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Nondestructive Techniques to Evaluate the Characteristics and Development of Engineered Cartilage

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Abstract

In this review, methods for evaluating the properties of tissue engineered (TE) cartilage are described. Many of these have been developed for evaluating properties of native and osteoarthritic articular cartilage. However, with the increasing interest in engineering cartilage, specialized methods are needed for nondestructive evaluation of tissue while it is developing and after it is implanted. Such methods are needed, in part, due to the large inter- and intra-donor variability in the performance of the cellular component of the tissue, which remains a barrier to delivering reliable TE cartilage for implantation. Using conventional destructive tests, such variability makes it near-impossible to predict the timing and outcome of the tissue engineering process at the level of a specific piece of engineered tissue and also makes it difficult to assess the impact of changing tissue engineering regimens. While it is clear that the true test of engineered cartilage is its performance after it is implanted, correlation of pre and post implantation properties determined non-destructively in vitro and/or in vivo with performance should lead to predictive methods to improve quality-control and to minimize the chances of implanting inferior tissue.

Introduction

Joint disease and cartilage defects are a major public health issue in countries with high life expectancies. Schulz *et al.* estimate that over 60 million people in the US will suffer from arthritis by 2020.^{1,45} Projections are for 572,000 primary total hip and 3.5 million total knee prosthetic joint replacements annually by 2030.^{3, 74} Tissue engineering is viewed as a promising approach for an alternate, biological repair or replacement of defective joint tissues. Based on the size of the potential patient population, cartilage tissue engineering has the potential of becoming one of the earliest and largest drivers of tissue engineering technology.

Tissue engineered (TE) cartilage can be viewed, in aggregate, as a laboratory-grown biomaterial to repair articular lesions. As such, it must be capable of withstanding all of the

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simultaneous environmental constraints experienced by native tissue. The consequence of applying joint-level conditions to tissue that is inadequate in any mode is failure.^{5, 42, 97, 101, 103, 104, 108, 138, 146, 153, 156, 167} A key obstacle to delivering reliable TE cartilage for implantation is the large (and still poorly understood) inter- and intra-donor variability in the performance of the cellular component of the tissue. This variability affects tissue engineering critical parameters, including the magnitude and timing of responses to growth factor treatment, the composition, physical properties and the timing of accrual of the extracellular matrix (ECM), and tissue metabolic rates, and thus sensitivity to mass-transport limitations. Put simply, from donor to donor, not all TE cartilage develops at the same rate, to the same endpoint, or with the same response to external cues. Within a given donor, variables such as degree of expansion of the cells also impact the outcome. Absent non-destructive evaluation, such variability makes it makes it near-impossible to predict the timing and outcome of the engineering process at the level of any one specific piece of engineered tissue. We believe that, in practice, TE requires understanding and predicting process failures. Importantly, if issues are caught early during the development stage, one could intervene to address them or decide to abandon a preparation to avoid implant failure and unnecessary costs.

Perhaps more importantly at this point in the evolution of cartilage tissue engineering, donor-based variability also makes it difficult and tedious to attribute the impact of changing TE regimens experimentally.

Commonly used evaluation approaches tend to be end-point tests that provide hindsight, but cannot easily be adapted to forecast tissue development towards a desired product. Ultimately, they are inconclusive since the tested sample becomes unsuitable for implantation. This leads to the inability to capture failures before clinical testing, as not all tissues mature at the same rate.⁹¹ Therefore, non-destructive technologies with predictive skill are needed for evaluating TE cartilage during development, and prior to and after implantation. The evaluation should be multimodal and multiscale, in real time, and (although this is not yet always possible) should not compromise the prospects for implantation.^{22, 96, 166} Comprehensively assessing TE cartilage, and indeed any engineered tissue, is an interdisciplinary undertaking, which requires expertise in subject areas such as molecular and cell biology, biomedical, chemical, mechanical, and electrical engineering, advanced imaging and computer modeling.^{22, 96}

Methods for evaluating engineered cartilage are described below. They span multiple size scales, from the whole tissue to the molecular level (Figure 1). Some have been applied to normal, osteoarthritic, enzyme treated, regenerated tissue and, to a lesser extent, to developing engineered cartilage in vitro. Although evaluation of developing TE cartilage has been limited, many of these methods are nondestructive and, therefore, they could, in principle, be extended to the evaluation of tissues in vitro. The cited literature is representative of the investigations on each area, but given the depth in each of these areas, it is not possible to cite all possible references. Some applications, output parameters, technologies, and tissue types studied are summarized in tables attached to the individual sections.

Mechanical Tests (Table 1)

Mechanical tests commonly used to evaluate cartilage include unconfined and confined compression, indentation, tensile, impact and tribological tests. Since these are well-established and have been described in numerous publications, they will not be reviewed here.^{40, 42, 94, 107, 138, 180} As these techniques and the resulting material properties have been used for more than thirty years, typical values are well-known and they are a “gold standard” against which engineered tissues may be compared. Historically, mechanical tests have been used to determine material properties in the lab, where issues such as contamination and potential mechanical damage as may be imposed in tensile or impact tests are not relevant. Because mechanical tests generally require physical contact with the specimen, it may be difficult, although not impossible, to perform them in a manner that preserves the tissue in a sterile state, which is essential if they are to be implanted. Thus, they may not be particularly suited to evaluating developing cartilage in vitro or in vivo.

In vivo, mechanical testing of cartilage using arthroscopic indenter probes is feasible and has been evaluated by several groups.^{18, 83, 90} This approach can be extended to cartilage implants and has the advantage of being able to compare the implant to adjacent host tissue. Limitations include calibration issues and the need to know the thickness of the material to determine stiffness meaningfully.¹⁶⁴

Ultrasound (Table 2)

Ultrasound (US) is an established method for non-destructive evaluation of materials and seems well-suited for in vitro evaluation of engineered cartilage. Acoustic properties such as reflection coefficient or attenuation have been used to characterize materials. Using ultrasound elastography strain fields through the thickness of a material can be determined, and inverse modeling can then be used to determine local material properties such as compressive modulus and Poisson’s ratio.^{30, 39, 43, 87, 124, 174, 193} This can be particularly valuable for characterizing tissue-engineered (TE) cartilage since, as noted above, it often develops non-uniformly through its thickness.

Acoustic properties, speed of sound (SOS), reflection coefficient (R), frequency dependent reflection coefficient, integrated reflection coefficient (IRC), ultrasonic roughness index (URI), attenuation and apparent integrated backscatter (AIB) have been used for characterizing normal, osteoarthritic, enzyme depleted and mechanically damaged articular cartilage, and to a lesser extent TE and spontaneously repaired cartilage.^{17, 47, 76, 77, 120, 129, 144, 175}

The reflection coefficient (R) is the ratio of the peak-peak amplitude of the reflected signal from the tissue-fluid interface and a fluid-air interface, that is, a reference amplitude. The frequency dependent reflection coefficient ($R_c(f)$) is the ratio of the frequency spectra of the signals used to compute R . The square of $R_c(f)$ expressed, in decibels, is the energy reflection coefficient ($R^{dB}_c(f)$), and the frequency-averaged integral of $R^{dB}_c(f)$ is known as the integrated reflection coefficient (IRC). Ultrasound has also been used to compute a cartilage surface roughness index (URI): the square root of the sum of the squares of the difference in the distance from the transducer to the cartilage surface relative to the mean

distance. Attenuation is characterized using three measures. Amplitude attenuation is

defined by $\alpha_{amp} = \frac{10}{h} \log_{10} \frac{A_1}{A_2}$ and integrated attenuation as $\alpha_{int} = \frac{1}{\Delta f} \frac{d}{df} \left(\frac{10}{h} \log_{10} \frac{A_1}{A_2} \right)$. The slope of the attenuation as a function of frequency is the broadband attenuation (BUA),

which is sometimes normalized by the tissue thickness (nBUA): $BUA = \frac{d}{df} \left(10 \log_{10} \frac{A_1}{A_2} \right)$. In these expressions, f is the frequency and A_1 and A_2 are peak-to-peak amplitudes of consecutive reflections and $A_1(f)$ and $A_2(f)$ are the corresponding spectra of the reflected signals. Another often-used acoustic parameter is the apparent integrated backscatter (AIB), computed as the integral, over frequency, of the apparent energy backscattered from the material. It is considered as a measure of features of sub-resolution scatters such as size, and acoustic impedance mismatch.^{6, 32, 47, 84, 117, 129, 140, 165, 170, 175}

Since changes in acoustic properties are indicative of changes in composition and morphology of cartilage, they have been used for evaluating engineered cartilage. For example, in surgically-created defects (4 mm diameter, 3 mm deep) filled with engineered cartilage R and AIB significantly decreased, while IRC and URI significantly increased when compared with intact cartilage close to the repair site.¹⁷⁰ In contrast, in the same study, surgical defects that were left to heal without an implant, only AIB had a significant decrease. A similar investigation using ultrasound to evaluate spontaneous repair of surgically created 6 mm diameter lesions that did not penetrate the calcified zone found that R was significantly lower in the lesion and URI was significantly higher when compared with intact tissue.⁷⁶

A different approach to quantifying the acoustic properties of cartilage, pioneered by Hattori and co-workers, uses wavelet maps to quantify reflected A-mode signals in terms of maximum magnitude and echo duration.^{56–59} In regenerated cartilage, wavelet analysis differentiated between preselected groups of surgically created lesions filled with hyaline-like engineered cartilage or fibrocartilage. However, only a weak correlation was found between histological grade and the maximum magnitude in this investigation.⁵⁸ In a related investigation of three different surgical models, maximum magnitude was significantly higher in spontaneous repair of a deep defect as opposed to superficial defects.⁵⁷

The investigations cited above have shown that acoustic properties can differentiate between native and engineered cartilage. However, acoustic properties alone are not indicators of the composition or mechanical properties of cartilage. There are, however, correlations between acoustic properties, and composition and mechanical properties. We used hydrogels as surrogates for cartilage to develop correlations between mechanical properties of cartilage and SOS, density and solid and fluid volume fractions.^{95, 173} Most such correlations have been identified in native, enzyme treated and osteoarthritic cartilage,^{69, 117, 129, 165, 175} but it is not unreasonable to suggest that such correlations will also exist for engineered cartilage. With respect to composition, proteoglycan, collagen and water content are significantly correlated with R, and SOS, IRC and URI.^{119, 129, 165}

Based on the idea that SOS is related to modulus and density, we have developed an approach to using ultrasound for evaluating the mechanical properties of TE cartilage while

it is contained in the sterile environment of a bioreactor.^{95, 173} Agarose hydrogels were used as surrogates for engineered cartilage since they can easily be made with consistent properties, and are considerably less expensive than engineered cartilage. We first showed that the aggregate modulus could be predicted from measured SOS and known gel concentration using a poroelastic model for wave propagation, i.e., the gel is modeled as having solid and fluid phases.¹⁷³ However, for a TE construct, the composition would not be known. Thus, we developed a statistical model that yielded an excellent prediction of modulus based solely on measured SOS.⁹⁵

The approaches described above give average mechanical properties of the material. However, it is well known that the properties of TE and native cartilage are depth-dependent. Optical methods have been developed for evaluating the depth-dependent properties of native cartilage, but as these use sections through the tissue, they are destructive.^{19, 20, 142, 143} Elastography, however, provides a promising approach for evaluating depth dependent properties of tissue in a bioreactor.^{35, 50, 102, 192} The potential value of US elastography was recognized by McCredie et al., who noted that the elastic properties of engineered cartilage were “reasonably depth-dependent.”¹⁰² We recently demonstrated the feasibility of using ultrasound elastography to evaluate depth-dependent deformation of engineered constructs in a sealed bioreactor.³⁵ We found that strains in interior regions were greater than those near the surface, which is consistent with the morphology of engineered cartilage: due to limits on diffusion, the interior of a construct often fails to develop cartilage-like tissue. Ultrasound elastography is dependent on the presence of acoustic inhomogeneities in the material that shift as the material is deformed. Therefore, a potential limitation of US elastography is that a tissue could be structurally, but not acoustically inhomogeneous.

Photoacoustics (Table 3)

Photoacoustic methods have several characteristics that make them attractive for evaluation of engineered cartilage. They are noninvasive and have been used to evaluate tissues *in vivo* and *ex vivo*, and, like ultrasound-based methods, are generally believed to be safe.^{53, 141, 160, 161, 186, 187}

Ishihara et al., were the first (to the best of their knowledge) to show that viscoelastic properties of material could be estimated using photoacoustic methods.⁶² This approach is based on the idea that the ratio of the viscous to elastic properties is related to the decay time of the stress wave. They used gelatin and tissue engineered cartilage to demonstrate the feasibility of determining viscoelastic material properties using photoacoustics. To establish feasibility, viscoelastic properties obtained from photoacoustic wave propagation were compared with those obtained from a “conventional measurement” of using a rheometer. Although light in the ultraviolet range was used, the investigators cautioned that this may not ideal for use with biological tissues.

This work was extended to include an in-depth evaluation of the viscoelastic and biochemical properties of tissue-engineered cartilage cultured from rabbit chondrocytes.⁶³ A strong inverse correlation (0.98) between viscoelastic properties ($\tan \delta$) and relaxation times

over a twelve week culture period was found. This suggests that the tissue was becoming more elastic and less viscous over the culture period. Relaxation time was also correlated strongly with biochemical composition: total chondroitin sulfate, the ratio of chondroitin 4 sulfate to chondroitin 6 sulfate, and total collagen. The ability of photoacoustic methods to identify a mechanical property and biochemical composition makes this a potential valuable non-invasive approach for evaluating the development of engineered cartilage prior to implantation.

The feasibility of evaluating engineered implants, *in vivo*, is suggested by photoacoustic tomography (PAT) imaging that has been used to evaluate normal and osteoarthritic cartilage.^{161, 185, 186} These investigations showed that PAT can identify joint structures as well as compositional differences between normal and osteoarthritic cartilage.

Optical Coherence Tomography (OCT, Table 4)

OCT has been used to evaluate normal and osteoarthritic cartilage and, to a lesser extent, regenerated or TE cartilage. However, the inherent characteristics of this approach suggest it has the potential to be a valuable technology for *in vitro* evaluation of engineered cartilage.⁹⁹ Like ultrasound, OCT is thought to be nondestructive. Although its depth of penetration, 1 – 2 mm, is small when compared to ultrasound or MRI, it is sufficient for evaluating the full thickness of cartilage on many joints and, for thicker cartilage constructs in a bioreactor, images can be formed from both sides of the sample, thus doubling the thickness that can be evaluated. Also, when compared with ultrasound, the axial resolution of OCT is better by one to two orders of magnitude. This places OCT between ultrasound (with lower resolution), and confocal and two-photon microscopy that have higher resolution.⁹⁹ OCT has been used to evaluate cartilage *in situ* through an arthroscope, and tissue explants.^{46, 99, 100, 113, 134, 162} In addition, OCT is sensitive to collagen orientation and fibrillation, which makes it well suited for evaluating osteoarthritic and repaired tissue.^{29, 34, 60, 92, 162} Using OCT areas of fibro-cartilage that are indicative of attempted but inadequate repair have been identified, although these might not be observed on visual arthroscopic examination of a joint.^{34, 60} Using OCT, differences in the ultrastructural features of regenerated cartilage, and the integration of host and regenerated tissue have been evaluated.⁴⁶

The speckle pattern in OCT images suggests that this approach could be coupled with elastography to determine internal deformation and mechanical properties of tissues. However, a PubMed search (June 2015) using the terms “elastography” “optical coherence” and “cartilage” produced only one review article suggesting that this could be used to evaluate cartilage.¹⁰⁰ In contrast, a search using the terms “elastography” and “optical coherence” resulted in 130 citations and searching “optical coherence” and “cartilage” produced 96 citations.

Reporter-based Imaging (Table 5)

Reporter gene-based imaging was developed to track molecular and cellular events.⁴⁵ Originally, reporter genes (e.g., luciferase) were driven by constitutively active promoters in the cells to be transplanted or implanted.⁸⁸ The imaging signal from luciferin acts as a

beacon, and is strong enough to be detected in large scale tissue constructs and even after implantation in small animals.⁸⁸ For medium or larger sized animals, a positron emission tomography (PET) imaging reporter can be used in place of luciferase for clinically relevant and quantitative live animal imaging.¹³² For example, mesenchymal stem cells (MSCs) implanted in a porcine model of degenerative intervertebral discs (IVD) to regenerate fibrocartilage¹³² have been reporter-gene transduced and then tracked by PET imaging. However, this type of imaging only demonstrates the targeted deposition and retention of the implanted cells. No information about the actions of these cells during repair or regeneration can be obtained.

For this, event-specific or tissue-specific reporters, rather than constitutive reporters are needed. For example, type II collagen (Col2) expression is an early event during articular cartilage development.⁵¹ Col2-GFP transgenic mice have been developed,³³ and a reporter gene with Col2 promoter-driven luciferase was developed⁸² to track chondrogenic differentiation with more experimental flexibility. Similarly, the transcription factor Sox 9 is a master regulator of MSC chondrogenic differentiation, and plasmids containing full-length or truncated Sox9 promoters have been developed. These have been used for studying campomelic dysplasia and autosomal sex-reversal; they can track Sox expression patterns during TE chondrogenesis.^{98, 18951, 66, 179}

An important aspect of cartilage TE is the balance between catabolic and anabolic signals in real time during the late-stage of cartilage formation or in newly formed tissue. Proteases and proteinases, such as the matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), play an important role in TE cartilage remodeling both pre- and post-implantation, and in many cartilage-related diseases. Among the MMPs, the principal neutral proteinases MMP-1, -3, -8 and -13 are of particular interest because they are capable of degrading native fibrillar collagens in the ECM.⁷⁰ MMP-13 promoter-driven reporters for imaging MMP-13 are available.^{106, 184, 191} Advantages of the MMP-13 reporter system are that destructive sampling can be avoided, and repeated imaging can be used to follow MMP-13 expression, which fluctuates during cartilage re-modeling. Similarly, reporters driven by the TIMP3 promoter are available.^{135, 182}

In the context of bioluminescent imaging (BLI) reporter systems, it is usually a lengthy process to identify and optimize a cartilage-specific promoter sequence because tissue-specificity usually depends on four to five enhancers interacting with (co-)activator(s) and co-repressors.¹⁶⁸ Non-coding RNAs hold great potential in diagnosis and treatment. Unlike promoter sequences, it is a potentially much simpler approach to use a miRNA-responsive reporter construct to track pathway regulation of chondrogenic differentiation, as this bypasses the need to validate which promoter/enhancer sequences or binding motifs to keep or cut. Furthermore, an imaging marker reflecting the miRNA expression can provide spatial localization as well as quantification. miRNAs from either synovial fluid or circulation have also been investigated in the process of chondrogenesis, and for disease diagnosis.¹¹⁰

In vivo reporter imaging devices, typified by the PERKIN-Elmer In Vivo Imaging Systems (IVIS) are capable of imaging fluorescent and bioluminescent reporters and fluorescent probes including fluorophores, fluorescent proteins, dyes and conjugates. The systems use

short cut-off filters and spectral unmixing algorithms to allow separation of multiple concurrent reporters. These imaging systems have the capability of tracking gene expression long-term after implantation; thus IVIS has been used to track implanted transfected mesenchymal stem cells for over 3 months.⁸⁹ Similarly, the infection of cells in-situ, using an intra-articularly injected AAV reporter has been demonstrated and could be monitored for up to a year.¹²⁸ Bioluminescent imaging (BLI) data can be co-registered with CT or MRI images to provide anatomical context, this is an integrated feature in newer IVIS systems.¹¹ IVIS has additional potential applications useful for evaluating the status of implanted TE materials. For example, Xie et al. have used hydrocyanine probes ⁷³ to measure reactive oxygen species produced in an OA model.¹⁸⁸ Further, Na et al. have demonstrated that IVIS can be used to monitor the sustained release of probe-conjugated drugs from implanted constructs over time (weeks).¹¹¹ In vivo, BLI provides fairly low anatomic resolution. For high (single cell) spatial resolution the BLI reporters can be replaced by fluorescent reporters to track cells within newly formed cartilage by two-photon imaging, or for histological identification by fluorescence microscopy.³⁷

Two-Photon Imaging (Table 6)

Two-photon imaging can identify labeled cells by imaging different fluorescent proteins (e.g., green fluorescent protein, GFP or red fluorescent protein, RFP) at the single cell level, thus overcoming the limited spatial resolution of BLI and PET/SPECT imaging. This bridges the gap between imaging and histological evaluation. As above (reporter-gene imaging), the cells can be labeled with beacon constructs or with event-specific constructs that allow tracking of differentiation steps, such as expression of specific genes.

Textural analysis of the ECM can also be utilized, as it has been applied to confocal images.⁶¹ Homogenous texture is characterized by high angular second moment, one of the 9 parameters for textural analysis based on Haralik's method.⁵⁴ In addition, principal components analysis can help to reduce the complexity of data-analysis for imaging collagen molecules, and to better quantitatively characterize type II collagen fibers or bundles of types I/II collagens relevant to cartilage quality evaluation.²⁵

Magnetic Resonance Imaging (MRI, Table 7)

MRI elastography (MRE) is also used to determine depth dependent properties of native and engineered cartilage.^{52, 55, 72, 86, 114–116, 190} Specialized pulse sequences are used to encode tissue deformation that are synchronized with externally applied loads or displacements.^{55, 114, 116, 190} Using MRE to evaluate developing TE construct requires specialized bioreactors, and the initial equipment cost is considerably greater than that for ultrasound systems. Operating costs (technician time, machine time, computer post-processing) for MRI are also greater than those for US, which is a potential disadvantage especially if a large numbers of samples are being evaluated. However, unlike ultrasound elastography, MRE can be used on tissues that are homogeneous, which could be an advantage as constructs mature.

MRI has been used to evaluate the biochemical composition (proteoglycans and collagen), cell density and water content in normal, osteoarthritic and regenerated

cartilage.^{48, 55, 72, 80, 85, 86, 114–116} GAGs are an essential part of the load carrying mechanism in articular cartilage and they are known to decrease in osteoarthritic cartilage. A potentially attractive feature of MRI for evaluation of TE cartilage is the ability to determine glycosaminoglycan (GAG) concentration using delayed Gadolinium Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC).⁹ dGEMRIC allows measurement of GAGs concentration since regions of high GAGs exclude gadolinium contrast agents during MR imaging.⁸

In engineered and repaired cartilage GAGs are typically lower than in native tissue, although they can increase to normal levels over long times.^{48, 181}

MicroCT (Table 8)

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.³⁶ Recently, the feasibility determining collagen orientation using micro-CT in conjunction with the labeling agents phosphotungstic acid and phosphomolybdic acid was demonstrated.¹¹⁸ The effectiveness of the labeling varied between human and equine cartilage.

The CT-based measurement of chondrocyte differentiation is possible using the clinically available CT contrast agent Hexabrix-320 (Mallinckrodt, Hazelwood, MO). Hexabrix contains ioxaglate, a negatively charged hexaiodinated dimer. After being preferentially absorbed by cartilage, it yields an equilibrium distribution, which can be measured by CT due to the radio-opacity of the contrast iodine that is inversely related to the density of the negatively charged sulfated GAG, similar to the mechanism of ionic GdTPA-2 used in dGEMRIC. Hexabrix has shown promising results with ex vivo or in situ imaging of animal joints using microCT to assess 3D cartilage composition and morphology,¹²⁵ and has been examined for use in detecting diseases associated with degeneration of articular cartilage.¹⁴⁸ The properties of the contrast agent and the mechanism of target binding for detection make this reagent suitable for use on extracted samples or tissue explants; in vivo imaging on live animal models remains challenging with this modality.

Nuclear Imaging (Table 9)

Challenges in applying nuclear and other imaging techniques to tissue engineering were summarized recently by Appel et al.⁴ The focus here is on imaging a specific target of interest. The radio-tracer, N-[triethylammonium]-3-propyl-[15]ane-N5 radiolabeled with ^{99m}Tc (^{99m}Tc-NTP 15-5), exhibits very high affinity for cartilage due to ionic interaction between its quaternary ammonium group and polyanionic glycosaminoglycans (GAGs) present in cartilage.¹²³ This tracer can be used for single photon emission computerized tomography (SPECT) imaging of GAG concentration and can be either a companion or alternative to dGEMRIC.

Uptake of another radiolabeled tracer, ^{99m}Tc-labeled glucosamine sulfate (GS), can be used as a direct and quantitative biomarker for stem cell derived chondrocyte function in cartilage regeneration via SPECT imaging.¹⁵² GS and chondroitin sulfate (CS) serve as substrates for

biosynthesis of GAGs. Because of transport issues with higher molecular weight hyaluronan, the lower molecular weight GS and CS are more effective tracers.⁷ Chondrocytes use GS for CS synthesis. Non-labeled native GS has therapeutic applications in osteoarthritis (OA) and radiolabeled GS was initially developed for scintigraphic detection of OA.^{150, 152} The uptake of radiolabeled GS is different from ^{99m}Tc-NTP 15-5, which binds to its target based on ionic interaction, charge does not play a major role for GS uptake, but facilitates charge-mediated diffusion of GS. Further investigation is required on the sensitivity and specificity of GS for use in assessing stem cell-based cartilage repair.

[^{99m}Tc]-RP805 is a highly sensitive MMP-targeted radiotracer that has been used in a murine model of post-myocardial infarction remodeling. It is designed for targeting MMP-2,¹⁵⁷ but actually has a greater affinity for MMP-13.¹⁵⁷ SPECT imaging of MMP-13 with this tracer has potential for imaging TE cartilage, and animal models of OA.

Spectroscopic Methods (Table 10)

Fourier transform infrared spectroscopy (FT-IR) and near infrared (NIR) spectroscopy have been used to evaluate tissue composition and collagen orientation in native, osteoarthritic, engineered and regenerated cartilage.^{1, 2, 10, 12–15, 23, 38, 68, 126, 131, 137, 154, 155, 178} The specificity and sensitivity of spectroscopic methods make them a potentially attractive approach for evaluating cartilage development. Using FTIR, for example, images of the distribution of tissue components can be made with a resolution of 6.25 μm .¹⁴ An in-depth review of FT-IR for evaluating native and engineered cartilage has been published by Boskey and Camacho.¹⁵ Here, we will focus here on applications of spectroscopic methods for the evaluation of engineered and regenerated cartilage.

The use of Fourier transform infrared spectroscopy imaging (FT-IRIS) to evaluate compositional and structural characteristics of regenerated cartilage was demonstrated by Bi et al.¹⁴ They showed that six months after microfracture in an equine model (knee) that PG is less than in surrounding native tissue and that, in general, collagen alignment does not follow the arcade-like structure of native cartilage. However, on the surface collagen appeared to be parallel to the joint surface, and had integrated with surrounding tissue.

FT-IRIS has also been used to evaluate PG and collagen content and distribution in TE cartilage.⁶⁸ In this case, cartilage was grown using chick chondrocytes in a hollow fiber bioreactor. Results showed that PG was greater near the inflow than outflow regions, while collagen was similar between inflow and outflow.

Near infrared spectroscopy (NIRS) is particularly attractive since it can penetrate the full thickness of engineered cartilage constructs, it is nondestructive and tissue composition can be determined in seconds rather than several hours as needed for biochemical analyses.¹⁰ Baykal et al, investigated the use of NIRS for evaluating matrix composition in developing engineered cartilage constructs.¹⁰ Using a broad spectral range (800 – 6000 cm^{-1} , 4 cm^{-1} resolution) showed that NIRS could be used to predict matrix development in engineered cartilage constructs.

Medium Analysis (Table 11)

We and others have reviewed the effects of medium formulations and optimization on in vitro chondrogenesis by stem cells.^{16, 21, 31, 41, 64, 93, 109, 127, 133, 177, 183} During TE, the culture medium provides key nutrients (oxygen, glucose), building blocks for ECM synthesis (amino acids), and signaling molecules which direct differentiation and ECM synthesis (TGF- β , FGFs, BMPs). Much information on the state of the cells can be derived from ongoing analysis of the culture medium during TE. Thus, deviations in the levels of nutrients and metabolites (e.g., lactate) can be predictive of the condition of the samples. Similarly, physiological rates (e.g., respiration rate) are critical for determining organism function.⁴⁴ Consumption or production rates for any molecule can be evaluated from mass balance by monitoring the medium outside of the tissue construct; monitoring the uptake/production of key biomolecules can provide dynamic, nondestructive, and minimally-invasive assessments of the status of the engineered tissue.^{49, 121, 127, 176}

Ultimately, the ECM produced by the cells determines the quality of the engineered implant. Cartilage development is characterized by gradual changes in the ECM's GAG composition and resulting PG architecture and the presence of specific collagen types.^{71, 130} Relative distributions of different GAGs in the ECM determines the type of cartilage, and can be predictive of its maturity and anabolic/catabolic and mechanical performance and durability.^{27, 28, 65, 67, 71, 75, 81, 105, 112, 139, 147, 149, 163, 171, 172} Generally, this is a destructive assessment of the ECM, however, methods have been proposed to non-destructively assess the ECM composition of engineered cartilage, including MRI and fluorescence spectroscopy coupled to ultrasound-backscattered microscopy.^{136, 159} These approaches estimate total GAG.²⁶ Interestingly, in culture, not all of the ECM components secreted by the cells are incorporated into the developing cartilage ECM.⁷⁹ Rather, a significant fraction of these structural molecules (and their processing enzymes) partition into the culture medium and can be quantified therein.⁷⁸ Thus, the maturity of the ECM and the chondrocyte's synthetic program can be assessed non-destructively over time by sampling and analyzing the culture medium.

Hybrid Methods

The methods described above have been combined or used with other technologies to enhance the evaluation of cartilage. For example, Sun et al. used time-resolved fluorescence spectroscopy (TRFS) and ultrasound backscatter microscopy (UBM) to simultaneously evaluate acoustic, mechanical and biochemical properties of normal and enzymatically degraded cartilage.¹⁵⁸ They found that TRFS was strongly correlated with collagen content and Young's modulus, while UBM was used to evaluate morphological properties. As these methods are nondestructive, they are potentially useful for evaluating engineered cartilage during development and prior to implantation. As mentioned above, BLI imagery can be co-registered with CT or MRI images for additional anatomical context.¹¹

Ultrasound has also been combined with OCT to enhance the evaluation of osteochondral and chondral defects.¹⁶⁹ US and OCT roughness indices, integrated reflection coefficients and apparent integrated backscatter (AIB and OBS) were computed. While there were

significant correlations between these two approaches, the better resolution of OCT provided “more reliable measurements of the cartilage surface integrity” while the greater depth of penetration of US provided more information about the inner structures of the regenerated tissue.

Summary

As described in this review, methods for evaluating a broad range of properties of native and osteoarthritic articular cartilage have been developed. To a lesser extent, these methods have been used to evaluate regenerated cartilage, and to an even less extent, developing tissue. However, with the increasing interest in engineering cartilage, methods are needed for nondestructive evaluation of tissue while it is developing and after it is implanted. A subset of the methods described here are applicable to nondestructive evaluation of developing tissues in vitro, and some can be used to monitor the state of the tissue post-implantation. Ultimately, the true test of engineered cartilage is its performance after it is implanted. In the future, correlation of pre and post implantation properties and performance will lead to predictive methods that should improve quality control and minimize the risk of implanting inferior tissue.

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Figure 1.

Approximate resolution ranges of the methods described in this review (in μm , log scale).

These methods span over 4 orders of magnitude

Table 1

Applications of traditional mechanical testing to the evaluation of native, engineered or regenerated cartilage

Output Parameter(s)	Technology	Tissue	Ref
• Compressive modulus / Permeability	• Unconfined compression • Confined compression • Indentation	• Normal equine cartilage	¹⁸
• Tensile properties	• Tensile test	• Bovine and rabbit TE cartilage	⁴⁰
• Friction wear and damage	• Tribological tests	• Rabbit engineered cartilage	¹⁸⁰

Table 2

Applications of ultrasound technology to the evaluation of engineered or regenerated cartilage

Output Parameter(s)	Technology	Tissue	Ref
<ul style="list-style-type: none"> • AIB • IRC 	<ul style="list-style-type: none"> • Acoustic microscopy • Evaluated ex vivo 	<ul style="list-style-type: none"> • Cartilage defects with cultured chondrocyte implants • Pig model 	47
<ul style="list-style-type: none"> • R • URI 	<ul style="list-style-type: none"> • US imaging • A-mode US • Evaluated in situ 	<ul style="list-style-type: none"> • Spontaneous repair of surgical defects • Pig model 	76
<ul style="list-style-type: none"> • Images • Dynamic mechanical modulus 	<ul style="list-style-type: none"> • US imaging • Mechanical testing • Evaluated in situ 	<ul style="list-style-type: none"> • Cartilage defects with cultured chondrocyte implants • Bovine model 	77
<ul style="list-style-type: none"> • Maximum signal magnitude from wavelet analysis 	<ul style="list-style-type: none"> • Wavelet analysis of A-mode US signals (Gabor mother wavelet) • Evaluated ex vivo 	<ul style="list-style-type: none"> • Cartilage defects, spontaneous repair or with cell based implants • Rabbit model 	57–59
<ul style="list-style-type: none"> • Strain and displacement through the thickness of the sample 	<ul style="list-style-type: none"> • US elastography • Evaluated in sterile bioreactor 	<ul style="list-style-type: none"> • TE cartilage constructs 	35
<ul style="list-style-type: none"> • Internal strain through the thickness of the sample • Elastic modulus 	<ul style="list-style-type: none"> • US elastography • Evaluated ex vivo 	<ul style="list-style-type: none"> • Stacked TE cartilage sheets 	102

Table 3

Applications of photoacoustic technology to the evaluation of engineered or regenerated cartilage

Output Parameter(s)	Technology	Tissue	Ref
• Viscoelastic material properties	• Propagation and attenuation of stress waves	• Chondrocyte seeded atelocollagen scaffold	62
	• Evaluated in situ	• Rabbit model	141
• Viscoelastic material properties	• Propagation and attenuation of stress waves	• Chondrocyte seeded atelocollagen scaffold	63
• ECM formation	• Evaluated in situ	• Rabbit model	

Table 4

Applications OCT technology to the evaluation of engineered or regenerated cartilage

Output Parameter(s)	Technology	Tissue	Ref
<ul style="list-style-type: none"> • Cartilage thickness, area of the repair tissue, surface roughness 	<ul style="list-style-type: none"> • OCT 	<ul style="list-style-type: none"> • Repaired goat cartilage in 3mm deep surgical defects 	²⁹
<ul style="list-style-type: none"> • Ultrastructure • Integration of repair and native tissues 	<ul style="list-style-type: none"> • OCT 	<ul style="list-style-type: none"> • Repair using cell seeded collagen matrix 	⁴⁶

Table 5

Applications reporter gene imaging for evaluation of native, engineered or regenerated cartilage

Output Parameter(S)	Technology	Tissue	Ref
• Live cell tracking after implantation	• BLI and PET imaging of triple fusion reporters (including <i>luc</i> and <i>tk</i>) transduced in MSCs	• MSC loaded ceramic cubes implanted s.c. in mice	88
• Live cell tracking after transplantation	• PET imaging of reporter <i>tk</i> transduced in MSCs	• MSCs transplanted in a porcine model of degenerative intervertebral discs (IVD) for fibro-cartilage regeneration	132
• Type II collagen (Col2) expression	• Fluorescent imaging of GFP driven by Col2 promoter	• Transgenic mice	33
• MSC chondrogenic differentiation	• BLI of <i>luc</i> expression driven by Col2 promoter	• Reporter transduced MSC pellets in bioreactor with differentiation medium	82
• IL-1 induced MMP-13 expression	• Luminometer reading of <i>luc</i> expression driven by MMP-13 promoter	• Human chondrosarcoma cell line	106
• Visualization of wound healing in live animal model	• BLI of <i>luc</i> expression driven by mouse MMP-13 promoter	• Transgenic mice	184
• Influence of PI3 kinase inhibitors and Akt siRNA on human TIMP-3 promoter activity	• Luminometer reading of <i>luc</i> expression driven by TIMP3 promoter	• Human knee articular chondrocytes	135

Table 6

Applications of two-photon microscopy to the evaluation of native, engineered or regenerated cartilage

Output Parameter(S)	Technology	Tissue	Ref
<ul style="list-style-type: none">Collagen contents, structure, orientation	<ul style="list-style-type: none">Two-photon	<ul style="list-style-type: none">Connective tissues freshly dissected from mice and other animals.	²⁴

Table 7

Applications MRI technology to the evaluation of engineered or regenerated cartilage

Output Parameter(s)	Technology	Tissue	Ref
• GAG concentration	• dGEMRIC	• Autologous chondrocyte transplantation	48
• Strain fields through cartilage • GAG concentration	• MRI	• Chondrocyte seeded agarose	114
• Shear stiffness	• Microscopic magnetic resonance elastography	• Cultured chondrocyte pellets	190

Table 8

Applications of microCT to the evaluation of native, engineered or regenerated cartilage

Output Parameter(S)	Technology	Tissue	Ref
• Proteoglycan content	• Contrast enhanced micro-CT using a gadolinium probe	• Bovine nasal cartilage	36
• Collagen orientation	• micro-CT with phosphotungstic acid and phosphomolybdic acid	• Equine cartilage	118
• Proteoglycan content	• microCT with contrast agent Hexabrix 320	• Joint tissue explant from 4-week-old calf • Postmortem femur from 3-month-old rabbit	125
• GAG contents	• Clinical peripheral quantitative CT with Hexabrix	• Normal and spontaneously degenerated adult bovine cartilage	148

Table 9

Applications radionuclide imaging for evaluation of native, engineered or regenerated cartilage

Output Parameter(S)	Technology	Tissue	Ref
• Polyanionic GAGs	• SPECT with [^{99m}Tc]-NTP 15-5 (binding to target GAG)	• In vivo (live human) cartilage	123
• Biosynthesis of GAGs	• SPECT with [^{99m}Tc]-GS (substrate for GAG synthesis)	• In vivo (live human) cartilage, OA	151, 152
• MMP-2, MMP-13	• SPECT with [^{99m}Tc]-RP805 (MMP ligand)	• Animal myocardium (with potential for animal models of OA)	157

Table 10

Applications FTIR and NIRS technology to the evaluation of engineered or regenerated cartilage

Output Parameter(s)	Technology	Tissue	Ref
<ul style="list-style-type: none"> PG content Collagen structure 	<ul style="list-style-type: none"> FT-IRIS 	<ul style="list-style-type: none"> Regenerated cartilage following microfracture Equine model 	14
<ul style="list-style-type: none"> PG content Collagen structure 	<ul style="list-style-type: none"> FT-IRIS FTIR spectral imaging 	<ul style="list-style-type: none"> Tissue engineered cartilage grown in a hollow fiber bioreactor 	68 131
<ul style="list-style-type: none"> Collagen orientation 	<ul style="list-style-type: none"> FT-IRIS Evaluated ex vivo 	<ul style="list-style-type: none"> Regenerated cartilage following microfracture Equine model 	12
<ul style="list-style-type: none"> Collagen and proteoglycan content 	<ul style="list-style-type: none"> NIRS 	<ul style="list-style-type: none"> Cultured chondrocyte seeded scaffolds 	10

Table 11

Applications of Medium analysis to the evaluation of engineered cartilage in culture

Output Parameter(s)	Technology	Tissue	Ref
• Uptake rates	• Biochemical analysis	• Engineered cartilage • Mesenchymal stem cells	122, 127
• ECM composition	• Biochemical analysis • MRI • Spectroscopy	• Normal cartilage • Engineered cartilage	26, 28, 65, 136, 159
• Partitioning of ECM components	• Biochemical analysis	• Engineered tissue	78, 79