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Synthesis and Characterization of a Lubricin Mimic (mLub) To Reduce Friction and Adhesion on the Articular Cartilage Surface

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Abstract

The lubricating proteoglycan, lubricin, facilitates the remarkable low friction and wear properties of articular cartilage in the synovial joints of the body. Lubricin lines the joint surfaces and plays a protective role as a boundary lubricant in sliding contact; decreased expression of lubricin is associated with cartilage degradation and the pathogenesis of osteoarthritis. An unmet need for early osteoarthritis treatment is the development of therapeutic molecules that mimic lubricin function and yet are also resistant to enzymatic degradation common in the damaged joint. Here, we engineered a lubricin mimic (mLub) that is less susceptible to enzymatic degradation and binds to the articular surface to reduce friction. mLub was synthesized using a chondroitin sulfate backbone with type II collagen and hyaluronic acid (HA) binding peptides to promote interaction with the articular surface and synovial fluid constituents. *In vitro* and *in vivo* characterization confirmed the binding ability of mLub to isolated type II collagen and HA, and to the cartilage surface. Following trypsin treatment to the cartilage surface, application of mLub, in combination with purified or commercially available hyaluronan, reduced the coefficient of friction, and adhesion, to control levels as assessed over macro- to micro-scales by rheometry and atomic force microscopy. *In vivo* studies demonstrate an mLub residency time of less than 1 week. Enhanced lubrication by mLub reduces surface friction and adhesion, which may suppress the progression of degradation and cartilage loss in the joint. mLub therefore shows potential for treatment in early osteoarthritis following injury.

Keywords

Viscosupplementation; Superficial Zone Protein (SZP); Hyaluronan (HA); Synvisc; Osteoarthritis Therapy

1. Introduction

Movement in vertebrates is made possible in part by joints of the musculoskeletal system. Synovial joints, including the knee and elbow, are characterized by articular cartilage at the

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ends of the long bones that provides a smooth sliding surface to facilitate joint movement. Natural lubrication mechanisms exist at the articular cartilage surface to ensure low friction and wear. Cartilage injury often leads to loss of surface lubrication, which is associated with the degenerative disease osteoarthritis.[1]

Osteoarthritis (OA) is a chronic joint disease in part characterized by the degradation of the extracellular matrix (ECM) in articular cartilage. Degradation is facilitated by matrix metalloproteinases and hyaluronidases that break down ECM molecules including type II collagen, hyaluronic acid (HA), aggrecan and lubricin.[2] Lubricin, a large proteoglycan found in the superficial zone of articular cartilage and the synovial fluid, plays a critical role in maintaining boundary lubrication at the articular surface [3–5] Loss of lubricin increases friction and wear on the cartilage, eventually leading to cartilage degradation and the pathogenesis of OA.[6]

Lubricin, also referred to as superficial zone protein (SZP) or proteoglycan 4 (PRG4), is encoded by the gene PRG4 and expressed by the cells in the surrounding synovium and cartilage.[7–9] Due to its role in reducing friction, it is not surprising that mechanical stimuli such as shear stress induce lubricin secretion into the synovial fluid; the fluid in turn acts as a reservoir from which the molecule is replenished at the cartilage surface.[9–11] In addition, biochemical factors including cytokines and growth factors, often released during excessive shear stress, injury and inflammmation, can downregulate lubricin synthesis and secretion from synoviocytes and chondrocytes.[12, 13]

At the joint surface, lubricin interactions with both type II collagen and HA help keep a molecular thin film of lubricin at the articular surface.[10, 14] Lubricin is typically more highly expressed in the anterior portions of the joint, which are the load bearing regions where cartilage is subjected to higher shear stresses.[15, 16] This protective lubricin layer is often disrupted following injury and disease;[6] and failure to quickly restore the thin film on the articular surface facilitates cartilage degradation. It has been shown that following injurious compression to the cartilage, delayed lubricin expression and secretion in the superficial zone was correlated with impaired healing.[11] Validation of the importance of re-establishing the lubricin film comes from animal models of joint injury that have shown that intraarticular treatment with lubricin resulted in a reduction of cartilage degradation.[4]

The introduction of supplemental lubricant injections into the synovial fluid to alleviate joint disease often termed *viscosupplementation*, has been recently implemented as a treatment for early OA The most common viscosupplement used clinically is a high molecular weight HA formulation (e.g. Synvisc). OA pain relief has been reported for up to 6 months, although the efficacy of Synvisc and other HA-based viscosupplements may be reduced by susceptibility to enzyme degradation and low residency time.[17] In an attempt to enhance the rentention of HA, recent studies have used a combination of lubricin and HA as a viscosupplmentation. [18]

In an effort to develop an improved lubricating therapy, we designed a functional mimic of lubricin (mLub). The lubricin mimic contains a chondroitin sulfate backbone conjugated to which are small peptide fragments that facilitate interaction with the ECM and that, in

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comparison to the full lubricin protein, contain fewer sites for enzymatic degradation making mLub potentially more stable in the joint environment. In addition, synthesis can be readily scaled up. The mLub was synthesized and optimized to contain a chondroitin sulfate backbone coupled to type II collagen [19] and HA binding peptides [20] to facilitate binding to the cartilage surface (collagen) and synovial fluid constituents (HA). Specific binding was confirmed to type II collagen and HA *in vitro*, as well as *ex vivo* and *in vivo* cartilage. We also confirmed the lubricating function of mLub, alone and in combination with HA-based viscosupplements, by measuring friction and adhesion on cartilage surfaces over multiple scales. Lastly, we studied the mLub residency time on the articular cartilage in a common animal model *in vivo*.

2. Materials and Methods

2.1 Synthesis of Lubricin Mimic (mLub)

The synthesis methods for mLub were modified from previous work done in our lab.[21] We have designed this molecule to resist enzyme degradation from hyaluronidase, MMPs and aggrecanase as described previously.[21, 22] However, it is difficult to eliminate all potential proteolytic sites from bioactive peptides; endopeptidases and limited expeptiases are still able to degrade the short peptides, but the degradation potential has been minimized. Vicinal hydroxyl groups on a chondroitin sulfate (CS) were oxidized with sodium periodate to create aldehyde functional groups. Briefly, 20 mg/ml CS (Sigma-Aldrich, St. Louis, MO) was dissolved in a 0.1 M sodium acetate buffer. For oxidation, the sodium periodate (Thermo Scientific, Waltham, MA) was added to allow for 21 adlehyde groups to form and allowed to react away from light. Gel filtration chromatography using an ÄKTA Purifier FPLC (GE Healthcare, Piscataway, NJ) was used to stop the reaction and purify the oxidized CS as previously described.[21, 22] A crosslinker, *N*-(β-Maleimidopropionic acid) hydrazide, trifluoroacetic acid salt (BMPH) (Pierce, Rockford, IL) was then added in excess along with sodium cyanoborohydride and allowed to react. Excess BMPH was then removed using gel filtration chromatography. The CS-BMPH was frozen and lyophilized before conjugating the binding peptides. The CS-BMPH was finally functionalized by the addition of an HA-binding peptide, GAHWQFNALTVRGGGC (GAH), and a type II collagen-binding peptide, WYRGRL (Genscript, Piscataway NJ). Initially, approximately 10 moles of each peptide was added to a single CS backbone (mLub10), later this ratio was changed to approximately 5 moles HA-binding and 15 moles type II collagen-binding peptides per mole of CS (mLub15). One GAH mole was biotinylated for characterization. The molecule was purified again and then frozen and lyophilized for storage at −80°C. The estimated molecular weight of mLub15, ~ 107 kDa, was calculated by summing the known molecular weights of the individual components of the molecule.

2.2. Characterization of mLub Binding In Vitro

The binding of mLub to HA was determined using a simple binding assay. 96-well Greiner plates were coated with 50 μg/ml HA (hyaluronic acid sodium salt from *streptococcus equi)* (Sigma-Aldrich) in 50 mM sodium carbonate and incubated overnight. After each step the wells were rinsed with PBS + 0.05% Tween. Wells were blocked with 1% BSA in PBS for 1 hour then rinsed. A 10:1 dilution of the lubricin mimic was added to the wells and incubated

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at 37°C for 1 hour and then rinsed. Streptavidin-HRP (R&D Systems, Minneapolis, MN) was added to each well and incubated for 20 minutes. After rinsing, a color solution was added to each well and incubated away from light for 20 minutes. 2N sulfuric acid was added to each well and the absorbance was read on a plate reader (SpectraMax, Molecular Devices) at 450 and 540 nm.

The binding of mLub to type II collagen was determined using a similar protocol with the HA. The well plate was coated with a 0.5 mg/ml solution of type II collagen from chicken sternal cartilage (Sigma-Aldrich) in 10 mM HCl and incubated overnight. The rest of the procedure is the same as described above.

2.3. Cartilage Harvesting and Treatments

Cartilage plugs were harvested with a 7 mm diameter cork borer from ~5-month-old bovine knee joints obtained 24 hours after slaughter (Dutch Valley Foods). Samples were taken from the anterior regions of the joint at the load bearing regions.[15] Samples were washed three times in Hank's Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA) with a protease inhibitor cocktail (PIC) (PMSF, EDTA, iodoacetamide, benzamidine hydrochloride hydrate (Sigma-Aldrich), penicillin-streptomycin (Cellgro)). To mimic osteoarthritis, randomly selected samples were treated with a 0.5% trypsin solution for 3 hours to deplete the cartilage of some peptidoglycans while others were left as WT samples.[23] The samples were then rinsed with HBSS/PIC and then washed with fetal bovine serum for 10 minutes to deactivate the trypsin. Samples were stored in the HBSS/PIC in 4°C and tested within 24 hours of harvest. It is important to note that the trypsin solutions were dissolved in HBSS plus a PIC. Despite the presence of PIC, evidence from the toluidine blue staining, friction differences and physical differences between WT and Trypsin, indicates the PIC only had minimal effects on inhibiting trypsin.

The lubricin mimic was dissolved to a concentration of 0.5 mg/ml in PBS, which is on the higher end of the range of concentrations found in synovial fluid.[24, 25] Synvisc (Genzyme, Ridgefield, NJ) was obtained and diluted to a concentration of 3 mg/ml to be a concentration closer to the actual HA concentration in synovial fluid. HA solutions were also made at a concentration of 3 mg/ml. Cartilage samples were treated with trypsin right before testing took place. Treatments were added on the surface of the cartilage twice, five minutes apart before the samples were rinsed and tested. If the treatment group included mLub and Synvisc/HA, mLub was added like described above, the cartilage was rinsed and then the Synvisc/HA treatment added and rinsed.

2.4. Characterization of mLub Binding to Articular Cartilage

After treatment and rinsing, cartilage plugs were frozen at −20°C in O.C.T compound (Tissue Tek) and sectioned at 10 μm thickness with a Shandon Cryotome FE (Thermo Scientific). Sections were allowed to dry at room temperature and stored at −20°C before being fixed in 4% paraformaldehyde in PBS and then briefly rinsed with PBS. For the detection of the biotin-labeled mLub, AF555-conjugated streptavidin (Life Technologies) was diluted 1:200 in a 10% donkey serum, 0.2% triton, and 0.02% sodium azide solution in PBS. DAPI was diluted 1:500 in the same solution to stain the nuclei. The sections were

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incubated 30 minutes in the dark, then rinsed and mounted before they were imaged under a Leica DMI6000 fluorescent microscope. This protocol was also followed after the in vivo cartilage harvesting with additional steps for SZP staining. After blocking, the *in vivo* cartilage samples were first incubated with 1:500 dilution of SZP primary antibody (mouse) for 1 hour. The sections were rinsed and then incubated with a secondary anitbody solution consisting of AF555-conjugated anti-mouse diluted 1:500, AF647-conjugated streptavidin diluted 1:200, and DAPI diluted 1:500 for 30 minutes in the dark.

To show the proteoglycan depletion by the 3 hour trypsin treatment, cryosections were stained with toluidine blue. The slides were stained with 0.04% Toluidine Blue (Sigma-Aldrich) solution in 0.1 M acetic acid buffer for 10 minutes. The slides were rinsed in milliQ water 3 times and then counterstained with 0.02% Fast Green FCF solution (Sigma-Aldrich). The slides were rinsed again and then dehydrated with 100% ethanol before they were imaged with a light microscope (Nikon Eclipse TS100).

2.5. Macroscale Measurement of Coefficient of Friction

To measure the COF of the cartilage, we adapted the procedure from previous studies.[26] To create a more unified radius, the cartilage samples were made into annuli by cutting a 3 mm diameter hole in the middle of the 7 mm diameter plug, creating a 2.6 mm effective radius (R_{eff}). The cartilage annulus was glued onto the center of a 20-mm flat rheometer geometry head (AR G2, TA Instruments). A glass microscope slide was taped to the bottom plate of the rheometer. The geometry head was lowered enough for the cartilage plug to barely touch the glass slide. HBSS/PIC was added to surround the cartilage to keep the plug from drying out. The samples were compressed at a rate of 0.002 mm/second until a 50 N normal force was measured. The plug then stayed at equilibrium for 60 minutes. Next, the samples were rotated with an angular velocity of 0.0873 rad/sec for 2 minutes. Torque (T) and normal force (N) were measured with the software. Static coefficient of friction (COF) was calculated by taking the maximum torque during the first 10 degrees (~2sec) and normal force and applying equation (1).

$$\mu=\frac{T}{R_{eff}N} \quad (1)$$

The kinetic COF of the cartilage was calculated by averaging the values collected over the course of the second rotation of the rheometer. It is important to note that, unlike previous studies by our group [27] [28], we did not determine the friction coefficient as a function of load due to our concern of surface treatment (e.g. mLub15) removal during repeated testing. Consequently, additional parameters, such as adhesion force during shearing under zero applied normal load, were not studied.

Differences in sample numbers (n) between groups are due to difficulty in maintaining the adhesion of the cartilage plug to the rheometer geometry head. If after raising the geometry head after a run it was observed that the plug separated from the geometry, then the data was not included due to the uncertainty of proper torque measurements.

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2.6. Microscale Measurement of Coefficient of Friction, Roughness, and Adhesion

Microscale measurements of the COF were taken using contact atomic force microscopy (contact AFM) with PicoView software. Silicon AFM probes with 2 um diameter borosilicate spherical tips (Novascan Technologies, Ames, IA) were used with a pre-calibrated spring constant of 0.6 N/m [29]. A lateral force calibration constant was calculated using Varenberg's improved wedge calibration method [30] and a TGF11 silicon calibration grating. This calibration constant allowed for converting the measured voltage to force (newtons).

The friction measurements were taken on 50 μm x 50 μm sections of the cartilage sample at a speed of 50 μm/sec. The area was scanned with a 5V set point applied (around 120 nN load). The set point (volts) was converted to normal force (Newton) by multiplying by the deflection sensitivity and spring constant. While observing the friction force plots during data analysis, areas of the surface where the controller was not detecting the specimen surface were observed. In order to take these areas out of the data analysis we programmed data analysis code areas with the presence of fibers and no controller interference. These areas were then used for analysis. The average area taken from each sample area was 971 μm^2 with a standard deviation of 280 μm^2. The friction voltage signal was averaged for both the trace and retrace scan and then the difference between the two divided by two. This value was then converted to friction force by multiplying by the calibration constant. Averaged friction force divided by normal force to calculate coefficient of friction.[27, 31]

Using the same areas taken for friction measurements, after removing the sample tilt surface RMS roughness (*R*) was calculated using the topography data in equation (2), where z = height.

$$R=\sqrt{\frac{1}{n}\sum_{i=1}^{n} z^2} \quad (2)$$

Adhesion force was also measured on the cartilage surface with the AFM.. 8×8 Micro-indentations, or so-called force-distance curves,[5] were sequentially applied over a 50 μm × 50 μm section with a trigger force of ~ 30 nN and load/unload speed of 14 μm/sec. The adhesion force was measured as the maximum deflection below the zero-deflection line when the tip retracts from the specimen (retracting portion of the force-distance curves). The measured adhesion forces were averaged over the area.

2.7. *In Vivo* Residency Study

An *in vivo* study was performed to measure the residency time of mLub in the synovial fluid and on the articular cartilage. In vivo studies were approved by the Purdue University Animal Care and Use Committee. Dunkin Hartley guinea pigs were chosen as a model due to their development of spontaneous OA.[32] The animals were allowed to age to 4 months before testing. The patella tendon was located on the hind legs of the animals for injection. 100 μL of 0.5 mg/ml mLub in PBS was injected into the synovial fluid behind the patellar tendon. The concentration was the same as used in the *ex vivo* studies, and falls within the

range of lubricin cnocentration in the synovial fluid and the volume was chose based on previous studies.[22] The contralateral knee was injected with 100 μL PBS for a control. The guinea pigs were allowed to move freely afterwards and until they were sacrificed. After 6 hours, 1 and 2 weeks, the animals were sacrificed and the femoral condyles were harvested and prepared in O.C.T. compound (Tissue-Tek) for cryosectioning like discussed in 6.4. Before this study, a 50 mg/ml Coomassie Brilliant Blue G-250 (Sigma-Aldrich) solution was injected for visualization to confirm accurate delivery into the synovial fluid. 15 μm cryosections were stained with the same protocol as stated previously.

3. Results and Discussion

3.1. Synthesis and Characterization of mLub

We developed a lubricin mimic (mLub) to lower the friction and adhesion of damaged cartilage. The basis of the molecule formulation originated from our previously developed aggrecan mimic[21] that contained a chondroitin sulfate (CS; 60 kD) backbone with ~20 covalently conjugated HA binding peptides (Figure 1a). The aggrecan mimic diffused into damaged cartilage and protected the cartilage in an environment that simulated the osteoarthritic joint.[22] However, in unpublished work with a type II collagen binding variant of the aggrecan mimic, we found that the molecule collected at the cartilage surface rather than diffusing into the tissue interior. In published work, we found that a variant of the lubricin mimic binds to another HA and type II collagen-rich matrix, the vitreous, and restored mechanical integrity and to some extent protected the matrix from proteolytic degradation.[33] Exploiting both the cartilage surface binding and the matrix protective properties of these type II collagen-binding molecules, and knowing that lubricin interacts with both HA and type II collagen to lubricate the cartilage surface, we investigated the potential benefit of HA and type II collagen-binding lubricin mimic to act as a cartilage lubricant.[26, 28]

mLub was designed to create a lubricating boundary on the cartilage surface by coupling a CS backbone to type II collagen- and HA-binding peptides (Figure 1b). The CS backbone is an integral part of lubricin and other cartilage proteoglycans as it provides the molecule with the important negative charges found on lubricating molecules for repulsive hydration forces. Moreover, CS has been reported to reduce the coefficient of friction on articular cartilage.[34] The addition of type II collagen binding peptides promotes site specific molecule binding to the cartilage surface. Inclusion of the HA binding peptide confers the ability of mLub to bind with HA on the cartilage surface as well as in the synovial fluid to further reduce friction.[26] Importantly, the design of mLub renders it less suceptible to enzymatic degradation than that the native structure of lubricin.[3] We tested mLub variants with either 10 moles each of HA and type II collagen binding peptides per mole of CS (mLub10) or 15 moles of type II collagen binding and 5 moles of HA binding peptides per mole of CS (mLub15). Peptide conjugation to the CS backbone was facilitated by first oxidizing CS to produce aldehyde groups and then conjugating the heterobifunctional molecule BMPS. Michael-type addition then facilitated conjugation of thiol containing peptides. After initial measurements of friction (Figure 2) (discussed subsequently), mLub15 was chosen for further study as mLub10 did not show a significant reduction in friction.

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Previous studies showed that lubricin, when combined with HA of varying molecular weights, still maintained the ability to lower the coefficient of friction on the cartilage surface.[35] Thus, it is possible that mLub will be able to reduce friction at the cartilage surface with fully intact HA in the synovial fluid as found in healthy patients, with Synvisc, or with reduced molecular weight HA that might be found in the joints of patients with OA.

We next investigated mLub15 for the ability to bind both HA and type II collagen (Figure 1c). On average, one of the five HA-binding peptide per CS backbone contained a biotin group, which enabled us to specifically analyze the binding characteristics and the spatial distribution of mLub by probing with streptavidin-fluorophore and measuring the resultant fluorescence. Using this method we confirmed that mLub binds to both HA and type II collagen as anticipated. The data also shows that HA binding is weaker than type II collagen binding, which was expected given the greater number of type II collagen binding peptides than HA binding peptides on the mLub molecule.

To evaluate the binding of mLub to the cartilage surface, an *ex vivo* bovine cartilage model was used. Articular cartilage from the load bearing regions of bovine knee joints was harvested and used for all *ex vivo* studies described herein. Following previous studies to mimic osteoarthritis by depleteing the cartilage of proteoglycans, the cartilage plugs were subjected to a 3 hour incubation in trypsin.[23, 36] Loss of proteoglycans was visually confirmed by Toluidine blue staining (Figure 1dii). The untrypsinized (wild type; WT) cartilage sample staining appeared more saturated throughout the tissue compared to the samples treated with trypsin. To confirm binding to the cartilage surface, a solution of mLub was added to the cartilage plugs; the plugs were then rinsed, cryosectioned and probed using fluorescent streptavidin (Figure 1di). The presence of red streptavidin on the surface of only the mLub-treated cartilage supported further investigation into possible lubricating function of the molecule.

3.2 mLub Reduces Coefficient of Friction at the Macroscale

To test the lubrication properties of mLub, the coefficient of friction (COF) on cartilage was first studied on a macroscopic scale. Importantly, the coefficient of friction of the cartilage surface correlates with OA severity.[16] A TA Instuments AR G2 rheometer was used to measure (T) and normal (N) force under constant load in order to calculate the COF (Figure 2a). The static friction of trypsin treated cartilage was significantly higher than WT cartilage ($p < 0.05$). No reduction in friction compared with trypsin-treated cartialge was seen following mLub10 treatment alone, mixed with or followed by a treatment of Synvisc (Figure 2b). Because these treatments were unable to fully restore the COF to WT values, we explored a different mLub formulation with fewer HA-binding peptides and more type II collagen-binding peptides, because we hypothesized that the interaction between HA in the synovial fluid and mLub10 was too strong and led to increased friction. Treatments of mLub15 (15 type II collagen and 5 HA binding peptides) alone or followed by Synvisc was studied. The static friction of the trypsin treated cartilage with treatments of mLub15 followed by Synvisc was restored to WT values and significantly lower than the trypsin treated cartilage alone ($p < 0.05$). The kinetic friction of the trypsin treated cartilage with a mLub15 followed by Synvisc treatment was significantly different than the other marked

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groups ($p < 0.05$). Neither the mLub15 nor Synvisc alone treatments completely restored the COF to WT values.

Since both the static and kinetic COF was restored after mLub15 and Synvisc treatment, the remaining studies were done with mLub15 rather than mLub10. A treatment of mLub15 followed by HA (instead of Synvisc) was also investigated to model mLub15 interaction with HA present in the native synovial fluid. We found that this treatment also restored the static COF on the cartilage (Figure 2b), which was consistent with our previous studies that utilized a combination of wild type lubricin and HA.[35] Interestingly, we see more changes among static friction groups (compared to kinetic friction groups), which may be due to changes observed in adhesion, but not roughness, at the surface (Figure 3).[37–39] [39]

To confirm that the mLub remained bound to the cartilage surface following the compression and shear stress applied to the cartilage during friction testing, fluorescent streptavidin was used to probe for and visualize biotinylated-mLub15 in cartilage samples cryosectioned following friction evaluation. The observed staining following friction testing demonstrates that mLub remained bound to the surface (Figure 2c). No comparison was made based on morphology change due to compression of the cartilage sample during testing.

Previous studies showed that briefly releasing the cartilage from loading enhanced the lubrication on the cartilage surface via lubricant reservoirs.[28] [10] Similar to the study by Chan et al. [28] that showed reduced friction using natural lubricin/SZP, reduced friction measured also in our study following mLub treatment suggests that both natural and synthetic molecules can be used to restore lubrication. Further, unlike the previous study, the cartilage in our experiments was not released from static loading before sliding and yet, lubrication was restored. However, because the friction coefficient is not necessarily a good indicator of wear resistance [40], additional studies that examine surface morphology or wear resistance following extended shearing times would provide additional insight into the efficacy and synergy of mLub15 treatment with Synvisc or HA.

Wild-type lubricin binds to the cartilage via the protein core while the mucin domain is free and creates a low-friction layer at the surface.[41, 42] Differences in surface binding configurations between mLub and native lubricin are important to note because they may create different packing densities to form an effective boundary lubricant layer. Although the packing density and the configuration of the molecules at the surface may be different, friction is still reduced at the surface through combination of mLub15 with Synvisc or HA (Figure 2b). It is possible that a sacrificial layer mechanism is maintained in mLub15 with the HA binding peptides allowing weakly bound HA to easily detached and rebind during contact and sliding.[10]

3.4. mLub Reduces Coefficient of Friction and Adhesion, but Not Roughness, at the Microscale

Following macroscale friction measurements, microscale surface features were investigated using atomic force microscopy (AFM). A colloidal probe was used for contact AFM to measure lateral force. Since a significant trend was observed for macroscale static and

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kinetic friction between WT, trypsin, and trypsin plus mLub and Synvisc treatments, only these three groups were investigated further. While measuring lateral force, a topography map was created to reveal cartilage fibers (Figure 3a), and COF was calculated by dividing the lateral (or friction) force by the normal load of the AFM probe. A COF trend matching the macroscale data was observed without statistical significance (Figure 3b). Additionally, no trend between the sample groups was observed for roughness. Adhesion forces measured on a different subset of cartilage samples (n=9), trypsin-treated cartilage was significantly greater than those measured on the WT ($p < 0.05$), and a trend of reduced adhesion force was observed on trypsin-treated cartilage following mLub + Synvisc treatment (Figure 3c).

The trends of microscale friction data are consistent with macroscale friction results. Similar to previous studies,[5] roughness remained unchanged following typsin treatment, although adhesion increased, which may be due to yet uncharacterized surface alterations following a ~12 fold increase in enzymatic incubation time. Also consistent with other studies, adhesion and friction measured by AFM are not necessarily related to roughness of the cartilage surface.[27] In light of the measured changes in adhesion, but not roughness, it is possible that adhesion was a dominant friction mechanism of articular cartilage for our experiments decribed, although other mechanisms (e.g. plowing[5]) were not specifically studied. Also, differences between friction measurements at macro and microscales was attributed in part to fluid pressurization largely lacking in AFM studies, as suggested by Park et al.[31], in addition to inherent different contact conditions that arise between the AFM tip and single asperities at the cartilage surface.

3.5. mLub Residency Time In Vivo is Less than One Week

Dunkin Hartley guinea pigs show signs of OA as early as four months,[32] thus guinea pigs approximately four months of age were used for initial binding studies (Figure 4). 100 mL injections of either 0.5 mg/mL mLub15 or PBS were done through the patella tendon on contralateral knees and animals were sacrificed at 6 hours, 1, or 2 weeks to probe for mLub15 on the cartilage surface. Cryosections of femoral condyles were stained with fluorescent streptavidin for presence of mLub, anti-SZP for native lubricin, and DAPI to identify nuclei within the cartilage (Figure 4c). mLub was found on the cartilage surface 6 hours following injection but not after 1 or 2 weeks, indicating that the residency time of mLub is less than 1 week. The natural turnover time for lubricin is unknown. Native lubricin (i.e. superficial zone protein, aka SZP) was found bound to the surface at 1 and 2 weeks and was present, but less so, at 6 hours suggesting that the mLub15 may have been replaced with native molecules over time.

The onset of osteoarthritis is correlated with a low expression of lubricin after some time.[6] Meanwhile, there is some evidence that at later time points lubricin expression is much higher than normal,[11, 16] which suggests that temporary replacement of lubricin may be sufficient to protect cartilage from the onset of degradation. Thus, introducing the mLub molecule during a transient period when lubricin expression is low, even with a residency time less than a week, may still be highly advantageous. Our one and two week *in vivo* data suggests that native lubricin replaced the mLub on the surface further supporting the idea that early and temporary lubricin replacement may be therapeutically relevant. This

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combined with the fact that the boundary layer naturally replenishes itself[10] supports the possibility that mLub can act as a place holder for the boundary layer. This data further supports preclinical investigations of mLub immediatly following an injury to the joint as a protective measure with regard to preventing OA pathogenesis.

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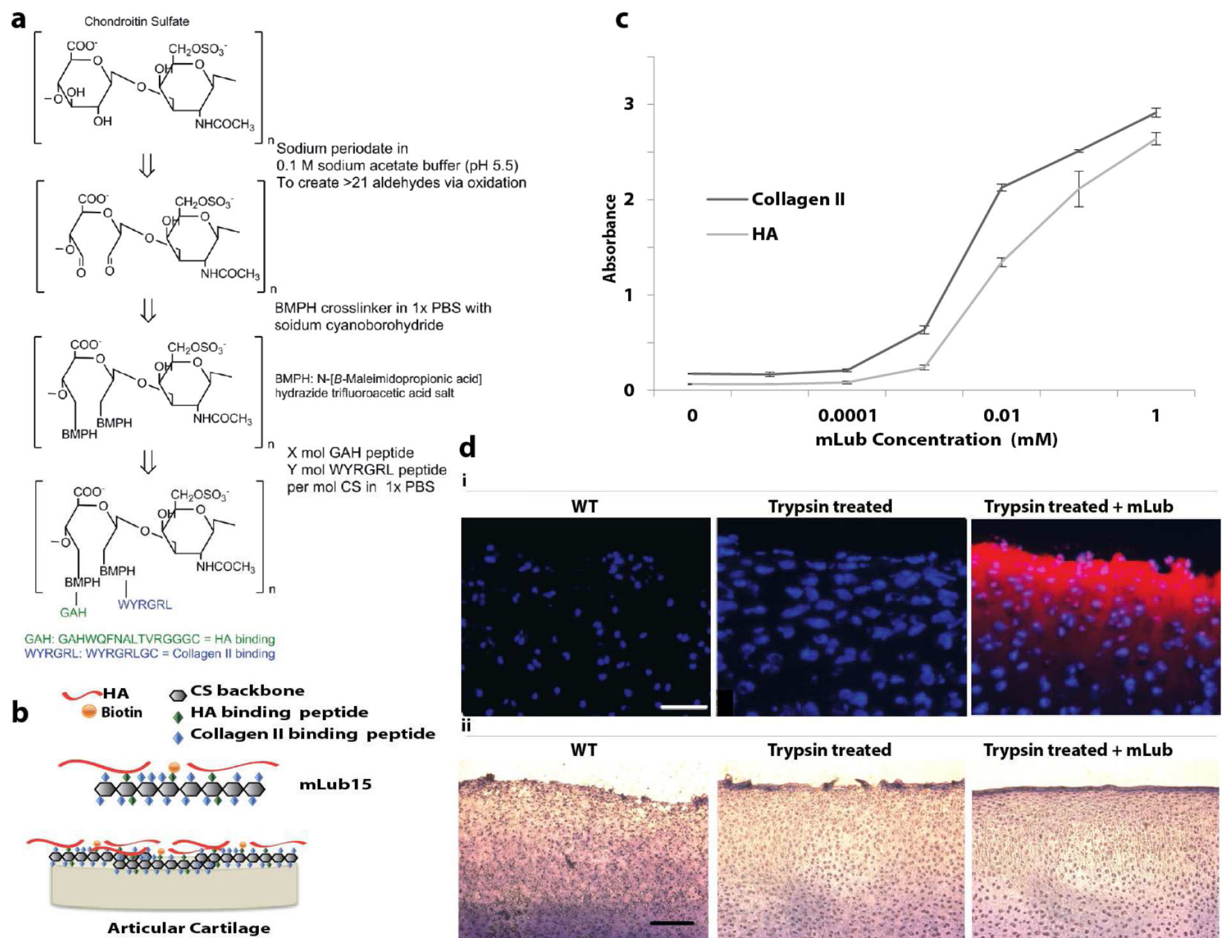

Figure 1. Synthesis of biomimetic lubricin molecule (mLub) bound to type II collagen and HA and spatially localized to cartilage surface

a, Reaction schematic for the synthesis of mLubX with the addition of HA and type II collagen binding peptides to a chondroitin sulfate backbone. **b,** Schematic of mLub15 binding to the articular surface and HA in the synovial fluid. **c,** mLub15 binding curves with HA and type II collagen on coated well plates. **d,** mLub15 binding on cartilage cryosections i) cartilage labeled with DAPI for cell nuclei (blue) and streptavidin for biotinylated mLub (red). ii) toluidine blue stained cartilage cryosections to show proteoglycan depletion by trypsin. White scale bar = 50um, black scale bar = 200um.

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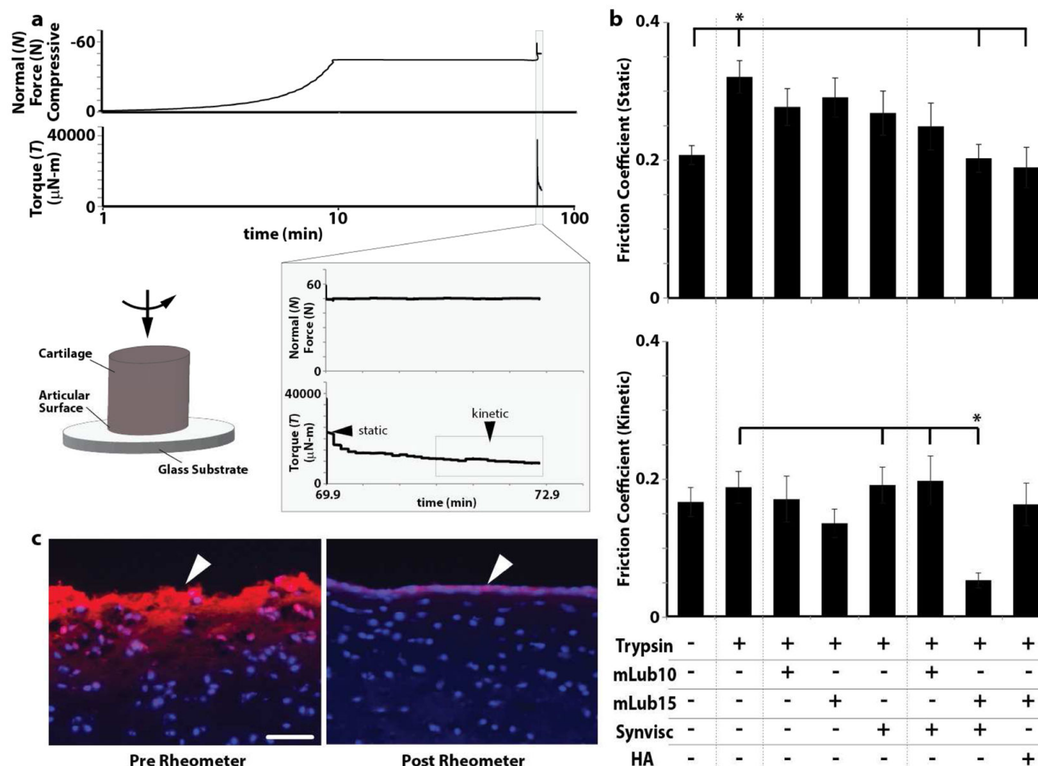

Figure 2. Treatments of mlub15 with Synvisc or HA on cartilage restores the macroscale coefficient of friction following trypsin treatment

a, Typical normal force and torque graphs during macroscale coefficient of friction testing using a rheometer. b, calculated static and kinetic coefficient of friction values of each treatment group (n 9). In static COF, there is statistical difference ($p < 0.05$) between the trypsin treated plug and the WT, mLub15 + Synvisc, and mLub15 + HA treatments. In kinetic friction, there is statistical difference ($p < 0.05$) between the mLub15 +Synvisc treatment and the trypsin, Synvisc, and mlub10 + Synvisc treatments. Standard error bars are shown. c, Fluorescent staining images of cryostections of cartilage with mLub probed with streptavidin (red) and DAPI for nuclei (blue). The left image represents a cartilage sample that did not go through the compression and shear movements on the rheometer while the right image was cyro-sectioned after the rheometer test. There is still mLub present after compression and shear movement. White arrows point to the mLub covered cartilage surface.

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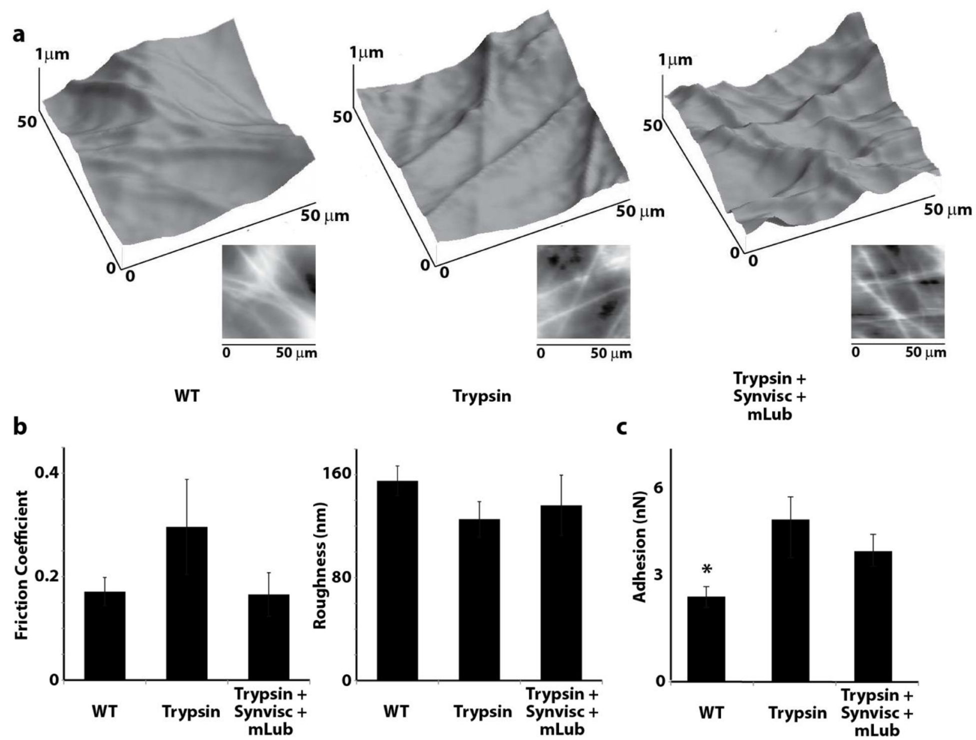

Figure 3. Cartilage surface properties measured by atomic force microscopy

a, 3D and 2D topography images of cartilage surfaces, WT, Trypsin treated, and Trypsin treated later followed by a mLub then Synvisc treatment. Images represent samples with roughness values near the values in b. b, Friction coefficient and roughness values of selected areas of cartilage surfaces ($n = 9$) ($p > 0.05$) Standard error bars are shown. c, adhesion values of a different subset of cartilage samples (n=8) ($p < 0.05$). Standard error bars are shown.

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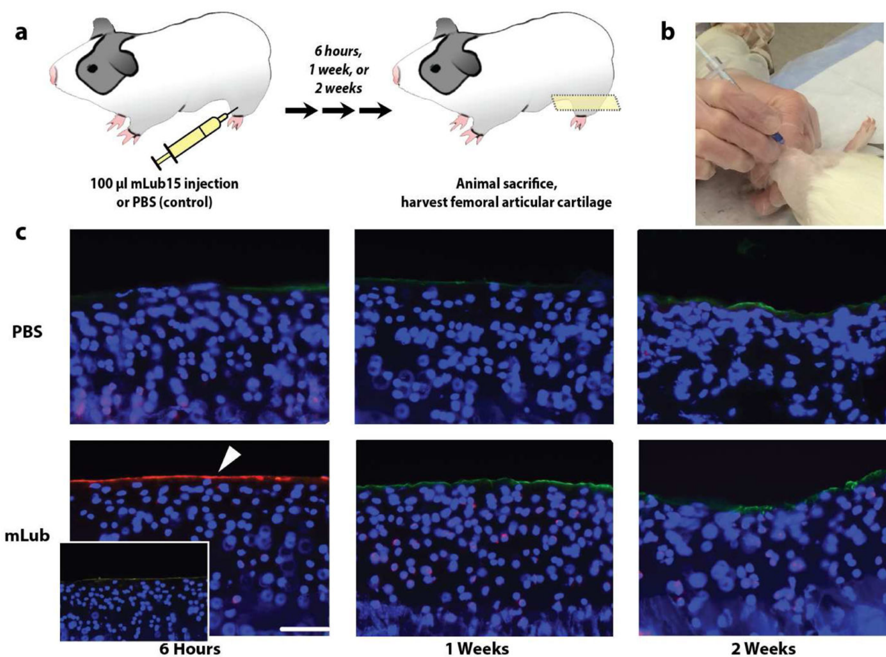

Figure 4. *In vivo* spatial localization of mLub to the articular cartilage surface with residency time of less than 1 week

a, Cartoon schematic of *in vivo* study. b, picture of injection through the patellar tendon and into the synovial fluid. c, fluorescent imaging of the cartilage of guinea pig joints injected with PBS (control) or mLub and harvested after 6 hours, 1 week or 2 weeks. Fluorescent streptavidin is bound to the biotinylated mLub (red), native SZP is labeled (green) and DAPI is bound to cell nuclei (blue). The scale bar represents 50 µm. An inset of native SZP staining alone after 6 hours is also shown.