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Microscale characterization of the viscoelastic properties of hydrogel biomaterials using dual-mode ultrasound elastography

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ABSTRACT

Characterization of the microscale mechanical properties of biomaterials is a key challenge in the field of mechanobiology. Dual-mode ultrasound elastography (DUE) uses high frequency focused ultrasound to induce compression in a sample, combined with interleaved ultrasound imaging to measure the resulting deformation. This technique can be used to non-invasively perform creep testing on hydrogel biomaterials to characterize their viscoelastic properties. DUE was applied to a range of hydrogel constructs consisting of either hydroxyapatite (HA)-doped agarose, HA-collagen, HA-fibrin, or preosteoblast-seeded collagen constructs. DUE provided spatial and temporal mapping of local and bulk displacements and strains at high resolution. Hydrogel materials exhibited characteristic creep behavior, and the maximum strain and residual strain were both material- and concentration-dependent. Burger's viscoelastic model was used to extract characteristic parameters describing material behavior. Increased protein concentration resulted in greater stiffness and viscosity, but did not affect the viscoelastic time constant of acellular constructs. Collagen constructs exhibited significantly higher modulus and viscosity than fibrin constructs. Cell-seeded collagen constructs became stiffer with altered mechanical behavior as they developed over time. Importantly, DUE also provides insight into the spatial variation of viscoelastic properties at sub-millimeter resolution, allowing interrogation of the interior of constructs. DUE presents a novel technique for non-invasively characterizing hydrogel materials at the microscale, and therefore may have unique utility in the study of mechanobiology and the characterization of hydrogel biomaterials.

1. Introduction

The importance of mechanical cues in the extracellular environment in directing cell behavior has been demonstrated conclusively over the past decade [1–3]. Mechanical signals combine with other cues, including soluble biochemical factors and cell–cell and cell-matrix adhesions, to regulate many important developmental, physiological, and pathological processes [4]. The realization that mechanical factors must be considered when seeking to understand cell function has led to an increasing interest in the field of mechanobiology, with an emphasis on determining how changes in matrix mechanical properties affect cell phenotype. However, the molecular mechanisms underlying mechanotransduction are not fully understood, in part because of the difficulty in robustly characterizing the mechanical behavior of biomaterials at the microscale.

Tissue engineering generally aims to enhance tissue regeneration through the use of cells and biomaterial scaffolds designed to mimic the properties of the extracellular matrix (ECM). It is well established that scaffold materials play a critical role in cell attachment, proliferation, and differentiation, and that their mechanical properties have significant effects on cell behavior [5]. Hydrogels are often used as scaffold materials because of their similarity to the ECM, cell compatibility, and ease of fabrication. These materials consist of water-swollen networks of cross-linked hydrophilic polymer chains derived from either natural, synthetic or hybrid materials [6], and have been used as biomimetic materials for skin [7], corneal [8,9], cartilage [10,11], and vascular [12] tissue engineering. Hydrogels have also been used to study cell-matrix interactions, including response to mechanical cues [13]. A key attribute of many hydrogel biomaterials is their viscoelastic nature [14], which mimics tissue behavior and has recently been shown to be important to the cellular response [15,16]. Therefore tuning and

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characterizing hydrogel mechanical properties are important when creating and maintaining engineered tissues. However, current methods for assessing material mechanical properties are generally limited by the need for invasive specimen preparation, difficulties in applying to soft and cell-seeded biomaterials, and the destructive nature of testing that precludes longitudinal studies.

Commonly used methods for measuring the mechanical properties of hydrogel biomaterials include uniaxial tensile testing [17], compression testing [18], and shear rheology [19,20]. In addition, more specialized testing using micro- and nano-indentation [21,22], bulge tests [23], magnetic force methods [24], and other contact-based approaches have been used to characterize the properties of hydrogel materials [25]. These conventional mechanical testing techniques typically deform a sample by stretching or compression applied directly to the sample by means of grips, platens or other physical fixtures [18,25]. Bulk material properties and stress–strain relationships can be generated to provide more insight into the material behavior, and time-dependent tests can be used to characterize basic viscoelastic properties. However, it is difficult to directly compare measurements obtained from different types of tests because the loading mode is different. For example, shear rheometry relies on tangential shear applied at the material surface (usually in torsion), whereas creep testing uses compression (or tension) to apply a force normal to the surface of the material. The values for the elastic and viscous components derived from these two orthogonal loading modes in soft materials are quite different. In addition, conventional approaches generally require physical contact with the samples, and are unable to determine internal strain distributions or spatial variation of the mechanical properties in materials.

Ultrasound techniques have the potential for non-invasive material characterization, because of their capability to penetrate and interact with cells and tissues at depth (recently reviewed in Ref. [26]). Ultrasound elastography [27] has been developed to detect tumors [28–30] by using ultrasound-generated images before and after tissue compression to derive information about sample stiffness [31,32]. In particular, acoustic radiation force (ARF) elasticity imaging techniques, such as acoustic radiation force impulse (ARFI) imaging [33,34], use the force associated with an ultrasound field to generate deformation within a body of material in a non-contact fashion [35,36]. While ultrasound elastography detects relative spatial variation of tissue stiffness, such as those caused by tumors, it does not generally provide a direct assessment of absolute material parameters or tissue viscoelastic properties.

An ultrasound technique called sonorheometry developed by Walker et al. [37]] for assessing blood coagulation [38,39] uses multiple ultrasound pulses to apply ARF to a sample, and measures the sample displacement over time. Relative elasticity and viscosity of the sample were derived by fitting the time–displacement curve with a Voigt model. Mauldin et al. [40] used a similar approach called monitored steady-state excitation and recovery (MSSER) to obtain displacement data before and after ARF application. These studies focused on non-invasively determining the relative mechanical properties of inclusions in tissues at depth. However, they worked solely with bulk displacements and did not provide information on microscale mechanical properties. In addition, these ARF-based techniques used the same ultrasound system for both force application and detection of displacement. While convenient, this configuration makes it difficult to generate the high intensity ultrasound pulses needed to generate sufficient ARF for tissue compression. Therefore for maximum performance, it is preferable to have separate deforming and imaging beams to optimize the characteristics of each type of ultrasound beam.

The goal of the present study was to demonstrate a versatile technique capable of non-invasive characterization of the viscoelastic properties of hydrogel biomaterials both in the bulk phase and at the microscale. We developed a dual-mode ultrasound elasticity (DUE) technique that uses focused ultrasound (FUS) pulses to induce compression in samples in conjunction with a co-linearly aligned high frequency ultrasound imaging system to detect sample deformation as a function of time and location at high resolution. The use of separate transducers for compression and detection provides the design flexibility to control loading conditions. We applied the DUE technique to representative hydrogel constructs to investigate the effects of biomaterial composition on viscoelastic properties, and to characterize the spatial variation of properties through the depth of these materials. Creep tests were performed by applying a constant load for a defined period of time and characterizing the compression and subsequent relaxation of hydrogel samples. Burger's viscoelastic model was applied to generate quantitative parameters that described the viscoelastic behavior. This study shows how DUE can be used to non-invasively characterize hydrogel materials, and how the spatial variation in mechanical and viscoelastic properties can be mapped using this technique. Such high resolution spatial information is expected to facilitate our elucidation of the principles of mechanobiology, and will aid in designing and developing engineered tissues.

2. Materials and methods

2.1. Preparation of hydrogel constructs

Four types of hydrogel constructs were tested: hydroxyapatite (HA)-doped agarose constructs (10.0 mg/ml), HA-doped collagen constructs (2.0 mg/ml and 5.0 mg/ml), HA-doped fibrin constructs (2.0, 4.0, 8.0, 10.0 and 12.0 mg/ml), and cell-seeded collagen constructs (collagen concentration 2.0 mg/ml) on day 1 and day 5. In acellular constructs, hydroxyapatite served as a scatterer that produced ultrasound signals, while in cell-seeded constructs the cellular component served as the scatterer. HA has been used as an additive in a variety of hydrogel biomaterials, for example to potentiate bone formation and to facilitate endothelial network formation [41].

Agarose solution (1.0% w/v) was prepared by dissolving agarose powder (Sigma Aldrich, St. Louis, MO) in distilled water via heating and stirring. Nano-grade hydroxyapatite (HA) (Sigma Aldrich) suspended in Dulbecco's modified Eagle's medium-low glucose (DMEM; Life Technologies, Grand Island, NY) was prepared at 200 mg/ml and placed in a sonication water bath for 1 h to disrupt aggregates [42]. Nano-HA stock solution was added to the agarose solution to obtain a final HA concentration of 10.0 mg/ml. The mixture was degassed and 250 μL was injected into a 48-well plate and allowed to gel at 4 °C for 30 min.

HA-doped collagen hydrogels of 2.0 mg/ml and 5.0 mg/ml were generated as previously described [43], with some modifications. Briefly, collagen type I (MP Biomedicals, Solon, OH) was dissolved at 4.0 mg/ml (for 2.0 mg/ml collagen hydrogel) or 10.0 mg/ml (for 5.0 mg/ml collagen hydrogel) in 0.02 N acetic acid and stirred overnight. HA-doped collagen hydrogels were generated by mixing 50% collagen type I, 20% 5X-concentrated Dulbecco's modified Eagle's medium (5X-DMEM; Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS; Invitrogen), 5% DMEM, 5% 200 mg/ml nano-HA stock solution, and 10% 0.1 N NaOH (Sigma Aldrich). This mixture (250 μL) was then injected into a 48-well plate and allowed to gel at 37 °C for 30 min. HA-collagen composites were 10 mm in diameter and 2–3 mm in thickness after fabrication and their dimensions remained stable over time.

HA-doped fibrin hydrogels of 2.0, 4.0, 8.0, 10.0 and 12.0 mg/ml fibrin were prepared by adding nano-HA solution to a fibrin gel