Viscoelastic Properties of Dental Pulp Tissue and Ramifications on Biomaterial Development for Pulp Regeneration

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Abstract
Introduction: A critical step in biomaterial selection effort is the determination of material as well as the biological properties of the target tissue. Previously, the selection of biomaterials and carriers for dental pulp regeneration has been solely based on empirical experience. Methods: In this study, first, the linear viscoelastic material functions and compressive properties of miniature pig dental pulp were characterized using small-amplitude oscillatory shear and uniaxial compression at a constant rate. They were then compared with the properties of hydrogels (ie, agarose, alginate, and collagen) that are widely used in tissue regeneration. Results: The comparisons of the linear viscoelastic material functions of the native pulp tissue with those of the 3 hydrogels revealed the gel-like behavior of the pulp tissue over a relatively large range of time scales (ie, over the frequency range of 0.1–100 rps). At the constant gelation agent concentration of 2%, the dynamic properties (ie, storage and loss moduli and the tanδ) of the collagen-based gel approached those of the native tissue. Under uniaxial compression, the peak normal stresses and compressive moduli of the agarose gel were similar to those of the native tissue, whereas alginate and collagen exhibited significantly lower compressive properties. Conclusions: The linear viscoelastic and uniaxial compressive properties of the dental pulp tissue reported here should enable the more appropriate selection of biogels for dental pulp regeneration via the better tailoring of gelation agents and their concentrations to better mimic the dynamic and compressive properties of native pulp tissue. (J Endod 2015;41:1711–1717)

Key Words
Biomaterial, compression, pulp, regeneration, tooth, viscoelastic

Pulp tissue is the only soft tissue in a tooth and serves primarily to maintain its own physiological functions as well as those of dentin through blood supply and nerves. Dental pulp tissue is a reservoir of multiple cell types including odontoblasts that reside on mineralized dentin surface in addition to abundant fibroblasts that are populated in a matrix of blood vessels and nerve endings. The extracellular matrix of dental pulp is also rich in terms of collagenous (collagens type 1: 56%, type 3: 41%, and type 5: 2%) and noncollagenous (chondroitin 4- and 6-sulfate: 60%, dermatan sulfate: 34%, keratan sulfate: 2%, and glycosaminoglycans as proteoglycans) proteins (1). The cells and the organic components of the dental pulp together determine the structural and functional nature of pulp tissue, with collagen type I likely contributing to its biomechanical properties, such as stiffness and strength (2), and proteoglycans mostly contributing to its viscoelasticity (1).

Despite reported clinical success, endodontically treated teeth become devitalized and brittle as well as susceptible to reinfections because of coronal leakage or microleakage, leading to considerable structural deformations, including removal of part of the enamel, dentin, and pulp during endodontic treatment, possibly resulting with tooth fracture and trauma (3). Because of extraction of the pulp tissue, endodontically treated teeth lose pulpal sensation and are unable to detect microbial challenges. If dental pulp can be regenerated, these complications may be avoided, and many teeth can be saved to function as native teeth.

Biomaterials for tooth regeneration need to be biocompatible and biodegradable; provide a suitable environment for cells that regenerate dental tissues; allow functionality for a variety of cells including ameloblasts, odontoblasts, cementoblasts, fibroblasts, vascular cells, and/or neural endings; be clinically applicable and easily handled by clinicians; and involve multiple structural characteristics because of diverse structures and functions of dental tissues. Although there are some insights for the selection of biomaterials for tooth regeneration based on experimental findings (4, 5), existing literature lacks benchmark data for the material properties of dental pulp tissue. The lack of data may stem from a lack of pertinent interest considering that dental pulp is confined and is shielded from direct exposure to mechanical stresses by several layers of hard mineralized tissues such as dentin, cementum, and enamel. However, many investigations have shown that the migration, proliferation, and differentiation of cells are intimately related to various physical properties of the scaffolding materials (6) including their viscoelasticity (7). Obviously, the viscoelastic material functions and biomechanical behavior of the native pulp tissue need to be known to allow their mimicry as a tool for biomaterial selection and development for regenerative dentistry. Such properties can then be used for the selection and

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tailoring of biogels to act as scaffolds for tissue engineering of dental pulp, and collagen, agarose, and alginate can be considered important candidates (3).

Collagen macromolecules, major constituents of the extracellular matrix, exist ubiquitously in diverse tissues including tooth (8) and have been used as scaffolding material for dental pulp regeneration (9). Chemical cross-linking by the addition of cross-linking agents such as glutaraldehyde or diphenylphosphoryl azide can enhance the mechanical stiffness of collagen scaffolds (10), yet only at the expense of cell survival and biocompatibility (9). One of the major challenges in tissue engineering is vascularization. Research showed that in vivo implantation of endodontically treated human teeth in mouse dorsum yielded recellularized and revascularized connective tissue upon delivery of the basic fibroblast growth factor and/or the vascular endothelial growth factor in a collagen carrier (11). Similarly, delivery of dental pulp stem cells and dentin matrix protein-1 with collagen scaffolds in mice led to ectopic formation of dental pulp–like tissue (12).

Another hydrogel that is considered as a scaffolding material for pulp regeneration and that can allow cell infiltration and growth is agarose (13–15). Agarose derives from seaweed and forms thermally reversible gels (9). Agarose was earlier used for the regeneration of prismlike enamel tissue (13). It was also investigated for the effect of mechanical loading on osteogenesis upon seeding with human dental pulp stromal cells (14). Findings suggested that mechanical loading through a bioreactor mimicking the biting force to enhance human dental pulp stromal cell osteogenesis in an agarose scaffold promoted bone formation and/or prevented bone resorption. Agarose hydrogels can also accommodate 3-dimensional neurite extension from primary sensory ganglia in vitro (15).

A third hydrogel widely used as a scaffolding material in tissue regeneration is alginate, which is a naturally derived polysaccharide. Alginate is biocompatible and permeable to small molecular-weight proteins (16). The mechanical strength of alginate can be modified by altering the calcium content and cross-linking density (17). Alginate hydrogel has been loaded with exogenous transforming growth factor beta 1 for regeneration of the dentin-pulp complex and found to promote odontoblast-like cell differentiation with subsequent secretion of tubular dentin matrix (18).

Thus, previous investigations using these 3 hydrogels have indicated that there is a significant degree of freedom in altering the properties of gels via compounding them with various bioactives at various concentrations, cross-linking to different degrees, and generation of tissue constructs using various cell lines. However, effective tailoring of biomaterials for tissue regeneration also requires the availability of the properties of the native tissues to allow the matching of the properties of the biomaterials. Here, the linear viscoelastic properties and uniaxial compression behavior of miniature pig dental pulp tissue were characterized and compared with those of collagen, agarose, and alginate gels.

**Materials and Methods**

**Dental Pulp Harvesting**

Pulp tissue was obtained from the mandibular canine of a 28-month-old miniature pig (single donor, n = 3) following a protocol approved by the Institutional Animal Care and Use Committee of Columbia University. Briefly, after the animal was euthanized, the tooth was extracted and cut into half approximately at equal distances from the coronal and apical regions using a saw (Fig. 1A). The fresh pulp...
tissue was removed by gently pulling the tissue out using forceps. Test specimens were then formed by punching cylindrical 8-mm-diameter discs, soaked into phosphate-buffered saline (PBS), and characterized within 4 hours.

Hydrogel Preparation and Characterization

**Alginate Gel Preparation.** Alginate acid salt (Cat#A2033; Sigma-Aldrich, St Louis, MO) from brown algae is a linear polymer composed of approximately 61% mannuronic and 39% guluronic acid, with a molecular weight range of 80,000 to 120,000. To make 2% (wt/vol) alginate, alginate acid sodium salt was first added at a 20-mg/mL concentration to PBS containing 150 mmol/L NaCl. The mixture was then stirred continuously until alginate was completely dissolved. Afterward, 50 mmol/L CaCl₂ was gently added at a 1:1 ratio by volume on top of the alginate-NaCl mixture, and CaCl₂ solution was left to diffuse into the solution for 30 minutes to complete gelation.

**Agarose Gel Preparation.** The 2-hydroxyethylagarose (Cat#A4018, Sigma-Aldrich [type VII, low gelling temperature agarose]) particulates were dissolved in PBS at a 20-mg/mL concentration by heating up to boiling 3 times. The solution was then left at room temperature to cool down to form 2% (wt/vol) agarose gel.

**Collagen Gel Preparation.** Two percent (wt/vol) collagen gel was prepared by mixing 10% PBS (10×), 0.23% 1 N NaOH, 69.77% dH₂O, and 20% collagen solution (10.12 mg/mL, Cat#354249; BD Biosciences, San Jose, CA) by volume basis. The contents were transferred to a cylindrical tube and vortexed for 1 minute. The solution was kept at 37°C for 1 hour to complete polymerization.

**Characterization of Viscoelastic Material Functions**

The samples were characterized in compression and oscillatory shear using the ARES rheometer (TA Instruments, New Castle, DE). The experimental setup used in this study was similar to that used earlier for the characterization of native cartilage tissue (19). Briefly, the specimen was inserted between 2 disks (8-mm diameter), which were also immersed in PBS solution kept at 37°C and 20% collagen solution (10.12 mg/mL, Cat#354249; BD Biosciences, San Jose, CA) by volume basis. The contents were transferred to a cylindrical tube and vortexed for 1 minute. The solution was kept at 37°C for 1 hour to complete polymerization.

**Small-amplitude Oscillatory Shear.** In small-amplitude oscillatory shear, the shear strain oscillates as a function of time as follows:

\[
\gamma = \gamma_0 \sin(\omega t),
\]

where \(\gamma_0\) is shear strain amplitude (ie, \(\theta/b\)), \(\theta\) is the angular displacement, \(b\) is the disk diameter, and \(t\) is the time. The shear stress, \(\tau\), response to the oscillatory deformation consists of 2 components related to the energy stored and energy dissipated as heat (ie, \(\tau = G'\omega \gamma_0 \sin(\omega t) + G''\omega \gamma_0 \cos(\omega t)\), where \(G'\omega\) is the shear storage modulus and \(G''\omega\) is the shear loss modulus). The ratio of \(G''\omega\) to \(G'\omega\) is tan \(\delta\). The oscillatory shear needs to be performed in the linear viscoelastic region at which the moduli are independent of the strain amplitude. The strain amplitude sweeps indicated that up to a strain amplitude of 100% the oscillatory shear deformation of the hydrogels and the native tissue took place in the linear region. The dynamic properties \(G'(\omega)\) and \(G''(\omega)\) were characterized as a function of frequency in the range of 0.1–1000 rps at 10% strain. Sweeping the frequencies enables the characterization of the linear viscoelastic response of the tissue over a range of time scales. In general, at relatively short characteristic times of deformation, the elastic response is emphasized, whereas the viscous flow behavior is emphasized at longer characteristic times. Time sweeps suggested that the samples were stable within the time scale of the experiments (typically less than 20 minutes of shearing for each sample).

**Biomechanical Characterization on Compression.** Uniaxial compression of the native tissues and hydrogels was performed in the strain range of 0%–10% at a constant compression rate of 0.05 mm/min. This strain level was previously used in biomechanical characterizations of native and engineered tissues (19). Here, the specimens were initially squeezed using a normal force of approximately 0.03 N to obtain full contact, and the compression test was then initiated. In a related set of experiments, the normal stress relaxation behavior of the specimens to the compressive loading was characterized upon application of step strains within 5 to 7 seconds.

**Statistics**

The multivariate analysis of variance test was used for the comparison of native pulp tissue and hydrogels in terms of peak force for relaxation experiments and the stress, modulus, and toughness for normal compression experiments. The P levels at which differences between groups are considered statistically significant is taken as .05 or less.

**Results**

**Strain Sweep Test**

The dynamic material functions, namely, the storage modulus (\(G'\)), the loss modulus (\(G''\)), and tan \(\delta\) (\(G''/G'\)) of the native pulp tissue and those of the hydrogels, were measured over the strain magnitude range of 1%–100% at 10 rps frequency. It was observed that the storage and loss moduli as well as tan \(\delta\) values remained independent of the strain amplitude over the range of strain amplitudes studied, and, thus, in this strain amplitude range both the native pulp tissue and the hydrogels exhibited linear viscoelastic behavior (Fig. 2A).

**Relaxation Behavior after Uniaxial Compression**

Figure 3A shows the response of the specimens of the native tissue and hydrogels upon 20% compression applied for a duration of 5 to 7 seconds. The decline in stress after imposing a nominal strain determines the time-dependent relaxation behavior. As shown in Figure 3A, the normal stress decreased with time at a similar rate as it was initially executed. The relaxation behavior of the samples was
characterized by a steady decrease in the normal stress as a function of
time up to about 1000 seconds after which the normal stress becomes
negligibly small. The results indicated that changes in stress took place
within comparable durations. Specifically, normal force reached
1.2 \pm 0.5 g, 1.2 \pm 0.9 g, 2.4 \pm 2.4 g, and 0.9 \pm 0.0 g for native tissue,
agarose, alginate, and collagen, respectively, in 1000 seconds. The peak
force for the native tissue, agarose, alginate, and collagen was deter-
mined to be 99.2 \pm 49.3 g, 101.5 \pm 15.9 g, 35.9 \pm 1.0 g, and
1.7 \pm 0.0 g, respectively. It is seen that the peak force generated by
20% compression of collagen is significantly smaller than that of native
pulp tissue, whereas agarose generated values comparable with the
pulp tissue (Fig. 3B).

Compressive Stress versus Strain Behavior

The compressive stress-strain behavior of the native pulp tissue
and hydrogels is shown in Figure 4A. The normal stress, Young’s
modulus, and the toughness (area under the stress-strain curve) of
the native tissue and the hydrogels determined at 10% strain are pre-
sented in Figure 4B. Specifically, at 10% strain, the normal stresses
were 7.3 \pm 3.0 kPa, 7.9 \pm 1.8 kPa, 2.8 \pm 1.0 kPa, and
0.3 \pm 0.0 kPa; Young’s modulus values were 0.8 \pm 0.4 kPa,
0.8 \pm 0.2 kPa, 0.3 \pm 0.1 kPa, and 0.03 \pm 0.0 kPa; and toughness
values were 37.7 \pm 19.1 kPa, 39.6 \pm 8.8 kPa, 13.8 \pm 3.0 kPa, and
1.4 \pm 0.0 kPa for native tissue, agarose, alginate, and collagen, respec-
tively. The results showed that at the 2% concentration used, the alginate

Figure 2. Linear viscoelastic material functions. (A) Storage modulus, G'; loss modulus, G"; and tan δ versus strain amplitude behavior of native pulp tissue at 10
rps and 37°C. (B) Frequency dependence of the storage modulus G', (C) the loss modulus G", and (D) tan δ of the native tissue and hydrogels at 10% strain
amplitude and at 37°C. Error bars represent standard deviation, n = 3.

Figure 3. (A) Normal stress relaxation to a compressive strain response of the native pulp tissue and the 3 hydrogels over 1000 seconds upon 20% compression at
0.05 mm/s and (B) peak values. *Significant difference at P < .05.
and collagen exhibited lower stress and toughness values compared with native pulp tissue, whereas agarose yielded comparable values.

**Discussion**

Dental pulp regeneration has so far focused on cell transplantation (20–22), using mostly gels as carriers, in which the selection of material is typically based on the ease of handling. Efforts to match the mechanical properties and viscoelastic material functions of the biomaterial with those of the native tissue have been lacking. The lack of efforts to match should be primarily caused by the data not being available for dental pulp tissue. This study, for the first time, offers viscoelasticity data for the dental pulp and suggests that a more realistic biomaterial selection approach can be based on the additional findings obtained from viscoelastic characterization of native dental pulp. Therefore, the current study is expected to set a benchmark in the development of clinically feasible biomaterials for such application.

The native pulp tissue obtained from the mandibular canine of a miniature pig and the 3 most commonly used hydrogels, namely, agarose, alginate, and collagen, were characterized in terms of their rheological properties under oscillatory shear and biomechanical properties under compression. The strain sweep experiments revealed that the storage modulus, $G'$, the loss modulus, $G''$, and the relaxation rate. Higher concentrations and molecular weights should lead to greater relaxation times (19, 24, 25). The native dental pulp tissue consists of collagen fibers, proteoglycans, and dental pulp cells together with water and electrolytes. The proteoglycans are in the form of networks that are able to store deformational energy (26), whereas the cross-linked fibrous collagen provides tensile stiffness and strength (27). The presence of these macromolecules (ie, collagen and proteoglycans) and their interactions with cells and other extracellular matrix components coupled with the hydrodynamics of the interstitial fluid flow regulate the viscoelastic behavior of connective tissue (28, 29). The permanent junction points formed by the cross-linked collagen fibers lead to a frequency independent gel-like behavior in the native pulp tissue. The tan $\delta$ values decreased from 0.24 to 0.12 in the 1- to 100-rps range yet did not approach the level of 0, the purely elastic behavior point.

Among the hydrogels characterized at a similar gelation agent concentration, collagen was observed to yield the closest linear viscoelastic material properties to those of native pulp tissue. However, it should be noted that the deviation in viscoelastic properties of agarose and alginate from the native pulp tissue can be minimized by altering the gelation agent concentration as well as the modifications of the degree of cross-linking and the incorporation of bioactives. If the biomaterials are used as tissue engineering scaffolds by seeding them with cells, the properties of the resulting tissue constructs before implantation will be affected by the proliferation and differentiation of the cells (19, 30), providing yet another avenue for the matching of the properties of the biogels with those of the native pulp tissue.

Compression relaxation experiments, showing the time-dependent behavior of the native pulp tissue and hydrogels, again point to the strong role of viscoelasticity. In Newtonian fluid behavior, the stress exerted would decline immediately upon the termination of the deformation. For a purely elastic material, however, the stress associated with a strain that is maintained would remain constant. In viscoelastic behavior, time-dependent stress relaxation occurs. For the native pulp tissue, in this study, the interconnectivity of the matrix and its integral viscoelasticity should play important roles to define its relaxation behavior. Generally, for polymeric biomaterials, it is the density of the entanglements between the macromolecules that controls the relaxation rate. Higher concentrations and molecular weights should lead to greater relaxation times (19).

Mechanical compression of the native pulp tissue and hydrogel specimens indicated that agarose hydrogel provided similar stress,
modulus, and toughness values compared with native pulp tissue. The area under the stress-strain curve obtained during application of the strain, until fracture, represents the toughness of the specimen. In this study, because the fracture of the specimen was not observed within the 10% strain range, the area under the stress versus strain curve would represent the total mechanical energy per unit volume associated with straining the specimen to the particular strain applied. Again, the response of hydrogels to mechanical deformation would be concentration dependent, which could further be optimized by changing the concentration.

Results of rheological and biomechanical characterization of the native dental pulp tissue and biomaterials imply that no single material at the studied (2% wt/vol) concentration can mimic the native pulp tissue. Collagen seems to be a good candidate in terms of viscoelastic properties, whereas agarose seems to be a better fit based on compressive biomechanical properties. Additional data involving different concentrations of gelation agents can be generated on candidates scaffolding biomaterials and further used to better match the physical properties of the scaffold or a hybrid composite with those of the native pulp tissue. In this regard, viscoelasticity is controlled by the density of the entanglements between the macromolecules, which, in turn, is influenced by the molecular weight distribution of the polymer. Indeed, a research performed using collagen gel and collagen thin film showed that there is minimal difference between the 2 samples in terms of storage and loss moduli (31). In addition, increased collagen concentrations of gels are known to improve biomechanical properties (32). Therefore, increasing the collagen concentration could lead to improved mechanical properties while keeping viscoelasticity at similar levels.

As noted earlier, cellularization of hydrogel biomaterials may have a significant effect on the mechanical/viscoelastic properties of samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density. It was previously found that seeding keratocytes (2 million cells/mL) in alginate samples as a function of culture time, cell type, and density.

Finally, the dental pulp constitutes a connective tissue with properties varying as a function of location in the cavity surrounded by the dentin. This suggests that the properties of the scaffolds can also be altered systematically in both the radial and axial directions as has been shown in various interface tissue engineering applications (34, 35) to generate improved scaffolds, which better mimic the complex elegance of the native pulp tissue.

Conclusions

In this study, linear viscoelastic properties and uniaxial compressive properties of native dental pulp tissue and some of the most commonly used hydrogels were investigated. Findings suggest that these properties of dental pulp tissue should provide valuable inputs for the selection of an appropriate biomaterial for dental pulp regeneration. In this regard, the current study is expected to set a benchmark for the upcoming similar research studies as well as in the development of clinically feasible biomaterials.

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