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Research Paper

Nanoscale viscoelasticity of extracellular matrix proteins in soft tissues: A multiscale approach

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ABSTRACT

It is hypothesized that the bulk viscoelasticity of soft tissues is determined by two length-scale-dependent mechanisms: the time-dependent response of the extracellular matrix (ECM) proteins at the *nanometer* scale and the biophysical interactions between the ECM solid structure and interstitial fluid at the *micrometer* scale. The latter is governed by poroelasticity theory assuming free motion of the interstitial fluid within the porous ECM structure. In a recent study (Heris, H.K., Miri, A.K., Tripathy, U., Barthelat, F., Mongeau, L., 2013. J. Mech. Behav. Biomed. Mater.), atomic force microscopy was used to measure the response of porcine vocal folds to a creep loading and a 50-nm sinusoidal oscillation. A constitutive model was calibrated and verified using a finite element model to accurately predict the nanoscale viscoelastic moduli of ECM. A generally good correlation was obtained between the predicted variation of the viscoelastic moduli with depth and that of hyaluronic acids in vocal fold tissue. We conclude that hyaluronic acids may regulate vocal fold viscoelasticity. The proposed methodology offers a characterization tool for biomaterials used in vocal fold augmentations.

1. Introduction

Extracellular matrix proteins (ECM) control the bulk (i.e., macroscopic) viscoelastic response of soft tissues under load. Biophysical interactions among ECM molecules occur over micro- and nanometer scales while the bulk tissue response is characterized over much larger scales. The goal of this study was to obtain models for the bulk viscoelastic properties of vocal fold tissue in terms of selected ECM components. Such models may allow predictions of the viscoelasticity of the target tissue during wound healing or following tissue engineering treatment. Specifically, the contribution of interstitial fluid flow to the viscoelastic properties of soft tissues was investigated.

Vocal folds, which are the subject of this study, undergo large quasi-static deformations during posture changes, superimposed with high-frequency deformations during phonation (Gray et al., 1999). The viscoelastic moduli of dissected vocal folds have been measured in both deformation regimes, using uniaxial traction testing (Alipour et al., 2011) and shear rheometry (Chan and Rodriguez, 2008). The viscoelastic properties were found to depend on the specimen configuration, due to presumably significant inhomogeneity and anisotropy of the tissue (Miri et al., 2012b). The problems associated with tissue inhomogeneity are reduced when measuring local properties using indentation testing (Chhetri et al., 2011). It is also easier to impose high-frequency loading in indentation testing than in other testing methods. This is important

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because phonation involves deformations at frequencies up to 1 kHz (Chan and Rodriguez, 2008).

Atomic force microscopy (AFM) allows probe scanning and force spectroscopy in biological soft tissues and biomaterials (Oyen, 2013; Tripathy and Berger, 2009). Compared to conventional indenters, AFM offers a greater sensitivity, which varies with the total mass of the cantilever, the system damping, and the cantilever stiffness (McConney et al., 2010). AFM-based indentation has been used for soft engineering materials (McConney et al., 2010), soft tissues (Nia et al., 2011), and cells (Mahaffy et al., 2004). In a recent study (Nia et al., 2013), a commercially available AFM with a spherical probe and a well-controlled piezoelectric substrate was used to perform nanoindentation on articular cartilage within a frequency range of 0.1–10 kHz. Spherical probes produce relatively large forces with minimal damage to the surface; hence, they are ideal for compliant materials, such as vocal folds. Colloidal force spectroscopy (McConney et al., 2010), based on the use of spherical probes and the Hertz contact theory, was used in a previous study (Heris et al., 2013) for the micro-indentation of vocal folds. The accuracy of AFM-based indentation testing for the prediction of the viscoelastic properties of vocal folds was assessed.

Mammalian vocal folds are multilayered structures. They include the epithelium, the lamina propria, and the muscle. The lamina propria is a hypocellular connecting tissue composed of fibrous proteins such as collagen and elastin entangled within interstitial molecules such as proteoglycan, glycoprotein and glycosaminoglycan (GAG). The viscoelasticity of the lamina propria must be within a specific range to allow proper phonatory function. Previous studies have hypothesized the role of collagen fibrils and elastin fibers in the viscoelasticity of the ECM structure (e.g., (Gray et al., 2000)). These fibrous proteins occupy a large fraction of the ECM volume in human vocal folds. They vary with depth within the lamina propria (Miri et al., 2012c). Negatively-charged aggrecans and GAG chains, on the other hand, are believed to control the mass transport of the interstitial fluid, due to their great swelling capacity (Gray et al., 1999). The vocal fold swelling-contraction characteristics imply a poroelastic behavior. The pores in the vocal fold tissue were found to be on the order of one micrometer. The ECM poroelasticity regulates the bulk viscoelasticity of the vocal fold tissue because the percolation of fluid volumes through the pores dissipates mechanical energy (Miri et al., 2012a). Linear poroelastic models have been used to simulate the dynamic mechanical behavior of vocal folds (Tao et al., 2009).

We hypothesize that mechanical energy dissipation in vocal folds results from two decoupled mechanisms: (I) the intrinsic viscoelasticity of the ECM solid framework at the nanometer scale and (II) the biophysical interactions between solid and the interstitial fluid at the micrometer scale. The length scale of the ECM components such as collagen, protoglycans and GAGs (Gray et al., 1999) is on the order of tens or hundreds of micrometers. A linear poroelastic model (Galli and Oyen, 2009) that isolates the contribution of solid–fluid interactions to tissue response from those of the ECM viscoelasticity was developed for indentation testing. In the present study, experiments were performed using porcine vocal fold lamina propria. Continuous microscale creep loading and nanoscale sinusoidal displacement were imposed by programming an AFM, following the testing procedure used by Nia et al. (2011). In creep tests, the AFM cantilever approaches the surface gradually, indents the sample, and remains at a constant force level for a specified time. Finite element analysis (FEA) was used to calibrate our formulation through estimation of the drainage length, a parameter describing fluid entrainment. The viscoelastic moduli of the ECM structure were determined from the oscillation tests. The proposed multiscale approach can be used to measure the nanoscale viscoelasticity of any other soft tissues.

2. Methods and materials

2.1. *Sample preparation*

Porcine vocal fold tissue samples ($n=3$) were prepared following the protocol used by Heris et al. (2013). A rectangular area of about 2 mm × 2 mm was excised with sharp blades from the central region of the vocal fold lamina propria along the sagittal plane. The tissue was flash frozen in OCT (Optimal Cutting Temperature Compound, Sakura Finetek, Dublin, OH) with no labeling or dehydration. The tissue samples were sectioned using a cryostat (Leica CM-3505-S). Starting from the superficial layer, 50 μm thick slices were removed at intervals of 100 μm. Three slices from each sample, down to a depth of about ~350 μm, were used for this study (nine in total). The general orientation of each slice was therefore in the sagittal plane, with the abscissa oriented along the anterior–posterior direction. The samples were mounted on microscopy glass slides. A very thin layer of nail polish was added to glue the tissue to the glass substrate.

2.2. *Atomic force microscopy*

A commercially available AFM (Multimode Nanoscope IIIa, Veeco, Santa Barbara, CA), equipped with a NanoScope V controller, was used to perform the indentation tests. Silicon nitride micro-cantilevers, with a nominal stiffness of 0.35 N/m, and colloidal probes (25 μm diameter; Novascan Tech Inc., Ames, IA) were used. Using nonlinear laser scanning microscopy data, Heris et al. (2013) showed that a 3–5 μm indentation depth with a 25 μm diameter probe (Fig. 1b) constitutes a representative volume for the bulk distribution of fibrous proteins.

The samples were submerged in a buffer solution with a pH of 7.6 to mimic physiological conditions. The deflection sensitivity of the piezoelectric cantilever was measured by probing the hard surface of the glass substrate. The probe spring constant was measured using the thermal tuning method (Heris et al., 2013). An area of approximately 100 μm × 100 μm was indented by the probe. Force–displacement curves that displayed insignificant noise and adhesion effects were selected for post-processing. Ideal force–displacement curves (McConney et al., 2010) may be decomposed into: (1) a straight