Biolubrication: Hyaluronic Acid and the Influence on Its Interfacial Viscosity of an Antiinflammatory Drug

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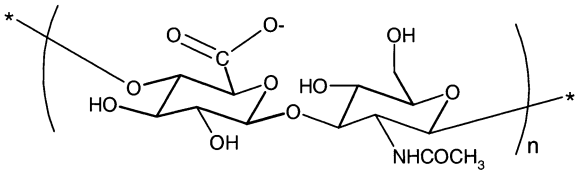
Introduction. How to understand the lubrication of animal joints? In seeking to dissect this complex problem into manageable components, it is interesting to consider hyaluronic acid (HA), more currently called hyaluron, a linear polysaccharide with the repeat unit of the disaccharide D-glucoronic acid and *N*-acetyl-D-glucosamine (see Figure 1a), which exists ubiquitously in most organisms with highest concentration in soft connective tissues, such as cartilage, synovial joints, and the vitreous body of the eye.[1,2] The most accepted structure is a 2-fold, tapelike helix motif with five hydrogen bonds per tetrasaccharide unit of HA, which helps explain the ubiquitous interactions of HA with lipids, membranes, and also itself.[1,3,4] In pharmacological applications, it is widely studied for use as a therapeutic agent in joint disease and related issues.[5] In parallel, it has caught the attention of engineers, and various controversial lubrication schemes have been proposed to explain its function in the lubrication of animal joints.[6−9] However, it is interesting to note that the pharmacological and the engineering communities have developed to be largely independent of one another. In the pharmacological community, the relation of chemical structure to biomechanical functions such as lubrication and shock absorbency is seldom considered.[1,5] Conversely, engineers recognize lubrication functions but seldom consider the possible influence on lubricating properties of physiological conditions, such as ionic strength and drug additives.[10−12] The present study is a modest attempt to begin to rectify this split by examining how the interfacial rheology of HA is modulated by the presence of an antiinflammatory drug, D-penicillamine (D-pen),[13] which is capable of hydrogen bonding to HA. We selected D-pen as a model system; as a drug, it is rarely prescribed these days.

In the mammalian body, the mechanical function of HA is surely complex because it coexists with plasma proteins. The viscosity properties of protein-free HA in bulk dilute solution, in the presence and in the absence of D-pen, were studied recently by Colby and coworkers.[14] The drug was found to cause a moderate reduction of the viscosity, and the reasonable hypothesis was advanced that the reason was disruption of intramolecular hydrogen bonding.[14] In the study below, HA was compressed between two surfaces such that the local concentration of HA much exceeded dilute. As will be seen below, this situation generates an effect of the opposite sign, an enhanced effective viscosity, and we hypothesize that this reflects the introduction of transient intermolecular cross-links via transient intermolecular hydrogen bonding.

To the best of our knowledge, no previous rheological study of HA was conducted in these conditions where HA was controllably confined to spacings as small as a few nanometers, thus potentially mimicking the condition the flow of synovial fluid between gaps in cartilage surfaces. As this comparison must ultimately fail because cartilage is softer than the solid surfaces that we studied, we restricted the comparison to low compressive loads, such that neither cartilage nor the model surfaces that we employed deformed appreciably. To put this in perspective, this study focuses on the liquid side of the solid−liquid interface.

a) Hyaluronic acid



b) Penicillamine

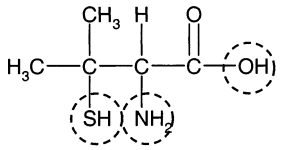


Figure 1. Structures of (a) hyaluronic acid (HA) and (b) D-penicillamine, whose three zones of potential hydrogen bonding are circled.

Experimental Section. The experimental platform was a home-built surface forces apparatus (SFA) modified to study the viscoelastic shear response of confined fluids as a function of frequency and shear rate.[15,16]

However, as mica is negatively charged in water and the negatively charged HA does not adsorb to it owing to charge−charge repulsion,[17] it was necessary to find a method by which to connect HA to the solid surface underneath. The strategy to modify the solid surface via a polymer connector was made with the following criteria in mind. First, the charge should be positive to promote adsorption onto it of the negatively charged polysaccharide. Second, surface coverage of the connector should be controllable in order to control the quantity of HA adsorbed. Finally, the thin-film viscoelastic responses of the connector itself should be known as a base of comparison. Given these considerations, supported soft connective layers were prepared by allowing the adsorption of quaternized poly(4-vinylpyridine) (QPVP), purchased as poly(4-vinylpyridine) from Polymer Source Inc., Quebec, Canada, and quaternized in our laboratory to a degree of 98%.[18] The weight-average molecular weight of the narrow-distribution samples after quaternization was 69 000 g mol^{-1}. Control experiments without added HA showed the final incompressible thickness of adsorbed QPVP−QPVP was 2 nm, corresponding to the adsorbed amount of <1 mg m^{-2}. From previous studies in this laboratory, we know the force−distance relations and thin-film viscoelastic shear responses of QPVP, which were qualitatively different[19,20] from the new data reported below. The adsorption concentration was kept low, 5 ppm, such that the polymer connector is known to adsorb in the thin "pancake" configuration.[19]

The sample preparation was as follows. Freshly cleaved mica sheets were glued onto cylindrical silica disks using Epon 1004, immersed into 0.005 mg mL^{-1}

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QPVP aqueous solution for 1−1.5 h at room temperature, rinsed with copious amounts of deionized water to remove excess nonadsorbed QPVP chains, and finally immersed into 0.13 mg mL^{-1} HA solution for 2 h. The HA was purchased from Aldrich (99.7% purity, molecular weight in the range $(3-5) \times 10^6$ g/mol). As the mechanical response of HA is known to be sensitive to small amounts of residual protein, we attempted to ascertain the amount of residual protein in HA that Aldrich obtained from animal (such as bovine vitreous humor, human umbilical cord) or bacteriological (streptococcus zooepidemicus) source; but the information could not be obtained. Control experiments showed no fundamental difference was observed between HA obtained from human umbilical cord and streptococcus zooepidemicus in our shear results. After HA adsorption, the disk was again rinsed well with deionized water before being mounted into the surface forces apparatus. A droplet of 0.1 M KNO_3 (Aldrich, >99.9% purity) aqueous solution, with or without 0.1 mg mL^{-1} penicillamine (Aldrich, 99.5% purity), was inserted between the two HA-coated surfaces after they were oriented in a crossed-cylinder geometry. Deionized water used in this study was prepared by passage through a purification system, Barnstead Nanopure II. Throughout the experiments, the apparatus chamber was humidified via a tray of deionized water in its bottom. The experiments were conducted at 25 ± 0.2 °C. The salt concentration of 0.1 M was selected to be similar to that of human plasma.

Control experiments, in which D-pen was added to QPVP layers, showed no change of thickness. This we interpret to indicate the D-pen did not associate with QPVP.

Results and Discussion. We started by measuring force−distance profiles, as illustrated in Figure 2, with and without the presence of penicillamine. In both cases the static forces were monotonically repulsive starting at the same spacing, $D \approx 60$ nm, and were reversible upon approach and separation, without adhesion. The decay length was 6 ± 0.5 nm for both cases, much larger than the Debye length of 0.96 nm at this ionic strength, which suggests that the repulsive forces at larger separations resulted from steric repulsion between HA chains. But although the pattern of monotonically repulsive force was nearly the same, the hard-wall thickness (the final incompressible thickness) differed, as illustrated in the inset of Figure 2; it was 9.0 and 4.8 nm for HA with and without penicillamine, respectively, indicating enhanced resistance to compression after penicillamine was added. From an educated estimate of the amount adsorbed (of the order 1 mg m^{-2}), it follows that the local concentration of HA was ca. 20 vol %. It is tempting to compare this to the D-pen concentration (0.1 mg mL^{-1}, thus nominally 0.01 vol %), but this is a lower bound as it is likely that D-pen partitioned to some extent into the HA adsorbed layers.

Additional comparisons of shear forces as a function of separation were made at fixed frequency (1.3 Hz) and oscillatory amplitude (0.3 nm) in the linear response regime. Despite the similar static force−distance profiles, the shear responses differed considerably. The expanded hard-wall thickness and the enhanced shear forces, after adding D-penicillamine, suggested a greatly stiffened mechanical response.

This hypothesis was further examined in shear studies as a function of frequency and shear rate. By discriminating the in-phase (elastic) and the out-of-phase (viscous) responses to harmonic shear, the frequency dependence of the viscoelastic spectra was quantified.[15,16] The main portion of Figure 2 illustrates shear responses of adsorbed HA layers at the same frequency but with thickness varied. The linear-response shear forces in the absence of penicillamine switched from predominantly viscous ($D > 10$ nm) to predominantly elastic (thin films, $D < 10$ nm). But in the presence of penicillamine, elastic and viscous forces were considerably larger in magnitude and nearly identical over the entire thick-film range, $D \sim 15-30$ nm. Other studies of the frequency dependence spectra of HA in penicillamine solution confirmed that the equality of elastic and viscous forces was general. Although an influence of penicillamine on the QPVP connecting layer cannot be ruled out (see comments below), this unusual response[16,21,22] appeared to reflect the complex structure of HA interacting with penicillamine.

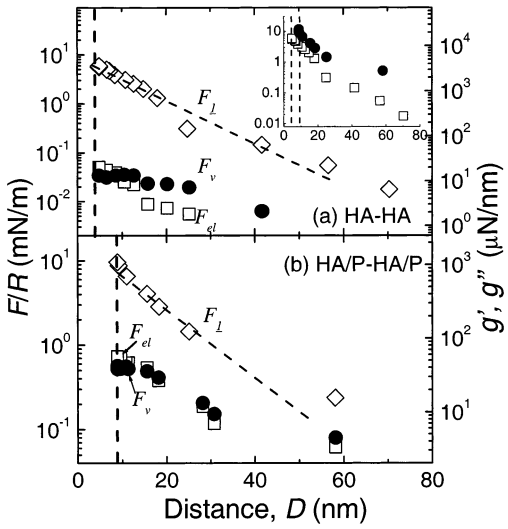

Figure 2. Forces $F(D)$ to compress the layers to a given spacing D, normalized by the mean radius of curvature, $R \approx 2$ cm, are plotted semilogarithmically against D for (a) adsorbed HA without penicillamine and (b) penicillamine added as described in the text. The dashed lines indicate the apparent decay length of the static forces, 6 ± 0.5 nm. Here, $D = 0$, measured by multiple beam interferometry, was defined as the contact of QPVP−QPVP layers (thickness ≈ 2.0 nm) calibrated independently by using two QPVP surfaces prepared under the same conditions but without HA added. The additional shear experiments, performed at 1.3 Hz and in the linear-response regime with 0.3 nm shear amplitude, yielded the elastic shear forces (squares), in-phase with the drive, and the viscous shear forces (circles), 90° out-of-phase with the drive. The inset illustrates the insensitivity of static forces to the presence of penicillamine (filled circles) or its absence (open symbols). The shear responses nonetheless differed, as also shown in the right-hand ordinate axis, showing in-phase (squares) and out-of-phase shear forces at 1.3 Hz in units of μN per nm deflection. The vertical dotted lines show "hard walls" past which it was not possible to compress further despite aggressive rise of the normal force, but quantitative data in this regime were not measured.

Consider films squeezed to the same mean normal pressure, 1.1 MPa. (Here we define normal pressure as force divided by the Hertzian contact area of the crossed cylinders, which flatten under compression.) The shear frequency was scanned by orders of magnitude while maintaining gentle sinusoidal shear deformations, amplitude ∼0.3 nm, so that the equilibrium structure of the films would not be disturbed by the measurements.

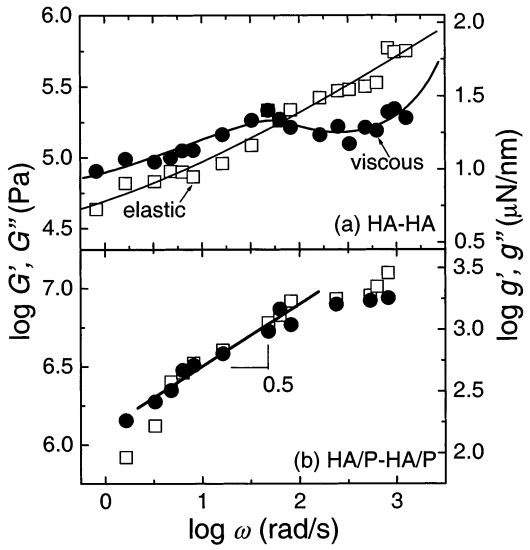

Figure 3. Shear responses after applying additional compressive pressure (1.1 MPa) in the hard-wall region, such that the crossed cylinder flattened at their apex to form parallel plates. The linear viscoelastic shear spectra spectra are plotted for (a) HA without penicillamine and (b) HA with penicillamine added as described in the text. On the left-hand ordinate scale are shown the viscous modulus, G'' (circles) and elastic modulus, G' (squares), plotted against angular frequency on log−log scales. On the right-hand ordinate scale are shown the equivalent viscous (circles) and elastic (squares) force constants in units of μN per nm of shear deflection.

In Figure 3, shear forces are normalized in two alternative ways. The right-hand ordinate scale shows viscous and elastic forces with units of force constant, force per nm of shear motion. Additional normalization for film thickness and contact area gave the effective loss modulus, $G''(\omega)$, and elastic modulus, $G'(\omega)$ (left-hand ordinate), where ω denotes radian frequency. Because the contact area was larger after flattening under compression, the force constants are larger than in Figure 2.

For HA without D-penicillamine (panel a of Figure 3), the peak of viscous response shows the existence of prominent low-frequency relaxation processes. Using procedures that are standard in the field of rheology, this can be analyzed in detail, but the main point is that the weak power laws indicate the confluence of a wide distribution of relaxation responses rather than a single one. Water and the polymer connector, QPVP, are simpler in their shear responses,[16,19,20] but similar complexity in the frequency response is also seen for adsorbed polymer melts,[23] presumably because that system similarly reflects the responses of adsorbed chains with a broad distribution of conformations. For comparison below with the case of penicillamine present, the longest relaxation time was quantified as ≈0.01 s from the reciprocal of the frequency, ω_0, where G' and G'' crossed in Figure 3. Thus, unlike the "solidity" generally observed when polymers are confined to be molecularly thin,[19,20,23,24] HA in the physiological condition of 0.1 M salt present still maintained fluidity. At lower frequencies than this inverse time, the confined HA films were more viscous than elastic.

With D-penicillamine present (panel b of Figure 3), the shear moduli were enhanced, by roughly 1 order of magnitude, and slower by more than 1 order of magnitude on the frequency scale, such that the confined HA films were predominantly viscous only at lower frequencies. The longest relaxation time was ≈1 s, 2 orders of magnitude slower than before; D-penicillamine acted as an effective cross-link. In addition, regardless of the method chosen to normalize them, the shear forces scaled as $\omega^{1/2}$ with equal amplitude for the viscous and elastic components over a substantial frequency range, 10−100 rad s^{-1}. Such an exceedingly broad spectrum of relaxation times is commonly associated with a system that contains strongly coupled structural components over many length scales.[16,21,22] Taken together, the comparison in Figure 3 suggests that the function of D-penicillamine was to form a hydrogen-bonded complex with HA because of its potential association with HA. Inspection of the chemical makeup of the molecule shows that this can occur at three distinct portions of the molecule: the sulfhydryl group (−SH), the amine group ($-NH_2$), and the hydroxyl group (−OH).

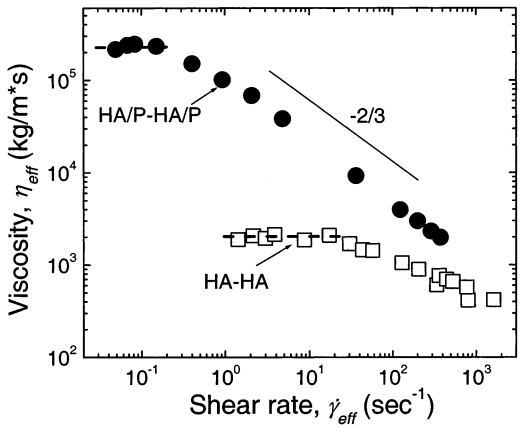

Figure 4. Effective dynamic viscosity in the hard-wall region and 1.1 MPa, in the case of (a) HA without penicillamine and (b) HA with penicillamine added as described in the text. The effective dynamic viscosity was measured by varying the displacement amplitude at 13 Hz such that the peak velocity was the product of frequency and amplitude and the shear rate was the peak velocity normalized by the film thickness, as discussed elsewhere.[31]

Such complexes are expected to be transient, associating and dissociating with time because the intensity of an individual hydrogen bond is weak. But the aggregate effect of many weak bonds is a strong one; multiple hydrogen bonds must act as effective (but labile and hence frequency-dependent) cross-links, as we observed. It is also possible that some permanent complexes may also have formed by the condensation of carboxylate groups of D-glucoronic acid ($pK_a \sim 3.2$)[25] on saccharide residues of HA, and amine groups of penicillamine (pK_a of amine group $\sim$ 10.7),[25] in these experiments conducted at pH ∼5.5. Yet although cross-linking appeared to tie together the HA chains, quantitative explanation remains a point to be clarified by future study, when one considers that the respective intensities of the three attachment points on the penicillamine molecule surely differed owing to their different pK_a.

This picture of a labile complex is supported by the observation of enhanced viscosity. It is known from the field of rheology that the effective dynamic viscosity is $\eta_{\text{eff}} \equiv G''(\omega)/\omega$. Figure 4 compares the effective viscosity of HA layers with and without penicillamine; the data are plotted as a function of the effective shear rate under conditions specified in the figure caption. One sees that the viscosity of HA/P in the linear regime, where

viscosity was independent of shear rate, exceeded that of HA by more than 1 order of magnitude. Moreover, the critical shear rate at the commencement of nonlinear responses was smaller—0.4 and 30 s^{-1} for HA/P and HA cases, respectively. This difference implies that the relaxation of adsorbed HA was further slowed by interaction with penicillamine. It is also intriguing to notice that shear thinning of HA showed a weaker dependence on shear rate than the shear thinning of HA/P. Indeed, the data for HA/P show a power law of −2/3 that has been predicted.[26] Thus, penicallimine appeared not just to thicken the near-surface adsorbed layer and to enhance the near-surface viscosity, but also to alter the character of shear-thinning.

Outlook. This is a study on a model system. It is not intended to suggest that the action of D-penicillamine is to concentrate in synovial fluid in the intercartilage gap and then to modify its rheological properties by increasing its viscosity; the drug is orally administered in small doses, and clinicians find that it usually takes 2−5 months before taking effect. A medical study shows that its mode of action, though not completely clear, is thought most likely to be via its effect on the autoimmune system, particularly its interaction with T-cells.[27] Furthermore, biological lubrication in mammalian joints, where cartilage surfaces at separations of order nanometers slide at velocities of order cm s^{-1}, takes place at shear rates of order 10^6 s^{-1} or higher. The lower shear rates in the present study might pertain to the startup of such motion, or to its turning points when motion reverses direction, but not to steady-state undirectional motion.

Another strong assumption of this study, which is open to legitimate question, is whether subsequent adsorption of HA might have caused the QPVP connector to lose the pancake configuration adopted when it first adsorbed. Tentatively, this we consider unlikely upon considering that, in the field of the layer-by-layer self-assembly of polyelectrolyte multilayers of alternate opposite charge,[28] it is known that kinetic barriers limit interpenetration to the dry thickness of one layer, when polyelectrolytes adsorb on top of one another. This dry thickness is only a few angstroms when dealing with polyelectrolyte multilayers in the dry state. Whether the interpenetration distance remains so small in solution is, to the best of our knowledge, not yet known, but an important question to answer when extending these preliminary findings. The strategy of causing an anionic polymer to adsorb in this manner has the advantage of simplicity and relative ease of implementation, but we recognize that an approach based on covalently bonded self-assembled monolayers of positive charge would, when implemented reliably, be conceptually more pure. The difficulty is that, in our hands, the current methods to graft amines onto mica[29] do not produce layers that coat the mica homogeneously.

With these caveats, it is provocative to notice the influence on biolubrication of intermolecular complexation with a drug, thus modifying its viscoelastic properties—an effect that has not been identified in previous literature of which we are aware. However, this finding is physically reasonable, as it is known that cross-linking to form a labile complex increases viscosity.[21,30] It suggests by rational extension other methods by which to accomplish similar purposes. While the issue of drug dosage has not been addressed here, the methods to do so are self-evident, so this study constitutes a proof of principle. Moreover, the thickening effect on polyaccharides of hydrogen-bonding smaller molecules may be useful not only for understanding biolubrication mechanisms in natural and artificial animal joints but also for other aspects of biolubrication, for example the blinking of the animal eye and the formulation of foodstuff products.

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