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Mechanical Testing of Cell-Material Constructs

A Review

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1. Introduction

The exponential growth in basic research and clinical trials involving tissue-engineered materials has generated a corresponding need for the evaluation of the material properties and functional performance of these constructs during development and/or after implantation. Applications focusing on musculoskeletal tissues, in particular, require detailed assessment of the biomechanical properties of neo-tissue constructs in vitro and in vivo *(1)*. Based on the known properties of normal tissues, investigators have identified a range of biological, biochemical, and biophysical end-point parameters that must be quantified to determine the potential for success of a particular tissue-engineering methodology. Such end-point assessment is critical to our understanding of the basic scientific approaches underlying tissue engineering. In addition, biomechanical assessment is crucial for the implementation of regulatory processes that are coupled to clinical practice.

When creating musculoskeletal tissue constructs, it is important to determine whether the constructs are capable of withstanding the forces associated with locomotion in vivo, and whether construct properties compare to the corresponding native tissue *(1,2)*. In some instances, it is required that the construct should be bioabsorbable, and measurement of material properties may help to quantify the mechanisms and kinetics of biodegradability. For tissue-engineering approaches in which cells must re-synthesize a functional extracellular matrix (ECM) within a scaffold, the mechanical properties of the construct will indicate whether the native structure is being replicated *(3)*. The ability to quantify the intrinsic mechanical properties of tissue constructs is

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also necessary to compare alternative techniques used to synthesize specific tissues and to compare approaches used by different research groups. Finally, the ability to monitor the mechanical properties of implanted constructs *in situ* can help to evaluate the degree of successful repair of injured or diseased tissues and organs *(4)*.

1.1. Native Tissue Properties Motivate Construct Evaluation

Musculoskeletal tissues are composed of cells surrounded by a porous, hydrated ECM (including a mineralized phase in the case of hard tissues). Biomechanical characterization of such tissues must reflect a variety of material properties, including the equilibrium behavior of the ECM and the time-dependent viscoelastic and poroelastic behavior of the tissue following deformation. For example, articular cartilage is often modeled as a poroelastic or biphasic material *(5,6)* with a porous solid phase and mobile interstitial fluid containing ionic *(7)* and other solutes. The mechanical properties are dependent on the behavior of the solid phase—which may be modeled as intrinsically elastic or viscoelastic *(8)*—as well as fluid–solid interactions that may accompany tissue deformation, limited by matrix porosity and electrical charge effects *(6,7)*. These fluid solid interactions give the tissue increased stiffness to loads that occur at higher rates (higher frequencies) *(9)*, a property that is critical to functional behavior in vivo. Therefore, investigators who study the biomechanical behavior of tissue-engineered cartilage constructs look to these cartilage-like properties as hallmarks of the potential for success upon implantation *(10–12)*.

1.2. Characterization of Constructs In Vitro

Cell-seeded constructs for tendon, ligament, meniscus, cartilage, and bone are being studied with the use of a variety of cell sources (e.g., primary cells, cell lines, stem cells) cultured in natural and synthetic scaffold materials *(13–15)*. Motivated by the tissue type and desired properties, methodologies have been developed to quantify construct properties in compression (confined and unconfined), tension, and shear. Although destructive non-sterile measurement techniques can be used to advantage, several incubator-housed testing instruments have recently been developed. Such devices enable the investigator to measure the time-dependent evolution of living construct material properties over a period of days, weeks, or even months in culture. These instruments can also be used for mechanical stimulation of cell-seeded constructs as a means of improving the functional mechanical properties of the end product.

1.3. Characterization of Repair Tissue In Situ

The use of tissue engineered constructs for musculoskeletal applications in vivo has necessitated the development of methods for quantifying the func-

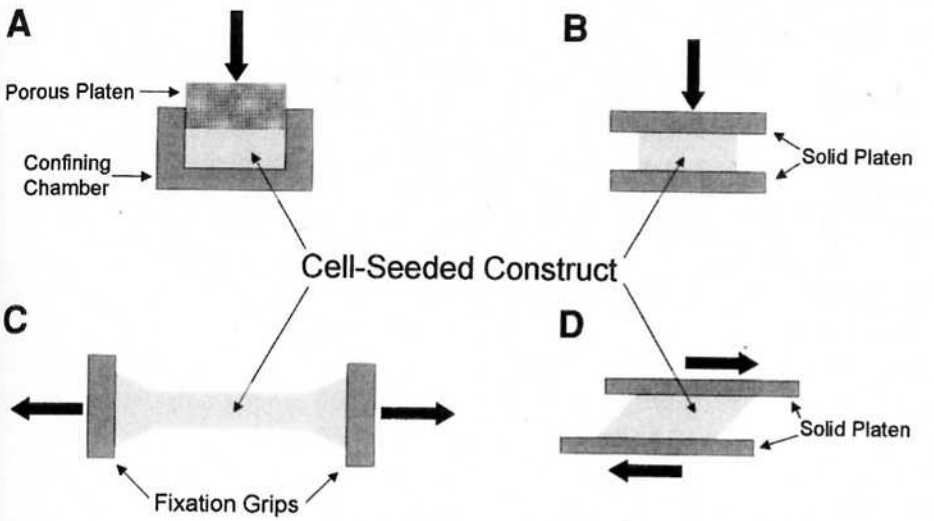

Fig. 1. Four testing configurations for measurement of intrinsic material properties of tissue-engineered constructs and cellular deformation in vitro: **(A)** Uniaxial confined compression. **(B)** Uniaxial unconfined compression. **(C)** Tension. **(D)** Shear.

tional biomechanical properties of the resulting implants as repair or unwanted degeneration ensues. After implantation into animals, it is often desirable to compare the properties of the repair tissue to those of adjacent normal tissue. Histological examination can provide valuable, qualitative information regarding the biochemical composition of the implant and tissue integration into the host. Non-destructive imaging modalities such as magnetic resonance imaging (MRI) can also provide compositional data during stages of construct development in vivo. However, it is extremely useful to have direct quantitative measurements of the biomechanical properties of the repair tissue that cannot yet be obtained by other modalities. Several new devices are now under various stages of development for direct *in situ* measurement of material properties, as summarized here.

2. Overview of In Vitro Biomechanical Evaluation

Upon implantation, tissue engineered constructs may be subjected to a complex physical environment. The objective of biomechanical testing in vitro is not to directly mimic *in situ* loading. Instead, mechanical tests utilizing compression, tension, or shear loading (**Fig. 1**) may be conducted *(2)* to establish the baseline intrinsic material properties of the construct (e.g., **Table 1** for cartilage). These values may be compared to those of native tissues to estimate whether the construct is suitable for implantation. The material properties of

Table 1
Mechanical Properties of Acellular and Chondrocyte-Seeded Constructs

Scaffold	Test mode	Time in culture	Modulus
Collagen-GAG Sponge *(22)*	Unconfined compression (equilibrium)	Acellular Type I Collagen Acellular Type II Collagen	145–730 Pa 730 Pa
Chondrocyte-seeded Agarose *(3)*	Confined compression (equilibrium)	0 d 70 d	20 kPa 150 kPa
Chondrocyte-seeded PGA *(20,21,30)*	Confined compression (equilibrium)	6 wk 7 mo	52 kPa 930 kPa
	Dynamic shear (frequency - 1 Hz)	7 d 56 d	0.8 kPa 15 kPa
PVA hydrogel *(25)*	Unconfined compression, (transient strain rate -1000%/min)	Acellular	1 MPa @ 10% strain 18 MPa @ 60% strain
	Shear (transient strain rate - 75%/min)		100 MPa @ 10% strain 450 MPa @ 60% strain
Scaffold-free monolayer *(28)*	Tension (transient strain rate - 12%/min)	8 wk	1.3 MPa

Values are summarized for examples of compressive, tensile, and shear testing in both equilibrium and dynamic modes.

various constructs may also be compared to evaluate the relative advantages of a particular scaffold material (*see* **Note 1**).

2.1. Equilibrium Biomechanical Properties

The equilibrium stress-strain behavior of constructs is determined by measuring the stress (load normalized to construct cross-sectional area) in response to an applied strain (change in tissue dimension normalized to the original dimension), or vice versa. Equilibrium properties may be evaluated by applying very slow ramps of load or displacement (e.g., at a low strain rate). Alternatively, a series of small increments in load (*or displacement*) may be applied, and the final steady-state displacement (*load*) attained after creep (*stress relaxation*) is used to compute the equilibrium stress-strain behavior. This stress-strain plot is used to calculate the equilibrium modulus. The simplicity of this testing protocol allows for measurements to be made using a simple-load cell and displacement system.

Constructs may exhibit an elastic region in which scaffold geometry is completely restored upon unloading. Native biological tissues are likely to be inhomogeneous and anisotropic, and may exhibit highly nonlinear stress-strain behavior. The initial deformation of tendons, for example, results in nonlinear increases in stress, the so-called "toe" region. The equilibrium stress-strain behavior beyond this toe region may be approximately linear, and is of interest in defining an equilibrium elastic modulus of the tissue—the slope of the linear stress/strain plot *(16)*. Similar behavior may be expected from cell-seeded constructs, although construct properties may be initially more homogeneous if cells are evenly seeded throughout the scaffold, especially at early stages of matrix deposition.

2.2. Dynamic Biomechanical Properties

Dynamic biomechanical measurements are important in characterizing construct response to periodic loading environments, such as that experienced by musculoskeletal tissues during locomotion. Thus, the rate or frequency of testing is motivated by physiological loading rates. The complex nature of dynamic testing requires more sophisticated instruments capable of feedback control of applied displacement or load. Sinusoidal, saw-tooth, pulse-like, or other waveforms are often used. Because of the poroelastic and viscoelastic properties of cell-seeded constructs, dynamic properties will depend on specimen geometry and testing conditions. In particular, dynamic properties are expected to depend on strain rate or frequency *(6)*. Rapid deformation also creates a proportional increase in hydrostatic pressure within a fluid-filled cell-seeded construct. In addition, the viscoelastic relaxation properties of the ECM are limited by rapid deformation, thereby increasing material stiffness. Test sample geometry may

also complicate the measurement of biomechanical properties. Cell-seeded constructs are often limited in size. As a result, clamping of the construct by the testing grips of the instrument can cause nonuniform strain distributions within the sample. Gardiner et al. *(17)* demonstrates an example of the effects of sample geometry on shear properties. Guidelines for optimal sample geometry are available from the American Society for Testing and Materials (ASTM).

2.3. Failure Testing

In addition to evaluating constructs in a non-destructive manner, failure testing may be used to identify the maximum load or strain that the construct may endure. For example, the strain at which a construct undergoes permanent deformation, and will not return to the original geometry upon unloading, is known as the yield strain, and the accompanying stress, the yield stress (or strength). Constructs tested in tension or shear may be deformed to the point when macroscopic fractures occur *(16)*, corresponding to the ultimate stress (or strength). Compressive ultimate strength testing is possible, but it is sometimes difficult to define failure, especially in softer tissues. Failure properties may be compared to the mechanical environment encountered in vivo in order to predict the structural stability of the implant.

Determining which failure parameter is the most relevant depends on the expected loading as well as the tissue surrounding the construct. For example, implantation of constructs into focal defects in articular cartilage can create an interface between native and construct materials with very different compressive stiffness. Without adequate integration at the interface, joint loading forces *(18)* can lead to failure at the interface, a very challenging problem for cartilage tissue engineering. Similarly, implantation of constructs for bone regeneration that occupy the entire cross-section of the bone must support total structural loading. Variation in construct strain can be predicted from applied stress. Construct failure analysis is based on the understanding of subfailure and failure properties of the material, utilizing the testing configurations outlined here.

3. In Vitro Biomechanical Methods

3.1. Confined and Unconfined Compression

Specimen geometry for compression testing (*see* **Notes 2–4**) is typically cylindrical disk or slab structures, with parallel surfaces to ensure even load distribution. Compressive testing is performed with samples held in a radially unconfined or confined geometry. In unconfined compression (**Fig. 1B**), samples are allowed to freely expand radially during uniaxial compression (*see* **Note 5**). Under ideal conditions, the slope of the measured equilibrium stress/

strain curve in the linear region gives the equilibrium compressive Young's modulus, E, of the construct. Specimen geometry is limited to a range of aspect ratios of sample height/width to prevent testing artifacts such as buckling.

Confined compression (**Fig. 1A**) requires specimens to be tested in a tight-fitting chamber to prevent any radial expansion. Typically, the specimen is compressed by a porous platen to allow free draining of the construct fluid at the platen-construct interface during compression (*see* **Notes 6,7**). Both the equilibrium-confined compression modulus, H, and the dynamic stiffness can be measured in this configuration. The dynamic stiffness includes contributions from hydrostatic pressurization within the construct associated with fluid-ECM frictional forces *(6,7)*. Extensive descriptions of methodological details are available in confined *(3,19,20–23)* and unconfined *(9,24,25)* geometries.

3.2. Tension

Tensile properties of constructs may be determined from both equilibrium and time-varying stress-strain measurements. The equilibrium Young's modulus, E, can be calculated from the linear region of the equilibrium stress-strain curve. Samples must be appropriately fixed within testing grips to prevent artefactual failure at the sample/grip interface. If the specimen size allows, test samples may be cut in a "dogbone" geometry (**Fig. 1C**) such that a large grip area relative to the working length *(26)* minimizes stress concentrations at the grip. Other fixation strategies are available for specific sample geometries (*16,27–29*). In all cases, failure of the sample within the working length is indicative of a properly fixed sample.

Tensile test sample lengths must be significantly greater than cross-sectional dimensions (*see* **Note 1**) to ensure uniform strain through the working length; *see* ASTM guidelines summarized in **ref.** *(2)*. Large working lengths may also minimize bending effects resulting from irregular samples or improper alignment in the testing apparatus. When a working length has not been defined, evaluation of strain must be representative of the working length. Extensometers, optical scanning, or other devices may be necessary to accurately evaluate strain in the region of interest.

3.3. Shear

Specimen geometry for shear measurements is similar to that for compression, in which flat, parallel surfaces are necessary for accurate testing. Samples are fixed between parallel platens so that shear deformation may be performed using rotational *(30,31)* or translational *(25,31–34)* displacement (*see* **Note 8**) (**Fig. 1D**). Translational displacements result in shear stress equal to the force normalized to specimen surface area. For rotational displacement, stress is calculated from the applied torque, sample radius, and surface polar moment of

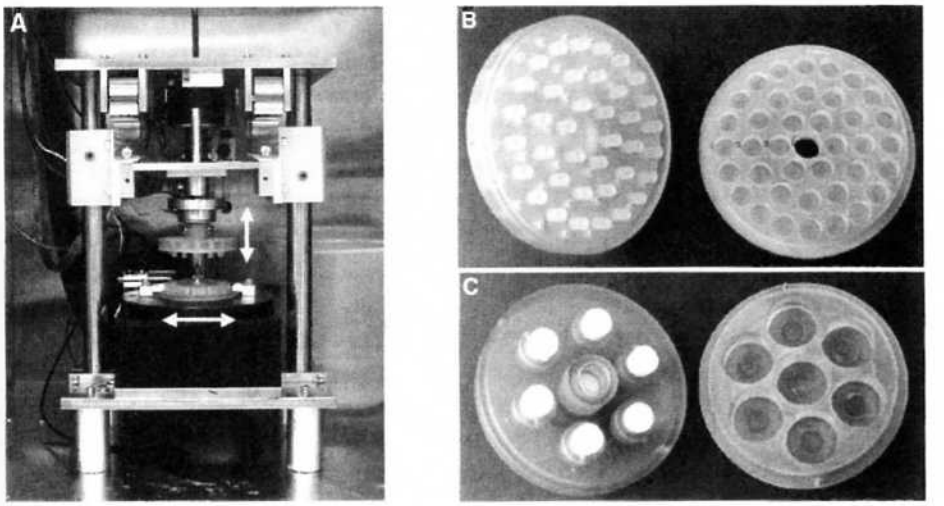

Fig. 2. **(A)** Example of an incubator-housed material testing instrument capable of measuring compressive, shear, and tensile properties, as well as studying the effects of applied mechanical loads on the development of tissue-engineered constructs ***(31)***. A testing chamber capable of loading 12 plugs in shear and/or compression is installed. **(B)** Loading chamber capable of testing or stimulating up to 38 samples in individual wells. Different well radii from the center allow three different levels of shear strain to be applied during a single loading event. **(C)** Chamber capable of compressive loading of up to six large cell-material constructs. A central spring ensures that the platen lifts off the samples during the unloading part of the cycle. Platens to compress the samples are porous to ensure adequate transport of feed media to center of constructs during prolonged loading.

inertia (e.g., *see* **ref. *32***), and shear strain is defined as the angle of deformation divided by the height of the sample. Both equilibrium and dynamic shear measurements are important for construct characterization. Under steady-state conditions, the equilibrium shear modulus G is calculated from the linear region of the stress-strain curve. The dynamic complex shear modulus, G*, includes the so-called storage (in phase) and loss (out of phase) moduli. For ideal, infinitesimal shear deformation, there is no fluid flow within the construct, and therefore, no fluid-solid frictional interactions. Thus, the dynamic G* reflects the frequency-dependent intrinsic viscoelastic properties of the ECM ***(34)***.

3.4. Biomechanics at the Cell and Nano-Molecular-Length Scales

Mechanical properties of cell-seeded tissue-engineered constructs are likely to be minimally influenced by the presence of cells. Cells typically occupy a

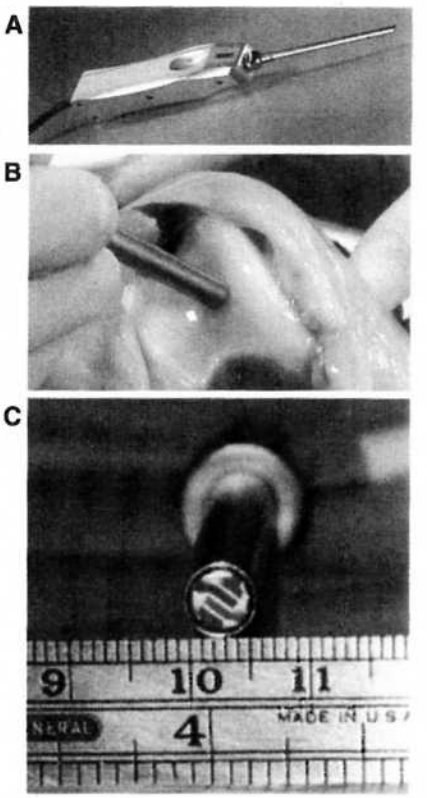

Fig. 3. **(A,B)** Devices capable of measuring material properties of constructs *in situ*. Artscan (Helsinki, Finland) probe capable of measuring cartilage compressive stiffness ***(4,49)***. **(C)** End view of sensors of surface electromechanical spectroscopy probe ***(51,52)*** capable of measuring impedance (electrical resistance) in cartilage. The impedance changes with tissue swelling and with changes in the content of charged GAGs. This probe is also capable of measuring electrical straming potentials and mechanical stress generated by a small electric currents (related to tissue content of GAG, tissue stiffness, hydraulic permeability, and other material properties).

small volume relative to overall scaffold geometry, and cell stiffness is typically low compared to that of the scaffold or newly synthesized. For example, a micropipet aspiration technique has been used to evaluate the elastic modulus of isolated chondrocytes, giving E ~ 0.6 kPa ***(35)***. Atomic force microscopy (AFM) indentation analysis ***(36)*** and magnetic bead rheometry ***(37)*** have provided values of fibroblast moduli E ~ 3–5 kPa and G ~ 20 kPa. In comparison, the moduli of cartilage ***(38)*** and ligament and tendon ***(39)*** are at least two orders of magnitude greater than that of the individual cells because of th

presence of ECM. Scaffold material properties vary, but are likely to be greater than that of the cells for practical handling. Therefore, material properties of cell-seeded constructs are likely to be dominated by accumulated ECM, with contributions from stiffer scaffold materials. Consequently, scaffold-ECM strain will be transmitted to the seeded cells in proportion to the deformation of the localized pericellular environment. Cell deformation has been visualized in chondrocyte-seeded agarose scaffolds. With no accumulation of ECM, compressive scaffold strains of 5–15% produced axial compression and lateral extension of the cells, changing cell morphology from spherical to elliptical *(40)*. Mechanical strain has also been observed to increase cell surface area and deform the nucleus of agarose-seeded chondrocytes *(41)*. These experiments illustrate the potential for regulation of biosynthesis in cell-seeded constructs by mechanotransduction. Static compression *(42,43)*, dynamic compression *(43)*, and dynamic shear loading *(44)* have been found to modulate ECM biosynthesis in cartilage explant culture. Static and dynamic compression is also a potent regulator of cell metabolism in chondrocyte-seeded agarose *(11,45)* and alginate *(46)*. Therefore, mechanical loading applied in vivo or during in vitro conditioning prior to implantation may be an important factor in the stimulation of an appropriate repair response using cell-seeded constructs.

3.5. Fatigue Testing

The mechanical tests previously described focus on testing of constructs in a nondestructive manner. However, in many instances it is important to know how a construct will perform over repeated loading cycles as well as the maximum stress it will bear before failure. Fatigue tests are common in the study of soft tissues and tissue replacements that are loaded in tension, such as tendons *(47)*. Fatigue during shear and compressive loading have also been addressed in detail *(34,47)*. A nominal target stress or strain in the physiological range is typically selected, and the sample is cycled between an initial state and the target value until rupture occurs. The number of cycles to failure is the fatigue life, and is usually dependent on the stress or strain applied each cycle as well as the frequency of loading (strain rate). If enough samples are tested using a range of target values, then a graph of "load vs number of cycles to failure" can be constructed (referred to as an S-N curve). This will allow researchers to predict the fatigue life of a tissue or construct given the expected loading regimen. Tissue failure for materials such as bone or bone substitutes may be obvious. For soft tissues, a clear definition of failure must first be identified. In cartilage constructs, for example, failure could be defined as the appearance of surface fissures. Tests to failure, rather than fatigue, are characterized by a single application of load at a desired strain rate, increased until failure occurs in tension, compression, or shear.

3.6. In Situ *Characterization of Graft-Repair Tissue*

The ultimate goal of tissue engineering is the implantation of the cell-material constructs into the body. If the construct becomes well-integrated with surrounding tissue and progressively achieves functionality like the original native tissue, it can be deemed a success. Assessment of the biomechanical properties of the evolving graft, as well as other measures (e.g., histological examination) is important for documenting the ultimate success of the repair. For clinical applications, there is a critical need for the development of nondestructive, minimally invasive biomechanical measurement techniques. In the case of bone-replacement constructs, X-rays can be used to evaluate trabecular structure. However, soft-tissue structure is not so easily imaged. Although MRI technology has advanced dramatically during the past decade, biomechanical assessment is not possible with this modality, and it is necessary to use minimally invasive contact methods for such *in situ* measurements.

Several indentation probes have been developed for clinical biomechanical assessment of cartilage during routine arthroscopic examination *(4,48)* (**Fig. 3A**). These probes are designed to characterize the mechanical stiffness of cartilage repair grafts and for the diagnosis of cartilage degeneration in osteoarthritis. The use of *in situ* indentation instruments for the estimation of the tissue's Young's modulus has recently been described *(49)*. Another probe that measures dynamic compressive and shear stiffness of cartilage has also been developed *(50)*. The choice of such a probe and the use of an indentation modality must be made with caution, since developing tissue constructs may not be able to withstand the force of indentation testing (*see* **Note 9**).

Other probes under development focus on the electromechanical and electrical impedance properties of tissue *(51,52)* (**Fig. 3B,C**). Tissue impedance is influenced by the concentration of charged molecules within the ECM (e.g., proteoglycans) and the tissue's porosity and water content, properties that change with construct growth, repair, and degeneration. Probe application of a small electrical current into tissue constructs may also induce a mechanical stress within the ECM that is measurable by the probe. The current induces intratissue fluid flow and micromechanical motions of the developing ECM, causing a current-generated stress that also depends on ECM charge density, hydraulic permeability, and mechanical properties. Multiple electrode contacts on such probes (**Fig. 3C**) allow current application at several spatial wavelengths across the construct surface, thus enabling evaluation of tissue properties at various depths into the tissue.

3.7. Summary

Material testing is a fundamental tool for evaluating the mechanical functionality of cell-seeded constructs with respect to development of neo-tissue,

or predicting structural stability when placed in a loading environment. The methods outlined in this chapter are designed to first allow the reader to select testing parameters that best represent the ultimate functional mode of the cell-seeded construct. Then, simple testing in compression, tension, shear, indentation, or electromechanical means may be performed to establish tissue stiffness and other physical properties associated with normal tissue function. Evaluation of mechanical properties *in situ* extends the characterization of construct development to environments in which ultimate failure or success will be determined. In this manner, cell-seeded construct development or degradation may be closely monitored at all time-points as an indicator of neo-tissue accumulation or remodeling in the cell/scaffold system. Diagnostic testing protocols may also be modified to apply non-destructive loading as a means of conditioning cell-seeded constructs in vitro. Cell signaling via mechanotransduction may be utilized to increase or modify biosynthesis, controlling ECM accumulation prior to implantation.

4. Notes

1. In vitro measurements may be performed using living tissue immersed in culture medium. Testing of previously frozen specimens requires the use of protease inhibitors to prevent degradation of the ECM during testing.
2. Micrometers or calipers may be used for dimensioning samples. However, accuracy may be compromised when samples are deformable. Feedback devices (e.g., resistance or voltage sensors) will help to identify when the measuring device is in contact with sample surfaces. The diameter of small cylindrical samples may be determined via a laser micrometer.
3. Sample thickness may also be determined using the testing apparatus. Zero strain can be defined by the position at which the testing platens produce a tare load in the specimen. Specimens must be completely immersed in appropriate medium or buffer for mechanical testing.
4. The upper platen may be fixed to the load cell if the weight of the platen affects the response of the sample to dynamic compression.
5. Unconfined compression: Test platens should be rigid and impermeable; low friction between specimen and platen will allow for appropriate radial expansion.
6. Confined compression: The upper platen must be porous, but should not deform during testing. Porous high-density or ultra-high mol-wt polyethylene (pore sizes of ~50–100 μm) are sufficient for most constructs.
7. Confined compression: Displacement control has an advantage over load control —stress relaxation is 4× quicker than creep. Although load control may mimic physiologic loading conditions, displacement and load control are equivalent for deriving intrinsic material properties.
8. For shear measurements, specimens are sometimes glued to platens to prevent slipping. However, glues are often toxic to cell-seeded constructs. Therefore, platens with a rough surface will be useful. In addition, a 5–10% static offset compression may be needed to grip the specimen.

9. Indentation tests may be needed for complex, in vivo tissue geometry (e.g., cartilage on bone in the intact joint). However, when using small-diameter indentors with non-ideal construct geometries, interpretation of time-dependent indentation data to derive intrinsic material properties may be difficult. The ability to remove specimens for in vitro testing is advantageous when possible.

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