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***In Situ* Friction Measurement on Murine Cartilage by Atomic Force Microscopy**

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Abstract

Articular cartilage provides a low-friction, wear resistant surface for the motion of diarthrodial joints. The objective of this study was to develop a method for *in situ* friction measurement of murine cartilage using a colloidal probe attached to the cantilever of an atomic force microscope. Sliding friction was measured between a chemically functionalized microsphere and the cartilage of the murine femoral head. Friction was measured at normal loads ranging incrementally from 20 nN to 100 nN with a sliding speed of 40 $\mu\text{m/s}$ and sliding distance of 64 μm . Under these test conditions, hydrostatic pressurization and biphasic load support in the cartilage were minimized, providing frictional measurements that predominantly reflect boundary lubrication properties. Friction coefficients measured on murine tissue (0.25 ± 0.11) were similar to those measured on porcine tissue (0.23 ± 0.09) and were in general agreement with measurements of boundary friction on cartilage by other researchers. Using the colloidal probe as an indenter, the elastic mechanical properties and surface roughness were measured in the same configuration. Interfacial shear was found to be the principal mechanism of friction generation, with little to no friction resulting from plowing forces, collision forces, or energy losses due to normal deformation. This measurement technique can be applied to future studies of cartilage friction and mechanical properties on genetically altered mice or other small animals.

Keywords

Scanning probe microscopy; Boundary lubrication; Lubricin; Tribology

1. Introduction

Articular cartilage serves as the bearing surface in diarthroidal joints, allowing joint motion and contact for decades with minimal friction and wear (Mow et al., 1992). The coefficient of friction of cartilage is one of the lowest of contacting bearing surfaces and has been reported to range from 0.002 to 0.03 (Ateshian and Mow, 2005; Dowson and Wright, 1976; Jin et al.,

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2000; Linn, 1968; Malcom, 1976; Unsworth et al., 1975). The mechanisms governing cartilage lubrication and frictional properties involve an intricate combination of biomechanical and biomolecular factors acting on a wide range of length and time scales.

For example, under some loading conditions, cartilage surfaces may be separated completely by mechanisms such as squeeze film lubrication (Hlavacek, 1993; Hou et al., 1992) and elasto-hydrodynamic lubrication (Dowson and Jin, 1986; Jin and Dowson, 2005). When cartilage-cartilage contact occurs, a large portion of the load on the tissue is borne by interstitial fluid rather than solid components of the tissue (McCutchen, 1959; Soltz and Ateshian, 1998). Interstitial fluid pressurization initially supports over 90% of the load and dissipates at a rate determined by the biphasic time constant of the contact (Soltz and Ateshian, 1998). A proportional increase in friction occurs as the load is transferred increasingly to solid-solid contacts (Krishnan et al., 2004), and thus the surface properties of articular cartilage are also believed to play a critical role in joint lubrication (Graindorge et al., 2005). Between points of solid-solid contact, boundary lubrication, by molecules such as lubricin (Swann et al., 1981) or its homolog superficial zone protein (SZP) (Flannery et al., 1999; Jay et al., 2001; Schmidt and Sah, 2007) serves to reduce cartilage friction and wear. The extensive glycosylations of PRG4 are necessary for lubrication (Jay et al., 2001), although the mechanisms of lubrication are not fully understood. Mutations in the gene coding for these glycoproteins (PRG4) cause camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome in humans (Marcelino et al., 1999), characterized by early non-inflammatory joint failure associated with a lack of lubrication (Jay et al., 2007) and chondroprotection (Rhee et al., 2005).

An improved understanding of the natural mechanisms governing friction and wear at the joint interface may provide new insights into the mechanical contributions to joint degeneration in diseases such as osteoarthritis, a debilitating disease which affects over 40 million people in the United States alone (Elders, 2000). While osteoarthritis is a multifactorial, complex disease with multiple etiologies (Mandelbaum and Waddell, 2005), recent studies have indicated that a loss of boundary lubrication associated with changes in PRG4 expression are associated with the onset of the disease (Elsaid et al., 2005; Young et al., 2006). To this end, there is a need for well-defined measurement of tissue frictional properties on molecular and supramolecular levels. In the genetic age, there is great potential for focused studies of cartilage lubrication on tissues from genetically altered animals. In particular, we would like to measure friction on cartilage from knockout and wild type mice. However, the size of these cartilage specimens precludes mounting in a standard friction tester. Colloidal probe microscopy (Ducker et al., 1991), a modified atomic force microscopy (Binnig et al., 1986) (AFM) technique, facilitates the measurements of such small samples, eliminating this limitation (Park et al., 2004).

In the present study, this technique, which predominantly measures boundary lubrication properties, was used to measure friction *in situ* on the cartilage of the murine femoral head. We demonstrate the feasibility of these measurements and validate the approach by comparing these results to AFM measurements on porcine cartilage. Roughness and indentation properties were also measured on each cartilage surface, providing further knowledge of the factors influencing cartilage friction in the boundary regime. Here we considered four possible contributions to boundary friction in more depth (Fig. 1).

2. Materials and Methods

2.1 Cartilage Samples

C57BL/6J mice (N=9, 20 weeks old) were sacrificed and frozen whole at -80°C until thawed for measurements. Both male and female mice were used. One femur was extracted from each mouse by carefully cutting away the surrounding tissue. The femur was then affixed to a polystyrene Petri dish by embedding its distal end in hot melt glue (Arrow Fastener Co., Saddle

Brook, NJ). The orientation of the femoral head was kept constant by placing both epicondyles of the knee flat on the base of the dish. Friction was measured on the anterior portion of the femoral head (Fig. 2).

Porcine cartilage used for this study was taken from the lateral section of the patella midway between superior and inferior regions. Fresh knees of female pigs (N=5, 2–3 years old) were obtained from a local slaughterhouse. Visually normal chondral samples approximately 1 cm in diameter were cut away, wrapped in gauze soaked with phosphate-buffered saline (PBS, Gibco, Carlsbad, CA), and frozen at -80°C until thawed for measurements. Sections were mounted to Petri dishes with cyanoacrylate glue. All samples were kept in PBS at room temperature throughout testing.

2.2 Atomic Force Microscopy

An MFP-3D AFM (Asylum Research, Santa Barbara, CA) was used for all measurements. All calibration constants were determined prior to measurements. Normal force spring constants were determined from the power spectral density of thermal noise fluctuations in air (Hutter and Bechhoefer, 1993; Walters et al., 1996) by the MFP-3D software provided by Asylum Research and normal photodiode sensitivity was measured as the slope of the constant compliance regime of the normal force curve on a glass slide (Cappella and Dietler, 1999). Lateral spring constants were calculated analytically from normal spring constants (Neumeister and Ducker, 1994) using cantilever dimensions obtained by optical and scanning electron microscopy. Lateral photodiode sensitivity was calculated by measuring the slope of the “stick-slip” region of a small friction loop (50–200 nm) on glass (Cain et al., 2000).

Friction was measured by tracking the lateral deflection signal as the probe scanned over the surface with a scan angle of 90° (Meyer and Amer, 1990), with trace and retrace signals averaged to eliminate any crosstalk between normal and lateral deflection. The probe was raster scanned across a 64 by 64 μm area of articular cartilage at 40 $\mu\text{m/s}$ with 16 scan lines and 512 points per scan line captured. Each area was scanned 5 times at applied normal loads increasing incrementally from 20 nN to 100 nN. Preliminary studies showed that repeated scanning under these conditions at applied normal loads up to 250 nN did not detectably influence measured friction. Three scan areas were measured per joint. The effects of scan rate and scan size on friction were also assessed. These tests were done in previously untested areas at an applied normal load of 100 nN.

2.3 Colloidal Probes

Friction and indentation measurements were performed by colloidal probe microscopy using triangular silicon nitride AFM cantilevers (spring constant $\approx 0.58 \text{ N/m}$, Veeco, Santa Barbara, CA) (Fig. 3). Borosilicate microspheres ($10 \pm 1 \mu\text{m}$ diameter, Duke Scientific Corporation, Palo Alto, CA) were attached near the free end of the AFM cantilever with Norland optical adhesive #81 (Norland Products inc., Cranbury, NJ). The spherical probe serves as a model of a single asperity and the contact area during sliding (estimated as $1\text{--}3 \mu\text{m}^2$ by Hertz contact analysis) is well within the range of asperity sizes seen in topographical images (see Fig. 9). Probes were functionalized with tri-ethylene glycol to provide uniform surface chemistry and minimize biofouling. To this end, they were coated with 45 nm of gold over a 5 nm adhesion layer of chromium in a thermal evaporator and soaked overnight in a 1 mM $\text{SH}-(\text{CH}_2)_{11}\text{-EG}_3$ (ProChemia, Poland) in ethanol to allow formation of tri-ethylene glycol terminated monolayers (Bain et al., 1989).

2.4 Roughness

Topography of the cartilage surface was measured concurrently with friction and a second-order flattening procedure was applied to eliminate topography variation from the curvature

of the femoral head and other orientation effects; furthermore, the root mean squared (RMS) roughness was recorded. As surface roughness measurements may depend on resolution, sample rate, sample size, and deformation (Poon and Bhushan, 1995), we individually assessed the influence of each of these factors. Colloidal probe measurements were compared to pyramidal tip measurements (with significantly smaller expected contact areas), sample rates and sample sizes were varied, and topography scans were compared over the full range of normal loads used for friction measurements.

2.5 Indentation

The elastic modulus of the superficial zone was determined using AFM indentation (Darling et al., 2007; Darling et al., 2006). Within each scan location, a 4×4 array of force-indentation curves was captured. A force trigger of 100 nN and an indentation rate of 1 μm/s was used for all indentations. Approach portions of force curves were analyzed by the Harding Sneddon model (Harding and Sneddon, 1945), a Hertzian contact equation modified to model indentation of a rigid sphere into a semi-infinite elastic solid (Eq. 1),

$$F = \frac{4ER^{1/2}}{3(1-\nu^{1/2})}\delta^{3/2}, \quad (1)$$

where F is the applied force, E is the compression modulus, R is the radius of the indenter, δ is the indentation, and ν is Poisson's ratio. Minimal adhesion was seen in the approach portion of the force curves, justifying the use of a Hertzian analysis (Fig. 4). Poisson's ratios of 0.2 and 0.03, recently determined for murine cartilage (Cao et al., 2006) and the superficial zone of porcine cartilage (Choi et al., 2007), were used for the fit.

Data was fit to a linearized version of Eq. 1 derived by raising both sides of the equation to the 2/3 power and substituting δ with an estimated indentation Δ that differs from the absolute indentation by a constant. This gives Eq. 2, which can be fit without knowledge of the constant since E is the only unknown term in the slope (Guo and Akhremitchev, 2006).

$$F^{2/3} = C^* - \left[\frac{4ER^{1/2}}{3(1-\nu^{1/2})} \right]^{2/3} \Delta. \quad (2)$$

The absolute indentation is obtained from Eq. 1 once the modulus has been found (Darling et al., In Press, Journal of Biomechanics). Thus, we have a consistent means of deriving the contact point based on information from the bulk sample rather than from the contact itself. Adhesion energy was calculated as the total area of the retract portion of each force curve below the zero force line (Fig. 5a).

2.6 Imaging

Samples were imaged in contact mode using pyramidal AFM tips (spring constant ≈ 0.12 N/m, Veeco, Santa Barbara, CA) functionalized with tri-ethylene glycol (see section 2.3). The applied load was limited to 5 nN. Other imaging parameters were varied to maximize image quality.

2.7 Statistical Analysis

Comparisons between the two data sets (murine vs. porcine) were made using a 2-tailed t-Test. Regression analyses were performed using StatView version 5.0.1. All results are reported as mean $\pm$ standard deviation.

3. Results

Friction force was nearly independent of scan rate from 10 to 100 $\mu\text{m/s}$ (Fig. 6a) with a trend toward higher friction at lower rates. Friction depended weakly on sliding distance for distances above 5 μm (Fig. 6b). Deviations from this trend at small sliding distances may have arisen because mechanical and chemical properties of the surface on those scales are not representative of the whole tissue. Regression analyses of data from scans with applied loads of 100 nN showed a statistically significant correlation with adhesion energy (Fig. 5, $p = 0.003$, $r^2 = 0.3$) but not with roughness ($p = 0.99$) or indentation depth ($p = 0.32$).

The measured roughness of mouse cartilage was 201 ± 76 nm (scan size: 64 μm , applied load: 20 nN) and was load dependent. Increasing the load to 100 nN decreased measured roughness by about 13%. Roughness measured on porcine cartilage (scan size: 64 μm , applied load: 20 nN) was 325 ± 114 nm, about 50% higher than on murine cartilage. Although the resolution and sample rate used here were sufficient to describe the system, a consistent increase in measured roughness as a function of scan size was observed. Approach portions of indentation curves were fitted well by the Hertz model, with correlation coefficients typically above 0.99. Compressive moduli were 354 ± 158 kPa and 44 ± 42 kPa on murine and porcine tissue, respectively.

The dependence of friction on applied load was linear within the scatter in all cases. Typical results are shown in Fig. 7. This system appears to be well described by Derjaguin's modification of Amonton's law (Derjaguin, 1934) giving the load on the system as a sum of the external load and the adhesive load,

$$F = \mu(L_0 + L) = F_0 + \mu L, \quad (3)$$

where F is the friction force, L is the normal load, and μ is the coefficient of friction. The subscript "0" indicates forces present at zero applied load. Coefficients of friction were thus interpreted as the slope of friction force plotted versus normal force and were 0.25 ± 0.11 for murine samples and 0.23 ± 0.09 for porcine samples (Fig. 8). F_0 values were 3.0 ± 3.5 nN on murine specimens and 2.8 ± 3.5 nN on porcine specimens. Coefficients of friction did not differ significantly ($p = 0.68$) between species.

The underlying fibrillar structure of the tissue was evident in AFM imaging (Fig. 9). It was not visible uniformly though, consistent with characterization of the cartilage surface as either "amorphous" or "fibrillar" by earlier researchers (Moa-Anderson et al., 2003).

4. Discussion

In this study, lubrication mechanisms other than boundary lubrication were minimized, allowing focused study of this mechanism. The time constant for interstitial fluid pressurization is proportional to square of the characteristic length of fluid flow (Mak et al., 1987), and thus scales directly with the contact area, as has been verified experimentally (Carter et al., 2007). The near absence of biphasic lubrication under microscale contacts (due to the small biphasic time constant, which is on the order of milliseconds) has also been experimentally demonstrated (Park et al., 2004), and motivates our study on the tribological properties of cartilage surfaces in absence of interstitial fluid pressurization. Furthermore, the measured frictional properties were generally independent of scan rate over a large range (Fig. 6a), which would not be expected if friction were measured in the presence of interstitial fluid pressurization (Soltz and Ateshian, 1998). Additional lubrication mechanisms potentially contributing to friction reduction *in vivo* are also expected to be minimized in this configuration. For example, hydrodynamic lubrication by PBS on cartilage is only expected at much higher speeds than

those used here (Gleghorn and Bonassar, 2007) and the prolonged sliding of the probe is not amenable to squeeze film lubrication.

A general model for boundary friction attributes friction to interfacial effects, arising from molecular interactions between surface components as they move over each other, and internal effects, due to inelastic deformation and recovery of the material (Moore, 1972). Additionally, plowing friction can result when a hard asperity sliding through a softer surface pushes the softer material forward, resulting in an asymmetric pressure distribution at the contact interface (due to either plastic or viscoelastic effects) (Lafaye et al., 2006). Finally, collisions between an AFM probe and the asperities of a rough surface can lead to an apparent dependence of friction on roughness that is not present at the macroscale (Sundararajan and Bhushan, 2000).

Neither plowing nor collision forces were seen to contribute significantly to friction measured in our system. Plowing friction, resulting from a rigid sphere sliding through a viscoelastic material, has been modeled and is expected to scale linearly with sliding velocity (Rennie et al., 2005). We observed little or even negative dependence on scan rate (Fig. 6a), which suggests that plowing friction likely contributes little to the overall friction. This result is consistent with the fact that the angle of attack of the spherical probe is relatively small. A plowing effect due to plasticity is improbable also since articular cartilage regularly withstands pressures much larger than those applied here (predicted by Hertz analysis to be tens of kPa) without damage (Hodge et al., 1986). Furthermore, there was no correlation between the frictional properties and cartilage roughness ($p=0.99$), suggesting that asperity collisions did not dominate friction.

Hysteresis between approach and retract portions of force curves indicated that tissue recovery after deformation was not completely elastic (Fig. 4a). The frictional component arising from inelastic deformation of the tissue was estimated from this hysteresis (Greenwood and Tabor, 1958) and was found to be only a small fraction of the total friction (see Appendix). Therefore we conclude that interfacial shear is the major component of the frictional forces measured here.

Adhesion energy can affect interfacial friction (McFarlane and Tabor, 1950), although the relationship is highly material dependent. Here, friction depended slightly on adhesion energy (Fig. 5). For many materials, adhesion hysteresis, the energy lost in the formation and breaking of adhesive bonds between surfaces, has been shown to affect friction more strongly than adhesion force or adhesion energy (Yoshizawa et al., 1993). Adhesion hysteresis may be velocity dependent since molecules at the contact interface are able to achieve more intimate and stronger interactions when they have more time to interact with each other. The high friction forces at very low sliding speeds (Fig 6A) may be due to the formation of stronger interactions between the sliding surfaces on those time scales.

The coefficients of friction measured on murine cartilage ($\mu=0.23$) are in good general agreement with microscale measurements of porcine cartilage ($\mu=0.25$, Fig. 8) or previous measurements on bovine cartilage ($\mu=0.15$) (Park et al., 2004). They also agree well with macroscale measurements of cartilage friction after significant time ($\mu = 0.14-0.57$) (Forster and Fisher, 1999; McCutchen, 1959; Park et al., 2004; Soltz and Ateshian, 1998). These findings suggest that cartilage frictional properties may not vary significantly among species, despite large changes in “scale”. This is consistent with other mechanical properties of cartilage, such as compressive and tensile moduli, which are relatively consistent among mammals ranging in size from mice to cows (Athanasίου et al., 1991; Cao et al., 2006; Flahiff et al., 2002). The ability to perform direct, *in situ* measurement of frictional and elastic mechanical properties, as shown here, upon genetically modified mice, will likely provide new detailed insight into the mechanisms that control cartilage lubrication in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic illustrating the four possible mechanisms of friction considered in this work: (a) Interfacial friction, (b) internal friction, (c) plowing friction, and (d) friction due to collisions with asperities.

Figure A1.
Typical force curve highlighting the region used for estimation of the elastic loss fraction.

Figure 2. Photograph showing the orientation of a mounted femur. The close-up highlights the region on which measurements were performed.

Figure 3. Scanning electron microscopy image of an AFM cantilever with a 10 μm glass sphere attached.

Figure 4.

(a) Sample indentation data. (b) Young's modulus calculated from a linear fit to indentation data raised to the 2/3 power. The contact point is taken as the point at which this linear fit crosses the x-axis.

Figure 5.

(a) Adhesion energy is calculated as the area of the retract portion of each force curve below zero force. (b) Friction force plotted as a function of mean adhesion energy for each scan location. The applied normal load is 100 nN.

Figure 6.

(a) Friction force measured on three different joints at various scan rates (applied load = 100 nN, scan size = 64 μm). (b) Friction force measured on three different joints at various scan sizes (normal load = 100 nN, scan rate = 40 $\mu\text{m/s}$). Only single error bars are shown, representing typical standard deviations of the 16 scan lines averaged for each data point.

Figure 7. Representative graph showing friction forces plotted as a function of load on one sample. Different symbols represent different scan locations. Error bars represent standard deviations of the 16 scan lines averaged for each data point.

Figure 8. Coefficients of friction measured on murine cartilage are comparable to those measured on porcine cartilage ($p = 0.68$).

Figure 9.

A representative AFM image taken with an EG₃-functionalized pyramidal probe shows the topography of the cartilage surface. (Scan rate = 70 $\mu\text{m/s}$, applied normal load $\approx$ 5 nN.)