Enhanced Lubrication on Tissue and Biomaterial Surfaces through Peptide-mediated Binding of Hyaluronic Acid

Anirudha Singh¹, Michael Corvelli¹, Shimon A. Unterman¹, Kevin A. Wepasnick², Peter McDonnell¹ and Jennifer H. Elisseeff¹*

¹Translational Tissue Engineering Center, Wilmer Eye Institute and Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD 21287, USA.

²Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218, USA

* To whom correspondence should be addressed: jhe@jhu.edu

Johns Hopkins University, Wilmer Eye Institute and Department of Biomedical Engineering, Smith Building Rm. 5035, 400 N. Broadway, Baltimore, MD 21231

Supplementary Methods

**Histology.** Morphological assessment of the cartilage samples was performed by a following procedure. In brief, the paraffin embedded cartilage samples were cut into 5 μm thick sections and placed onto a glass microscope slide after keeping for a few seconds in a 40 °C water bath. The glass slides with these sections were kept overnight at 40 °C on a hot plate. To rehydrate the samples, sections were rinsed sequentially, first with xylene followed by 100%, 95%, 80% ethanol, and deionized H₂O. Slides were soaked for 3 minutes in a solution of Fast Green (Sigma) then for 10 seconds in 1% glacial acetic acid (Sigma) solution. Post-drying, sections were placed for 10 minutes in 0.1 % Safranin-O solution (ScholAR Chemistry 9466802). After staining, samples were dried by washing with deionized H₂O, 80%, 95%, 100% ethanol and xylene. Samples on a cover slip were mounted with a Permount mounting solution and dried for 24 h. Gross topography of the cartilage samples for the severity of damage was studied by application of India Ink (Becton Dickson, MD).
Samples were embedded in either paraffin wax or Tissue Embedding Media (Thermo Scientific, Logan, UT) after freezing in liquid nitrogen, and sectioned with a Cryo-mill (Leica). Samples were imaged with Zeiss Discovery V2 dissection imaging microscope.

**Lubrication testing: supplementary experimental details.** After “zero-ing” the instrument (RFS-3 rheometer, Rheometric Scientific), we calculated the initial heights of the cartilage samples with an electronic caliper followed by loading the samples on the rheometer. The samples were glued with cyanoacrylate glue to the top and bottom rheometer fixtures in parallel plate configuration. Only a thin layer of glue bound to the cartilage and metal fixture surface. The 8.0 mm sample surface was positioned on top of the 12.0 mm surface. The top sample was lowered and pressed against the bottom sample until a load value of ~0.01 N to avoid insufficient contacts between the sample surfaces, load value fluctuations and minimize the errors in height measurements. The corresponding recorded height, which was automatically sensed by rheometer, was taken for strain calculation. The instrument was programmed to record the total cartilage thickness and calculate the height for 18% compression. The total thickness of the human cartilage sample was in the range of 4.5 ± 0.5 mm, which were tested in a bath of fluid (10 mL) covered with protecting lid to prevent desiccation. Each sample was checked for proper alignment and surface irregularity, and the experiment was performed on samples with flat surfaces. The samples were bathed in the test lubricant, compressed to 82% of their original combined height and preconditioned by rotating two revolutions in each direction at an effective sliding velocity of 0.3 mm/s, which is defined as the angular velocity times the effective radius of the annulus \( R_{\text{eff}} = \frac{2}{3}[(R_o^3-R_i^3)/(R_o^2-R_i^2)] \).

This preconditioning was repeated twice more, followed by a 3600 seconds stress-relaxation period to allow the pressurization of the fluid in the compressed cartilage to fully subside. The equilibrium normal stress data recording and measurement was performed for each experimental group with a typical profile highlighted in Fig S3. Lubrication testing was performed in 14 stages. The first two stages were considered negligible and used as a clearing or pre-shear stage. Stages 3, 6, 9 and 12 were performed to analyze the effect of different durations of relaxation. Samples were allowed to relax between tests for 1200, 120, 12 and 1.2 seconds. Lubrication data were recorded during stages 4-5, 7-8, 10-11 and 13-14; each stage was in a different direction.
of rotation and at a constant shear rate. During each test, torque (τ) and normal force (N) were measured, and instantaneous measurements of µk, the kinetic friction coefficient, were determined from the following equation: µk = τ/(Reff * N). Instantaneous µk values were averaged over the second revolution in each direction to produce an average <µk> that was used for comparison. Static friction coefficients were calculated as the instantaneous µs = τmax/(Reff * N) at the maximal torque value found during the startup period of the test. After experimentation a central indentation due to 18% compression on the cartilage surface was confirmed.

Semi-quantitative analysis for bound HA on cartilage samples. We performed a semi-quantitative experiment with a static reservoir and cartilage explants (native vs. treated for lubricin removal) to estimate the quantity of bound HA on the cartilage tissue surfaces. In a 96 well plate, cartilage explants were incubated in 50 μL of HA solution (with and without HABpep). After 2 h, cartilage explants were transferred to wells with 50 μL of PBS (for washing) during various time intervals until 18 h when a negligible fluorescence signal was observed. A plot of fluorescence intensity vs. time was created. A calibration curve of different dilution of initial HABpep/HA solution (fluorescence intensity vs. concentration) was created and corresponding fluorescence with time was converted into concentration of the solution that remained with cartilage, which on extrapolation resulted in retained HA on the cartilage surface (approximated for only surface area of the samples; height was considered negligible).

Spectroscopy techniques. NMR (1H, DMSO-D6 or D2O) and FTIR-ATR experiments were performed on Bruker Advance 300 MHz or 400 MHz and Bruker, Vector 22 with a Pike Miracle ATR attachment, respectively.

Statistical analyses.

Statistical analysis was performed with SPSS software (IBM), specifically using a one-way ANOVA Tukey's test. The analysis took into account all averaged coefficients at each time point (1.2 s - 1200 s) between each condition in each group. Statistical significance threshold was 95% (p ≤ 0.05) and is labeled with a specified asterisk (*) (Figs. 3b-e). Similarly, for contact lens samples (n =3), the rates of water evaporation values were
considered statistical significance for $p \leq 0.05$. Statistical analysis on friction data for Figs. 3f-i was performed with Prism 6.0 software, specifically using an unpaired t-test with Welch correction method and 95% confidence interval ($p \leq 0.05$).

**Supplementary Results & Discussion**

Native and polymer-modified cartilage surfaces were assessed for lubrication using a modified rotational test protocol developed by Schmidt and Sah\textsuperscript{53} (Fig. S2a). Specifically, 8 mm cartilage annuli were compressed against 12 mm cartilage disks to 82% of original height, and rotated against each other at a physiologically relevant sliding velocity. Cartilage explants were bathed in HA solutions (5.0 mg/mL of ~975 kDa HA) to model both, healthy and diseased tissue, and PBS without HA served as a negative control (Figs. S2b,c). Coefficients of friction were derived from torque and normal force data (example raw data found in Tables S1a&b). Our aim was to investigate the lubrication efficacy of HABpep-HA coating on the articular cartilage surface in a standard lubrication experimental set up. Although a constant contact area and complete opposition of the cartilage samples suggested boundary lubrication (Fig. S2d), complete elimination of lubrication effects arising from fluid pressurization was possibly not achieved. During the test, the sample was compressed to 18% of initial height with a rate of 0.2 mm/s and allowed to equilibrate for 1 h (Fig. S3a,b). The axial force equilibrates and an equilibrium force is established within 1 h of the experiment. This data was recorded for a human OA cartilage tissue in a PBS reservoir ~10 mL. Although axial force remains approximately constant during different ramps/presliding times, the axial force values jumped from equilibrium to another value due to the sample rotation while friction measurement (Fig. S3c). These data recordings were typical of all experimental groups tested.

Prior to the tribological studies, an accurate and reliable assessment of the cartilage degeneration is a prerequisite to the identification of stages and mechanisms of damage and the resulting treatment plan. Histological analysis provides visual details of the internal structural and physiological information of cartilage deterioration, serving as a gold standard for validation of other potential assessment tools. Similar to a method developed by Ateshian\textsuperscript{42}, we categorized the severity of OA in human cartilage explants with varying levels of...
cartilage damage. However, we classified OA samples broadly in 4 subgroups to null the hypothesis that increasing stages of OA offer higher friction values (stage 1-2: mild, stage 2-3: moderate and stage 3-4: severe).

Histological evaluation of OA samples, specifically in stage 3-4 (Fig. S2e vs. Fig. S2q) showed large tears and deformations of the surface compared to the normal cartilage sample that had normal smooth surface with almost non-existence of the superficial zone. The mid stages of OA (stages 1-2 and 2-3, Figs. S2i & S2m) showed relatively even surface topography with no obvious tears and presence of superficial zone. These classifications along with their mechanical properties provide us vital information on the characteristics of OA stages, more importantly the lubrication effect of HA on various topographical surfaces for understanding of HA as a possible OA therapeutic.

Normal human cartilage friction in both static and kinetic movement was affected by HA. Normal human cartilage had an average static friction ($<\mu_s>$) of 0.028 in PBS and 0.018 in HA (Fig. S2f), a reduction of 38%. Kinetic friction values ($\mu_k$) in normal human cartilage (Fig. S2g) were also affected by the addition of HA; average friction values were recorded as 0.014 in PBS and 0.008 in HA, an average reduction of 46%. The corresponding static and kinetic lubricity values ($<\mu>$ in PBS - $<\mu>$ in HA) were 0.010 and 0.006, respectively (Fig. S2h). Stage 1-2 human OA cartilage (Fig. S2i) during tribological testing recorded an average static friction ($<\mu_s>$) of 0.046 in PBS and 0.028 in HA, a reduction of 39% (Fig. S2j). Average kinetic friction values for stage 1-2 human OA cartilage measured 0.024 in PBS and 0.012 in HA, which correlated to an average reduction of 50% (Fig. S2k). The corresponding static and kinetic lubricity values ($<\mu>$ in PBS - $<\mu>$ in HA), were 0.016, and 0.010 (Fig. S2l), respectively. Stage 2-3 human OA samples (Fig. S2m) recorded $<\mu_s>$ values of 0.066 in PBS and 0.022 in HA (Fig. S2n), correlating to an average reduction in static friction between PBS and HA of 69%. Kinetic friction values recorded an average value of 0.030 in PBS and a value of 0.012 in HA, equaling an average reduction between PBS and HA in stage 2-3 of 62% (Figs. S2n,o,p). Severely diseased articular cartilage sample (Fig. S2q) recorded an average static friction value of 0.096 in PBS and 0.036 in HA, an average lubricity value of 0.060, correlating to an average 61% reduction in static friction between PBS and HA. Kinetic friction values in the same sample (stage 3-4) measured an average of 0.044 in PBS and 0.018 in
HA, an average lubricity of 0.026, which determined an average reduction of kinetic friction of 60% between PBS and HA systems (Figs. S2r,s,t). We also confirmed that the coating itself did not enhance the lubrication of articular cartilage by comparing a coated cartilage sample to an uncoated sample in PBS with no HA (Figs. S4d,e). OA stage 3-4 cartilage samples with no HA-binding coating and no HA recorded an \(<\mu_s>\) of 0.096 and an \(<\mu_k>\) of 0.044, which decreased to an \(<\mu_s>\) of just 0.036 and an \(<\mu_k>\) of 0.018, when tested with HA and no coating, respectively (Figs. 3d,e). On application of HA-binding coating and bound HA on OA stage 3-4 cartilage samples had an \(<\mu_s>\) of just 0.028 and an \(<\mu_k>\) of 0.016 (Figs. 3d,e). Cartilage treated with HA-binding coatings and pre-incubated in HA were able to improve the surface lubrication compared to native cartilage without treatment. The lubrication data suggest that the static and kinetic friction values of severe OA cartilage samples with HABpep coating and bound HA on the surfaces are 60-70% less than that of without peptide in PBS (Figs. S4f,g). The \(<\mu_s>\) for OA stage 3-4 surfaces treated with HABpep-polymer and tested in PBS was ~30% less compared to the untreated cartilage tested in the presence of HA lubricants (0.028 vs. 0.036). (Fig. S4f). Moreover, the magnitudes of \(\mu_k\) for OA stage 3-4 surfaces treated with HABpep-polymer and tested in PBS were slightly less or similar to native, untreated cartilage tested in the presence of HA lubricants (Figs. 3c,e).

To estimate the binding levels of HA on cartilage surfaces fluorescence was semi-quantitatively evaluated using an experiment with a static reservoir and cartilage explants. Bound HA on cartilage samples treated with HABpep treatment was almost 2.2 times more than the native cartilage with no HABpep treatment. Cartilage samples that were treated to remove lubricin bound 1.3 times more HA via HABpep than the samples with no HABpep treatment although there was possibly higher bulk absorption of HA in cartilage samples treated to remove lubricin that reduced the overall percentage difference. However, the bound HA due to HABpep treatment on both native cartilage and treated cartilage samples to remove lubricin remained unchanged. Although this method to quantify bound HA on cartilage surface \textit{in vitro} is not directly related to \textit{in vivo} HA retention, it provides an insight for HABpep treatment efficacy on cartilage surfaces.
Supplementary Figures

Figure S1 | 1H-NMR and FTIR-ATR spectra of the HA binding polymer. Chemical analysis was performed to confirm the conjugation at various stages of product synthesis: **a**, 1H-NMR spectra- appearance of a peak at 2.9 ppm for thioether CH₂ on conjugation of azlactone with thiolated PEG (i & ii); and peaks 6.8-8.0 ppm for collagen binding peptide conjugated through azlactone functionality confirm the attachment to HABPep-PEG (iii); **b**, FTIR-ATR spectra of the products at different stages of synthesis.
Figure S2 | Friction measurements and lubrication analysis for articular cartilage surfaces. a, Lubrication of articular cartilage was evaluated using a cartilage disk and annulus rotated in opposite direction. Cartilage samples were isolated and incubated in either control PBS (b), or an HA lubricant solution (c). Samples were compressed 18% and rotated 720° in each direction. d, Forces occurring during lubrication testing between the two articular cartilage surfaces. Red arrows indicate opposite force applied from the coated surface. Cartilage tissue samples were divided into different subgroups from normal to severely damaged samples for lubrication analysis. Representative histology images and gross topography, graphs of static friction, kinetic friction and lubricity vs. pre-sliding time (s) for each of the subgroups in the respective order: e, f, g & h for Normal healthy cartilage samples; i, j, k & l for mild OA samples; m, n, o & p for moderate OA samples; q, r, s & t for severe OA samples. u, Static friction percent reductions of all OA stages in PBS vs. HA solution. v, Kinetic friction percent reductions of all OA stages in PBS vs. HA solution. Comparison of static, w, and kinetic, x, lubricity values (<μ>PBS - <μ>HA) of a severely damaged OA sample with a healthy cartilage sample.

Figure S3 | Equilibrium axial force and torque values during rheological testing. Tissue samples were compressed to 18% of initial height at a rate of 0.2 mm/s with a limit of 20 N (rheometer limit). Once 18% compression was reached, the sample was allowed to equilibrate for 1 h (a, linear scale and b, log scale). During this period the axial force equilibrates and an equilibrium force (Neq) is established. This data was recorded for a human normal cartilage tissue in a PBS reservoir ~10 mL. Axial force (N) at various presliding times after 1 h of sample compression for force to reach equilibrium remained approximately constant. c, A uniform and constant axial force (N) was recorded in presliding time range. d, Torque (N-mm) values at a representative presliding time of 120 s (ramp 10) for a human cartilage sample tested in PBS. e, Angular position of the sample while rotating after 1 h of axial force equilibrium after different presliding times (1.2 s, 12 s, 120 s, 1200 s).
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Figure S4 | Tissue surface modification with an HABpep-polymer system.  

**a & b**, Comparison of static and kinetic lubricity values ($\mu_{\text{PBS}} - \mu_{\text{HA}}$) of normal and severe OA samples coated with HABpep.  
**c**, Fluorescence microscope images of the normal and severe OA cartilage samples before and after tribological testing with HABpep coating and HA-rhodamine (scale bar = 500 μm).  
**d & e**, Representative graphs of static friction and kinetic friction vs. pre-sliding time (s) for the normal cartilage sample in PBS with and without coating of HABpep. The resulting lubrication had a $\mu_s$ of 0.028-0.036 vs. 0.064-0.068 and a $\mu_k$ of 0.014-0.016 vs. 0.030-0.032, suggesting that the surface modification without HA resisted the surface movement.  
Percentage reduction of static friction and kinetic friction values with pre-sliding time (s) for cartilage samples at OA stage 3-4 (**f & g**).
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Figure S5 | Bound HA via HABpep on human normal cartilage and on cartilage treated to remove lubricin. Fluorescence studies were performed to calculate the amount of bound HA (ng/mm²) with HABpep treatment or no HABpep treatment on native cartilage samples and cartilage samples treated to remove lubricin (n=5).  

**a**, Semi-quantitative analysis of fluorescence study showed 2.2 times more bound HA on native cartilage samples via HABpep treatment than the samples with no HABpep treatment.  

**b**, Cartilage samples that were treated to remove lubricin bound 1.3 times more HA via HABpep than the samples with no HABpep treatment.  

**c**, The bound HA due to HABpep treatment on both native cartilage and treated cartilage samples to remove lubricin remained unchanged.