Lipid-hyaluronan synergy strongly reduces intrasynovial tissue boundary friction

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Abstract

Hyaluronan (HA)-lipid layers on model (mica) surfaces massively reduce friction as the surfaces slide past each other, and have been proposed, together with lubricin, as the boundary layers accounting for the extreme lubrication of articular cartilage. The ability of such HA-lipid complexes to lubricate sliding biological tissues has not however been demonstrated. Here we show that HA-lipid layers on the surface of an intrasynovial tendon can strongly reduce the friction as the tendon slides within its sheath. We find a marked lubrication synergy when combining both HA and lipids at the tendon surface, relative to each component alone, further enhanced when the polysaccharide is functionalized to attach specifically to the tissue. Our results shed light on the lubricity of sliding biological tissues, and indicate a novel approach for lubricating surfaces such as tendons and, possibly, articular cartilage, important, respectively, for alleviating function impairment following tendon injury and repair, or in the context of osteoarthritis.

1. Introduction

Efficient lubrication at sliding biological surfaces is often a key to the well-being of the associated organs. Examples include eyelids sliding past the cornea (or past contact lenses)[1,2]; articulating cartilage surfaces in joints[3,4]; and intrasynovial tendons gliding within their sheaths[5,6]. Breakdown of such lubrication is related to pathologies, such as dry eye syndrome, or irritation preventing contact lens usage[7]; cartilage damage leading to osteoarthritis (OA) in the case of the major synovial joints[8,9]; or the impairment of tendon function following tendon injury and repair[10]. Understanding the origins of such lubrication and being able to prevent its failure is clearly desirable, and has been studied extensively[11], particularly the boundary friction of articular cartilage (see recent review in Ref.[4]), due to the large health and socio-economic burden of OA[12]. The main molecules believed to play a role in the lubrication of synovial joints, all of which are ubiquitous in synovial fluid and in the cartilage, as well as at intrasynovial tendon surfaces[11], are the linear polysaccharide hyaluronan (hyaluronic acid, or HA)[13-16], the glycoprotein lubricin[17-20], and phospholipids[21-25]. Recent surface force balance (SFB) studies[26,27] on model (mica) surfaces have shown that phosphatidylcholine (PC) lipids complexed with surface-attached HA form robust, strongly-lubricating boundary layers, providing the low levels of friction characteristic of healthy articular cartilage, up to the high pressures associated with the major joints. Based on such model experiments, it was proposed that HA-PC complexes, attached to the cartilage surface via lubricin, form the boundary layers responsible for the efficient lubrication of articular cartilage[26-28]. In this proposed scenario, these molecules (HA, PCs, lubricin) act together synergistically, each with a different role, to provide the very low boundary friction. The ultimate microscopic origin of this low friction is the hydration lubrication mechanism[29-31], acting at the highly-hydrated phosphocholine head-groups of the PCs exposed at the slip-plane between the tissue surfaces as they slide past each other. The ability of such HA-PC complexes to lubricate biological tissue has not however been demonstrated.

Here we provide evidence of strong HA-PC lubrication synergy at a sliding biological tissue surface, that of an intrasynovial flexor tendon sliding within its sheath (using an analog of the flexor digitorum profundus (FDP) from a domestic chicken digit). Such tendons, including that of the chicken toe tendon as used in the present investigation, have compositions and are surrounded by sheath fluid broadly resembling, respectively, that of articular cartilage and synovial fluid in joints[11], and their physico-chemical characteristics, have been studied extensively[34]. It is important to bear in mind when considering the implications of our results on tendons for the case of articular cartilage that the loads, and the maximal pressures at articular cartilage surfaces in the major joints, such as hips and knees, are much higher[35] than those at tendon surfaces. At the same time, there are indications, as has been earlier pointed out[10], that "whether the gliding is cartilage on cartilage or tendon on tendon sheath, the basic biologic strategies are very similar". For this reason, results obtained on tendon/sheath friction may also be of relevance for cartilage lubrication, though the differences in loads and pressures at the two tissues should be borne in mind. Tendons were treated with PC lipids (in the form of liposomes) or with HA, or in combination, while HA itself was used either in the native form or was functionalized with a collagen-binding group, to bind the polysaccharide covalently to the tendon surface. Fluorescence-labelling of the liposomes shows that the presence of added HA strongly enhances the attachment of the lipids to the tendon, and significantly more so when the HA is functionally-bound to the tendon surface. Corresponding to this, we find that treatment with either HA or the PC liposomes alone reduces the tendon/sheath friction coefficient by some 40-50% relative to the saline control, but that their combination reduces it up to a full 5-fold. Our results demonstrate that sliding biological tissues may be efficiently lubricated by a boundary layer comprising an HA-PC complex, as recently proposed[26,27]. They have clear implications for novel approaches to reducing friction at such surfaces, including those of articular cartilage, as well as of tendons as studied here.

2. Materials and methods

2.1 Materials

Water used was purified by Barnsted NanoPure systems to 18.2 MΩ cm resistance with total organic content levels of < ca.1 ppb. All phosphatidylcholines lipids were purchased from Lipoid, GmbH (Germany). Phosphate buffer saline, pH 7.4 (PBS), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C_{18} or DiI) and DOPA were purchased from Sigma (Israel), and HA of Mw = 1.5 x 10^6 Da was bought from Lifecore (Minnesota, USA).

2.2 Tendon specimen preparation

The third digit of a domestic chicken was dissected[32]. First, the skin was removed and the tendon sheath was revealed. Then, the second phalanx (out of four) was separated from the rest of the digit through an arthrotomy of its proximal and distal joints. The analogs of the flexor digitorum profundus (FDP) was cut 1.5 cm distal and 1.5 cm proximal to the distal and proximal joint, respectively. Next, the analog to the flexor digitorum superficialis was removed along with its insertion to the phalanx. The vinculum of the FDP was removed allowing the FDP to glide within its tendon sheath. Lastly, the proximal half of the phalanx was removed leaving the distal part (0.5 cm) with the distal pulley and tendon. The specimen was fixed to a plastic plate with Dermabond and 4-0 nylon sutures that were suture to pre-drilled holes in the plate. Both ends of the tendon were sutured with Nylon 4-0. Nylon sutures were tied to the FDP tendon ends connected it to the sensors. Each tendon was used only once, for one treatment. The tendons and their sheaths were generally freshly prepared on the same day that their friction was measured, and kept in PBS to avoid drying of the tissue. Tendons were kept at room temperature (in PBS) till they were used on the day of surgery. In those cases (around 25%) where tendons, prepared later in the day, were not measured in the tribometer on the day of preparation, they were kept overnight in PBS at 4-8 ^{o}C, and measured the following day. However, all tendon/sheath samples were measured within 24 h of surgery. We note that

measurements on tendons used after such overnight storage in PBS were identical within the scatter to those performed on tendons on the day of their fresh preparation. This indicates that tendons were stable for at least 24 h with respect to subsequent treatment and lubrication properties. Further details are given in Supplementary Information (SI).

2.3 Liposome preparation

Small unilamellar vesicles (SUVs) were prepared as described earlier[44]. Briefly, multilamellar vesicles prepared by hydrating hydrogenated soy phosphatidylcholine (HSPC) at 65 ℃ in PBS were downsized to form SUVs, ~100 nm in diameter, by stepwise extrusion through polycarbonate membranes starting with a 400-nm and ending with 50-nm-pore-size membrane, using a Lipex 20 mL extruder system (Northern Lipids, Vancouver, Canada). For the fluorescence and confocal measurements, the SUVs of HSPC liposomes were labeled using DiI as following: DiI was dissolved in EtOH for a stock solution of 5 mM. 1 mL of the DiI stock solution was mixed with 0.375 g of HSPC lipid at T = 65 ℃ until getting a homogenous solution, giving DiI to lipid molar ratio of 0.1%. Then, 9 mL of PBS solution was added followed by sonication and heating to 65 ℃ for 15 minutes and the liposomes were downsized using an extruder, as described above. In order to remove free DiI molecules from the suspension, the solution was dialyzed for 2-3 days against PBS using a dialysis membrane of MWCO 3500 (Membrane Filtration Products, Texas, US). Liposomes were characterized for size distribution by Dynamic Light Scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd., UK), which indicated liposome diameters ≈ 100 nm in the bulk PBS solution, for both HSPC and HSPC/DiI liposomes.

2.4 HA-DN synthesis

HA and dopamine (DOPA or DN) conjugates (HA-DN) were prepared following ref. [57]. Briefly, dopamine was coupled to the carboxyl group of HA via 1-ethyl-3-(3’-dimethyl aminopropyl) carbodiimide (EDC) coupling reaction (fig. S2a).

Full details of the synthesis and characterization of the HA-DN by UV/Vis spectroscopy and ^{1}H-NMR analysis are given in the SI. Analysis of the UV/Vis spectra and, independently, of the ^{1}H-NMR spectra (fig. S2b) revealed the degree of dopamine substitution in the synthesized HA-DN conjugate to be 18 mol% and 19 mol%, values closely consistent with each other.

2.5 Tendon surface modification

The following tendon treatment solutions were prepared: a) PBS (the control solution); b) HA; c) HSPC-SUV; d) HSPC-SUV + HA; e) HSPC-SUV + HA-DN. The HA and HA-DN solutions were 0.4 mg/mL, and the HSPC-SUV dispersion was 11 mM, all in PBS except for the HA-DN, which was prepared and kept in pure water (pH 5.8 - 6) prior to use to enhance material stability (no color change was observed, indicating little or no oxidation of the DOPA had occurred[58] over the storage time, typically up to 2 h). After mounting the tendon in the measurement cell in the tribometer and performing a zero-load calibration in PBS at 37 ℃, the PBS solution was replaced by one of the different treatment solutions (except where measurements in PBS itself were required). The tendon was allowed to incubate in the treatment solution at 37 ℃ for 20 minutes, following which the solution was exchanged by fresh PBS and friction measurements were performed. The relatively high HSPC concentration (11 mM) was used to ensure efficient surface attachment of the liposomes to the tissue surface over the relatively short incubation time prior to replacing the solution by pure PBS. At the same time the HA concentration (somewhat lower than the lower range, 1 mg/mL, of HA in normal SF) was used to ensure that the solution was not too viscous or gel-like so that it could be efficiently removed when replacing with pure PBS.

For treatments by mixtures of HSPC-SUV and HA or HA-DN, the mixture components were kept separately and only before use were mixed by combining the two components, and then poured to cover the tendon in its cell.

2.6 Fluorimetry measurements

Sample preparation: 4 mm long sections were cut from the tendon and immersed in three different treatment solutions for 20-24 h at 37 ℃. Then, each section was washed in 8 mL of PBS in a 6-well plate, thoroughly mixed by shaking and transferred to a 24-well black micro-plate (to avoid cross-talk) for fluorimetry measurements (Ibidi, Germany), or was placed on a cover slide for confocal microscopy. The three treatment solutions used DiI-HSPC-SUV; DiI-HSPC-SUV +HA; and DiI-HSPC-SUV + HA-DN, prepared at concentrations as described earlier (save that DiI-labelled liposomes were used). Quantitative fluorescence measurements were performed using a BioTek Synergy HT Fluorometer. Filters used were 528±20 nm for excitation and 560±20 nm for emission.

2.7 Spinning disk confocal microscopy measurements

We used a laser confocal unit CSU-W (Yokogawa Electric Corporation, Japan)) which enabled imaging at 400 to 850 nm, a confocal unit supporting two pinhole wheels that allowed confocal imaging using high NA with small working distance objectives as well as long working distance water immersion objectives. We used the Andor laser at 561 nm. Light was collected and imaged using the Andor iXon Ultra EMCCD camera, characterized by low light sensitivity and fast imaging, allowing up to four fluorophores examination at a video rate. The system is equipped with the CRISP autofocus unit (ASI Applied Scientific Instrumentation, USA) enabling long experiments and compensating for small temperature drifts by activating an ASI piezo Z-stage. Samples were placed on a motorized ASI XY stage that provided the ability to run montage scans and large image areas at high resolution, and to perform multi-well experiments. The entire sample handling on the BX61 (Olympus, Japan) is within an environmental enclosure (Okolab, Italy) that controls the sample temperature. ImageJ software was used for image analysis.

2.8 Friction measurements

Different methods have been used to measure friction forces in tendons, among them is the pin-on-plate tribometer[6] and a system which measures the friction at the tendon-pully interface[59]. In this study we use a modified CETR© tribometer, UMT model with a low load (high sensitivity) sensor enabling normal loads in the range of 0 - 1000 g to measure directly the friction forces between tendons and their sheath by applying different normal loads, as described in the Results section. Fig. 1 shows the set-up schematically. Up to 6 independent measurements (different tendons) were carried out for each treatment. The mean pressure P on the tendon was evaluated as $P = L/(\pi R^2)$ where L is the load and R (= 3 mm) the radius of the rod transmitting the load, pressing on the sheath as shown in fig. 1. For the two load values used (0.4 and 0.8 N), $P \approx 0.15$ and 0.3 atm.

2.9 Statistical Analysis

The mean values of columns in fig. 3, 5 and 6 were determined using an Excel spreadsheet program, where the error bars represent standard deviations from the mean.

3. Results

We examined the attachment of PC-lipids to the tendon surface via fluorescence labelling, using confocal fluorescence microscopy and fluorimetry; and measured the sliding friction of the tendons in their sheaths, using a purpose-modified tribometer. Measurements were carried out following treatment of the tendon/sheath couple by immersion in different combinations of lipids and HA. We used PBS as a control, and for washing and as the immersion medium following the different treatments. Treatments consisted of incubating the tendon in its sheath in PBS solutions of either HA or SUVs of hydrogenated soy phosphatidylcholine (HSPC-SUVs), or of their combination, (HSPC-SUV + HA), followed by washing away of the treatment solution. The washing away and replacement by pure PBS was done to ensure as far as possible that any effect on the friction would arise from surface-attached species alone rather from their presence in the surrounding medium. In addition, we examined treatment in

solutions of (HSPC-SUV + HA-DN), where HA-DN is an HA functionalized with 3,4-dihydroxy-L-phenylalanine, (DOPA or DN) to further promote its attachment to collagen at the tendon surface. Full details are given in Materials and methods 2.4.

3.1 Determination of lipid attachment to the tendon surface via fluorescence labelling

HSPC-SUVs labelled with the lipophilic dye 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C_{18} or DiI), designated DiI-HSPC-SUV were used to monitor the liposome attachment to the tendon surface. Fluorescence microscopy measurements of tendons treated with different solutions: DiI-HSPC-SUV; (DiI-HSPC-SUV + HA); and (DiI-HSPC-SUV + HA-DN), in all cases followed by washing, were carried out using a spinning disk confocal microscope. They provide a qualitative indication of the relative lipid adsorption on the tendon surface. Typical images are presented in fig. 2.

Treatment with the labelled liposomes on their own results in some lipid adsorption, fig. 2A, while treatment with DiI-HSPC-SUV/HA solutions, fig. 2B, shows that the presence of HA promotes significantly higher adsorption. A yet-larger increase in the liposome adsorption onto the tendon relative to immersion in PBS alone is seen when the treatment solution consists of liposomes with the modified HA-DN, fig. 2C. Quantitative fluorimetry measurements, using a microplate reader for these same treatments, are shown in fig. 3.

In line with the qualitative indications by confocal microscopy in fig. 2, the normalized intensity shows a significant increase (~70%) in the adsorption of liposomes on tendons from a (DiI-HSPC-SUV + HA) solution (fig. 3B) as compared to its adsorption from saline alone (fig. 3A). When adsorbed from a (DiI-HSPC-SUV + HA-DN) solution, fig. 3C, the normalized lipid adsorption increases relative to its value with the unfunctionalized HA, and is a full 3-fold greater compared to the lipid adsorption from saline in the absence of HA (fig. 3A). These results show clearly the effect of HA in promoting the PC-SUV attachment to the tendon surface, while the increased surface coverage of HA-DN, figs. 2C and 3C (relative to HA alone,

figs. 2B and 3B), arising from the interactions of DN with surface amine groups, promotes yet denser lipid attachment.

3.2 Friction measurements

The friction force on the tendon sliding in its sheath was determined using a modified UMT tribometer (Methods), by measuring the force required to glide the tendon within its sheath over cycles of back-and-forth motion while applying either a zero load or different loads onto the sheath, compressing the gliding tendon within. The amplitude of the tendon motion within its sheath was 4 mm, generally at a sliding speed v_s of 2 mm/sec, and friction was monitored up to 500 cycles (2000 sec). Sliding friction was measured for five treatment groups (followed by washing in PBS): PBS; HSPC-SUV; HA; (HSPC-SUV + HA); and (HSPC-SUV + HA-DN). As a control, the friction force under zero load $F_{s,0}$ (following 50 cycles) was measured in PBS for all tendons prior to treatment in the different solutions. We found little significant difference (within the scatter) for a given tendon/sheath couple between the zero-load resistance-to-motion $F_{s,0}$ (magnitudes of $F_{s,0}$ were in all cases in the range 0.011±0.002 N). Moreover, the zero-load values $F_{s,0}$ in PBS did not increase (within the scatter) following multiple back-and-forth motion up to 500 cycles. As a further control, the effect of varying v_s was measured both under zero-load and under a load of 0.16 N; the shear force $F_s(v_s)$ did not vary under a four-fold change in v_s (0.5 mm/sec and 2 mm/sec). Normal loads F_n of either 0.4 N or 0.8 N (40 and 80 g wt) were then applied on the sheath, compressing the enclosed tendon. While data on the normal force on flexor tendons *in vivo* have not been identified in the literature, these loads (0.4 and 0.8 N) are comparable with the 1 N normal load estimated perpendicular to the tensile region of a hand extensor tendon[6]. The resultant mean pressures P between tendon and sheath in our experiments were ca. 0.15 and 0.3 atm respectively for the two loads.

Typical variation in the resistance to sliding F_s (for a load 0.4 N) with the number of back and forth cycles are shown in fig. 4, taken from traces as shown inset to fig. 4B. Forces F_s thus

measured for the different treatments are shown in figs. 5A and 6A for the two respective loads, while figs. 5B and 6B show the *increase* in resistance to sliding in the two cases relative to the zero-load values $F_{s,0}$ (i.e. the net sliding friction). The data (fig. 5B and 6B) show clearly that this sliding friction coefficient relative to untreated tendons following 500 cycles, is reduced by ca. 40±10% following incubation in the single-component HA or HSPC-SUV solutions, while incubation in (HSPC-SUV + HA) and (HSPC-SUV + HA-DN) solutions leads to a much larger reduction of up to 5-fold at the two loads. Over the 500 cycles, moreover, the net friction for the untreated and the single-component-treated tendons (HA or HSPC-SUV) increases continuously and markedly (by ca. 30 - 50%), while when treated with the (lipid + HA) or (lipid + HA-DN) solutions, it does not increase at all within the scatter.

4. Discussion

The main finding of this study is that a combination of PC lipids (HSPC-SUVs) together with HA attach synergistically at the surface of a relevant biological tissue, an intrasynovial tendon sliding past its sheath, to form a robust, highly-lubricating boundary layer. Such a boundary layer was recently proposed, based on SFB experiments on model surfaces bearing HA complexed with PC lipids, either dipalmitoylphosphatidylcholine (DPPC) or HSPC, as responsible for the low boundary friction of articular cartilage in synovial joints[26,27] (though these are subject to much higher maximal loads[35] than the tendon/sheath system). The origin of the low frictional dissipation by such a PC/HA layer was attributed to hydration lubrication by the highly-hydrated phosphocholine groups exposed at its outer surface. It has not been clear, however, that results from the nanotribological SFB studies, on boundary layers coating atomically-smooth, rigid mica substrates in a single-contact configuration, would apply to lubrication of complex biological tissues. Our finding that this applies not only for mica surfaces, but also at the much softer and rougher intrasynovial tendon surface sliding past its sheath, validates the conclusions concerning the boundary lubrication ability of these PC/HA complexes derived on the basis of the model experiments using smooth, rigid substrates.

To consider the present results it is appropriate to describe briefly the tendon composition, particularly at its surface. Tendons consist of a network of (mostly type I) collagen and elastin embedded in a proteoglycan-water matrix, with collagen accounting for 65-80% and elastin ~1-2% of the dry weight of the tendon[36]. The collagen is surrounded by proteoglycans, glycosaminoglycans (GAGs, of which HA constitutes some 6%), structural glycoproteins including lubricin, and other small molecules. The outer surface of intra-synovial tendons within their sheaths (such as the FDP used in the present study) is a membrane (the epitenon), comprised largely of collagen whose cells resemble synovial cells and secrete matrix components (including fibronectin), much as they do in articular cartilage[37-40]. The extracellular matrix of the epitenon resembles the superficial zone of articular cartilage, including collagen, HA, proteoglycans, and phospholipids (though the epitenon is mostly type 1 collagen while articular cartilage is mostly type 2 collagen). The boundary layer exposed at the outer surface of the epitenon contains HA, phospholipids and proteoglycans, including lubricin, and may also contain fibronectin[11,41-43] (components in common with those in the boundary layers coating articular cartilage) and is believed to provide the high lubricity of healthy intra-synovial tendons as they slide within their sheaths. Finally, the tendon-sheath space is permeated by a fluid whose composition resembles that of synovial fluid in joints, including phospholipids, HA and lubricin[5,40,41]. These features help to appreciate our results.

Fluorescence microscopy shows qualitatively that after incubation in the HSPC-SUV dispersion, followed by washing, lipids (in the form of liposomes) attach at (relatively) low surface density to the tendon surface (fig. 2A), but that they attach significantly more densely from a (HSPC-SUV + HA) solution (fig. 2B), and more densely still from a (HSPC-SUV + HA-DN) solution (fig. 2C). This is seen more quantitatively in the corresponding fluorimetry measurements (fig. 3), and is explained as follows. PC liposomes expose zwitterionic phosphocholine groups at their outer surfaces, and are known to adsorb to negatively charged surfaces or polymers via a dipole-charge interaction[26,44]. It is likely therefore that their attachment to the tendon is through their attraction to negatively-charged groups present on its

outer surface. Such negative charges reside for example on the molecular components of the boundary layer noted above, notably on HA. However, surgical removal of the tendon from its *in-vivo* environment and of its surrounding synovial fluid, and its subsequent handling, washing and incubation in PBS prior to use (Materials and methods 2.2), may have led to the depletion of some of these charged surface components from the boundary layer. Addition of HA to the PC liposomes in the tendon-treatment solution then enables replenishment and thus a higher coverage of the HA on the tendon surface (for example, through the known binding of HA to fibronectin or lubricin, also present at the tendon surface[40,41]). When the HA is further functionalized as HA-DN in the treatment solution, where the dopamine group binds to amine groups (as on collagen 1 or GAGs) at the tendon surface, it binds yet more densely at the tendon surface. We attribute the progressively higher liposome coverage, as seen in fig. 2A – 2C and 3A – 3C, to this increasing HA coverage, since such PC vesicles are known to adsorb strongly on surface-attached, negatively-charged HA through the dipole-charge interaction[26,27].

The relative frictional behavior following the different treatments may likewise be understood in the light of the different HA - and corresponding PC-vesicle – coverage revealed by these fluorimetric measurements. Layers formed by PC-lipid/HA complexes have been identified, through direct measurements on model surfaces[26,27], as highly efficient boundary lubricants, and the results of the friction measurements in the present study (F_s vs. number of cycles) reflect this also for the tendon tissue, as in fig. 5 and 6. Resistance F_s to motion arises due to energy dissipation, as the tendon slides within its sheath in our configuration (Materials and methods 2.8), in a number of ways. These include the mechanical distortion, first one way then the other, of the soft sheath itself surrounding the tendon, as the latter moves back and forth. This represents a systematic contribution to F_s quite apart from any relative sliding motion between them, as indicated by the fact that the zero-load resistance $F_{s,0}$ is similar for all tendons and does not vary with number of cycles. In contrast, in the presence of load, boundary friction and fluid-film drag are expected to be the major contribution to the energy dissipation during actual relative sliding motion between tendon and sheath, as do viscoelastic losses within the

soft sheath tissue as the tendon slides past it. Since $F_{s,0}$ arising from the tissue distortion is taken to be a systematic resistance to motion unrelated to dissipation while sliding, it may be subtracted from F_s to get a better measure of the net sliding friction itself. This is shown in fig. 5B and 6B, where the corresponding sliding friction coefficient is defined as:

$$\mu = (F_s - F_{s,0})/F_n. \quad (1)$$

We emphasize that in each case the value of $F_{s,0}$ being subtracted corresponds to the particular tendon/sheath couple for which it was determined, ensuring consistency in accounting for this systematic effect.

In the presence of PBS alone the initial net sliding friction (e.g. fig. 4A and B), as reflected by the higher initial value of $(F_s - F_{s,0})$, corresponds to $\mu \approx 0.04$ - 0.05. As the surfaces slide past each other, we attribute the progressive rise in F_s with sliding to this relatively high friction, which causes wear of any lubricating boundary layer, leading to increased frictional dissipation with cycle number. After 500 cycles the friction coefficient has risen to $\mu \approx 0.06 – 0.08$ (fig. 5 and 6). Following treatment with HSPC-SUV alone leads to reduction in friction relative to the PBS, as the sparsely-attached liposomes (see fig. 2A and 3A) provide some lubrication via their highly-hydrated phosphocholine headgroups. The increase in F_s over the 500 cycles is attributed to removal of some of the lipid vesicles and consequent. Likewise, treatment with solutions containing HA alone results in HA attachment to the tendon surface with similar initial friction, attributed to partial replenishment of the lubricating boundary layer on the epitenon. HA by itself is not a good boundary lubricant at pressures above a few atm[26,45,46], but at the low contact pressures (ca. 0.15 – 0.3 atm) exerted by the loads in our experiments, there is evidence that the steric, hydration and electrostatic double-layer repulsion reduces the friction between HA-bearing surfaces[45,46]. As before, we attribute the increase in friction with progressive sliding to wear of the lubricating layer. Both HSPC-SUV alone and HA alone, therefore, result in friction increase of ca. 30% – 50% following 500 back-and-forth sliding cycles, to friction coefficient values $\mu \approx 0.04$.

Incubation in solution containing both HA and the PC-lipids, however, and even more so when HA-DN is used with the lipids, strongly reduces the friction coefficient, by some five-fold relative to PBS (fig. 5B and 6B). Friction coefficients down to $\mu \approx 0.012$ are observed, and the friction does not increase within the scatter up to 500 back-and-forth cycles. We attribute this to the formation of HA/PC-lipid complexes at the tendon boundary, with resulting very low friction as seen with similar layers in earlier model studies. The low friction arises from the hydration lubrication effect due to the strongly hydrated phosphocholine groups exposed at the slip plane. The stable value of the friction force is attributed to the robustness of the HA-lipid complexes, together with the low friction, which at least over 500 cycles does not appear to lead to wear of the boundary layer, thereby maintaining its lubricity. We recall that the experiments were carried out over a period of 2000 seconds (500 cycles) at the lower load (0.4 N), and then over another 2000 seconds (500 cycles) at the higher load (0.8 N); as noted, for the combinations of HA and HA-DN with liposomes, the lubrication was stable over this period (fig. 4). Moreover, in a number of experiments, following measurements at the lower (0.4 N) and then higher load (0.8 N), the load was decreased again to 0.4 N, and friction measurements carried out again. These showed the low-load behavior reversibly reproducing itself within the scatter. These observations provide a significant measure of the stability and retention of the lubricating properties of the boundary layers on the treated tendons. We note also that, at the low velocities of our measurements, the contribution of fluid drag to the sliding friction is minor, as indicated by the absence of an increase in F_s when the sliding velocity was increased by 4-fold (as noted also in earlier studies[4]), indicating that boundary friction is the dominant mode.

The question of the tendon surface roughness is also of interest. We have not identified in the literature direct measures of this, though preliminary atomic force microscopy scans that we carried out on the fresh chicken intrasynovial tendon surface under saline suggest a roughness of a few hundred nm (with the caveat that such scans of a soft material under PBS may be subject to artefacts arising from distortion by the AFM tip). However, if we assume that the

epitenon roughness, and effective compressive modulus normal to the surface, are roughly similar to those of the outer surface of articular cartilage, their values would be roughly 0.5 μm and 10^5 N/m^2 respectively[47,48]. In that case, even under the low 0.3 atm pressure in our study, considerable deformation of the tendon asperities would be expected, as considered in detail in ref. [47], so that close-to-affine contact would occur between the sliding surfaces.

It is of interest to briefly compare our results with earlier studies of intrasynovial tendon friction, and in particular with studies in which the tendon surface was treated to modify the friction. Such modifications have been extensively studied[6,40,42,43,49-54] and reviewed[5,10,11,55], and have demonstrated the friction-reducing properties of different tendon surface treatments, including functionalized HA[16] which attaches strongly to the tendon surface, either by itself or with other components[42,49-54,56], such as gelatin and lubricin. We note that these surface-modification studies were often carried out on extrasynovial tendons[49,51-53,56] which *in vivo* are not encased within a tendon sheath, and which have a different structure and a generally higher friction coefficient than intrasynovial tendons (the present work), when sliding against the tendon pulley. One study[50] showed that friction between an unmodified rabbit flexor (intrasynovial) tendon and its sheath in HA solution was reduced relative to saline alone, increased on removal of the tendon membrane, and returned to its previous level (intact tendon + HA alone) when treated with (HA + DPPC). In general, the lowest values of sliding boundary friction coefficient μ that were reported in all these studies after 500 – 1000 cycles of back-and-forth motion, following treatment of tendon surfaces with HA or in combination with other components, were in the range $\mu \approx 0.02 - 0.03$. These values are comparable with or slightly higher than in our study with the (lipid + HA) or (lipid + HA-DN) treatments, though direct comparison is not straightforward as the methods for measuring μ are rather different. We note that the low values of these friction coefficients were also obtained at quite low pressures, as in our study.

5. Conclusion

We have shown that HA can complex with a PC lipid on the surface of an intrasynovial tendon to provide a highly lubricious boundary layer at the interface with the tendon sheath. The large reduction in friction coefficient, by some 5-fold relative to saline alone, and some 2 – 3-fold relative to treatment by either lipid alone or by HA alone, is attributed to a synergy between the HA and the PC-lipid forming a dense complex exposing the highly-hydrated phosphocholine groups. This reduces the boundary friction through the hydration lubrication mechanism, as suggested by a study on model surfaces bearing such HA/lipid complexes[26,27]. This is the first clear indication that HA/PC-lipid complexes can strongly reduce friction at a sliding biological surface to which they are attached. It supports the proposed structure[4,26] of lubricating boundary layers on articular cartilage, though with the caveat, noted earlier, that articular cartilage may be subject to much higher loads than between tendon and sheath. This finding thus has implications for lubricating biological surfaces such as tendons and, possibly, articular cartilage, important, respectively, for alleviating function impairment following tendon injury and repair, or in the context of osteoarthritis.

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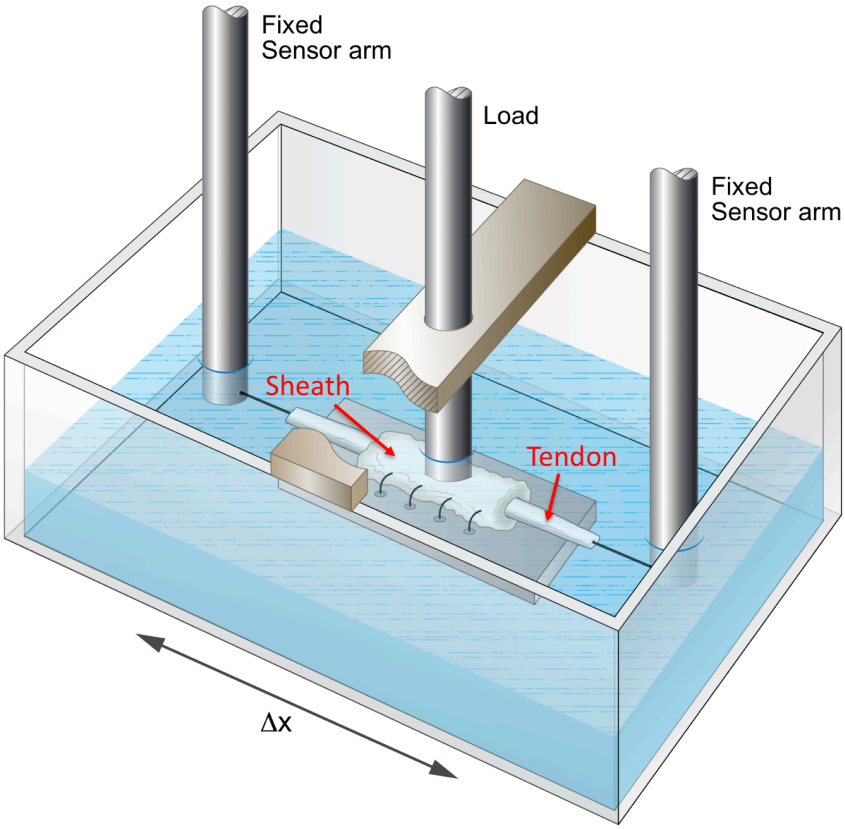

Fig. 1. Schematic of the modified tribometer used for the tendon-sheath friction measurements. The tendon is within its sheath, which is fixed on the plate via nylon sutures (see Supplementary Information fig. S1e). The plate in turn is rigidly mounted in the bath. Loads are applied via the vertical rod as shown. The tendon ends are attached as shown to the two fixed sensor arms via nylon sutures. As the bath moves back and forth in the x direction within the tribometer, the tendon slides within its sheath and sliding resistance is measured directly by the high-sensitivity sensor arms of the tribometer.

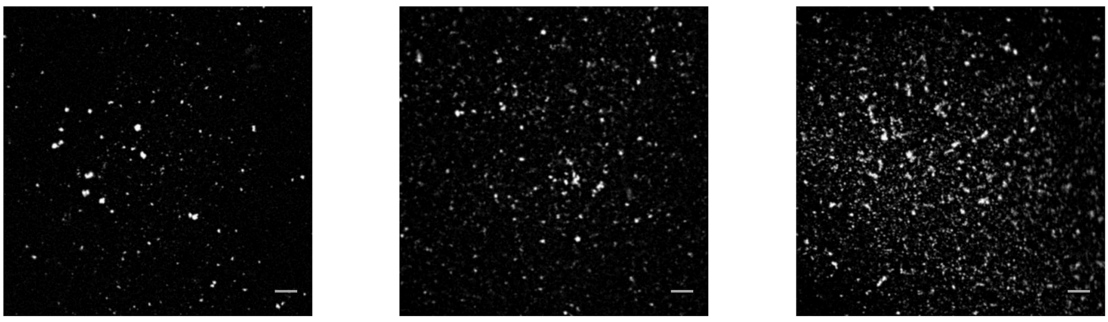

Fig. 2. Spinning disk confocal microscopy images of tendons following different treatments with DiI labeled species. (A) HSPC-SUV. (B) HSPC-SUV with HA (C) HSPC-SUV with HA-DN (18%). Scale bar is 10 μm.

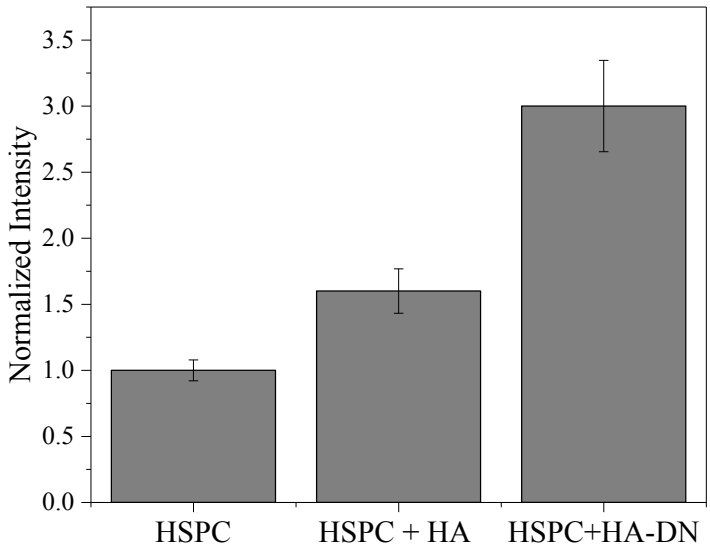

Fig. 3. Normalized fluorescence measurements (relative to HSPC-SUV alone, left column) of tendon surface treated with DiI labeled species: A) HSPC-SUV; B) HSPC-SUV + HA; C) HSPC-SUV + HA-DN.

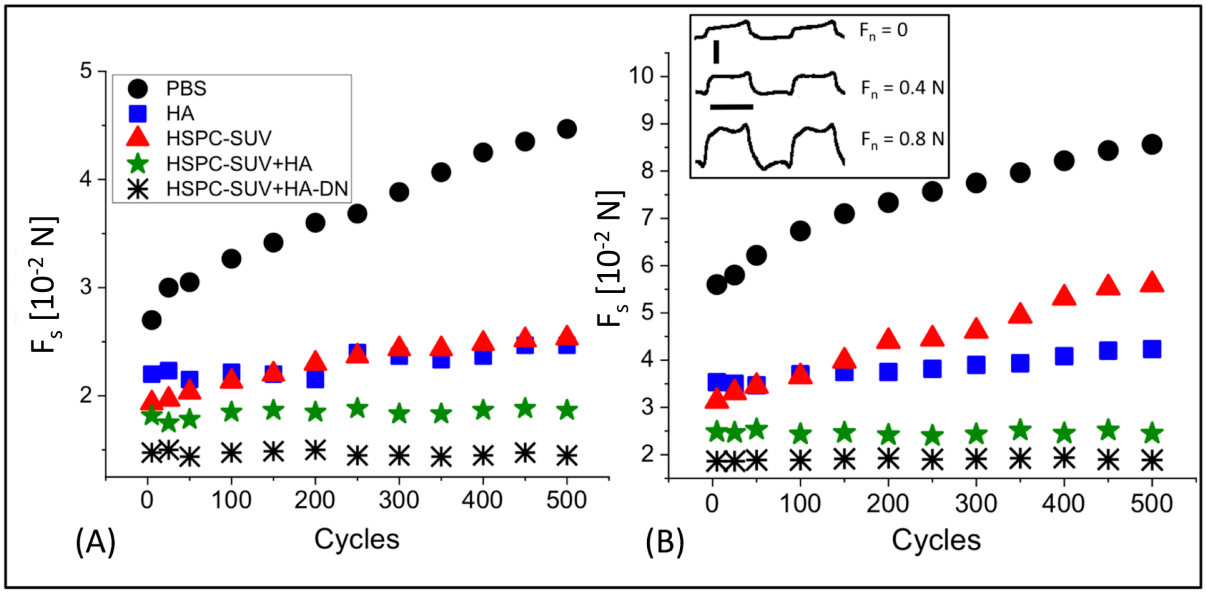

Fig. 4. Typical friction values as a function of number of back-and-forth cycles between a tendon and its sheath under a normal load of (A) 0.4 N and (B) 0.8 N, for the five different treatments (symbols as indicated in the legend): a) PBS; b) HA; c) HSPC-SUV; d) HSPC-SUV + HA; and e) HSPC-SUV + HA-DN. The inset to fig. 3B shows typical time (t) vs. F_s (N) traces at loads zero, 0.4 N and 0.8 N; vertical and horizontal scale bars correspond respectively to $F_s = 0.04$ N and $t = 2$ sec. The friction force is evaluated as ½ the difference in force between trace plateaus.

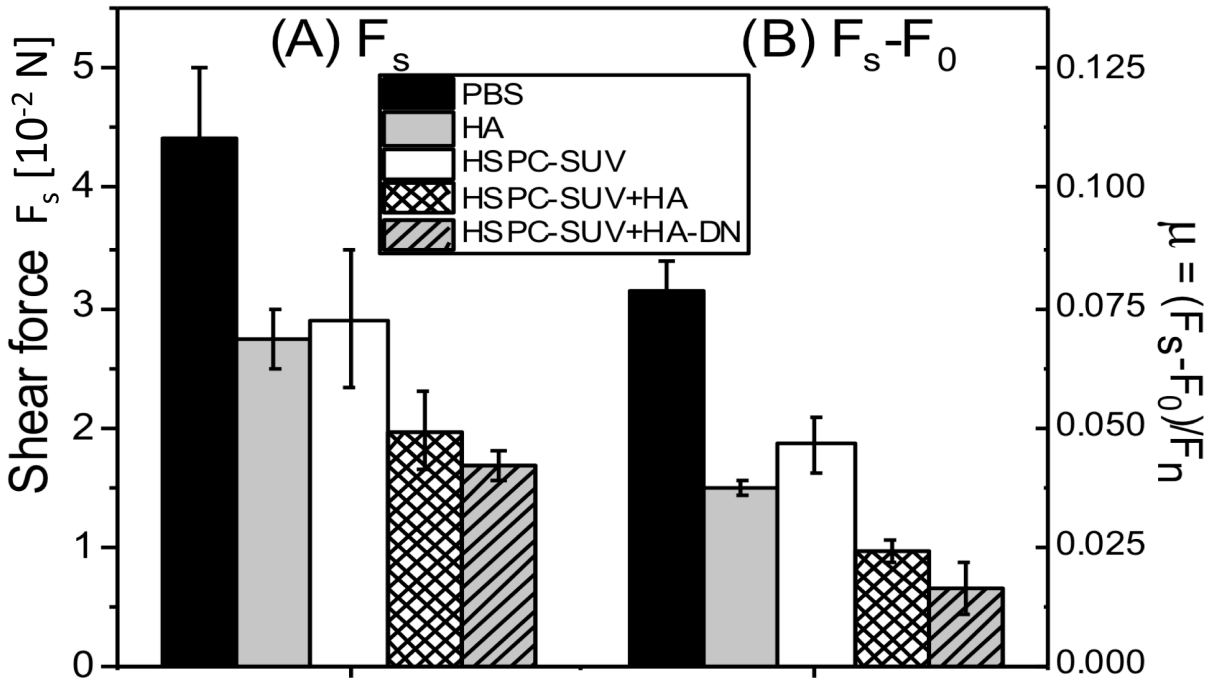

Fig. 5. Summary of measurements of resistance to shear under load of 0.4 N for tendon-sheath sliding following the 5 different treatments, as indicated in legend: a) PBS; b) HA; c) HSPC-SUV; d) HSPC-SUV + HA; and e) HSPC-SUV + HA-DN. (A) and (B) are the as measured resistance and following correction by the zero-load value. Values represent average of measurements of up to 6 independent tendons per treatment, and error bars are the standard deviations. The corresponding friction coefficients are shown on the RH scale.

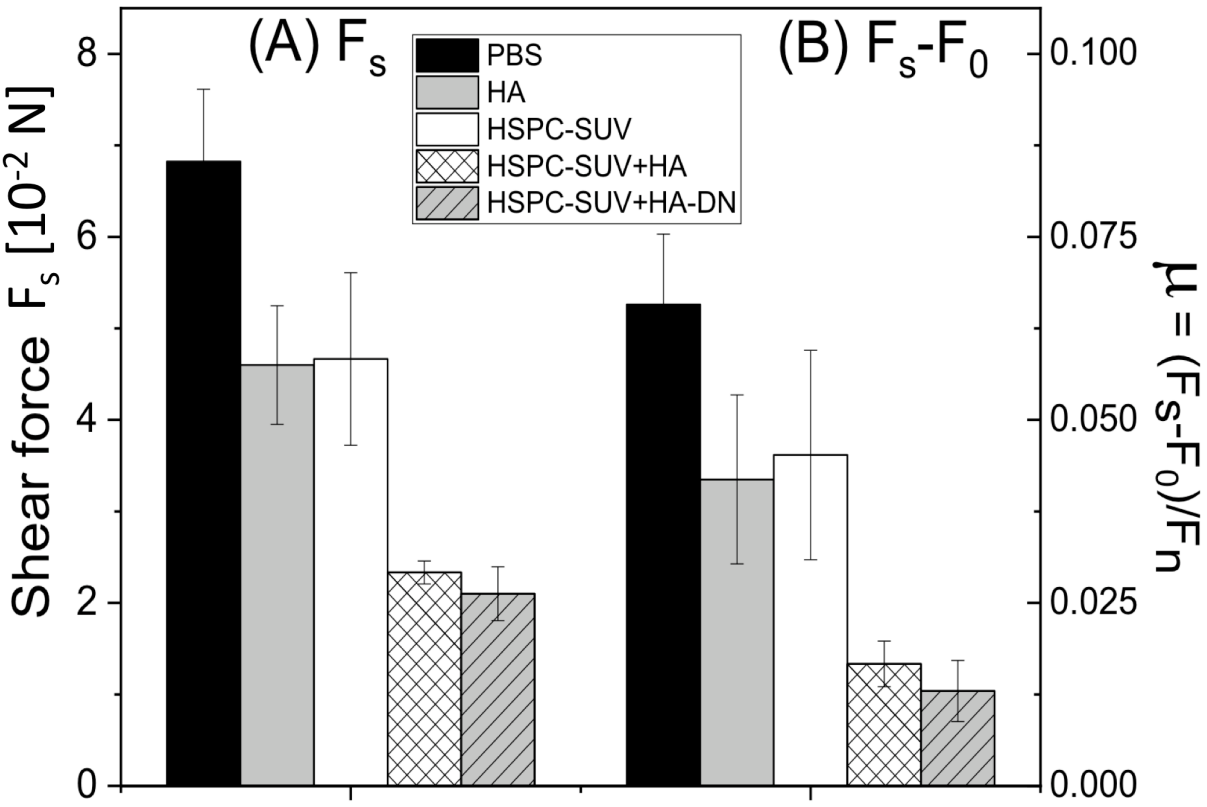

Fig. 6. Summary of frictional force measurements under load of 0.8 N for tendon-sheath sliding following the 5 different treatments, as indicated in legend: a) PBS; b) HA; c) HSPC-SUV; d) HSPC-SUV + HA; and e) HSPC-SUV + HA-DN. (A) and (B) are the as measured resistance and following correction by the zero-load value. Values represent average of measurements of up to 6 independent tendons per treatment, and error bars are the standard deviations. The corresponding friction coefficients are shown on the RH scale.

Supplementary Information for

Lipid-hyaluronan synergy strongly reduces intrasynovial tendon boundary friction

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S1. Tendon specimen preparation

The third digit of a domestic chicken was dissected[1]. First, the skin was removed and the tendon sheath was revealed (fig. S1a). Then, the second phalanx (out of four) was separated from the rest of the digit through an arthrotomy of its proximal and distal joints (fig. S1b). The analogs of the flexor digitorum profundus (FDP) was cut 1.5 cm distal and 1.5 cm proximal to the distal and proximal joint, respectively. Next, the analog to the flexor digitorum superficialis was removed along with its insertion to the phalanx (fig. S1c). The vinculum of the FDP was removed allowing the FDP to glide within its tendon sheath (fig. S1d). Lastly, the proximal half of the phalanx was removed leaving the distal part (0.5 cm) with the distal pulley and tendon. The specimen was fixed to a plastic plate with Dermabond and 4-0 nylon sutures that were suture to pre-drilled holes in the plate (fig. S1e). Both ends of the tendon were sutured with Nylon 4-0. Nylon sutures were tied to the FDP tendon ends connected it to the sensors (fig. S1e). Each tendon was used only once, for one treatment. The tendons and their sheaths were generally freshly prepared on the same day that their friction was measured, and kept in PBS to avoid drying of the tissue. Tendons were kept at room temperature (in PBS) till they were used on the day of surgery. In those cases (around 25%) where tendons, prepared later in the day, were not measured in the tribometer on the day of preparation, they were kept overnight in PBS at 4-8C, and measured the following day. However, all tendon/sheath samples were measured within 24 hours of surgery. We note that measurements on tendons used after such overnight storage in PBS were identical within the scatter to those performed on tendons on the day of their fresh preparation. This indicates that tendons were stable for at least 24 hrs with respect to subsequent treatment and lubrication properties.

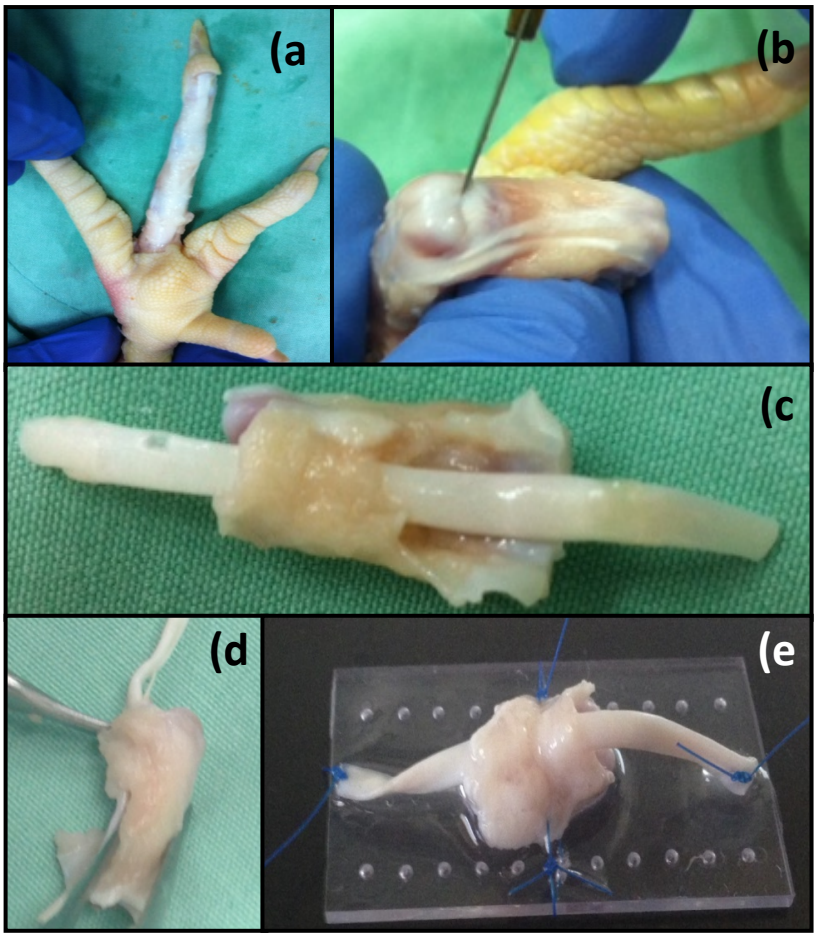
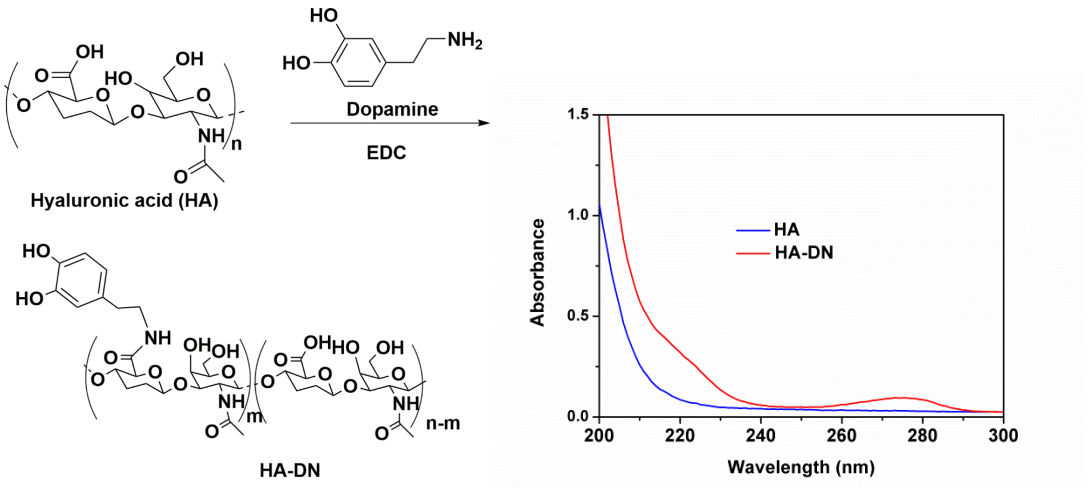


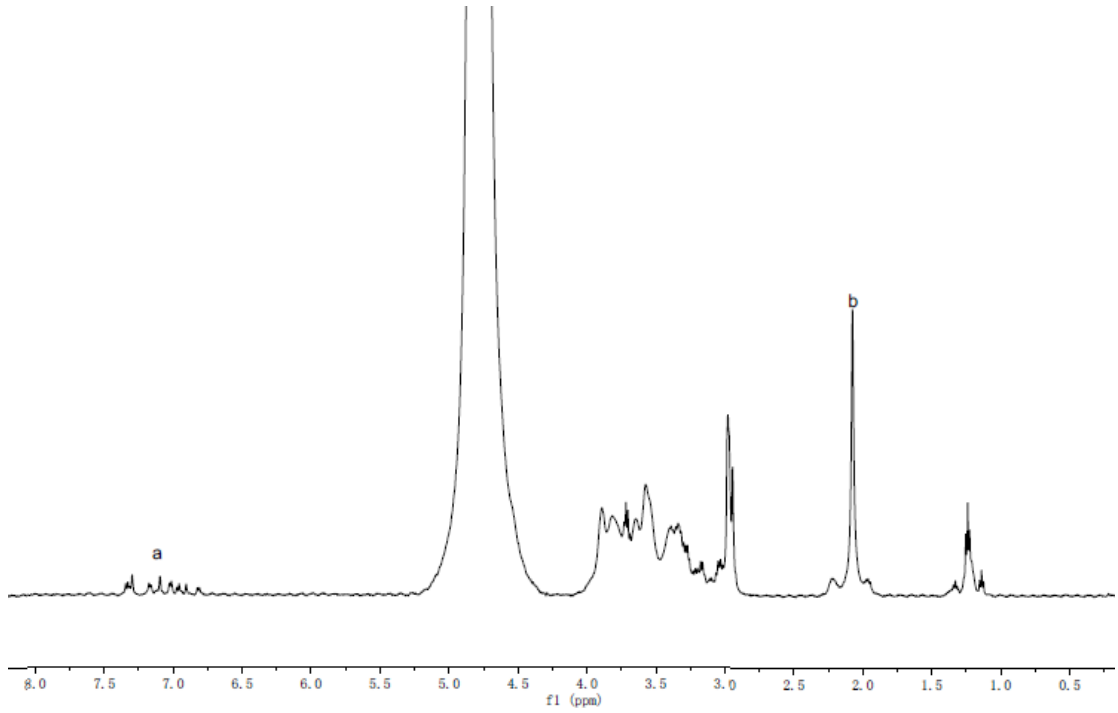
Fig. S1. The tendon specimen preparation and mounting procedure (see S1).

S2. HA-DN synthesis

HA and dopamine (DOPA or DN) conjugates (HA-DN) were prepared following ref. [2]. Briefly, dopamine was coupled to the carboxyl group of HA via 1-ethyl-3-(3'-dimethyl aminopropyl) carbodiimide (EDC) coupling reaction (Figure S2a). 0.5 g of HA was dissolved in 50 mL of PBS solution and the pH was adjusted to 5.5 using 1 N HCl solution. 40 mg (0.05 mmol) of EDC and 94 mg (0.05mmol) of dopamine hydrochloride were added and the pH of the reaction solution was maintained at 5.5 for 2 hours with 1.0 N HCl and 1.0 N NaOH. Then, the solution was dialyzed against water for 2 days and was subsequently lyophilized, which resulted in a white powder. DN was analyzed by Ultraviolet (UV) Spectrophotometry and Nuclear Magnetic Resonance (NMR) analysis of HA-DN. For UV analysis, a solution of 1mg/mL in water was prepared. For 1H-NMR, the sample was dissolved in deuterated water (D_2O) for 3 hours at concentrations of 2 mg/mL. The spectra were recorded at 298 K and 500 MHz for ^{1}H-NMR analysis. Figure S2b shows the results obtained by UV-Vis spectroscopy. It can be seen that at approximately 280 nm one band appears for the HA-DN conjugate, which was not observed for the pure HA. From this it was determined [2] that the concentration of dopamine units in the HA-DN solution was 0.075 mg/mL. The degree of dopamine substitution in the synthesized conjugate was 19 mol%. The catechol content (mol%) in HA-DN was determined from the integral area ratio calculation f = a/b, where a is the integral area of the peaks at around 7 ppm, which corresponds to the amount of H in the aromatic rings of grafted catechol moieties, and b is the integral area of the peaks at about 2.0 ppm, which represents the amount of H in the methylene of polymeric backbone (Figure S2c). The mole ratio of dopamine conjugated in the resultant polymer was about 18 % as determined by this method, which is consistent with the result of the UV analysis.



(a) (b)



(c)

Fig. S2. (a) A schematic representation of preparing HA-DN; (b) UV-Vis spectra of the conjugate (HA-DN) and the control (HA);(c) ^{1}H-NMR spectra of HA-DN.

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