Nanomechanical Properties of Individual Chondrocytes and Their Developing Growth Factor-Stimulated Pericellular Matrix

Laurel Ng\textsuperscript{a}, Han-Hwa Hung\textsuperscript{b}, Alexander Sprunt\textsuperscript{c,g}, Susan Chubinskaya\textsuperscript{d}, Christine Ortiz\textsuperscript{e}, Alan Grodzinsky\textsuperscript{a,b,c,f*}

\textsuperscript{a}Biological Engineering Division, \textsuperscript{b}Center for Biomedical Engineering, Departments of \textsuperscript{c}Mechanical Engineering, \textsuperscript{e}Material Science & Engineering, and \textsuperscript{f}Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge MA 02139

\textsuperscript{d}Department of Biochemistry and Section of Rheumatology, Rush University Medical Center, Chicago

\textsuperscript{g}currently at ATA Engineering, San Diego, CA

*Correspondence and requests for materials should be addressed to:

Correspondence to:
Alan J. Grodzinsky
77 Massachusetts Ave. NE47-377
Cambridge, MA 02139
Phone: (617) 253-4969
Fax: (617) 258-5239
Email : alg@mit.edu

Keywords: cartilage, chondrocytes, nanomechanics, nanoindentation, growth factors

Word Count: 3,527 (Introduction through Discussion)

For Submission to Journal of Biomechanics as an Original Article
ABSTRACT

The nanomechanical properties of individual cartilage cells (chondrocytes) and their aggregcan and collagen-rich pericellular matrix (PCM) were measured via atomic force microscope nanoindentation using probe tips of two length scales (nanosized and micron-sized). The properties of cells freshly isolated from cartilage tissue (devoid of PCM) were compared to cells that were cultured for selected times (up to 28 days) in 3-D alginate gels which enabled PCM assembly and accumulation. Cells were immobilized and kept viable in pyramidal wells microfabricated into an array on silicon chips. Due to the nonconventional but known geometry of the microfabricated wells, Hertzian contact mechanics as well as finite element analyses were employed to estimate apparent moduli from the force-depth curves. The effects of culture conditions on the resulting PCM properties were also studied by comparing 10% fetal bovine serum vs. medium containing a combination of insulin growth factor-1 (IGF-1) + osteogenic protein-1 (OP-1). While both systems showed increases in matrix stiffness with time in culture between days 7 to 28, the IGF-1 + OP-1 combination resulted in a higher stiffness for the cell-PCM composite by day 28, and a higher apparent modulus of the PCM. These studies give insight into the temporal evolution of the nanomechanical properties of the pericellular matrix relevant to the biomechanics and mechanobiology of tissue engineered constructs for cartilage repair.
1. Introduction

Chondrocytes occupy only 3-5% of the volume of adult articular cartilage and, hence, do not contribute significantly to the bulk mechanical properties of the tissue (Stockwell and Meachim 1979). However, they are responsible for the synthesis, maintenance, and turnover of the tissue’s extracellular matrix (ECM). Mechanical loads and deformations applied to cartilage in vivo and in vitro are known to regulate chondrocyte synthesis and catabolic degradation of ECM macromolecules (Fitzgerald et al., 2004; Guilak et al., 1994; Kim et al., 1994; Valhmu et al., 1998). The mechano-regulation of chondrocyte metabolism in tissue engineering gel scaffolds depends partly on the cell’s environment and the development stage of the newly synthesized, cell-associated pericellular matrix (PCM) (Buschmann et al., 1995).

The 2-4µm thick PCM contains a high percentage of typeVI collagen and proteoglycans (PGs) (Poole et al., 1992; Poole et al., 1988a) and is critically important to biochemical and biomechanical cellular function (Petit et al., 1996).

The PCM transfers loads from the ECM to the cell and its cytoskeleton and intracellular organelles during physiologically-induced deformations in compression, shear, and tension. Thus the mechanical properties of individual chondrocytes with and without their PCM have been measured using micropipette aspiration (Guilak 2000), cytoindentation (Koay et al., 2003), and in unconfined compression (Leipzig and Athanasiou 2005). Apparent moduli of isolated chondrocytes were reported in the range 0.6-4kPa from micropipette aspiration and compression of cells in agarose (Freeman et al., 1994). The PCM in adult cartilage has a higher modulus than the cell (~60-70kPa) as measured via micropipette aspiration (Alexopoulos et al., 2003; Guilak et al., 1999), compression of chondrons in agarose (Knight et al., 2001), and AFM indentation (Allen and Mao 2004).
The PCM may also act as a regulator of cell signaling. Scaffolds seeded with chondrons containing intact PCM accumulated proteoglycans and typeII collagen more rapidly than parallel cultures of enzymatically isolated chondrocytes initially devoid of PCM (Graff et al., 2003). Chondrocytes treated with insulin-like growth factor (IGF-1) and osteogenic protein-1 (OP-1) showed increased PCM accumulation compared to chondrocytes maintained in fetal bovine serum (FBS) (Flechtenmacher et al., 1996; Loeser et al., 2003; McQuillan et al., 1986; Nishida et al., 2000; van Osch et al., 1998). Architecture and morphology of this newly developing PCM differ from adult chondron PCM as seen in immunohistochemistry of type VI collagen which revealed a compact structure in adult chondrons but had a diffuse appearance in immature tissue (Lee and Loeser 1998). Taken together, efforts to create tissue engineered cartilage require detailed understanding of pericellular microenvironments.

In this study, we examined the mechanical properties of immature bovine cartilage chondrocytes and their newly developing PCM using atomic force microscope (AFM)-based indentation (i.e., measurement of force, F, versus indentation depth, D, on loading and unloading at two length scales via a nanosized tip (end-radius, \( R_{\text{tip}} \approx 40 \text{nm} \)) and a micron-sized tip (\( R_{\text{tip}} \approx 2.5 \mu m \)). The use of AFM to probe the mechanical properties of individual living cells has been reviewed (A-Hassan et al., 1998; Lehenkari et al., 2000a; Radmacher 1997) and applied in the context of mechanotransduction (Charras and Horton 2002), disease (Chasiotis et al., 2003), drug effects (Rotsch and Radmacher 2000), and lysis kinetics (Hategan et al., 2003). To apply this technique to phenotypically round, nonadherent chondrocytes, we first developed microfabricated surfaces with wells to immobilize individual cells while keeping them viable. Freshly isolated chondrocytes, and cells released from 3D alginate gels after selected times in culture, were immobilized on these microfabricated surfaces, and studied nanomechanically to determine the effects of the newly developing PCM on cell-PCM stiffness. Cultures were
supplemented with either FBS or the combination of IGF-1+OP-1 to compare the effects of these anabolic stimulants on PCM development. The temporal evolution of cell-PCM biomechanical properties was compared to total GAG and collagen accumulation over time in culture by alginate-released cells. Apparent cell and PCM moduli were estimated from nanoindentation data using Hertzian contact mechanics and finite element analyses (FEA).

2. Materials and methods

2.1 Cell isolation and culture

Chondrocytes were isolated from femoral condyle cartilage of 2-3 week old bovine calves using sequential 0.2% pronase (Sigma) and 0.025% collagenase (Boehringer Mannheim) digestions as described (Ragan et al., 2000). Cell viability after isolation, assessed by trypan blue (Sigma) exclusion, was >95%. Cells were seeded at 20x10^6 cells/ml in 2% w/v alginate (KelcoLVCR) in 0.9% NaCl. Beads ~3mm diameter were formed through polymerization of droplets of alginate dispensed from a 22-gauge needle into 102mM CaCl_2 solution. At selected times in culture, cells were released from alginate beads by depolymerization in a calcium chelator (Hauselmann et al., 1992;Petit et al., 1996), 55mM NaCitrate, as described (Masuda et al., 2003). In one series of experiments, cell-seeded beads were maintained in hi-glucose DMEM with 10% FBS, 20µg/ml L-ascorbic acid (Sigma), and 1% antibiotic-antimycotic (Sigma). In a second series, cells were cultured in hi-glucose DMEM supplemented with 100ng/ml recombinant human IGF-1 (PreproTech), 100ng/ml recombinant human OP-1 (Stryker Biotech, Hopkinton, MA), mini-ITS (Benya and Padilla 1993)(containing 5nM insulin (Sigma) to minimize stimulation of the IGF-1 receptor, 2µg/ml transferrin (Sigma), 2ng/ml selenous acid (Sigma), 420/2.1µg/ml linoleic acid-albumin from bovine serum albumin (Sigma)), 55µg/ml L-ascorbic acid), and 1% antibiotic-antimycotic. Seven alginate beads were cultured in 3ml
medium per well (12 well plate); medium was changed every other day.

2.2 Histology and immunohistochemistry of type VI collagen

Cells released from alginate were resuspended in culture medium (1x10^6 cells/ml) and fixed in 2%(v/v) glutaraldehyde solution (Polyscience) buffered with 0.05M sodium cacodylate (Sigma), and containing 0.7%(w/v) of ruthenium hexaamine trichloride (RHT, Polyscience) to minimize loss of PGs during fixation (Hunziker et al., 1982). Fixed cells were mounted onto glass slides using a Cytospin (1,400rpm for 10min), air dried, and stained for sulfated-PGs (Toluidine BlueO, Sigma) and collagen (phosphomolybdic acid followed by aniline blue (Rowley Biochemical))(Luna 1968). In addition, cells from day39 culture were released, mounted on glass slides, dried for 3hours, treated with 2mg/ml hyaluronidase (Sigma) in 0.1M Tris(hydroxymethyl)aminomethane-HCl, pH 5.8, for 2.5 hours at 37°C to expose typeVI collagen epitopes, then blocked with 5% donkey serum in PBS, pH 7.1, for 4 hours. The antibody for typeVI collagen (Chemicon) was incubated on the slides overnight (1:10 dilution in 1% donkey serum in PBS), then incubated with a secondary rhodamine-conjugated antibody (1:50 dilution in 1% donkey serum in PBS) for 4 hours. Slides were rinsed with PBS after each step; fluorescently labeled cells were viewed using a Nikon TE300 microscope. Cell viability after release was >90% as assessed using fluorescein diacetate (0.2mg/ml) and ethidium bromide (10µg/ml) (Sigma).

2.3 Cell appearance and pericellular biochemical composition

Dimethyl methylene blue dye binding (DMMB)(Farndale et al., 1986) and hydroxyproline(Woessner 1961) assays were used as measures of sulfated-GAG and collagen content, respectively. Optical micrographs of cells released at each time point were obtained to measure cell diameter and to aid in estimating PCM thickness.
2.4 Atomic force microscope imaging

Tapping mode AFM (TMAFM) images were taken of chondrocytes adsorbed on mica (SPI Supplies, West Chester, PA) in ambient conditions using a Multimode Nanoscope IIIa (Veeco, Santa Barbara, CA) and Olympus AC240TS-2 rectangular Si cantilevers ($k \sim 2\text{N/m}$, $R_{\text{tip}} < 10\text{nm}$).

2.5 Microfabrication of Silicon Substrates for Single Cell Immobilization

Microfabricated substrates were prepared from silicon wafers at MIT’s Microsystems Technology Laboratory and contained an array of inverted square pyramidal wells to hold a single cell in each well (Fig. 1a,b). Substrates with well side-dimensions of 15, 18, 20, or 22µm were designed to hold freshly isolated cells and cells with associated PCM.

2.6 Nanoindentation

Silicon substrates were cleaned in piranha solution (3:1 concentrated $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2(30\%)$) rinsed with acetone and DI water, and then immersed in DI water for 2 days. Culture medium was allowed to coat the silicon surface for 5 minutes. Then 100µl of cell suspension was dropped onto the surface just prior to nanomechanical testing. The silicon wells, cells, and cantilever probe tip could be visualized with a 10× optical microscope attached to the AFM (Fig. 1b). An AFM probe tip was used to push a cell laterally into a silicon well and then to perform cycles of nanoindentation (Fig. 1c). The cell-containing well was identified and indentation was then repeated on the same cell using a second probe tip of different size (Fig. 1c). The Picoforce NanoscopeIV AFM (Veeco) was used to obtain indentation (F-D) data at z-piezo displacement rates of 200nm/s, 500nm/s, 1µm/s, 3µm/s, 5µm/s, and 10µm/s. No significant change in load-unload hysteresis was observed up to 1µm/s. Above 1µm/s, the area enclosed by the hysteresis loop increased in a logarithmic fashion. Therefore, to limit the contribution of cellular
visco/poro-elastic effects to the measurements, data obtained at 1µm/s are the focus of this paper (Appendix A). A standard Si₃N₄ AFM square pyramidal tip (Veeco, Rₜip~50 nm, k~0.06N/m) and a colloidal probe tip were used. The colloidal probe tip was prepared using the AFM by attaching 2.5µm radius silica beads (Bang Labs) onto tipless cantilevers (Veeco, k~0.06N/m) with low viscosity epoxy (SPI, MBond610). Cantilever spring constants were calibrated individually by the thermal oscillation method (Hutter and Bechhoefer 1993).

2.7 Cell stiffness

Apparent elastic moduli were estimated from the nanoindentation data using 3 models: the Hertz model for a conical tip and a spherical (colloidal) tip, the slopes of stress/strain curves in the small strain region, and elastic FEA simulations. Analysis of cell moduli was limited to indentation depths less than 10% of the cell diameter, a small strain regime that minimized artifacts introduced from substrate effects (Tsui and Pharr 1999).

FEA were carried out with ABAQUS (Providence, RI). ¼ of the tip and cell were modeled; the well walls remained fixed in all directions, a frictionless boundary condition between the elastic cell and rigid well wall was used, and the displacement of the tip occurred only in the z-direction (normal to the cell) to ensure symmetry (see Appendix B for details).

2.8 Statistics

For PCM thickness and force-indentation curves of individual cells (n=5 replicate loading cycles applied to the cell), data are reported as mean±SD. When averaging loading curves for multiple cells, with each cell subjected to five replicate loading cycles, data are reported as mean±SE. Changes in PCM thickness were tested using ANOVA. The effects of depth and tip radius or culture time on indentation force were tested using two-way ANOVA. When significant effects (p<0.05) were detected, comparisons between groups were performed using the Tukey post hoc test.
3. Results

3.1 Characterization of pericellular matrix

TMAFM images in air show that freshly isolated cells (day0) had no distinct PCM (Fig. 2a). After day6 (10% FBS culture), a PCM layer was clearly observed (Fig.2b is a typical image for day11). By day18 (Figs. 2c,d), type II collagen could be identified in the PCM as reported previously in alginate gel beads using immunohistochemistry and gel electrophoresis (Petit et al., 1996). Here, TMAFM showed fibril diameters of 59±9nm with a prominent small banding periodicity of 22±2nm, n=10, likely associated with the 0.4D overlap zone within the primary D=67nm periodicity (Eyre 2005;Hodge 1967;Ortolani et al., 2000).

Images of fixed cells (Fig.3) confirmed that freshly isolated cells (day0) had no visible accumulation of PG or collagen. By day7, PGs stained uniformly around the entire cell for both the FBS and IGF-1+OP-1 cultures. Collagen staining was minimal for FBS but distinct for IGF-1+OP-1 cultures, both around the cell membrane and as a diffuse halo extending outward from the cell. By day14, PG staining increased in diameter and intensity for both cultures, and collagen staining appeared around FBS cultured cells. By the third and fourth weeks in culture, no substantial changes in PG staining were observed, but collagen staining increased slightly in intensity and extent for both cultures. PG staining generally appeared greater with IGF-1+OP-1 compared to FBS at all time points. While pericellular collagen staining appeared to extend further from the cell with IGF-1+OP-1, staining intensity remained diffuse. Cell diameter (excluding PCM) was 7.65±0.85µm (mean±SD). PCM thickness by optical microscopy was ~3-4µm between days7-28, and did not change significantly during this time (Fig.4a). Instances of dividing cells sharing matrix were observed but were not used for nanoindentation. Cell viability in gel culture remained above 80%, and chondrocytes retained their spherical phenotype (Fig.4b).
Type VI collagen was visible in the PCM of FBS-(Fig.4c) and IGF-1+OP-1-cultured cells (not shown) processed at day 39, similar to that found in related experiments with same-aged bovine calf chondrocytes on day 7, 14, and 21 in agarose gel (DiMicco et al., 2005). Thus, these cells were capable of synthesizing and depositing this important component of the PCM in gel culture. Biochemical characterization showed that GAG and collagen content increased rapidly between days 0 and 14 in both cultures (Fig.5). Between days 14-28, total collagen content did not appear to increase in either culture; GAG content continued to increase but was lower in FBS compared to IGF-1+OP-1 cultured cells up to day 28.

3.2 Indentation of freshly isolated cells

Indentation tests performed on single freshly isolated cells (day 0) using both the nanosized and micron-sized probe tips consisted of 5 sequential loading-unloading cycles averaged at one location at a displacement rate of 1 µm/s (Fig.6). A nonlinear increase in repulsive force with indentation depth was observed; the small standard deviations for the 5 cycles indicated reversibility of deformation after each cycle. Subsequent comparisons between days and culture conditions utilized only the loading component of the cycle. For day 0 cells, the micron-sized probe tip produced significantly different forces than the nanosized probe tip at the same indentation depth (p<0.05) (Fig.7). The Hertz model predicted an apparent cell modulus between 0.7-1 kPa (Appendix B). FEA simulations accounting for cell and tip geometry and boundary conditions (Fig.8a) predicted an apparent cell modulus between 2.3-3 kPa for the micron-sized probe tip (Fig.8b) (Appendix B).

3.3 Indentation of cells with newly developing pericellular matrix

For cells in 10% FBS, both probe tips revealed stiffening of the cell-PCM composite with time in culture up to day 28 (p<0.05) (Fig.9a and b). For the nanosized probe tip, this stiffening
with time was apparent for data in the range D>700 nm, while for the micron-sized probe tip, stiffening was pronounced throughout most of the indentation range. Interestingly, the cell-PCM composites were always less stiff than the freshly isolated day0 cells devoid of PCM (p<0.05) (Fig.9a,b). For day28 cells, the nanosized and micron-sized tips showed similar nanoindentation behavior for D<900 nm (Fig.9c), while for D>900nm, the nanosized tip force exceeded the micron-sized tip at the same indentation depth.

Cell-PCM composites in IGF-1+OP-1 also stiffened with time, observed with nanosized and micron-sized tips, and to a greater degree than the FBS-cultured cells (Fig.10). By day 28, cell-PCM composites were stiffer than freshly isolated day0 cells (p<0.05)(Fig.10). A modified FEA model of a cell with a surrounding elastic (PCM) shell, indented by the micron-sized tip, was used to estimate apparent moduli of the PCM. Using the apparent modulus of day0 cells with no PCM (2.75kPa, Fig.8b) the shell modulus was varied until the model output matched the experimental data. For day21 FBS cultured cells, the shell modulus was 0.1kPa and by day28, the shell modulus increased slightly to 0.17kPa. For the day 21 IGF-1+OP-1 cultured cells, the shell modulus was 10× stiffer than the FBS cultured cells at 1kPa and by day28, the shell modulus was 26× stiffer at 4.15kPa (Fig.8d). See Appendix B4 and Table A1 for more details.

4. Discussion

4.1 Mechanical properties of freshly isolated (day 0) cells

While the micron-sized probes interrogate cellular-scale properties, the small radius of curvature of nanosized probes are expected to be more sensitive to local cellular structures such as cytoskeletal elements and intracellular organelles. Nevertheless, the estimated modulus measured at both length scales was of the same order. This is in contrast to previously reported measurements on intact cartilage where the stiffness was 100× greater with a micron-sized probe
compared to a nanosized probe (Stolz et al., 2004). Hysteresis observed during the sequential, reversible loading/unloading cycles (Fig.6) suggested the presence of time-dependent material behavior (see Appendix B.5 for estimates of viscoelastic properties).

4.2 Pericellular matrix development

Developing PCM was confirmed and characterized using AFM imaging, measurement of collagen and GAG content, histological assessment of collagen and sulfated proteoglycans, and immunofluorescence labeling of type VI collagen. The appearance of the PCM structure (e.g. a dense network of collagen fibrils) observed by TMAFM imaging (Fig.2) was similar to characteristic features of protease-digested native cartilage by TEM (Jurvelin et al., 1996) and AFM (Stolz et al., 2004). GAG and collagen accumulation with time in culture (Fig.5) were qualitatively similar to that previously reported (Ragan et al., 2000), and the enhanced accumulation of GAG resulting from IGF-1+OP-1 compared to FBS treatment was consistent with previous studies (Loeser et al., 2003). Histology showed a larger collagen-stained region for IGF-1+OP-1 compared to the FBS-fed cells, though likely more diffuse and containing less total collagen at later times, as suggested by Fig.5b. Type VI collagen (Fig.3c), characteristic of the PCM, appeared as a diffuse halo around cells as seen by Lee and Loeser (1998), compared to the compact and more uniform PCM of fully developed chondrons (Poole 1997).

4.3 Mechanical properties of cells with developing PCM

Cell cultures supplemented with FBS or IGF-1+OP-1 produced cells with a developing PCM that increased in stiffness from day 7 until day 28 in culture (Fig.9). While the estimated thickness of the PCM did not change significantly after day 7 (Fig.4a), GAG and collagen levels increased substantially from day 7 to day 14, suggesting an increase in PCM density consistent with increased PCM stiffness (Fig.9). However, IGF-1+OP-1 cultured cells showed increases in
stiffness despite a leveling of PGs and collagen. One explanation may be that the PCM is undergoing molecular organization of the small PGs and collagen, as well as collagen crosslinking (Chang and Poole 1997; Eyre et al., 1987; Poole et al., 1988b). In addition, OP-1 has been found to help chondrocytes retain their developing PCM after release from alginate (Nishida et al., 2000). While the developing PCM appears to accumulate quickly in culture, the molecular structure and collagen architecture within the PCM may not resemble that of fully developed adult chondrons.

For the FBS fed cells, it was notable that the stiffness of the cell-PCM composite even after day28 in culture was markedly less than that of freshly isolated cells, consistent with formation of a soft PCM of GAG, collagen, and other matrix molecules loosely organized around the cell membrane. As seen previously by immunofluorescence to the keratin sulfate antibody 5D4 (Lee and Loeser 1998), newly developing matrix has a diffuse appearance. Thus, the early-time PCM structure, when probed via indentation, had an apparent modulus less than the cell itself. Contrary to the freshly isolated cells (Fig.7), the same amount of force was generated with the micron-sized tip and the nanosized tip up to D~900 nm (Fig.9c). One hypothesis is that the sharp nano-tip penetrated the developing PCM layer and came into closer contact with the stiffer cell membrane, thus generating as much force as seen by the larger micro tip, which may not have easily penetrated the PCM and thereby sensed bulk PCM properties.

In comparison, the IGF-1+OP-1 cultured cell-PCM composites released on day28 showed a significant increase in stiffness over day0 cells with both the probe tips (Fig.10). Because the FEA had difficulty converging at depths greater than 200nm for the nanosized tip due to the sharpness of the tip, we focus on FEA analysis with the micron-sized tip. For the first 600nm, the shell model predicted the experimental data well and gave a PCM modulus of 0.16kPa for the day28 FBS cells (Fig.8c), a value much lower than freshly isolated cells. The
shell model compared well to the full range of data for the IGF-1+OP-1 supplemented cells (shown in Fig.8d for first 600nm) giving a PCM modulus of 4.15kPa, higher than that of freshly isolated cells (2.75kPa). By comparison, the moduli of newly developing PCM for both cell treatments was much lower than that reported for native adult chondron PCM (Allen and Mao 2004; Guilak et al., 1999) possibly due to the more mature PCM structure in adult tissue.

IGF-1 alone has been shown to increase proteoglycan content in newly developing PCM (van Osch et al., 1998). OP-1 has also been shown to increase proteoglycan (Nishida et al., 2000) as well as collagen synthesis and accumulation in newly developing PCM (Flechtenmacher et al., 1996). The combination of IGF-1+OP-1 has been shown to increase cell division as well as promote proteoglycan content in the newly developing PCM (Loeser et al., 2003). IGF-1+OP-1 treatment may cause additional modifications in PCM ultrastructure, including changes in macromolecular packing density and organization as well as increased collagen cross-linking.

4.4 Concluding remarks

In conclusion, a microfabricated surface was created to immobilize individual chondrocytes and their newly developing PCM to maintain a spherical phenotype during indentation tests. Apparent cell and PCM moduli were estimated from nanoindentation data using Hertzian contact mechanics and FEA. Temporal evolution of the cell-PCM composite in response to IGF-1+OP-1 and FBS supplements in cell cultures showed that the former led to a greater GAG accumulation as well as a significant increase in stiffness observed by nanoindentation measurements, indicating perhaps are more developed PCM ultrastructure. For both cell cultures, the developing PCM properties showed increasing stiffness over a culture period of one month. A longer term study may reveal the kinetics by which continued increases in PCM stiffness could lead to the properties of fully developed chondrons, such as that measured via micropipette aspiration (Guilak et al., 1999; Jones et al., 1999) and AFM (Allen and Mao 2004).
Acknowledgements: This work was supported by NSF-NIRT 0403903, NIH Grant AR33236, and a Whitaker Foundation Fellowship (LN). The authors would also like to thank Dr. Eliot Frank for contributions to viscoelastic modeling, and to the MIT Institute for Soldier Nanotechnologies funded through the U.S Army Research Office for use of instrumentation.

5. Appendix (Supplementary Material)

Appendix A. Experimental quantification of the rate-dependence of indentation hysteresis of individual chondrocytes

Freshly isolated cells were probed with the nanosized and micron-sized tips at indentation rates of 200nm/s, 500nm/s, 1µm/s, 3µm/s, and 5µm/s. The area enclosed by the hysteresis loop (e.g., Fig. 6) is related to the strain energy dissipated during cyclic loading of the cell, which is most likely associated with viscoelastic and/or poroelastic cell behavior (Leipzig and Athanasiou 2005). The hysteresis loop area was calculated by fitting the loading and unloading curves to polynomials, integrating, and subtracting the area under the unloading from that of the loading curve. Hysteresis was seen at all rates and normalized to the area obtained at 1µm/s (Fig. A1). In this study, an increase in hysteresis was not seen between 200nm/s to 1µm/s indentation rates, indicating a pseudo-steady state within this loading regime, giving an estimated relaxation time constant of ~1s (based on a rate of 1µm/s). A previous study involving cytoindentation of single adult bovine chondrocytes reported a time relaxation constant of 1.32s (Koay et al., 2003). Similar hysteresis behavior was reported previously for skeletal muscle cells for which hysteresis did not decrease below 500nm/s (Collinsworth et al., 2002). We therefore used 1µm/s z-piezo displacement for data collection. Hysteresis was thereby minimized in order to reduce the contribution of cell visco- and poroelastic dissipation and thereby calculate the apparent elastic properties of the cell (A-Hassan et al., 1998).
Appendix B. Theoretical modeling of indentation of individual chondrocytes for the estimation of apparent elastic moduli

B.1. Hertz model (isotropic elastic half-space)

Using the small strain region (\(\varepsilon<0.05\)) corresponding to an indentation depth up to 300 nm for the data of Fig.7, the Hertz model was used to calculate an apparent modulus for the nanosized and micron-sized tip. The modified Hertz model of a rigid conical probe tip in contact with an elastic material was used for the nanosized tip indentation (Radmacher 1997):

\[
F = \frac{\pi}{2} \frac{E}{(1-\nu^2)} D^2 \tan \alpha \tag{A1}
\]

where \(F\) = force (nN), \(D\) = indentation depth (\(\mu\)m), \(E\) = Young’s modulus (cell, kPa), \(\nu\) = Poisson’s ratio (taken as 0.4 (Freeman et al., 1994), and probe tip angle \(\alpha = 35^\circ\). The Si\(_3\)N\(_4\) tip (modulus~400GPa (Petersen 1982)) can be assumed rigid compared to the cell (modulus ~1kPa). This model gave an average modulus \(E = 0.85\) kPa (Fig. A2a). Matching the Hertz model to the upper and lower limits of the standard error gave a modulus range between 0.7kPa to 1.0kPa (Table A1).

For the micron-sized tip, the Hertz model of two contacting spheres was used assuming a rigid colloidal tip and an elastic cell (Johnson and Greenwood 1997):

\[
F = \frac{4}{3} \frac{E}{(1-\nu^2)} \left[ \frac{1}{R_1} + \frac{1}{R_2} \right] D^{3/2} \tag{A2}
\]

where \(R_1\) = radius of micron-sized tip (\(\mu\)m) and \(R_2\) = radius of cell = 7.65 \(\mu\)m. The Hertz model compared well to the experimental data with a modulus of 1kPa (Fig. A2b). The upper and lower limits matched the standard error with moduli of 1.12kPa and 0.82kPa, respectively.
The apparent moduli of 0.70 - 1.00 kPa and 0.82 - 1.12 kPa for the nanosized and micron-sized tips, respectively, were significantly different (p<0.05, unpaired t-test). These values are in the range between 0.65 - 2.47 kPa reported previously using micropipette aspiration (Jones et al., 1999) and unconfined compression (Leipzig and Athanasiou 2005). Interestingly, the apparent elastic cell moduli estimated from our nanosized tip data were significantly lower than those estimated from the micron-sized probe tip data. Previous studies have also emphasized differences in matrix (Stolz et al., 2004) and cellular properties (Lehenkari et al., 2000b) measured at nano- versus and micron- length scales. Of course, all such elastic models are based on the simplifying assumption that the cell can be represented as a homogeneous elastic solid. It should be noted that while we assume a simple elastic model, the hysteresis seen with both probe tips (Fig. 6) indicated that more complex viscoelastic and/or poroelastic behavior was present. Ongoing studies are focused on estimation of poro-viscoelastic cell parameters from these data.

B.2. Stress-strain

A stress-strain (σ-ε) curve was calculated from the force-indentation depth data of Fig. 7 using the 0 - 200 nm depth range for the micron-sized tip. The stress was calculated by dividing force by a contact area that increased with indentation depth.

\[
\sigma = \frac{F}{A} \quad \text{and} \quad \varepsilon = \frac{D}{2R_2}
\]

where \( A = 2\pi R_1 D \). A modulus of 1.96 kPa was calculated from the slope of the \( \sigma-\varepsilon \) curve (Fig. A3), which was linear in this range. The Hertz model and stress-strain estimation for the micron-sized probe tip yielded similar apparent moduli for freshly isolated chondrocytes. Due to the complex contact interaction between the sharp nanosized tip and the cell, an accurate stress-strain curve could not be estimated.
B.3. Finite element analysis: Effect of mesh density and boundary conditions

Finite element analysis was used only for the micron-sized tip as the nanosized tip simulations had difficulty converging at depths larger than 150nm due to its small radius of curvature relative to the indentation depth. The fixed parameters were probe tip end-radius of 2.5µm, cell diameter of 7.6 µm, and Poisson's ratio of 0.4 (Freeman et al., 1994; Jones et al., 1999). The fitting parameter was the cell modulus. Contact between the indenter and cell was frictionless. A 20-node quadratic brick mesh (C3D20 in ABAQUS element library) was seeded with a higher density in the vicinity of the contact region of the probe tip where larger stresses and strains occurred. To verify that the mesh was dense enough to obtain accurate results, two different densities of meshes were implemented and showed agreement of the resultant F-D curves. Boundary conditions may also influence the FEA results. The exact interaction between the cell and well wall are unknown, so the two extremes of no-slip and frictionless boundary conditions were implemented. Interestingly, a no-slip boundary condition between the elastic cell and rigid wall resulted in a lower modulus (by ~25%) compared to the frictionless boundary condition. This implies that if the wall is frictionless, then the tip has to indent more of the cell to obtain an equally large resultant force to that of the no-slip wall, which may be due to the cell “slipping” into the well.

B.4. Cell-PCM elastic two-layered finite element analysis

The elastic cell, 7.6µm diameter, was modeled with a modulus of 2.75kPa. The elastic PCM was modeled as being 3.65µm thick with the modulus used as the fitting parameter. A PCM modulus of 4.15 kPa was needed to match the experimental data, consistent with the observation that IGF-1+OP-1 treatment produced much stiffer cells-PCM composites by day 28. At an indentation distance of 860 nm, the cell was more deformed (0.155 µm vs. 0.014 µm)
when surrounded by the stiffer PCM. The softer PCM absorbed the indentation force and did not cause cell deformation as readily as the stiffer PCM. Interestingly, the deformation of the PCM by the tip was approximately the same for both the 0.1 kPa and 4.15 kPa PCM stiffness (0.63 \( \mu \text{m} \) vs. 0.59 \( \mu \text{m} \), respectively).

B.5. Viscoelastic and poroelastic models

Quasi-static viscoelastic models to predict creep and stress relaxation behavior and to extract instantaneous and equilibrium moduli, viscosity, and a relaxation time constant have been developed in the area of nanoindentation of polymeric films such as polystyrene, semicrystalline polyvinyl alchol (Cheng et al., 2005), and poly(methyl methacrylate) (PMMA), and poly (dimethylsiloxane) (PDMS) (Oyen and Cook 2003; VanLandingham et al., 2005). A similar approach has been used along with unconfined compression tests to estimate parameters for individual chondrocytes (Leipzig and Athanasiou 2005; Shieh and Athanasiou 2005). By applying small sinusoidal oscillations (2-50 nm amplitude at 0.1 - 300 Hz), dynamic viscoelastic models have been used to calculate storage and loss moduli of PMMA and PDMA films via nanoindentation (White et al., 2005). Dynamic testing has also been performed via AFM with a colloidal probe tip for single cells such as fibroblasts (Mahaffy et al., 2004), alveolar epithelial cells (Rico et al., 2005), and smooth muscle cells (Smith et al., 2005), and using a nanosized pyramidal tip for lung epithelial cells (Alcaraz et al., 2003). In these approaches, the form of the elastic Hertz model was extended to incorporate time-dependent behavior and thereby derive viscoelastic material parameters. Poroelastic models, which include the spatial and temporal dependence of fluid-solid frictional interactions and intracellular pressure gradients to characterize time dependent cell deformation behavior, have also been used to predict the effective equilibrium elastic modulus and the hydraulic permeability of single chondrocytes,
modeled as homogeneous, linear, isotropic biphasic media in unconfined compression (Leipzig and Athanasiou 2005). The recent experimental identification of non-equilibrium intracellular hydrostatic pressure gradients within the cytoplasm, or blebs, of filamin-depleted melanoma cells (Charras et al., 2005) have, indeed, suggested that such poroelastic behavior may be intrinsic to transient or dynamic cell deformation.

Motivated by the observed hysteresis in our nanoindentation experiments, we first used the simple three-element viscoelastic solid model of Eq. (A4) (Leipzig and Athanasiou 2005) to calculate effective viscoelastic parameters corresponding to the micron-sized tip data of Fig. 6b:

$$\frac{1}{E(t)} = \frac{1}{E_\infty} + \frac{E_\infty - E_0}{E_0 E_\infty} e^{-t/\tau} \quad (A4)$$

Using a value for the relaxed modulus, $E_\infty = 1$ kPa, corresponding to effective equilibrium modulus predicted from the Hertz model derived from the data of Fig. A2b, the hysteresis loop area at a loading rate of 1 $\mu$m/s data (i.e., Fig. 6b) was reasonably well matched using parameter values for the instantaneous modulus, $E_0 = 2$ kPa and the time constant $\tau = 0.1$ s. Interestingly, these values of $E_\infty$, $E_0$, and $\tau$ are all within a factor of 2-3 of those reported by Leipzig and Athanasiou, 2003. We note, however, that these calculated values were found to vary with loading rate, as the measured hysteresis (Fig. A1) did not increase proportionally with loading rate. Therefore, our ongoing studies focus on extensions using dynamic viscoelastic models to calculate frequency-dependent storage and loss moduli ($G'(\omega)$, $G''(\omega)$) describing the relaxation spectrum as well as poroelastic models that incorporate both time and space dependence as well as nonlinear behavior.
BIBLIOGRAPHY


expression patterns and involves intracellular calcium and cyclic AMP. Journal of Biological Chemistry 279, 19502-19511.


Woessner, J.J.F., 1961. The determination of hydroxyproline in tissue and protein samples containing small amounts of this imino acid. Archives of Biochemistry and Biophysics 93, 440-447.
Fig. 1. (a) Schematic of micron-sized square pyramidal wells in a silicon substrate used for cell immobilization and nanomechanical measurements. (Indentation of chondrocytes was attempted on mica but the flat surface was not suitable as the cells rolled away from the tip.) The wells were etched with a 20% KOH solution using a silicon oxide hard mask of circles with diameters of 15, 18, 20, and 22 µm. The production of inverted square pyramids from circular mask openings is a consequence of anisotropy: (100) and (110) crystal planes are etched much more quickly than (111) planes, so self-terminating features bound by (111) planes are produced, forming planes 55 degrees from the vertical (Kovacs et al., 1998). The masking oxide was thermally grown on 100 mm diameter single crystal silicon wafers and patterned with a Buffered Oxide Etch (BOE) using a photoresist mask. The photoresist was then stripped and the wafers placed in an 80°C bath of 20% KOH for ~15 min. until the etch self-terminated. The oxide mask was stripped with a second BOE and the wafer was singulated with a die-saw. The silicon surface was reusable after removal of organics with piranha solution (3:1 concentrated H₂SO₄/H₂O₂(30%)) followed by heat sterilization. (b) A 10× optical microscope image of a single chondrocyte on a microfabricated silicon substrate and a 0.06 N/m Si₃N₄ cantilever used to maneuver an individual cell into a 15µm inverted square pyramidal Si well. (c) After the cell was seated in a well, indentation was performed with the (e.g., nanosized) tip and then repeated on the exact same cell using the micron-sized tip.
Fig. 2. Tapping mode amplitude AFM images in air of calf chondrocytes adsorbed on mica substrates. (a) freshly isolated (day 0), (b) chondrocyte released from alginate culture at day 11 where the PCM is clearly distinguishable from the cell body, (c) chondrocyte released from alginate culture at day 18 shows single collagen fibrils emanating out of the dense fibrillar network of the PCM, (d) a higher resolution image of the dense network which exhibit fibril diameter characteristic of type II collagen fibrils (Lodish et al., 2000). Morphological features and cell surface roughness are likely altered by their sample preparation (for a review see (Shao et al., 1996)).
Fig. 3. Optical microscopy images (10×) of individual living calf chondrocytes and images (40×) of fixed calf chondrocytes released from alginate at different times in culture using fixed and stained for PG and collagen (Methods). (a) FBS supplemented medium and (b) IGF-1+OP-1 supplemented medium. The top rows of (a) and (b) images were taken in culture medium. The middle rows show toluidine blue staining for PGs after day 7, which covered the entire cell surface, and extended over a larger diameter for cells cultured in IGF-1+OP-1 compared to FBS supplemented medium. The bottom rows show aniline staining for collagen, which was generally not as uniform and intense as the PG stain.
Fig. 4. Characterization of the PCM of living calf chondrocytes cultured in alginate using either FBS or IGF-1+OP-1 supplemented medium. (a) An increase in PCM thickness (mean ± SD) measured from optical microscope images was observed from day 0 (freshly isolated cells) up to day 7; after day 7, PCM thickness did not change significantly (ANOVA, p < 0.05; n = number of cells measured.) PCM thickness was calculated as the average diameter measured at each time point minus the average diameter of freshly isolated cells (day 0) cells divided by 2. The error bars represent one standard deviation as calculated by a pooled sample variance. (b) Fluorescein diacetate and ethidium bromide showing live (green) and dead (red) cells on day 28 indicated >80% viability (n=20). (c) Type VI collagen (immunohistochemistry) was present around both FBS (shown) and IGF-1+OP-1 fed day 39 cells.

Fig. 5. Biochemical characterization of the PCM of calf chondrocytes released from alginate culture at designated time points corresponding to days that nanoindentation experiments were conducted. Total GAG content of the PCM (by DMMB) was higher for cells supplemented with IGF-1+OP-1 compared to FBS. However, collagen content (by hydroxyproline) was similar for both cell cultures. Data were collected from 3 alginate beads pooled per condition per time point.
Fig. 6. Typical indentation curves of individual freshly isolated (day 0) calf chondrocytes. The tip-cell contact point was identified by a running average of the slope of every group of five data points until the calculated slope was greater than zero. Each plot shows 5 loading-unloading cycles (mean ± SD) for one cell immobilized in a silicon well using a z-piezo displacement rate of 1 µm/s with (a) a nanosized square pyramidal Si$_3$N$_4$ probe tip ($R_{tip} \sim 40$ nm) and (b) a micron-sized colloidal probe tip ($R_{tip} \sim 2.5$ µm).

Fig. 7. Nanoindentation (loading component) of freshly isolated calf chondrocytes (day 0) using both the nanosized (mean ± SE for n=25 cells with 5 loading cycles/cell, $R_{tip}$~40 nm) and micron-sized (mean ± SE for n=17 cells with 5 loading cycles/cell, $R_{tip}$~2.5 µm) probe tips. (These 17 cells were also sequentially tested using the nanosized tip.)
Fig. 8. (a) Elastic FEA model of nanoindentation experiment on chondrocytes with a micron-sized probe tip. (b) FEA model predictions using an apparent cell modulus of 2.75 kPa compared well to experimental data for indentation on loading of freshly isolated (day 0) cells (n=17 cells, 5 loading cycles per cell) with micron-sized probe tip ($R_{tip}=2.5 \mu m$). (c) Using a cell modulus of 2.75 kPa, shell thickness of 3.65 \mu m, $R_{cell}= 7.6 \mu m$, and Poisson’s ratio = 0.4, the FEA shell model with a shell modulus of 0.16 kPa compared well with experimental data for day 28 FBS cultured cells (n=6 cells, 5 loading cycles per cell). (d) Using a cell modulus of 2.75 kPa and a shell thickness of 3.65 \mu m, the FEA shell model with a shell modulus of 4.15 kPa compared well with the experimental data for day 28 IGF-1+OP-1 cultured cells (n=5 cells, 5 loading cycles per cell). Data are mean ± SE. Estimation of the cell modulus using FEA was limited to strains <10%. All analyses were done using a consistent indentation depth of 400. Based on the data, the modulus used in the FEA simulations was chosen to bracket the data of the force-indentation curves.
Fig. 9. Average indentation curves (mean ± SE of 5 loading cycles per cell on n cells) on loading of individual chondrocytes with their cell associated PCM after release from alginate at different times in culture with 10% FBS. (a) From the relative slopes of the F-D curves, the stiffness of the cell-PCM composite increased steadily from day 7 (n=5), to days 14 (n=4), 21 (n=5), and 28 (n=6), for the case of the nanosized probe tip ($R_{tip}$~40nm). Even by day 28, however, the stiffness of the cell-PCM composite was lower than that of freshly isolated (day 0) cells devoid of PCM. (b) Similarly, F-D curves obtained with the micron-sized probe tip ($R_{tip}$~2.5µm) showed
stiffening of the cell-PCM composite with each week in culture, from day 14 (n=4) to days 21 (n=5) and 28 (n=7) (c) With day 28 cells, the F-D behavior with the micron-sized tip was similar to that of the nanosized tip for the first ~900 nm (nanosized probe tip: n=6; micron-sized probe tip: n=7).
Fig. 10. Average indentation curves (mean ± SE of 5 loading cycles per cell for n cells per time point) on loading of individual calf chondrocytes with their PCM after released from alginate at different times in culture with IGF-1+OP-1 supplemented medium. (a) Nanosized probe tip ($R_{tip} \sim 40$ nm) data compared to 10% FBS data of Fig 9a; the IGF-1+OP-1 fed cells showed a marked increase in stiffness from day 21 to day 28 (n=5), and a higher force than cells in 10% FBS. (b) Micron-sized probe tip ($R_{tip} \sim 2.5\mu m$) data show an increase in stiffness from day 21 (n=5) to day 28 (n=5). In contrast to FBS cells of Fig. 9, day 28 IGF-1+OP-1 cultured cells were stiffer than freshly isolated (day0) cells as measured by the micron-sized probe tip, and for the nanosized tip (a) in the range $D>700$ nm.
Fig. A1. Average hysteresis area (mean ± SE) normalized to the hysteresis area measured for cells using a displacement rate of 1µm/s with the nanosized tip (n = 6 cells, each with five sequential loading and unloading curves) and micron-sized tip (n = 4, each with five loading and unloading curves). Minimal change in hysteresis was observed between displacement rates of 200nm/s to 1µm/s.

Fig. A2. Comparison of experimental data up to 400 nm indentation distance (from Fig. 7, freshly isolated calf chondrocytes) to the predictions of the Hertz model using , R_{cell} =7.6 µm, Poisson’s ratio=0.4, for (a) the nanosized and (b) the micron-sized tip, respectively. Indentation curves on loading (mean ± SE) taken with both the nanosized (average of 25 cells with 5 loading cycles/cell, R_{tip} ~ 40nm) and micron-sized (average of 17 cells with 5 loading cycles/cell, R_{tip} ~ 2.5µm) probe tips. Based on the data, the modulus used in the Hertz model was chosen to bracket the data of the force-indentation curves.
Fig. A3. Stress-strain curves for nanoindentation data shown in Fig. 7 for loading (mean ± SE) for freshly isolated calf chondrocytes (day 0) taken with micron-sized probe tips (average of n=17 cells with 5 loading cycles/cell, $R_{tip} \sim 2.5 \mu m$) resulting in an average $E=1.96$ kPa with a range of 1.76-2.19 kPa.

Table A1. Summary of apparent cell moduli obtained by comparison of experimental data of Figure 10b with three different models. For nanosized tip, a modulus could not be calculated with the stress-strain curve due to the complex contact interaction of the sharp tip radius and the cell. The FEA simulation with the nanosized tip also had trouble converging due to the probe’s small radius of curvature relative to the indentation depth. For the day 21 and day 28 cells, the cell modulus was set to 2.75 kPa while the PCM was varied until the FEA F-D matched the experimental data.