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MECHANICAL DESIGN CRITERIA FOR INTERVERTEBRAL DISC TISSUE ENGINEERING

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Abstract

Due to the inability of current clinical practices to restore function to degenerated intervertebral discs, the arena of disc tissue engineering has received substantial attention in recent years. Despite tremendous growth and progress in this field, translation to clinical implementation has been hindered by a lack of well-defined functional benchmarks. Because successful replacement of the disc is contingent upon replication of some or all of its complex mechanical behaviour, it is critically important that disc mechanics be well characterized in order to establish discrete functional goals for tissue engineering. In this review, the key functional signatures of the intervertebral disc are discussed and used to propose a series of native tissue benchmarks to guide the development of engineered replacement tissues. These benchmarks include measures of mechanical function under tensile, compressive and shear deformations for the disc and its substructures. In some cases, important functional measures are identified that have yet to be measured in the native tissue. Ultimately, native tissue benchmark values are compared to measurements that have been made on engineered disc tissues, identifying measures where functional equivalence was achieved, and others where there remain opportunities for advancement. Several excellent reviews exist regarding disc composition and structure, as well as recent tissue engineering strategies; therefore this review will remain focused on the functional aspects of disc tissue engineering.

Keywords

Intervertebral Disc Mechanics; Motion Segment; Annulus Fibrosus; Nucleus Pulposus; Tissue Engineering

INTRODUCTION

The disc is a fibrocartilage that lies between bony vertebral bodies, conferring flexibility, load transfer, and energy dissipation to the spine. It is comprised of the central gelatinous nucleus pulposus (NP) surrounded circumferentially by the annulus fibrosus (AF) (Fig. 1). The hyaline cartilage endplate forms an interface between the disc and adjacent vertebral bodies. The NP

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CONFLICT OF INTEREST STATEMENT

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is structurally and mechanically isotropic and contains a network of type II collagen interspersed with proteoglycans, resulting in a high water content within the tissue. The osmotic swelling that results is a defining feature of NP mechanics. Each lamella of the multi-lamellar AF consists of highly aligned collagen fibers whose orientation alternates above and below the transverse axis of the spine by approximately 30° in adjacent lamellae (Fig. 1) (Cassidy et al., 1989; Marchand and Ahmed, 1990). While the AF can be approximated as an angle-ply laminate ring, its true architecture is more complex: lamellae are circumferentially discontinuous and traversed by fibrous elements that run radially outward (Pezowicz et al., 2005; Schollum et al., 2008).

Cell Biology of the Intervertebral Disc

Cells of the disc possess regionally distinct phenotypes, reflecting the compositional heterogeneity of their ECM. Mature NP cells have a rounded morphology *in vivo* and express chondrogenic markers such as Sox-9, type II collagen, and aggrecan (Sive et al., 2002). However, NP cells are distinct from chondrocytes; they harbor specific phenotypic traits suited for survival in the unique microenvironment of the NP, which includes lower pH, oxygen, and glucose levels, and higher osmolarity than what is physiologic for most tissues (Boyd et al., 2005; Maroudas et al., 1975; Risbud et al., 2006; Tsai et al., 2007; Uchiyama et al., 2008). Work to better understand these adaptations is important for the determination of NP-specific phenotypic markers and to distinguish between notochordal and NP cells. Considerably less work has been performed on the biology of AF cells, which are most frequently discussed as fibrochondrocytes, due to their expression of markers typical of both fibroblasts (e.g. type I collagen) and chondrocytes (type II collagen, aggrecan). An advanced understanding of the *in vivo* function of AF and NP cells will be instrumental in developing stem cell-based tissue engineering strategies where differentiation into a disc-like phenotype is necessary. A second population of cells, notochordal cells, have been observed within the NP during adolescence in humans, however their function and the reason for their eventual disappearance are unknown (Trout et al., 1982).

Degeneration of the Intervertebral Disc

Disc degeneration is an aberrant, cell-mediated response to progressive structural failure, whereby changes in structure and composition give rise to mechanical dysfunction (Adams and Roughley, 2006; Miller et al., 1988). Degeneration is also thought to play a dominant role in the development of low back pain (Bogduk, 1991; Miller, et al., 1988). Clinical treatments for discogenic back pain and disc degeneration are focused on the alleviation of symptoms, while restoration of function remains largely unaddressed. Fusion is the surgical standard for the treatment of axial low back pain (Deyo et al., 2005). This treatment is highly invasive and is intended to stop pain by eliminating motion across the joint space. Despite the frequency of its practice, fusion often fails to alleviate pain and may accelerate degenerative changes in adjacent discs (Harrop et al., 2008; Levin et al., 2007). Total disc arthroplasty is a recently approved surgical option that aims to maintain segmental motion; however, its long term efficacy has not been established and mechanical wear may challenge its the long-term success (Resnick and Watters, 2007; van Ooij et al., 2007). NP partial disc implants are under development; however, while they may restore disc height and mechanical support, they do not aim to repair AF damage incurred during implantation or due to degeneration (Di Martino et al., 2005). Given the current treatment options, there is tremendous need for a tissue engineering strategy that alleviates pain and restores spine function. Therefore, it will be of critical importance that engineered replacement tissues overcome the harsh mechanical challenges of an extended lifetime in the intervertebral joint space. An advantage of this approach over arthroplasty is that living tissues, both native and engineered, retain their capacity for adaptive remodeling.

FUNCTION OF THE INTERVERTEBRAL DISC

The disc is mechanically quite complex. Therefore, in order to establish the design criteria for engineered replacement tissues, we must first establish the functional properties of the disc and its component tissues, the AF and NP. In this section, we will discuss the complex mechanical behaviors of the AF, NP, and motion segment. In doing so, we will use published data to generate quantitative functional benchmarks for disc tissue engineering (Table 1). Brief discussion of testing modalities and their relative importance is also included (Fig. 2).

Given the inherent complexity of the disc and the variability in most mechanical measures, it is nearly impossible to establish discrete, “hard and fast” benchmark values. Therefore the values reported here are culled from various studies in the literature and provide a range of standards against which engineered constructs can be compared. Because different animal models are employed in the study of disc mechanics, benchmarks are further obfuscated by inter-species variability (Beckstein et al., 2008; O’Connell et al., 2007b). Here, we will discuss results obtained for human tissue (when available). Also, this review will focus the discussion to the overall tissue properties of the NP, AF, and disc, without explicit reference to local variations that are present within each. Nonetheless, it is important to note that these properties vary with region, and functional heterogeneity is quite relevant to discussions of both disc pathogenesis and certain specific applications in disc tissue engineering.

Discussion of mechanical properties will be limited to elastic and some viscoelastic measures. Much of the tissue engineering literature has emphasized failure properties such as ultimate stress and percent elongation at failure. These properties are instructive for material characterization, but have limited relevance for *in vivo* function. Deformations that occur after the yield stress/strain but prior to failure are irrecoverable, meaning that when the external loads are removed, the initial geometry does not return. Naturally, this is problematic for *in vivo* implantation, where the engineered tissue would be loaded repeatedly. Moreover, many materials become weaker after yielding, making further damage probable. Therefore, measures characterizing the post-yield behavior of materials and engineered tissue constructs are omitted here.

Mechanics of the Annulus Fibrosus

AF architecture and composition combine to produce an anisotropic, nonlinear, and viscoelastic tissue, uniquely suited to withstand the complex mechanical loading experienced *in vivo*. Consequently, extensive studies have been performed on the AF using multiple loading modalities and spanning many length scales, and studies are still ongoing to fully characterize its behavior.

Uniaxial Tension—The AF is subjected to large tensile stresses along the circumferential direction of the disc. In uniaxial tensile testing, loads are applied along a single axis while all surfaces parallel to the loading axis are stress-free (Fig. 2). Due largely to the technical difficulty associated with isolation of single lamellae, only a handful of studies have investigated mechanics at this level (Holzapfel et al., 2005; Skaggs et al., 1994) (Table 1). The single lamella presents two important functional behaviors that are mirrored by the AF at larger length-scales: anisotropy and stress-strain nonlinearity. Tensile nonlinearity is a common feature of many fiber-reinforced soft tissues, and is often characterized by a small-strain or toe-region modulus, a linear region modulus, and a transition strain at which the transition from toe to linear region occurs (Fig. 2). While often overlooked, transition strain is important to tissue function: implanting an engineered tissue with too high a transition strain would result in laxity and instability, while too low a transition strain will limit motion and overload the surrounding tissues. The AF undergoes large elastic deformations during physiologic function

[25, 26]. It is therefore important that replacement tissues also deform elastically over a large range before yielding (ϵ_{yield} , Table 1).

Due to AF anisotropy, it is necessary to consider how tensile behavior varies with orientation within the AF. The uniaxial tensile modulus of the AF is one to two orders higher along the circumferential direction than in the axial and radial directions (Fig. 1, Table 1). This level of anisotropy should be preserved by an engineered tissue in order to avoid failure under multi-axial loading *in vivo*.

The high water content and intrinsic material composition of the AF introduce viscoelastic, strain-rate dependent behavior to its tensile properties (Kasra et al., 2004); however, these have received considerably less attention than compressive and torsional rate dependence. *In vivo* deformations of the disc can range from slow or quasi-static loads (body weight) to high frequency deformations such as those experienced during running or the operation of large machinery (Virtanen et al., 2007). Consequently, it is important to understand the relation between loading rate and mechanics for engineered tissues and how this relation compares to the native AF. It is possible that an engineered tissue that replicates the static tensile modulus of AF may still prove inferior when loaded at physiologic rates, limiting its *in vivo* function.

Biaxial Tension—While uniaxial extension is a simple and valuable testing modality, the AF has no free boundaries *in vivo*. Biaxial testing permits rigorous mechanical testing in a format that replicates the constrained boundaries encountered *in vivo* (Fig. 2) (Sacks and Sun, 2003). By simultaneously loading along two orthogonal directions, an abundance of data can be generated from a single test. This also poses a drawback, however, in that it is not straightforward to determine what subset of this information is most relevant for tissue characterization or comparison of engineered and native AF. Multiple combinations of strain ratios can be applied, ranging from fixed boundary tests to equibiaxial tension. While it remains unclear what combination of strain ratios most closely approximates *in vivo* deformations of the AF, two commonly considered scenarios are circumferential extension with fixed axial boundaries and equibiaxial tension (O'Connell et al., 2007a). Although these two special cases are useful as benchmarks of biaxial function, constitutive modeling may be necessary to process complex datasets in a fashion that enables meaningful comparisons between engineered and native tissue (Bass et al., 2004; Billiar and Sacks, 2000; Humphrey et al., 1990; O'Connell et al., 2009).

Compression—Compressive loading of the disc simultaneously produces narrowing of the disc height and outward bulging of the NP, placing axial and radial compressive stresses on the AF. Consequently, the AF has been well characterized in compression (Best et al., 1994; Drost et al., 1995; Iatridis et al., 1998; Klisch and Lotz, 2000; Perie et al., 2005; Perie et al., 2006; Yao et al., 2002). While properties can be probed in unconfined compression, confined compression is more amenable to theoretical analyses, and more closely approximates *in vivo* loading. In confined compression, the compressive load is applied via a porous (water permeable) platen to samples that are confined so as to prevent lateral expansion of the solid phase and to restrict fluid flow to the loading axis (Fig. 2). While the mechanical properties discussed thus far have treated the AF as an elastic solid, the high water content of the AF strongly influences its behavior in compression. As such, many of these studies have described AF compressive properties in a biphasic framework. Under small strains, biphasic materials are described by an aggregate modulus (H_A) and a fluid permeability (k). Biphasic theory has been generalized to include large deformations such as those experienced by the AF *in vivo* by the use of additional properties describing the strain dependence (nonlinearity) of the modulus (β) and permeability (M) (Ateshian et al., 1997). Analyses of AF nonlinear biphasic properties (Iatridis, et al., 1998; Perie, et al., 2005) provide similar values of H_A and k_0 to the linear case (Best, et al., 1994), although the nonlinearity parameters M and β are nonzero, indicating that

under large deformations the AF behaves nonlinearly (Table 1). Despite the strong tensile anisotropy of the AF, confined compression indicates no variation in properties with orientation, suggesting that the fibrous components of the AF have little to no contribution to compressive properties in the absence of tensile strains (Iatridis, et al., 1998). Although swelling is typically a behavior reserved for discussions of NP mechanics, confined compression experiments have measured considerable swelling pressures in the AF, and have found this propensity for tissue swelling to play an important role in the compressive properties (Perie, et al., 2005; Yao, et al., 2002). While less has been done to characterize the role of swelling pressure in tensile mechanics of the AF, there is evidence that hydration plays an important role in tension as well (Hirsch and Galante, 1967). Despite its role in native AF mechanics, swelling has remained largely unaddressed by tissue engineering.

Shear and Torsion—The AF experiences considerable shear stresses during torsion and bending of the spine. Because application of pure shear is often difficult, AF shear tests have been performed on cylindrical, cubic, or planar samples under compressive or tensile preloads (Fig. 2). In shear, the AF is anisotropic (Fujita et al., 2000; Jacobs et al., 2010; Yoder et al., 2009) and viscoelastic (Iatridis et al., 1999). Under tensile pre-stress representing physiological AF loading, shearing in the lamellar plane is resisted by stretching of the collagen fibers, resulting in larger shear moduli than measured in the absence of this preload (Yoder, et al., 2009). Torsional shear studies have been used to measure dynamic viscoelastic behavior, resulting in estimates of complex shear modulus ($|G^*|$) and phase shift (δ), which characterize dynamic stiffness and energy dissipation, respectively (Iatridis, et al., 1999). Because torsion of the spine produces prominent shearing of the AF in the radial plane, circumferential shear properties (Figure 2, Table 1) may be most crucial for replication in engineered tissues.

Inter-lamellar and Sub-lamellar Mechanics—The macroscopic response of the AF is a unique product of its microscopic organization and associated mechanical properties. As such, assessment of the functional viability of engineered replacement tissues necessitates an understanding of how AF function evolves over a broadening length scale. Relatively little work has been done at the sub-lamellar scale to characterize nano-scopic mechanics of the disc. In the only such study to date, AFM indentation tests revealed isotropic and heterogeneous behavior (Fig. 2, Table 1) (Lewis et al., 2008). Much work remains to investigate sub-lamellar mechanics and how they relate to the aggregate behavior of a single lamella.

Although very little is known about the functional role of the inter-lamellar matrix, theoretical models predict large interlamellar shear stresses (Iatridis and ap Gwynn, 2004). Moreover, recent work suggests that interlamellar properties may be important to macroscopic AF mechanics (Michalek et al., 2009; Nerurkar et al., 2009a). Direct measurement of interfacial strength at the interlamellar surface via lap or peel tests (Fig. 2) would provide an improved understanding of how single lamellar mechanics relate to the overall behavior of the AF. No such data are available in the native AF.

Mechanics of the Nucleus Pulposus (NP)

NP mechanics are central to disc function and the overall flexibility and stability of the spine. Mechanical function of the NP is largely dictated by its composition: an ECM comprised primarily of type II collagen and proteoglycans. A high fixed charge density provided by sulfated glycosaminoglycans generates considerable osmotic pressure and causes the NP to imbibe water. Swelling of the NP is constrained by the AF and endplates, establishing a hydrostatic pressure in the absence of external loads. As a result, mechanical properties of the NP share physical traits of both a solid and a fluid (Iatridis et al., 1996). The NP is isotropic and subject primarily to compressive and shear stresses *in vivo*. Consequently, studies of NP

mechanics have focused on these properties, accounting for the importance of the fluid-based effects through either a multi-phasic or lumped-parameter viscoelastic framework.

Compressive Mechanics—Compressive properties of the NP have been investigated via local indentation (Causa et al., 2002; Umehara et al., 1996) and unconfined (Cloyd et al., 2007) or confined (Best, et al., 1994; Johannessen and Elliott, 2005) compression. Indentation methods are instructive in obtaining information about mechanical heterogeneity; however, indentation produces tensile stresses transverse to the loading axis, resulting in a complex loading scheme not experienced by the NP *in vivo*. Likewise, compression-induced fluid flow and lateral expansion of the NP are restricted *in vivo* by the surrounding AF and endplate cartilage, resulting in an increase in intra-discal pressure that is necessary to support axial spine loads. As a result, it is difficult to reconcile unconfined compression testing with physiologic function. Therefore, confined compression is the key functional benchmark for tissue engineering of the NP. While it is quite soft in unconfined compression, results in confined compression show the NP to withstand much larger loads, attributed directly to the considerable role of fluid pressurization in NP mechanics (Johannessen and Elliott, 2005). Linear biphasic material parameters for the NP are provided in Table 1.

Shear and Torsion—*In vivo*, torsion of the spine generates shearing about the axial direction of the disc. Because this is superimposed upon compressive stresses, NP torsional shear properties have been investigated under axial compression (Fig. 2). Because there are very pronounced rate-dependent effects in shear (Iatridis, et al., 1996), studies of torsional shear have primarily focused on viscoelastic behavior (Iatridis et al., 1997) (Table 1). One important observation is that under constant shear deformation, the shear stress of NP relaxes almost completely, similar to its behavior in unconfined compression (Johannessen and Elliott, 2005). This unique, fluid-like behavior poses an interesting hurdle for NP tissue engineering.

Mechanics of the Motion Segment

A motion segment consists of the intact disc along with its adjacent vertebral bodies (Fig. 1B). Typically the term “motion segment” may or may not include other surrounding substructures (e.g., facet joints, longitudinal ligaments). Each paper should be evaluated for its definition. For studies related to disc tissue engineering, the interactions between the engineered constructs and the bony interface are of great interest; therefore, motion segment studies that introduce the additional complexity of facet joints are not as relevant to establish structural benchmarks. Because functional analyses at this level can be easily considered in the context of physiologic loading, motion segment mechanics have been well studied for many decades. Due to our focus on benchmarks for disc tissue engineering, many details of this complex mechanical system are not discussed here; the reader is directed to the referenced work for detailed accounts of motion segment mechanics.

The spine is compressed axially due not only to body weight and daily activity, but also due to loads from the musculature that surrounds the spine. The macroscopic response of the motion segment relies on the properties of both AF and NP substructures. Similarly, torsional properties of the motion segment are important for stability and flexibility of the spine for a broad range of daily activities. Compressive and torsional properties of the human motion segment are provided (Table 1). These measures define perhaps the most important benchmarks in disc tissue engineering, as they represent the macroscopic response that must be obtained by an engineered disc, or by a segment of remnant disc when coupled with an engineered replacement such as an annular patch. Replicating of the properties of isolated AF and NP does not ensure replication of motion segment mechanics when the two are combined; therefore it is important to consider the overall response of engineered AF and NP in concert.

If the resulting material does not compare favorably with motion segment benchmarks, the material may fail regardless of constituent properties.

Like the AF and NP, the motion segment also has rate dependent behavior. There are many approaches for analyzing creep and stress relaxation experiments, and consequently there is no one established method or metric to describe this complex behavior (Beckstein, et al., 2008; Keller et al., 1987). Therefore, while such studies are undeniably important for understanding disc function, an in depth analysis of multi-parameter rheological models is beyond the scope of the current work. Dynamic compressive stiffness of the motion segment has also been measured, and depends significantly on the frequency of loading (Costi et al., 2008). This rate-dependence is dominated by fluid-solid interactions (poroelastic effects) rather than the intrinsic rate dependence of the solid ECM (viscoelasticity).

Theoretical Modeling of Intervertebral Disc Mechanics

Theoretical models have a history of application in disc research (Hickey and Hukins, 1980; Spilker, 1980; Wu and Yao, 1976). Constitutive models, mathematical relationships between tissue deformation and stress, have been developed to better understand and characterize the structure-function behaviors of the AF (Eberline et al., 2001; Elliott and Setton, 2001; Guerin and Elliott, 2007; Guo et al., 2006; Klisch and Lotz, 1999; O'Connell, et al., 2009; Sun and Leong, 2004; Wagner and Lotz, 2004; Yin and Elliott, 2005) and have recently been employed to generate quantitative measures of functional growth of engineered AF constructs (Nerurkar et al., 2007; Nerurkar et al., 2008b). Constitutive models provide insight into the structure-function relationships of native tissues in order to guide tissue engineering approaches, and provide quantitative benchmarks that unify multiple testing modalities. Accurate constitutive models can be incorporated into finite element analyses to perform simulations of mechanical function that account for the complex loading and geometry of the tissue that comprise the disc. These can then provide insight into the stress distributions and concentrations that result from a range of physiologic motions (Martinez et al., 1997; Shirazi-Adl, 1994; Shirazi-Adl et al., 1984) and have been used to assess the ability of total disc replacements and injectable NP reinforcements to dissipate stresses within the spine (Denoziere and Ku, 2006; Rohlmann et al., 2008). For tissue engineering, these models can predict the local stress-strain environment that the construct will encounter upon implantation. Finally, through the use of poroelastic, viscoelastic, and biphasic constitutive laws, finite element models can be used to characterize complex behaviors such as dynamic mechanics of the motion segment under combined loading conditions like compression, bending and torsion (Ferguson et al., 2004; Laible et al., 1993; Williams et al., 2007).

TISSUE ENGINEERING OF THE INTERVERTEBRAL DISC

While the study of disc mechanics has spanned several decades, tissue engineering of the disc is a relatively new field of research. As such, while many promising approaches have been developed using various biomaterials and cell sources, little has been done to evaluate their functional equivalence with native tissue (2008;Butler et al., 2000). Recent reviews are available that summarize progress in disc tissue engineering from a cellular and biomaterials perspective (Kandel et al., 2008;O'Halloran and Pandit, 2007). Therefore, here we will focus on landmark disc tissue engineering studies and emphasize, whenever possible, the measurement of mechanical properties of engineered constructs (Table 2). A central aim of this review is to identify in the literature where well-crafted pursuits have produced viable disc-like tissue, but mechanical properties have yet to be assessed and/or functional benchmarks have not yet been met.

Early disc-related tissue engineering work was concerned primarily with establishing culture systems in which the phenotypes of AF and NP cells could be preserved. One early study

probed the effect of various combinations of growth factors on the fibrocartilaginous phenotype of AF and NP cells seeded in a cross-linked type I collagen/hyaluronan scaffold (Alini et al., 2003). Proteoglycan and collagen synthesis were maintained by various combinations of growth factors, with TGF- β 1 yielding the most pronounced anabolic response by both cell types. Although native tissue composition was not achieved and mechanics were not assessed, this study first indicated that tissue engineering may indeed hold some potential for the treatment of disc degeneration. In the years following this work, AF and NP cells have been successfully cultured on a wide array of synthetic and natural polymers (Chang et al., 2007; Chang et al., 2009; Gruber et al., 2009; Mizuno et al., 2006; Nerurkar, et al., 2009a; Rong et al., 2002; Sato et al., 2003b; Sato et al., 2003c; Shao and Hunter, 2007; Wan et al., 2007).

Tissue Engineering of the AF

Numerous groups have demonstrated the capacity for AF cells to attach to and proliferate on a range of scaffolding materials. In many such studies, AF cells retained phenotypic stability and elaborated an ECM that compositionally resembled native AF (Chang, et al., 2007; Chou et al., 2008; Gruber, et al., 2009; Nesti et al., 2008; Sato, et al., 2003b; Wilda and Gough, 2006; Yang et al., 2008). A particular challenge for AF tissue engineering has been to replicate the multi-scale architecture that distinguishes the AF from other soft tissues. In one recent study, unidirectionally aligned alginate/chitosan fibers (Fig. 3A, Table 2) were synthesized to mimic the collagen alignment within a single lamella of AF and seeded with AF cells (Shao and Hunter, 2007). Although cells adhered to these fibers and deposited fibrocartilagenous ECM, mechanical properties and ECM organization were not investigated, and due to the large size of scaffold fibers, cells maintained a rounded morphology. Recently, several groups have investigated electrospinning for AF tissue engineering (Fig. 3D) (Gruber, et al., 2009; Nerurkar, et al., 2007; Nerurkar, et al., 2008b; Nerurkar, et al., 2009a; Yang, et al., 2008). Electrospinning is a scaffold fabrication method whereby a large electrostatic gradient is used to draw a polymer solution into nanofibers by harnessing the competing forces of charge repulsion and surface tension (Li et al., 2005; Mauck et al., 2009). These electrospun fibers closely approximate the scale of native collagen fibers found in most soft tissues. Collection onto a rotating mandrel results in the formation of an aligned mesh of nanofibers. The macroscopic mechanical behavior of these meshes results directly from their organization, and has been well characterized (Courtney et al., 2006; Li et al., 2007; Nerurkar, et al., 2007). When seeded onto aligned nanofibrous scaffolds, AF cells adopt an elongated morphology, aligning themselves parallel to the underlying scaffold (Baker and Mauck, 2007; Nerurkar, et al., 2008b). During disc development, alignment precedes ECM deposition by AF precursor cells (Hayes et al., 1999; 2001). Indeed, AF cells on aligned nanofibrous scaffolds deposit an aligned, collagen-rich ECM *in vitro*, suggesting that the electrospun scaffold serves as a template for new tissue formation (Nerurkar, et al., 2008b). In one recent study, uniaxial tensile properties of aligned poly(ϵ -caprolactone) nanofibrous scaffolds seeded with bovine AF cells were measured, and doubling of the tensile modulus from 25 MPa to 50 MPa was observed over 8 weeks of *in vitro* culture (Table 2). While this remained below the fiber-direction modulus for a single lamella of native AF (80-120 MPa, Table 1), it represents the highest tensile modulus achieved by a single lamellar engineered AF construct.

Others have synthesized a novel biodegradable polymer for AF tissue engineering, and demonstrated maintenance of fibrocartilagenous phenotype by rat AF cells seeded onto the material (Wan, et al., 2007). Although a compressive modulus was measured (Table 2), it was several orders of magnitude lower than native AF. Some studies have also successfully replicated the multi-lamellar organization of the AF. Recently, one such study developed a biphasic scaffold, where an inner AF region was constructed from a concentrically wrapped bio-polymer sheet seeded with chondrocytes, and an outer AF was fashioned from demineralized bone matrix (Fig. 3E) (Wan et al., 2008). Compressive and tensile tests of the

engineered AF constructs were performed at fabrication; however, only failure properties were reported. Additionally, while this study mimicked the multi-lamellar architecture of the AF, intra-lamellar collagen synthesis and organization was not reported.

In an alternative approach, oriented electrospun scaffolds seeded with mesenchymal stem cells (MSCs) have been used to generate bi-lamellar constructs with opposing collagen orientations of $\pm 30^\circ$ (Nerurkar, et al., 2009a). With 10 weeks of *in vitro* culture, these biologic laminates closely replicated the circumferential tensile modulus of native AF (Table 2) (Nerurkar, et al., 2009a). Moreover, this study identified a unique role for the interlamellar matrix in reinforcing the tensile response of angle-ply tissues like the AF. Studies like this one demonstrate the inherent value of engineered tissues as structural and functional analogs for their complex native counterparts, where studying the simpler system may provide insight into the structure-function relations of the native tissue as well.

Tissue Engineering of the Nucleus Pulposus

Gene expression and ECM production by NP cells has been characterized under a number of culture conditions (Yang and Li, 2009). From these studies, it is evident that, like chondrocytes, ECM production and phenotypic stability of NP cells requires culture in a three-dimensional format, where rounded cell morphology can be maintained (Chou et al., 2006; Chou and Nicoll, 2008; Chou, et al., 2008; Gruber et al., 1997; Gruber et al., 2003; Reza and Nicoll, 2009b; Roughley et al., 2006; Wang et al., 2001). Similar to the AF, mechanics have not been widely investigated for engineered NP tissue, and often times such measures are only made at the time of scaffold fabrication. Therefore, functional changes due to ECM deposition are generally not examined. While to our knowledge, confined compression properties of engineered NP have not yet been reported, a number of studies have reported findings in unconfined compression for various hydrogel-based constructs (Baer et al., 2001; Chou et al., 2009; Cloyd, et al., 2007; Reza and Nicoll, 2009b). Because in unconfined compression the NP is quite soft (~ 5 kPa), many of these studies report comparable properties to native NP prior to any matrix deposition (Table 2). However, the critical role of swelling pressure and the biphasic properties that define the time-varying behavior of NP may be more challenging to achieve.

One noteworthy study examined the compressive and torsional shear properties of alginate hydrogels seeded with porcine inner AF cells and cultured for up to 16 weeks *in vitro* (Baer, et al., 2001). Although the NP-like phenotype of cells was preserved, dynamic shear modulus declined with culture duration, and remained an order of magnitude below native NP. Additionally, the phase angle increased to within 5° of native. While these values demonstrate a gap between engineered and native tissue mechanics, this work is among the few to exhaustively measure mechanics for engineered NP. One recent study measured the dynamic shear properties of type I collagen gels for NP tissue engineering, and while phase angle was lower than native NP, the dynamic shear modulus compared quite favorably (Table 2) (Bron et al., 2009).

Tissue Engineering of the Intervertebral Disc

While many groups have sought to engineer replacements for either the NP or AF, replacement of only one or the other may prove insufficient for cases beyond mild degeneration. However, few groups have focused on engineering an entire intervertebral disc. The union of two tissues as disparate as the fibrous AF and gelatinous NP poses formidable challenges in tissue engineering, and will likely require a combination of biomaterials, cell types, and chemical and mechanical factors. Additionally, diffusion limitations in free swelling culture may preclude the growth and vitality of cells within large, dense tissues. This is problematic for engineering discs that have the appropriate geometries for implantation both clinically and in translational studies using large animal models.

Despite these challenges, in an elegant recent study, a full disc was formed by seeding NP and AF cells into a composite formed from a central alginate gel surrounded by a fibrous PGA mesh (Fig. 3F) (Mizuno, et al., 2006). To address diffusion limitations associated with *in vitro* free swelling culture, engineered discs were implanted subcutaneously in athymic mice. Cells within the construct proliferated and deposited ECM. Most notably, ECM deposition resulted in measurable changes in mechanical function: compressive equilibrium modulus increased by nearly four-fold over 16 weeks while hydraulic permeability decreased. However, the magnitude of equilibrium modulus obtained in this work (~kPa) remained below the axial compressive modulus of the motion segment (~MPa) and the engineered AF region within the construct lacked the angle-ply organization of native AF. Nonetheless, this work represents a landmark achievement in disc tissue engineering. Further, it emphasizes the importance of functional metrics - and not simply histologic or biochemical assessment - as indicators of success.

Another recent study coupled hyaluronic acid (HA) hydrogels with electrospun nanofibrous scaffolds to form engineered discs seeded with human MSCs (Nesti, et al., 2008). MSCs were encapsulated in HA and injected into the center of nonaligned nanofibrous meshes that had also been seeded with MSCs. This method produced distinct NP and AF zones (Fig. 3G). Cells residing within these regions appropriately achieved chondrogenic and fibrous phenotypes, respectively. Nonetheless, mechanical function was not assessed and the microstructural details of native AF organization were not achieved. However, this work demonstrates the ability to generate disc-like tissue from MSCs obtained from human donors, representing an important clinical advancement of the field.

Finally, disc analogues that replicate the microstructural organization of the AF have been developed using aligned nanofibrous scaffolds and hydrogels. These engineered discs possess a multi-lamellar, angle-ply AF region with alternating $\pm 30^\circ$ fiber orientations, and a central NP-like core (Mauck, et al., 2009; Nerurkar et al., 2008a). In preliminary acellular experiments, axial compressive modulus (~2 MPa) - but not torsional shear modulus (~250 kPa) - closely approximated native disc values (Table 1). Short-term culture of these constructs, seeded with bovine AF cells, revealed evolution of a collagen matrix that replicates the $\pm 30^\circ$ fiber organization of the AF (Fig. 3H). While this approach shows promise for replicating both form and function of the AF and coupling with a hydrogel NP, long term studies investigating the effect of ECM deposition on compressive and torsional properties must be performed to determine whether functional growth can be achieved with clinically relevant sizes and geometries.

Challenges in the Future of Disc Tissue Engineering

While significant progress has clearly been made toward engineering a functional replacement for the disc, there remain numerous challenges that must be addressed if tissue engineering is to be implemented clinically. Our discussion thus far has been limited to the NP and AF; however, the cartilaginous endplate plays an important role in nutrient supply and waste removal and forms the interface between the disc and vertebral body. Engineering an interface between two disparate tissues is indeed a complicated problem, and one which is being studied in other tissues (Lu and Spalazzi, 2009; Spalazzi et al., 2006; Yang and Temenoff, 2009) as well as the disc (Hamilton et al., 2006). Upon successful engineering of the disc, it will be important to develop a functional interface with which the construct can be implanted into the disc space without risk of dislocation. Moreover, transmission of force across the interface from vertebral bodies to the disc is essential if the disc is to function in its load bearing capacity. If, for instance, the fibers of the AF are not anchored within the vertebrae and endplates, they cannot bear tensile loads and will, as a result, fail to stabilize the spine. As more is learned

about the composition, architecture, and function of the endplate, it may be possible to engineer discs that include this tissue and can interface with the vertebral bodies themselves.

Avascularity of the disc is thought to play a prevalent role in the onset of disc degeneration, as efficient nutrient delivery and waste removal does not occur (Urban et al., 2004). So too, is this a challenge for engineered discs. Small engineered constructs, suited for instance to the disc geometry of small animal models such as mouse and rat, may mature *in vitro* and obtain mechanical properties commensurate with the native disc. However, scaling up this approach is likely to prove quite challenging, as the diffusion distance increases with increasing geometry. Therefore, while small geometries may serve as a sound starting point, it will be important that strategies can be scaled to sizes of clinical relevance, matching the length scale of the human adult disc. While it is unlikely that diffusion alone will suffice under these conditions, it is possible that convective transport may facilitate nutrient transport. Therefore, either flow or deformational loading bioreactors may be necessary. Alternatively, subcutaneous implantation provides an *in vivo* environment that may be more nourishing than *in vitro* culture, and has been shown to promote ECM deposition and functional growth in engineered discs of small to moderate size (Mizuno et al., 2004; Mizuno, et al., 2006). These challenges demonstrate that, while achieving functional parity with the disc is important, and itself a formidable task, there remain significant obstacles in the future of disc tissue engineering.

Other Regenerative Approaches

It is important to note that tissue engineering is not the only treatment for disc disease that is being actively researched. Although disc allografts have had moderate success in canine and nonhuman primate models (Luk et al., 2003; Matsuzaki et al., 1996), availability of healthy human disc tissue is very limited. Moreover, differences in mechanics, composition, geometry, and immunoreactivity preclude interspecies allografts from being a viable alternative. While tissue engineering relies on a scaffold or carrier for the delivery of cells, some groups have recently investigated the protective effect of direct injection of cells into the disc (Crevensten et al., 2004; Sakai et al., 2003). While MSC delivery by bolus injection has reduced degenerative changes such as GAG depletion and loss of disc-height, these studies are typically carried out in animal models where degeneration (which normally progresses pathologically over several years) is emulated rather abruptly by injury, injection of some agent, or mechanical stimulation (Boxberger et al., 2006; Elliott et al., 2008; Hoogendoorn et al., 2007; Hsieh et al., 2009; Imai et al., 2007; Wuertz et al., 2009). Injection of growth factors and anabolic agents into the disc has also been considered, with promising results observed for molecules such as OP-1 and BMP-2 (Li et al., 2004; Masuda et al., 2003; Masuda et al., 2006). Alternatively, some groups have sought to revive cells within the ailing disc via gene therapy. This is a promising approach, that could potentially induce disc cells to generate protective or anabolic signals themselves, eliminating the need for repeated injections of growth factors or other therapeutics (Nishida et al., 1998).

These minimally invasive, injection-based therapies - be they cells, proteins, or gene therapy - will likely be most effective when implemented early in degeneration, before structural breakdown of the AF has occurred. However, because discogenic low back pain develops quite late during the degenerative process, these therapies might not be a substitute for structural tissue engineered replacements. However, it is possible that the success of tissue engineering treatments may require commingling with one or a combination of these other regenerative techniques.

CONCLUSIONS

Our current understanding of the intervertebral disc is built upon decades of careful analysis of disc structure, composition, and cell biology. This rich bedrock of mechanical investigation has provided insight into how the disc can function in such a demanding environment, and some of the mechanisms by which it fails when structure and function give way to degeneration. Disc tissue engineering has now advanced to the point where comparisons to native disc composition and mechanics are an essential consideration. It will be critically important to evaluate constructs for their ability to replicate the mechanical role of the disc. This is especially true as disc tissue engineering moves toward animal models and ultimately toward application to treat degeneration and back pain. In this review, we have outlined a number of functional benchmarks that capture the mechanical complexity of the native tissue. These values (and corresponding measurement techniques) provide the means to assess engineered tissue function in direct comparison to native tissue. We provide values for each property currently measured in the disc literature, from the sub-lamellar level, to that of the entire motion segment. As each scale of functionality builds one upon the other, resulting in function at the highest order, it is likely that some or most of these values will have to be achieved if motion segment mechanics are to be restored with an engineered disc replacement. The benchmarks outlined in this review provide a quantitative means to identify ‘successes’ at each level of disc hierarchy and to recognize the remaining ‘challenges’ on the path to clinical implementation.

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Figure 1.

A) Schematic representation of the multi-scale architecture of the intervertebral disc indicates the primary geometric axes that are regularly referred to in the text: r = radial direction (from the nucleus pulposus outward), z = axial direction (the spinal long axis), and θ = circumferential direction (parallel to the lamellae as they wrap around the disc). Image modified from Guerin and Elliott (Guerin and Elliott, 2006b). B) MRI of a motion segment, with disc substructures as labeled: AAF = anterior annulus fibrosus, PAF = posterior annulus fibrosus, NP = nucleus pulposus, VB = vertebral body.

Figure 2.

Schematic representations are shown for the testing modalities discussed (Left), along with typical stress-strain profiles associated with each (Right). E = modulus; toe/lin = toe region/linear region; ϵ^* = transition strain; ϵ_y = yield strain; G = shear modulus; S_{int} = interfacial/lap strength; $\sigma_{\text{peak/equil}}$ = peak/equilibrium stress.

Figure 3.

An array of strategies for disc tissue engineering. A) hybrid alginate/chitosan fibers synthesized for AF tissue engineering (Shao and Hunter, 2007). B) Carboxymethylcellulose gel seeded with NP cells (Reza and Nicoll, 2009a). C) Atelocollagen honeycomb scaffolds engineered from natural ECM (Sato et al., 2003a; Sato, et al., 2003b). D) Aligned, electrospun, nanofibrous scaffolds seeded with mesenchymal stem cells (Nerurkar, et al., 2007; Nerurkar, et al., 2008c; Nerurkar, et al., 2009a; Yang, et al., 2008). E) Engineered multi-lamellar AF constructed from poly(polycaprolactone-triol-malate) seeded with chondrocytes, and surrounded with a demineralized bone matrix (Wan, et al., 2008). Composite whole-discs constructed from an NP cell-encapsulated alginate hydrogel surrounded by an AF cell-seeded PGA mesh (Mizuno, et al., 2006). G) Disc formed from a composite hyaluronic acid/nanofibrous scaffold seeded with human mesenchymal stem cells (Nesti, et al., 2008). H) Polarized light microscopy of Picrosirius Red stained section from disc-like angle-ply structure formed from aligned nanofibrous scaffolds surrounding an agarose NP (Nerurkar et al., 2009b). Published images appear with permission from John Wiley & Sons (A, C), and Elsevier (E, F); additional images were provided as acknowledged below.

Table 1

Summary of native tissue benchmarks related to mechanics of the AF, NP, and motion segment. E = modulus; ϕ indicates angle relative to the prevailing collagen orientation; S_{int} = interfacial strength; toe/lin = toe region/linear region of stress-strain curve; θ, z , and r indicate the loading axes along the disc circumferential, axial, and radial directions as indicated in Fig. 1A; ε^* = transition strain indicating the transition from toe to linear region of the stress-strain curve; ν = Poisson's ratio; ε_{yield} = yield strain; G = shear modulus; $|G^*|$ = complex shear modulus; δ = phase shift; P_{swell} = swelling pressure; H_{Ao} = aggregate modulus; β = modulus nonlinearity parameter; k_o = permeability; M = permeability nonlinearity parameter; G_{app} = apparent shear modulus; $\tau_{1/2}$ = time constants describing short/long term creep response.

TISSUE Scale	Testing Modality	Benchmark	Native Value	Reference
Annulus Fibrosus Sub-lamella	Nanoindentation	E	0.6 – 1.2 MPa	Lewis, et al., 2008
Annulus Fibrosus Single Lamella	Uniaxial Tension	E ($\phi=0^\circ$)	80 – 120 MPa	Skaggs, et al., 1994 Holzapfel, et al., 2005
		E ($\phi=90^\circ$)	0.22 MPa	
		ν ($\phi=0^\circ, 90^\circ$)	N/A	
Annulus Fibrosus Multiple Lamellae	Lap Testing	S_{int}	N/A	N/A
	Uniaxial Tension	E_θ (toe/linear)	2.5/18-45 MPa	Acaroglu et al., 1995 Ebara et al., 1996 Fujita et al., 1997 Guerin and Elliott, 2006a Elliott and Setton, 2001 Kasra, et al., 2004 Guerin and Elliott, 2007
		ε_θ^*	0.06	
		E_z (toe/linear)	0.27/0.82 MPa	
		E_r (toe/linear)	0.19/0.45 MPa	
		$\nu_{\theta z}$	1.77	
		$\nu_{\theta r}$	0.33	
		ε_{yield}	20% - 30%	
	Biaxial Tension	Axial Fixed E (toe/linear)	9.8/27.2 MPa	O'Connell et al., 2010
		Equibiaxial E (toe/linear)	<u>Circumferential</u> 16.5/43.3 MPa <u>Axial</u> 10.7/ 26.8 MPa	
	Planar/Torsional Shear	$G_{z\theta}$	20 – 125 kPa	Iatridis, et al., 1999 Fujita, et al., 2000 Jacobs, et al., 2010
		$ G^* $	75 – 200 kPa	
		δ	17° – 20°	
	Confined Compression	P_{swell}	110 - 130 kPa	Best, et al., 1994 Iatridis, et al., 1998 Perie, et al., 2005
		H_{Ao}	440 - 750 kPa	
		β	2.7	
		k_o	1.6 – 2.3 ($\times 10^{-16}$ m ⁴ N ⁻¹ s ⁻¹)	
		M	1.5	
NUCLEUS PULPOSUS	Confined Compression	P_{swell}	0.138 MPa	Johannessen and Elliott, 2005

TISSUE Scale	Testing Modality	Benchmark	Native Value	Reference
		H_A^{eff}	1.0 MPa	
		k_o	$9.0 (\times 10^{-16} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1})$	
	Torsional shear	$ G^* $	7.4 – 19.8 kPa	Iatridis, et al., 1997
		δ	23° – 30°	
MOTION SEGMENT	Axial Compression	Stiffness	1.73 kN/mm	Nachemson et al., 1979
		E	3 – 10 MPa	Shea et al., 1994 Beckstein, et al., 2008
	Torsion	G_{app}	2 – 9 MPa	Elliott and Sarver, 2004 Abumi et al., 1990 Haughton et al., 2000 Beckstein et al., 2007
	Static Viscoelasticity	Various multi-parameter rheological models; see referenced literature for details.		Keller, et al., 1987 Beckstein, et al., 2008
	Dynamic Viscoelasticity	Dynamic Stiffness	3.5 – 5.1 kN/mm (0.001 – 1 Hz, respectively)	Costi, et al., 2008

Table 2

Summary of recent advances in functional tissue engineering of the AF, NP, and disc.

	Cell Source	Scaffold Material	Major Finding	Mechanics Measured		Native Benchmark	Reference
AF	AF cells (lapine)	Atelocollagen honeycomb (Fig. 3C)	AF cells retained fibrocartilage phenotype and produced more ECM in 3-D culture than in monolayer	N/A		N/A	Sato, et al., 2003b
	AF cells (canine)	Alginate/chitosan hybrid fibers (Fig. 3A)	AF cells attached to fibers and deposited ECM containing types I and II collagen and aggrecan	N/A		N/A	Shao and Hunter, 2007
	AF cells (rat)	Poly(1,8-octanediol malate)	AF cells proliferated and expressed type II collagen; constructs were nonimmunogenic upon subcutaneous implantation	Compressive modulus	0.12 – 0.25 kPa	440 – 750 kPa	Wan, et al., 2007
				Ultimate tensile stress	7-15 MPa	N/A	
	AF cells (bovine)	Electrospun PCL (Fig. 3D)	AF cells oriented parallel to aligned nanofibers and deposited aligned collagen matrix, resulting in improved tensile mechanics	Uniaxial tensile modulus	50 MPa	80 – 120 MPa	Nerurkar et al., 2008b
	Chondrocytes (lapine)	Bone matrix gelatin and poly(polycaprolactone triol malate) (Fig. 3E)	Cells survived within multi-lamellar constructs, deposited disc-like ECM, and constructs survived 100 cycles of compression without permanent deformation	Ultimate tensile stress	1.3 MPa	N/A	Wan, et al., 2008
				Ultimate compressive stress	3.5 MPa	N/A	
NP	MSCs (bovine)	Electrospun PCL	Bi-lamellar constructs replicated $\pm 30^\circ$ angle-ply collagen architecture of AF; opposing fiber orientations enhanced tensile response over parallel fiber families via inter-lamellar shearing	Uniaxial tensile modulus	14.5 MPa	18 MPa	Nerurkar, et al., 2009a
	Inner AF (bovine)	Alginate	Cells survived and expressed fibrocartilage markers, but compressive and torsional properties declined with culture duration despite matrix accumulation	Compression and torsion	$\delta = 6^\circ - 14^\circ$ $ G^* = 0.2 - 0.6 \text{ kPa}$	23 - 30° 7.4 – 19 kPa	Baer, et al., 2001
	NP cells (bovine)	Calcium polyphosphate substrate	Cells deposited ECM that matched native proteoglycan content but not collagen content, resulting in improved properties in unconfined compression	Unconfined compression	$E_{eq} = 8.3 \text{ kPa}$	5.4 kPa	Seguin et al., 2004
	N/A	Type I collagen gel	Gel formation was tailored to replicate mechanical function of the NP in dynamic shear	Torsional shear	$\delta = 6.5^\circ - 8.5^\circ$ $ G^* = 2 - 10 \text{ kPa}$	23 - 30° 7.4 – 19 kPa	Bron, et al., 2009
	NP cells (bovine)	Photo-crosslinked carboxymethylcellulose (Fig. 3B)	Hydrogel processing produced tunable mechanical properties; NP cells survive when encapsulated	Unconfined compression	$E_{eq} = 4.3 \text{ kPa}$	5.4 kPa	Reza and Nicoll, 2009b

	Cell Source	Scaffold Material	Major Finding	Mechanics Measured		Native Benchmark	Reference
	NP cells (bovine)	Photo-crosslinked alginate	Photocrosslinking improved proteoglycan accumulation by NP cells, resulting in increased compressive properties with culture duration	Unconfined compression	$E_{eq} = 4.3 \text{ kPa}$	5.4 kPa	Chou, et al., 2009
IVD	AF and NP cells (ovine)	PGA (AF) and Alginate (NP) (Fig. 3F)	Successfully formed AF-NP composites with ECM accumulation and increased compressive properties after subcutaneous implantation	Unconfined compression	$H_A = 50 \text{ kPa}$ $k = 5 (\times 10^{-14} \text{ m}^2/\text{Pa s})$	3 – 10 MPa	Mizuno, et al., 2004 Mizuno, et al., 2006
	AF cells and MSCs (bovine)	Electrospun PCL (AF) and agarose (NP) (Fig. 3H)	Nanofibrous reinforcement enhanced agarose mechanics; cells adopted opposing $\pm 30^\circ$ orientations and deposited collagen into multi-lamellar angle-ply organization of native AF	Unconfined compression	2 MPa	3 – 10 MPa	Nerurkar, et al., 2008a
	MSCs (human)	Electrospun PLLA (AF) and hyaluronic acid (NP) (Fig. 3G)	Human MSCs adopted AF and NP like phenotypes and accumulated disc-like ECM	Torsion	250 kPa	2 – 9 MPa	
				N/A	N/A	N/A	Nesti, et al., 2008