral injury, and rates greater than 1000 MPa/s may cause osteochondral fracture [27,31-36]. In normal joints, with low friction, it is expected that the shear loads will be two to three orders of magnitude less that the compressive loads.

Given the range of loading conditions on a joint, it is unlikely that one method of biomechanical evaluation will yield a complete picture of the tissue's functionality. Methods that have been used to determine properties of normal cartilage, and pathologic cartilage are also used to compare native and tissue-engineered cartilage. The majority of methods used for biomechanical evaluation of cartilage fall into either of two categories: those that determine material properties, or those that give an indication of performance under conditions that approximate normal joint function. Tribological tests fall into the latter category. Relevant material properties are defined by models of mechanical behavior. Although elastic and viscoelastic models have been used to describe cartilage [37-43], biphasic theory developed by Mow and co-workers has become the standard for describing the mechanical behavior of cartilage [24]. Biphasic theory models cartilage as a mixture of a solid and fluid. The solid component includes the collagen network, proteoglycans, cells, lipids and noncollagenous proteins. In its most basic form, the solid is modeled as an isotropic, incompressible elastic material, and the fluid is modeled as incompressible and inviscid. Fluid-solid interaction in the model gives rise to time-dependent behavior and governs load carriage and deformation. The basic form of the biphasic model includes material properties of the solid matrix (two independent constants, for example, aggregate modulus and Poisson's ratio) and a fluid flow property (permeability). Tests under confined compression, unconfined compression, and indentation have been developed in conjunction with model-based data analysis techniques that are used to extract material properties [24,44-48].

If cartilage behaved as an isotropic elastic solid, then only two independent properties would be needed to specify its mechanical properties. However, the tissue is not this simple: it is porous-elastic and not isotropic. However, even if all the mechanical properties were known, they would not necessarily be sufficient to characterize the quality of an implant. A simple analogy illustrates this issue. All steel alloys have essentially the same Young's modulus, but they have vastly different yield and ultimate strengths. Even if the yield and ultimate strengths were known, these would not necessarily give an indication of the friction and wear properties of the tissue. For these reasons, it is unlikely that a single test will be sufficient to indicate that a piece of cartilage is adequate for implantation.

Early investigations showed that the biphasic model did not fit data from some tests, *e.g.*, stress relaxation in unconfined compression [46]. Guided by the structured collagen architecture in native cartilage, anisotropic fibril reinforced models, and models that incorporate transverse isotropy have been developed [44,49-54]. Shirazi *et al.,* developed a particularly detailed model that includes three zones with anatomically inspired fibril orientation: a superficial zone simulating randomly distributed in-plane fibrils, a transitional zone without a dominant fibril orientation, and a deep zone simulating fibrils perpendicular to the cartilage-bone interface [55]. These modeled orientations were chosen to mimic those in native tissue [56]. Other models have investigated the effects of transverse isotropy alone, which mimics the deep zone of articular or growth plate cartilage [44,54]. Another important contribution to modeling was the incorporation of different mechanical properties in tension and compression [45,57]. Models incorporating fibril reinforcement and tension-compression nonlinearity have been particularly successful in mimicking the behavior of native cartilage under a wide range of conditions. Given the structured collagen architecture and distribution of proteoglycans, it is not surprising that inhomogeneous and anisotropic

models are needed to capture the complete response of cartilage, and that its overall behavior will depend on composition and structure [58].

The applicability of these enhancements to TE cartilage remains to be determined. For example, anisotropic and fibril reinforced models might not be directly applicable to TE cartilage, which lacks the well-defined collagen structure of native cartilage. If the structure is isotropic, the solid matrix of TE cartilage might be well-described by two independent material properties (for example, aggregate modulus and Poisson's ratio) and permeability. Absence of an organized structure does not, however, eliminate the possibility of having different properties in tension and compression. For the remainder of this review, mechanical properties will be described in terms of the isotropic biphasic model, and unless otherwise noted, the methods described below are implemented under conditions of small strain, where material behavior is linear.

2.1 Material property tests

2.1.1 Compression—In confined compression, a cylindrical sample is placed in an impervious chamber and loaded across its entire circular surface through a porous filter [44,59,60]. In this configuration, the only component of cartilage displacement is in the same direction as the compression. Fluid flow is perpendicular to the circular face of the sample. This test can be performed in either under constant load (creep) or under constant displacement (stress relaxation). The intrinsic compressive stiffness of the solid matrix (aggregate modulus) and permeability are determined by fitting model predictions to the measured output (deformation or stress).

In unconfined compression, the sample is placed between smooth impermeable platens while its cylindrical surface is unconstrained [46,54,61]. In this configuration, compression is applied perpendicular to the sample's circular face, as it is in confined compression, but the sample is deformed in vertical and radial directions. Fluid flow is only in the radial direction.

Both confined and unconfined compression tests can be run in either creep or stress relaxation modes. In these tests, the time to reach equilibrium is typically in the thousands of seconds. Permeability and either aggregate modulus (confined compression) or Young's modulus (unconfined compression) can be determined by fitting a biphasic model of the experiment to the complete time-dependent experimental data. Alternatively, if only compressive modulus is desired it can be computed from the slope of equilibrium stress *vs.* equilibrium strain.

2.1.2 Indentation—In an indentation test, a small portion of a sample's surface is loaded through a small circular indenter (typically 0.9 mm to 1.5 mm in diameter), which puts the tissue under a more complex state of stress than in confined or unconfined compression [10,47,48,62-66]. Indentation tests are typically performed under constant load, and run until displacement comes to equilibrium, which is typically in the thousands of seconds. An advantage of indentation over other tests in compression is that small samples can be evaluated *in situ*. For example, indentation can be performed on native or repaired cartilage in the rabbit knee [66-69]. Due to the joint's curvature, and thin cartilage, it would be impossible to get uniformly thick cylindrical samples from the rabbit knee for use in confined or unconfined compression tests. However, finite element simulations have shown that curvature has negligible effects on indentation results, at least for the conditions studied [70]. Curvature does affect the diameter of the indenter. In a joint like rabbit knee, with a relatively small radius of curvature, it is advisable to use a smaller diameter indenter (≈ 1 mm) than would be used with larger joints [67]. Larger diameter indenters require greater displacement to get full contact with the cartilage, and larger displacements invalidate the

linear models used for data analysis. Using a biphasic model-based analysis approach, two properties of the solid matrix (for example aggregate modulus and Poisson's ratio) and the permeability are determined from one test [47,48]. Typical values for human medial femoral condyle are in the range of 0.59 ± 0.11 MPa for aggregate modulus, 0.074 ± 0.84 for Poisson's ratio, and $1.14 \pm 0.16 \times 10^{-15}$ $m^4/N \cdot s$ for permeability [9].

2.1.3 Shear—Understanding tissue behavior in shear is an integral part of evaluating TE cartilage, as joint motion induces shear deformation. In theory, testing cartilage in shear can be used to determine the intrinsic behavior of the solid matrix component, as poroelastic theory predicts that there should be no fluid flow under pure shear and small strain [71]. However, tests have shown that the matrix behaves viscoelastically. Nevertheless, modeling the matrix as elastic solid and ascribing the time dependent behavior to diffusive flow of fluid through the solid matrix is relatively common [44,54,61,72-74].

The viscoelastic behavior of the solid matrix has been characterized using both transient and dynamic measurements. Transient measurements (creep or stress-relaxation) may be used to determine a (reduced) relaxation function or creep compliance, while dynamic measurements (oscillating stress or strain) result in the complex modulus and loss angle. Equilibrium shear modulus is in the range of 0.1-0.4 MPa, the dynamic modulus $|G^*|$ is between 0.2 and 2.0 MPa, while the loss angle δ is about 9-15° [13,42,71,75-78].

A common practical problem with all of these tests is gripping the sample. One approach is to clamp the sample between porous or roughened surfaces to prevent slippage when the sample is sheared. Alternatively, a sample may be glued to smooth flat platens using cyanoacrylate.

2.1.4 Tension—Tension tests are typically performed under constant strain rate or constant load. When native samples are tested, it is common to align them such that tension is applied either parallel or perpendicular to the split-line pattern on the articular surface [12]. Because of the indistinct boundary to the subchondral bone, which needs to be removed for tensile tests, almost no attempts have been made to test full-thickness cartilage in tension. Results have shown that tensile properties (typical tensile moduli 0.3 to 15.7 MPa) depend on depth from the articular surface, orientation relative to split lines, and the presence or absence of an open physis [12,79]. Evaluation of healthy and proteoglycan digested cartilage has shown that the collagen network provides strength and stiffness, while proteoglycans control the time dependent tensile deformation at high strains by impeding rotation of collagen fibrils [80-83]. Tensile properties of native and TE cartilage can be compared at several levels. Using constant strain rate tests, strength, and stiffness as functions of depth is one comparison. Under constant load (creep tests) the duration of the initial and late phases of creep can be compared. In TE cartilage, which is generally collagen deficient, we would expect both stiffness and strength to be much reduced relative to native tissue [84-89]. This is currently a major barrier to clinical use of TE cartilage.

2.1.5 Depth-dependent properties—A limitation of the above tests is that they yield average values across the entire thickness of the test sample. While it is likely necessary that TE cartilage should have similar average properties to native cartilage, it is not clear that this is sufficient to ensure adequate repair of a defect. In native cartilage, morphology, chemical composition, and mechanical properties vary as a function of depth from the articular surface [90-93]. For example, in the bovine patellofemoral groove, stiffness increases with depth, with the deepest layer being more than 6 times stiffer in compression than the surface layer [92]. It is likely that these variations are functionally important and therefore should be considered when evaluating TE cartilage. In TE cartilage, mass-transport limitations result

in tissue where the surface is more mature morphologically and biochemically, and stiffer than the deeper tissue. This is exactly the opposite of what is seen in native cartilage.

One approach to determining depth-dependent properties is to cut multiple sections thorough the thickness of cartilage, and test each of these [12]. Depending on the test, less accurate results are likely as the thickness of samples diminishes. For example, in confined compression tests, the assumption that the surface of the porous plunger is flat breaks down when the sample thickness decreases to where the roughness of the plunger is a large percentage of the sample thickness [94]. Testing of cartilage slices is also the usual approach for tensile tests (see section.2.1.4, above).

Other approaches include optical and (see below) acoustic measurement methods. Using optical imaging methods, fiducial markers such as fluorescently labeled cells or other features can be tracked during deformation to get a map of local strain fields [90-93,95-98]. Depth-dependent compressive and shear stiffness can then be computed from the strain distribution and known stress. Digital image correlation is a similar method that tracks user-defined regions of interest rather than tracking individual cells to determine displacement fields [99-101].

For shear, a novel and highly accurate method tracks the deformation of photobleached lines drawn across the thickness of a fluorescently labeled sample. In this case, the sample is first uniformly stained with a fluorescent dye such as 5-(4,6-dichlorotriazinyl) aminofluorescein, (5-DTAF) that labels all amino acids in the tissue. Lines are then photobleached, perpendicular to the direction of the applied shear displacement, on the stained tissue using the confocal microscope laser. The slope of the deformed lines (the shear strain) and the known shear stress are used to determine local shear stiffness.

2.2 Tribological tests

One of the more remarkable features of native articular cartilage is its low friction and wear. A thorough description of the tribology (friction, wear and lubrication) of synovial joints is beyond the realm of this review and the reader is referred to many excellent publications in this area [72,102,103]. Although simple hydrodynamic lubrication in synovial joints is unlikely, other modes, some of which are unique to cartilage, have been proposed including boundary [17,19,104-107], weeping [108,109], boosted [110,111], microelastohydrodynamic [112-114], and biphasic lubrication [14,109,115-118]. Current evidence favors a combination of boundary lubrication in conjunction with biphasic or microelastohydrodynamic lubrication.

Tribological evaluation of engineered tissue may provide a better measure of the ability of a construct to function *in vivo* than measurements of mechanical properties. Our own tribological investigations of TE cartilage have shown, at least for a particular set of scaffold-free constructs, that engineered tissue is easily damaged under combined compression and sliding shear, while the same conditions did not produce any noticeable damage to native cartilage [98,119,120]. Failure under these conditions might not have been predicted by mechanical properties. We also observed an increase in friction over time that is characteristic of biphasic lubrication, a mode of lubrication in which compressive joint force is carried by interstitial fluid pressure in the matrix (an approximately 15-fold increase, from 0.02 to 0.3 in coefficient of friction over 20,000 seconds has been reported) [14,109,117]. In addition, boundary lubrication, which arises from molecules adhered to the articular surface, is believed to be important for protecting cartilage. One such molecule named Lubricin has been isolated from synovial fluid and shown to affect friction in joints [104,105,121-131]. It is produced by synoviocytes, and related molecules are produced by chondrocytes in the superficial zone of articular cartilage [132]. Other evidence suggests that

surface-active phospholipids may act as boundary lubricants of articular surfaces, and that they may be part of the lubricin molecule [15,133,134]. It is likely that TE cartilage will need a source of boundary lubricants for long-term survival. Since shear upregulates production of boundary lubricating molecules in native tissue, it may be necessary to apply shear to engineered constructs during their development [135-138].

2.3 Sample preparation

Sample preparation is an important and potentially time-consuming process. Confined and unconfined compression, torsional shear, and tribological tests typically use cylindrical samples with parallel circular faces. Cylindrical samples can be obtained using a coring tool or biopsy punch. Getting parallel faces often requires shaving the surface using a microtome. Since samples are inherently thin, typically 1 to 3 mm for human articular cartilage, freezing or adhesive is needed to hold them in the microtome. In tensile tests it is necessary to achieve a region of uniform tensile stress. Roth and Mow developed a dog-bone shape for this purpose, but it was nearly 23 mm long, which is longer than typical TE cartilage constructs [12]. In contrast, for indentation tests, only minimal sample preparation is required. An osteochondral specimen can be cored or a whole small joint can be tested without additional preparation.

3. Biological and biochemical considerations

In addition to mechanical evaluation, biochemical and imaging evaluation of the engineered cartilage can be revealing. Biochemical evaluation is generally performed on homogenized tissue, resulting in average values for the test sample. Imaging approaches are exemplified by histological assessment and scoring. This approach provides a more localized look at the tissue and may provide visual correlates to the depth-dependent or anisotropic properties described above. Histochemical and immunohistochemical approaches can provide spatial distribution of the cells and extracellular matrix ECM components. Quantitative three dimensional tissue properties can be obtained using morphometric approaches, but unless an entire sample is serially sectioned, 3-D extrapolations from two-dimensional sections carry considerable uncertainty due to cartilage tissue inhomogeneity even over very short distances. In the past couple decades, three-dimensional imaging techniques including MRI, micro-CT, bioluminescent imaging (BLI), and ultrasonography have provided a more comprehensive look. The tradeoff is that the spatial resolution of these techniques is generally much less than with light microscopy. A further consideration which can dictate the choice of approach is that most of these are inherently destructive end-point tests, whereas some, *e.g.*, BLI, MRI, or ultrasound can potentially be performed on live samples as a part of a quality control program to develop release criteria.

3.1 Biochemical composition

The composition of cartilage has been characterized at a number of scales. At the highest level, the overall composition of cartilage has been characterized by, *e.g.*, Anderson as: water 70-85%, mineral salts 4-7%, and organic substances 10-15% [139]. More detailed characterizations of the “organic substances” compartment reveal an extraordinarily complex tissue. For example, in native cartilage, composition varies in a depth-dependent fashion between the surface layer and the calcified cartilage layer adjacent to the subchondral bone. Variations in composition give rise to variations in other properties; thus, there is a positive depth-dependent correlation between proteoglycan (PG) concentration and stiffness, and a negative correlation between PG content and permeability [92,140,141]. A recent review of the composition of normal and OA cartilage provides ranges of normal values for the major constituents of the tissue [142]. In TE cartilage, the composition can vary as a function of the distance from the free surface of the construct as a result of mass-

transport limitations. Although this review focusses on articular cartilage and cartilage TE for joint repair, the constituents of cartilages from various anatomical sites (*e.g.*, ear, trachea, *etc.*) varies considerably [63].

3.1.1 Water content—Bulk water content (~ 70-85%) can be determined by from the weight difference between a fresh sample and the same specimen after freeze-drying. The T_1 relaxation rate using MRI might be used to obtain a more localized assessment of the water distribution [143]. This provides a useful point of comparison between TE and native cartilage. In native tissue, mechanical properties correlate with water content; with higher water content, permeability increases, while stiffness decreases [144].

3.1.2 Mineral content—Mineral content (mostly calcium salts) is generally given in the 5% range for articular cartilage, however in the zone of calcified cartilage adjacent the subchondral bone it can approach 30% [145]. It has been postulated that mesenchymal stem cell (MSC)-based chondrogenesis is an analog of the early phases of fracture-healing; the tendency of these cells to undergo hypertrophy and possibly mineralization late in differentiation is consistent with this line of thinking [146,147]. Therefore assessing mineral content of TE cartilage should be considered. Global mineral content can be estimated destructively by ashing or by dedicated colorimetric calcium assays, more localized approaches include alizarin red stain for calcium, von Kóssa stain for (usually calcium) phosphate or carbonate, and backscatter electron imaging [145,148-150].

3.1.3 Organic substances

3.1.3.1 Cells: The cellularity of a tissue sample can be determined by several methods. Values in the literature for normal cartilage vary widely between species and anatomical location, in the range of 14,000 to 330,000 cells per mm^3 [22,23]. Classical histomorphometry can be used to determine the number of cells in a reference volume. To be accurate, this requires strict adherence to stereological precepts, and can be quite time consuming [151,152]. However, if information about the regional distribution of cells in a tissue sample is needed, this may be the best approach. Vital stains, *e.g.*, fluoresceine diacetate/propidium iodide or calcein/ethidium homodimer can be used to determine cell viability and distribution of the cells throughout the tissue [153].

An estimate of bulk cellularity can be obtained by measuring DNA content as described previously [154]. In this approach, the tissue is digested with papain [155], and total DNA content is measured using a dye-binding assay. The assay is obviously destructive. Non-destructive bulk assays include measures of metabolic activity, such as resazurine (Alamar blue) or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays, glucose or O_2 consumption, *etc.* [156-159]. It should be noted that using these assays strictly as a measure of cell numbers is risky. Results can be influenced greatly by changing metabolic activity of the cells, diffusion of the salts into, and the dye product out of the tissue, *etc.,* and thus should be used circumspectly.

Other cell behaviors are relevant to tissue quality, including apoptosis and chondrocyte “cloning”, the latter a histologic feature of articular cartilage degeneration in OA that refers to clusters of chondrocytes formed by clonal expansion of the cell in its lacuna [160]. Similar behavior can be seen in TE cartilage [161,162].

In mature and differentiating TE cartilage, cell proliferation is generally very slow. In, for example, MSC-based cartilage TE, it is customary to start the process with a solid mass of cells (see, *e.g.*, aggregate culture). Cell density in these types of culture and in scaffold-based TE is very high, on the order of 80 to 100×10^6 cells per ml, then drops over time [163]. Changes in cellularity are largely due to cell death and/or increases in ECM synthesis,

which reduce the volumetric density of the cells. In TE cartilage, regions distant from the free surface tend to be metabolically challenged, make less ECM, and therefore can be cell-rich and ECM-poor compared to the more differentiated surface regions [98,119].

3.1.3.2 Glycosaminoglycan (GAG) content: GAGs constitute a major component of cartilage ECM. GAGs consist of long polysaccharides, composed of a repeating disaccharide, in N- and/or O-sulfate groups and are typically unbranched. Cartilage GAGs include chondroitin sulfate (CS, 60-90%), keratan sulfate (KS), hyaluronic acid (HA), dermatan sulfate, heparin, heparan sulfate. Most cartilage GAGs are covalently linked to a core protein to form a bottlebrush-like structure (proteoglycans, PGs). PGs account for 5-10% of tissue wet weight in normal cartilage [142]. HA stands out, as it is neither sulfated nor covalently attached to a core protein; it does, however, complex non-covalently with PGs in the ECM. GAGs are highly negatively charged molecules, which contribute to the low compressibility and the lubricity of cartilage. In solution, GAGs occupy a large volume, but in cartilage, their expansion is constrained by the collagen fibril network resulting in a swelling pressure in the tissue [164]. Thus, adequate GAG content is likely required for satisfactory function of TE cartilage *in vivo*.

The GAG content of native or TE cartilage lysates can be determined using dye-binding assays. Sulfated GAGs can be measured using 1,9-dimethylmethylene blue. The binding of the sulfated GAGs to the dye induces a metachromatic absorption spectrum shift proportional to the amount of sulfated GAGs; which can be quantified spectroscopically. The assay detects sulfated GAGs in aggregate, but not the unsulfated HA. Sulfated GAG content makes up 1 to 10% of cartilage wet weight, however it varies with age, joint, position in the joint, depth relative to the surface, health of the cartilage, and proximity to the cells [165-167]. The safranin-O dye-binding assay of Carrino *et al.* detects all GAGs including HA [154,168]. Proteoglycan content can also be normalized to, *e.g.*, tissue mass, DNA or protein content.

In lysates, the spatial information is lost. GAG can be localized histochemically using Safranin O or Toluidine Blue O staining (see below). Histological and biochemical assessment of GAG content do not necessarily correlate. Orth *et al.*, for example, reviewed several histological scoring systems (see below) and found poor correlation between the scores and GAG content determined by biochemical methods [169]. Thus, the two approaches are complementary. Establishing a fingerprint of the ECM composition may be useful in evaluating TE cartilage against normal cartilage. For example, the ratios of CS to KS change as a function of age and in disease [170,171].

Specific antibodies against core proteins and antibodies against saccharide patterns can be used to identify PGs by immunohistochemistry or by blotting [172-180]. This type of analysis is rarely done in TE cartilage but should be considered as it has led to important observations. For example, lumican, a small leucine-rich glycoprotein is overexpressed in TE cartilage and may be responsible for the low collagen deposition [84]. Although not to our knowledge used in cartilage, biotinylated HA binding protein can be used to probe for HA in tissue [181-183]. Other methods for Glycoprotein and proteoglycan analysis include LC-MS and FACE gel electrophoresis. This exceeds the scope of the present review, but see, *e.g.*, Estrella *et al.* [184].

3.1.3.3 Proteins: Proteomic analysis has identified nearly 200 proteins in cartilage ECM [185,186]. The composition varies regionally and zonally, which should be considered if comprehensively evaluating TE cartilage [187,188]. Collagens make up 60 to 70% of the dry weight of cartilage with type II being predominant [142]. Other collagen types are present in smaller amounts (Reviewed in [189]). Collagen architecture varies through the

depth of the tissue. In TE cartilage collagen content is generally 50% or less than what would be expected, which has implications for tensile strength and load-carriage [84-89]. For a more detailed overview of the composition of normal cartilage, including, minor protein components, growth factors, cytokines, and proteases, see [190].

3.2 Histological Evaluation

3.2.1 Commonly used staining protocols for cartilage—Schmitz, *et al.* recently reviewed the most common methods of histopathological assessment of cartilage, with detailed protocols [191]. Detailed protocols, including for immunohistochemistry (IHC) or *in situ* hybridization can also be found in An and Martin [192]. The techniques are applicable to TE cartilage, particularly after implantation.

Overview stains include the use of India ink to stain for surface imperfections, and standard Hematoxylin – Eosin (H&E) stains [193,194]. Staining approaches such as Safranin O and Toludine Blue O highlight GAG deposits efficiently. Fixation and decalcification lead to proteoglycan loss; if a more quantitative assessment is desired, a cationic dye should be included in these solutions [195-197].

Less frequently used stains include tartrazine, van Gieson, and the Masson and Goldner trichome stains. Picrosirius red F3B (Direct Red 80) stains collagen fibers, as originally described by Puchtler *et al.* [198]. Under polarized light, collagen fibers exhibit a bright birefringence that is specific for collagen [199]. The method has been used on articular and on TE cartilage and provides information on structural anisotropy [200].

3.2.2 Immunohistochemistry—IHC-qualified antibodies for many ECM constituents are available through collaborations or from commercial vendors or repositories. Protocols vary by antibody and target, and this discussion exceeds the present review. A common feature of IHC in both native and TE cartilage is the need for unmasking by digestion of the tissue sample using a protease or lyase, or some other form of antigen retrieval. See also An and Martin [192].

3.2.3 Histological scoring systems—Histological quality is an important outcome measure in evaluating the success of TE cartilage. The staining protocols above provide a subjective assessment of tissue quality as well as morphological, localization and distribution information. Scoring systems derive a global scalar value by assigning numerical values to various combinations of assessments of cell morphology, matrix staining, surface regularity, structural integrity, thickness/defect filling, osteochondral junction, adjacent bonding, basal integration, cellularity, clustering/distribution, adjacent cartilage degeneration, mineral, blood vessels, subchondral bone, viability of the cell population, inflammation, and cartilage plug quality. Scoring systems for pathological cartilage have been in use since Collins and McElligot developed a macroscopic classification of OA cartilage in 1949 and Mankin developed the Histopathological-Histochemical Grading System (HHGS) in 1971 [201-203]. Scoring schemes for TE cartilage have been suggested and in part validated by a number of investigators and task forces. Commonly used ones include the scoring system of O'Driscoll *et al.* and the “Bern score” for TE cartilage [204]. The HHGS and OARSI scores, or variants thereof, are currently most frequently used for OA cartilage. O'Driscoll *et al.* have published scoring systems for repair cartilage and these scoring systems have continued to be refined including in Pineda, *et al.,* Wakitani, *et al.,* Peterson, *et al.,* and the ICRS II scores [68,205-207]. The Bern score has been used to assess TE cartilage in aggregate culture [208-212]. Histological scores have been used to validate novel cartilage quality analyses by imaging methods [213-216]. Observer bias can be reduced by having the sections examined by multiple,

blinded investigators, alternately, computer-assisted scoring systems are under investigation [217]. Importantly though, where cross-validations have been attempted, biochemical assessments of tissue quality did not all correlate well with histological scores, thus these remain complementary approaches [169]. The scoring systems have recently been reviewed extensively in Rutgers, *et al.*, who also suggest an algorithm for selecting the most appropriate scheme for the tissue at hand [218].

4. Destructive *vs.* non-destructive testing of tissue – implications for implantation release criteria

Decades of work have demonstrated that cartilage-like tissue can be grown in the lab and can be implanted, but we lack implantation release criteria, which predict success of an implant *in vivo*. Implanting an inadequate piece of tissue into a joint, where it must immediately withstand repetitive loads of several times body weight results in catastrophic failure [219]. Gold-standard histological or biomechanical assessments are described above, but these tests are generally destructive, and a construct tested by such methods is no longer suitable for implantation [12,13,44,47,48,50,90,92,96,220-222]. Endpoint evaluation could be performed on redundant test samples, but limited cell availability, cost, and time considerations do not make this a viable high throughput approach [47,48]. As a result, the specific TE construct to be implanted is not usually evaluated before surgery, creating an unmet need for non-contact, non-destructive quality control methods.

Cartilage can be characterized non-destructively in multiple ways. These include, *e.g.*, magnetic resonance imaging (MRI) or micro-CT techniques, which can be used to visualize GAG concentration and water distribution, bioluminescent imaging (BLI), which can image cellular processes, mechanical NDT, and ultrasound (US) [223-225].

4.1 MRI techniques

MRI can be used effectively to gauge cartilage thickness *in vivo*, which will likely have applications for implanted TE cartilage [226-228]. The GAG component of cartilage can be imaged using delayed gadolinium-enhanced MRI of cartilage (using Gd-DTPA^{2-}, dGEMRIC) by T_1-relaxation time measurements after penetration of the gadolinium contrast agent. The approach exploits the fact that mobile ions will distribute in cartilage tissue to reflect the local GAG concentration, as GAGs carry abundant fixed negative charge. More of the gadolinium agent distributes into areas with low GAG (or that are deleted of GAG) than in areas of high GAG concentration. T_1 images then reflect the tissue GAG concentration. The approach has been used in native cartilage, repair cartilage and tissue engineered cartilage [229-231].

4.2 Micro-CT

Cartilage signal by micro-CT is weak, as cartilage does not attenuate X-rays very well. However, contrast enhanced micro-CT using a gadolinium probe has shown promising results on cartilage explants and could be applicable to TE cartilage [232].

4.3 Mechanical NDT

Methods developed for mechanical evaluation of cartilage *in vivo* could, in principle, be adapted for evaluating TE cartilage at any stage in its development [233-237]. Most often, these methods are based on indentation, are implemented arthroscopically, and out of necessity are performed under sterile conditions, which is highly desirable for TE applications. Indentation properties are estimated from the known indentation depth, and applied force. The depth of indentation is controlled by the design of the tip of the arthroscopic probe or is derived from cartilage thickness measured ultrasonically. Force is

measured using a load cell that is integral to the arthroscopic instrument. These methods might be best suited for use as a quick screening tool rather than final release criteria since they are not able to identify internal inhomogeneities, will give average properties of a construct, and do not give the intrinsic biphasic properties of the tissue.

4.4 Direct US measurements

Among NDT methods, ultrasound (US) has been used to evaluate native, degenerated and tissue engineered cartilage. Many investigations have focused on correlating cartilage health with acoustic properties. For example, US speed of sound (SOS), attenuation, reflection coefficients, and US roughness indices has all been shown to correlate with cartilage quality [238-242]. Wavelet analysis has been used extensively to evaluate characteristics of cartilage using reflected US signals (signal intensity, echo duration, and interval between signals of cartilage), and, like other approaches, differences in cartilage quality can be identified [243-246]. However, it has been suggested that the simpler time domain methods (*e.g.*, reflection coefficients and roughness indices) may be as effective in predicting tissue quality as the more computationally-intensive frequency domain methods such as wavelet analysis [241]. Acoustic measurements have also been correlated with mechanical properties through combined US and mechanical measurements [236,247,248]. However, although theoretically possible, mechanical properties of cartilage have not been determined directly from reflected signals and estimates of the physical properties of a tissue.

Models of wave propagation have established mathematical relationships between speed of sound, stiffness, and density of a material. Experimentally, SOS can be measured, density can be estimated, and therefore stiffness can be determined. We previously tested the feasibility of using US to estimate cartilage mechanical properties quantitatively, on hydrogel surrogates. Processing ultrasound SOS measurements using a poroelastic model for wave propagation, we were able to estimate mechanical properties of the gels accurately and rapidly [249]. Currently, we are working to extend this approach to cartilage and TE cartilage. We have also observed that TE cartilage is acoustically inhomogeneous, while healthy native cartilage is essentially homogeneous at the wavelengths typically used in US. This observation is consistent with histological results, which has led to an M-mode imaging approach that is currently under investigation [250]. Although qualitative, M-mode imaging may provide a simple method for following the development and eventual release of TE cartilage for implantation.

4.5 Analysis of Chondrogenic Differentiation

Under appropriate conditions, MSCs undergo chondrogenic differentiation within a few weeks. Monitoring the progression of the cells along the chondrogenic lineage can be important in many types of experiments. The expression of cartilage markers can be used as evidence of the chondrogenic differentiation. For example, constructs initially contain type I collagen and no cartilage-specific molecules but the cells rapidly produce abundant ECM containing cartilage-specific markers such as type II collagen and aggrecan. In TE cartilage, lineage progression beyond the “hyaline” phenotype to a hypertrophic phenotype is also a consideration [146,251,252]. Such markers can be assessed at the gene expression level by harvesting total RNA from the developing tissue. Parenthetically, the abundant ECM makes it almost impossible to obtain good RNA yields after a few days of differentiation using affinity column-based kits; TRIzol yields better results. As described above, histologic and biochemical analyses can also be used to track differentiation markers. All these tests are destructive.

Currently, there are very few effective non-destructive ways to track the differentiation of stem cells. Marker genes can be selected for their association with a differentiation event.

These can be used to monitor stem cell differentiation by creating a reporter system, in which regulatory elements from the marker gene are used to drive the expression of a reporter gene. The best studied of these approaches uses the Type II collagen promoter as driver (reviewed in [186] for chondrocytes, but equally applicable to MSC-based TE). Other regulatory sequences have been studied in a similar manner, *e.g.*, aggrecan, [253]. A selection of cartilage-specific candidate genes could be derived from the literature [185,186]. These reporter constructs can be introduced into cells by transfection or infection [254]. The reporter gene expression can, of course, be assayed histochemically, by RT-PCR, or by *in situ* hybridization, but with an appropriate reporter, it can be imaged and quantified non-destructively (including *in vivo)* [255]. Luminescent reporter gene constructs can be used either as constitutive beacons to track cells, or as inducible reporters to monitor specific gene expression events. This approach has been demonstrated using transgenic mouse models that expressed green fluorescent protein, controlled by an osteoblast lineage-specific promoter to image osteogenic differentiation and bone development. These experiments required histological analysis [256,257], but using bioluminescent imaging (BLI) with luciferase reporter genes, it is possible to repeatedly image cells to monitor events in an *in vitro* or *in vivo* setting [258]. The latter approach is under development for chondrogenesis [259-262].

5. Conclusions and future directions

A host of methods have been developed for evaluating the mechanical, biochemical and biological properties of native cartilage across the whole spectrum from normal healthy to profoundly pathologic tissue. The properties of TE cartilage in most of these tests can be expected to fall somewhere along this continuum. Most of these assessment methods are therefore easily and meaningfully applicable to TE cartilage. Despite the richness of available assays for evaluating TE cartilage we really don't yet know what properties are essential for clinical success. For example, we and others speculate that “mechanical properties” are important to the success of an implant, but we don't know specifically what the critical properties are. For example – is it sufficient to know aggregate modulus alone, or must we know the shear modulus as well? Or, a TE construct could be mineralizing, in which case it would appear stiff in compression and shear, but have terrible tribological properties. Similarly, TE cartilage is notoriously collagen-poor. Is this an insuperable problem, or might it not really matter? Must we replicate the zonal architecture to create a durable implant? For a putative critical property, what also remains to be determined is, where along the continuum of values does TE cartilage have to fall to be good enough for implantation?

For practical reasons, once the critical properties have been identified, we feel that efforts should be made to develop and validate non-destructive and even non-contact assays for these properties. In this way, the exact piece of tissue that is intended for implantation could be subjected to rigorous quality control before being released to the operating room.

Acknowledgments

Supported by a grant from the National Institutes of Health P01-AR053622 (JMM & JFW).

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