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Ultra-low friction between boundary layers of Hyaluronan-phosphatidylcholine Complexes

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Abstract

The boundary layers coating articular cartilage in synovial joints constitute unique biomaterials, providing lubricity at levels unmatched by any human-made materials. The underlying molecular mechanism of this lubricity, essential to joint function, is not well understood. Here we study the interactions between surfaces bearing attached hyaluronan (hyaluronic acid, or HA) to which different phosphatidylcholine (PC) lipids had been added, in the form of small unilamellar vesicles (SUVs or liposomes), using a surface force balance, to shed light on possible cartilage boundary lubrication by such complexes. Surface-attached HA was complexed with different PC lipids (hydrogenated soy PC (HSPC), 1,2-dimyristoyl-sn-glycero-3-PC (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-PC (POPC)), followed by rinsing. Atomic force microscopy (AFM) and cryo-scanning electron microscopy (Cryo-SEM) were used to image the HA-PC surface complexes following addition of the SUVs. HA-HSPC complexes provide very efficient lubrication, with friction coefficients as low as $\mu \sim 0.001$ at physiological pressures $P \approx 150$ atm, while HA-DMPC and HA-POPC complexes are efficient only at low P (up to 10 – 20 atm). The friction reduction in all cases is attributed to hydration lubrication by highly-hydrated phosphocholine groups exposed by the PC-HA complexes. The greater robustness at high P of the HSPC ($(C_{18})_2$ tails) complexes relative to the DMPC ($(C_{14})_2$) or POPC (C_{16} , $C_{18:1}$) complexes is attributed to the stronger van der Waals attraction between the HSPC acyl tails, relative to the shorter or un-saturated tails of the other two lipids. Our results shed light on possible lubrication mechanisms at the articular cartilage surface in joints.

Keywords: Synovial-joint lubrication; phospholipid-hyaluronan complexes; hydration lubrication; biological lubrication.

1. Introduction

The major synovial joints such as hips or knees constitute unique tribological systems[1]. The articular cartilage layers coating the joints can slide past each other at local contact pressures as high as 100 atm or more[2, 3] over a wide range of shear rates[4], with sliding friction coefficients μ (defined as (force required to slide)/load) as low as $\mu \approx 10^{-3}$ (see e.g. references in [5]). This remarkable lubrication has been attributed to both fluid film mechanisms (as for example in weeping lubrication and interstitial fluid pressurization models [6-9]) and to boundary lubrication [1, 10-13]. In boundary processes the friction arises as the opposing surfaces – or molecules coating them – slide past each other, and frictional energy is lost via irreversible processes such as the breaking of molecular bonds or local hopping events at the slip plane between them[14]. The overall friction likely arises from a so-called mixed regime, where both fluid film and boundary-contact processes play a role[1]. The boundary lubrication properties of several molecules present in the synovial fluid and in cartilage have been studied, prominently hyaluronic acid (HA)[15-18], lubricin[11, 12, 19-21] and phospholipids[22-25], as well as others. A recent review[10] considers many of these studies. Since nanotribology of living cartilage is very challenging, as described in ref. [10], direct measurements have focused on model systems, probing the friction between boundary layers of different molecules. Such studies have shown that, on their own, the molecules most often implicated in the very efficient boundary lubrication of articular cartilage, including those noted above, do not provide such good lubrication (i.e. $\mu \approx 10^{-3}$) anywhere near the maximal pressures (> 100 atm) in living joints (see also extended references in review[10]).

A recent study by Seror et al.[26] demonstrated that a boundary layer consisting of

surface-attached HA together with phosphatidylcholine (PC) vesicles (i.e. liposomes), could provide extremely efficient lubrication ($\mu \approx 10^{-3}$) up to contact pressures $P > 100$ atm, well within the range of maximal pressures in joints[3, 27]. Moreover, this low friction persisted over extended sliding periods and was little changed over several orders of magnitude in sliding velocity, indicating its largely boundary origin (as opposed to fluid film lubrication where the friction might be expected to increase roughly linearly with sliding velocity). Based on these results, and on earlier studies showing the remarkable ability of PC lipid assemblies to reduce friction via hydration lubrication arising from their exposed, highly-hydrated phosphocholine head-groups[28-32], Seror et al.[26] proposed that a synergistic mechanism was responsible for the efficient boundary lubrication of articular cartilage. According to this proposal, HA, PC lipids and lubricin act together, each with a different role, to provide the lubricating boundary layer: Lubricin, present at the outer superficial zone of cartilage and at its surface from the synovial fluid, attaches HA at the cartilage surface, and PC lipids complex with the HA via a charge-dipole interaction to expose the hydrated phosphocholine groups that ultimately provide the boundary lubrication at the slip plane[10].

While our earlier study[26] focused on the lipid dipalmitoylphosphatidylcholine (DPPC), ubiquitous in joints, with a phosphocholine headgroup and two C_{16} acyl tails ($(C_{16})_2$), added in the form of small unilamellar vesicles (SUVs) to the surface-attached HA, many other PC lipids are present in joints[33, 34]. The question of whether PC attachment with surface-bound HA, and the corresponding efficient boundary lubrication, is common to other PC lipids, and how the structure of lipids and their corresponding phase behavior relates to their interaction with HA, has not to date been investigated. In the present study, therefore,

we extend the earlier work to three additional PC lipids (HSPC ($C_{16(15\%)}, C_{18(85\%)}$), DMPC ($(C_{14})_2$) and POPC ($C_{16}, C_{18:1}$)), with varying acyl chain length and saturation, and with their solid-ordered (SO) to liquid-disordered (LD) transition temperatures T_M both higher and lower than that of DPPC. We construct the HA surface layers as described in the previous work and use atomic force microscopy (AFM) and cryo-scanning electron microscopy (cryo-SEM), together with the surface force balance (SFB), respectively to examine the surface structure, and the subsequent lubrication ability following addition of the different PC lipids. We find (as earlier briefly noted[26]) a dramatic decrease of the friction in the case of HSPC added to the HA-bearing surfaces, with values of μ as low as or lower than those in healthy joints at the maximal physiological pressures ($P \gtrsim 100$ atm). In contrast, addition of the DMPC and POPC lipids to the surface-attached HA results in poor lubrication already at pressures well below these maximal values, an effect attributed to the more fluid nature of their lipid layers relative to the HSPC, with a consequent poorer resistance to pressure and shear.

2. Experimental Section

2.1. Materials.

Avidin from egg white (A9275) was supplied by Sigma Aldrich. Biotinylated HA (bHA) was made as described in references[26, 35]; its molecular weight is 1.3 MDa, a value comparable to the lower range of HA molecular weights in synovial fluid [36]. Three lipids, hydrogenated soy phosphatidylcholine (HSPC), 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Lipid

(Ludwigshafen, Germany) and used as received. The water used throughout both for the sample preparation and subsequent measurements was from a Barnstead NanoPure system, with total organic carbon (TOC) <1 ppb and resistivity 18.2 M Ω -cm (so-called conductivity water). Highly purified salt (KNO₃) was purchased from Merck (99.995% purity, Suprapur Merck Batch #105065) The mica used was from S&J Trading (ruby muscovite grade I, New York, NY) and was cleaved to sheets of 1.5–3.5 μ m thickness, atomically smooth on both side, and melt-cut into ~ 1 cm $\times$ 1 cm facets. The glue used to attach mica to the lenses was EPON 1004 (Shell Chemicals).

2.2. Liposome preparation

SUVs of each PC were prepared using standard procedures[29, 37]. Firstly, lipids were dispersed in water and bath-sonicated for 15 min at temperature above the phase transition of each lipid. An extruder (Northern lipid Inc, Burnaby, BC, Canada) was used to downsize the MLVs through polycarbonate filters with defined pore size starting from 400 nm (4 cycles), 100 nm (4 cycles) and ending with 50 nm (7 cycles). Then, the size distribution of PC-SUVs was characterized by dynamic light scattering (DLS) at a scattering angle of 173°.

2.3. Experimental procedure

The HA-coated mica surfaces were prepared as previously described[26]. In brief, a layer of positively charged avidin was adsorbed from solution on the negatively charged mica surfaces and the lightly biotinylated HA (bHA) was attached to the avidin layer via the avidin-biotin interaction. At each stage (bare mica; avidin-coated mica; avidin-bHA coated mica) force profiles were measured as a control, as described in detail in ref.[38]. Each

surface was then immersed in 10 ml of pure water, followed by addition of 400 μ l of 15 mM PC-SUV solution. Following incubation at room temperature for HSPC and POPC, and 4 °C for DMPC overnight, the surfaces were rinsed in pure water (lens held and shaken in the beaker with 400 ml pure water for 3 minutes, then remounted in SFB) and measured in the SFB. For experiments at high salt, the water was removed from the SFB boat following the pure water measurements (leaving a meniscus between the surfaces), and salt solution added to 0.15M concentration and well mixed before equilibration and further measurements.

2.4. Atomic force microscopy (AFM)

An MFP-3D SA (AFM) instrument (Asylum Research, Santa Barbara, CA) was used to image the structure on the mica surfaces in intermittent contact (AC mode) under conductivity water. The AFM probe (SNL, Bruker) has a silicon tip with a nominal radius of 2 nm and a triangular silicon nitride cantilever having a nominal spring constant of 0.35 N/m).

2.5. Cryo scanning electron microscopy

Cryo-SEM samples of the HA-PC complexes, prepared as described above, were rapidly frozen by plunging into liquid ethane. Then the frozen samples were mounted on a holder and transferred to a BAF 60 freeze fracture device (Bal-Tec AG, Liechtenstein). Water was sublimed at -80°C for 2 h. Samples were coated with 3 nm Pt by double axis rotary shadowing at an angle of 45°. Samples were transferred to an Ultra 55 SEM (Zeiss, Germany) using a VCT 100 vacuum-cryo transfer system (Bal-Tec AG, Liechtenstein) and observed at -120°C and 2 kV using an in-lens secondary electron detector.

2.6. Surface force balance (SFB)

The surface force balance (SFB) technique and the detailed experimental procedure to measure normal and shear interactions between mica surfaces have been described elsewhere[39, 40]. In brief: two atomically smooth mica surfaces (half-silvered on their back-sides) are mounted on top of two cylindrical lenses in a crossed-cylinder configuration. The distance D between two mica surfaces (optimally to 0.2-0.3 nm) and the mean radius of curvature R (≈ 1 cm) of the surfaces are measured by the white-light multiple-beam interferometry. The lower lens is mounted on a horizontal leaf spring (spring constant $K_n = 127$ N/m), and the normal force is calculated as $F_n = K_n \Delta D$, where ΔD is the bending of the normal spring. The upper lens is mounted on a four-sectored (+ 1 internal sector) piezoelectric tube (PZT). By applying opposite potentials to opposite sectors, precisely controlled and highly parallel lateral motion (Δx_0) of the upper surface is obtained relative to the lower surface. Shear forces $F_s(D)$ between the surfaces are monitored via bending of a set of lateral springs ($K_s = 300$ N/m) and determined via changes in an air gap capacitance. The shear force $F_s = K_s \Delta x$, where Δx is the bending of the shear springs, can therefore be measured directly; typical results are shown later in the paper as time traces $F_s(t)$. A schematic of the interacting surfaces in the SFB is inset in fig. 3.

Normal and shear interactions between two surfaces were first measured between the bare mica surfaces in water, to establish the zero of contact, then following the adsorption of each layer (avidin, followed by HA), as controls, and finally with the added PC-SUVs. Shear forces were typically measured as the surfaces approached, so that second approaches at the same contact point are representative of surfaces that had been sheared up to high pressures

already on the first approach, and can thus reveal changes arising as a result. The mean contact pressure P is evaluated as $P = F_n/A$; A is the contact area, given by $A = \pi a^2$, where a is the mean radius of the circular or elliptical contact measured from the flattening of the interference fringes. When there is no clear flattening (at lower loads), the Hertz relation[41] was used to evaluate the contact radius a , where $a^3 = F_n R/K$, where $K = (5 \pm 2) \times 10^9 \text{ N/m}^2$ is the effective elastic modulus (determined separately) of the mica/glue combination.

2.7. Statistical analysis

Error bars in the force profiles indicate estimated uncertainty arising from scatter in the individual data points, due largely to thermal drift during the measurements and intrinsic small uncertainty (see above) in the measured surface separation values. Data were expressed as mean $\pm$ standard deviation (SD), for example of final separations D_f and pressure P , see text.

3. Results

3.1. Cryo-SEM and AFM characterization.

Following addition of the PC-SUVs to the HA-covered surfaces, using freshly-cleaved test mica surfaces, samples were imaged by AFM and cryo-SEM. The main figures 1A-C are cryo-SEM micrographs of mica coated by an avidin-bHA layer and incubated, respectively, in dispersions of HSPC-SUV, DMPC-SUV and POPC-SUV (followed by rinsing, see Experimental section). Insets 1 and 2 to fig. 1A show respectively (on the same scale as the main figure), an image of HSPC-SUVs adsorbed on bare mica, taken from ref.[29]; and cryo-SEM images of avidin-bHA coated mica following incubation in dispersions of SUVs of Dipalmitoylphosphatidylcholine (DPPC-SUVs), taken from ref.

[42]. The main figure 1A shows entities resembling ‘hollowed-out’ vesicles (of characteristic dimensions ca. 20 – 50 nm) connected in thread-like structures on the mica surface. We attribute these thread-like complexes to the HSPC vesicles that adsorbed – via dipole-charge interactions - to the HA on the mica surface. These vesicles were in part degraded, ruptured or hollowed out by the rather aggressive sample preparation protocol associated with cryo-SEM, which involves rapid plunging into liquid ethane (hence high shear fields at the surface prior to freezing of the surface film) followed by sublimation of the frozen water. It is of interest that when bare mica is incubated in the HSPC-SUV dispersion, inset 1 to fig. 1A, the resulting structure is very different and shows intact (not ruptured or hollowed out) liposomes close packed on the mica surface. We attribute the difference to the more robust nature of the vesicles when they are close-packed on a surface (where they buttress each other), as opposed to being attached in a thread-like manner on the negatively-charged bHA polymer extending from the avidin-covered mica. This additional robustness on the bare mica surface makes them more resistant to the cryo-SEM sample preparation procedure, so that they retain their full and rounded shape. Since loose liposomes on the close-packed vesicle layer on mica (inset 1 to fig. 1A) do not appear to be hollowed out, it may also be that the adsorption of vesicles on the HA promotes their hollowing-out during the sublimation stage. Moreover, we note that similar thread-like complexes of hollowed out or degraded vesicles were obtained also with DPPC vesicles added to HA-covered mica (studied in our earlier work[26]) as shown in inset 2 to fig. 1A.

In contrast, Figures 1B, C, show cryo-SEM images following the incubation of the avidin-bHA coated mica in dispersions of DMPC-SUVs and POPC-SUVs, respectively, that are very different to main fig. 1A, and show few traces of liposomes on the surface (as are

seen for the HSPC), whether degraded or otherwise. Both in fig. 1B and (faintly) in fig. 1C there are indications of thin (ca. 10 nm) thread-like structures on the surface which may be HA complexed with residual lipids, but this cannot be resolved unambiguously from the micrographs. We attribute the essential absence of liposomes to the much more fluid state of the DMPC and particularly POPC bilayers in the respective vesicles. These presumably are therefore much more readily ruptured and removed from the anchoring mica-bound HA molecules during the (aggressive) cryo-SEM sample preparation procedure, for example during the plunging into liquid ethane. We consider the cryo-SEM evidence further in the Discussion section.

Figure 2A – D shows AFM micrographs for the different added PC-SUVs, imaged under water. Fig. 2A shows HSPC-SUVs on bare mica, imaging by AFM the close-packed structure also revealed by cryo-SEM (e.g. inset 1 to fig. 1A). While the lateral dimensions (ca. 70-80 nm) of the vesicles are consistent with the DLS results, their heights (around 8-10 nm) probably reflect their squeezing down by the AFM tip in the AC mode[43] (from a true height of ca. 20 nm, determined hydrodynamically[29]). Figs. 2B-D are AFM images of mica coated by an avidin-bHA layer following incubation, respectively, in dispersions of HSPC-SUV, DMPC-SUV and POPC-SUV. All three images are rather similar, showing structures which appear pearl-necklace-like, of width ca. 10 nm, which greatly resemble the structure, seen in our earlier study[26], of avidin-HA coated mica that had been incubated in a DPPC-SUV dispersion (shown for comparison as inset to fig. 2D). Contours of one “pearl necklace” are outlined in green in Figure 2B. We note that the AFM micrographs are very different to the cryo-SEM images of the same layers, figs. 1A-C. We attribute this to the

rather different conditions under which the surfaces were prepared and the images taken, as well as to the better height resolution of the AFM approach, as considered further in the Discussion section.

3.2. Normal surface interactions

Prior to measuring the surface force profiles $F_n(D)/R$ vs. D with the PC-SUVs, control measurements at each stage building up to the final layer were carried out for each and every independent experiment: normal and shear interactions were first measured between two bare mica surfaces; then between the avidin-coated surfaces; then avidin-bHA bearing surfaces; and finally, if all controls were satisfactory (i.e. revealed the progressive buildup of the layers), between the surfaces following incubation in the PC-SUVs as described above. Satisfactory control profiles were generally similar to those shown (for mica-avidin and mica-avidin-bHA) in ref.[38] and will not be shown again here. Non-satisfactory controls revealing contamination or other artifacts led to termination of experiments. Results shown are based on 7 independent experiments, with at least 2 for each set of conditions (including several independent contact points within each experiment)

$F_n(D)/R$ vs. D profiles between two avidin-bHA-HSPC bearing mica surfaces are shown in Figure 3. The long ranged repulsion for the first approaches (above the level of scatter in the data at ca. 0.05 – 0.1 mN/m) starts at surface separations around 250 nm (black filled symbols in Figure 3), similar to that for the case of avidin-bHA-DPPC-SUV layers in our earlier study[26], while repulsion on a second approach at the same contact point (crossed blue symbols in Figure 3) sets on at shorter range. At the highest loads, up to contact pressures ca. 150 atm, the two surfaces approach to a final (“hard wall”) separation $D_f =$

$32.5 \pm 3.2 \text{ nm}$ for both the first and second approaches, suggesting little damage of the surface layers during first approach and shear. The overall behavior is suggestive of interactions between extended layers, initially - on a first approach - of liposomes attached to a loose HA layer, as also indicated by the cryo-SEM image (fig. 1A). On a second approach, following shear on the first approach, the profiles indicate less extended layers, suggesting that liposomes had been removed or ruptured, and may have been replaced by more compact lipid complexes with the surface-attached HA. Scatter in the data may arise from the rather heterogeneous surface structure, as indicated in fig. 1A. Red symbols in fig. 3 are normal force profiles of the avidin-bHA-HSPC bearing surfaces in 0.15 M KNO_3 solution, showing somewhat shorter ranged repulsion relative to the pure water case (possibly arising from screening of long-ranged double layer electrostatic interactions at the higher salt).

Normal force profiles for avidin-bHA-DMPC bearing surfaces are shown in Figure 4. Following incubation in the DMPC-SUV dispersion (incubation at 4°C overnight, well below its transition temperature ($T_M(\text{DMPC}) = 24^\circ\text{C}$), followed by rinsing), $F_n(D)/R$ vs. D profiles were determined at 20°C , when DMPC is still some degrees below its T_M . For the first approaches, repulsions appear somewhat shorter ranged (onset of repulsion at ca. 150 nm) than for the HSPC case. In contrast to the HSPC, however, both the receding profiles (empty symbols) and profiles on a second approach (blue data) show significantly longer-ranged repulsion than on first approach, while at the highest compressions the “hard wall” separation between the two surfaces increases from $D_{f1} = 11.9 \pm 1.7 \text{ nm}$ on first approaches to $D_{f2} = 28.4 \pm 4.9 \text{ nm}$ for the second approaches. This behavior strongly suggests that, already on a first approach (and shear), the HA-DMPC complexes undergo damage, resulting in debris formation and longer ranged interactions on second approaches, as earlier discussed for

DMPC layers on bare mica[30]. The shorter onset range relative to the HSPC case (fig. 3), ca. 150 nm compared to ca. 250 nm (though there is significant scatter in both cases) may indicate rupture or removal of the DMPC vesicles already when the opposing layers first interact on initial approach.

In the present work, POPC is the only phospholipid in its liquid phase at the measurement temperature ($T_M(\text{POPC}) = -2^\circ\text{C}$). The force profiles between avidin-bHA-POPC bearing mica surfaces are shown in Figure 5, showing that monotonic repulsion on first approaches onsets around 200 nm. Both the receding profiles and second approaching profiles are somewhat further out for $D \lesssim 100$ nm relative to the first approaches, with the ‘hard-wall separation’ $D_f = 11.1 \pm 1.0$ nm and 29.2 ± 4.8 nm for first and second approaches respectively. This may be interpreted as arising from the damage to the surface complexes occurs on strong compression and shear because of the fluid nature of the POPC which makes it less able to resist shear at high compressions. This leads to lipid-debris formation between the surfaces, as previously seen for the DMPC[30], resulting in higher D_f and a stronger steric repulsion at shorter range ($D \lesssim 100$ nm).

3.3. Shear force measurements

Shear force measurements between the avidin-bHA-PC bearing surfaces were carried out by laterally moving the top surface with respect to the lower one, as seen in shear force traces below. The top triangular waveform in a set of traces show the back-and forth lateral motion $\Delta x_0(t)$ applied to the upper surface as a function of time, while the lower traces are the corresponding shear forces $F_s(t)$ transmitted to the shear springs at different compressions. The plateau region in the F_s traces represents the sliding regime, yielding the sliding friction

coefficient. The separations and corresponding pressure are shown to the right of the traces. Since absolute values of friction measured in the SFB are typically small (as are the loads), it is most instructive to discuss in terms of mean contact pressures and friction coefficients.

Typical $F_s(t)$ traces for sliding between the avidin-bHA-HSPC bearing surfaces at increasing loads F_n (and P values) are shown in Figure 6: We observe smooth sliding at lower pressures ($P < 25$ atm, upper set of traces in fig. 6) and stick-slip sliding at higher pressures ($P > 60$ atm); the friction data is summarized, in fig. 7 as F_s vs. F_n , for both water (no added salt) and 0.15M KNO_3 solution. At low loads (and low P) the friction coefficient is relatively high on a first approach of the surfaces. This is attributed to substantial energy dissipation when the HSPC-SUV's, or lipid complexes, which are attached to the HA molecules extending from the surface, first overlap and slide past the similar opposing surface layers, and was observed also in our earlier study on DPPC lipids[26] (see shaded region in fig. 7). On second and subsequent approaches (blue data in fig. 7), when loosely-attached liposomes have presumably been sheared off, the energy dissipation due to shearing the extended layers of HA-lipid/liposome complexes is significantly lower, and consequently the friction on initial onset of interactions is also substantially lower. At higher loads (and P) the friction increases moderately but the friction coefficient becomes very low, tending to $\mu \approx 10^{-3}$ or lower as the pressure approaches and exceeds 100 atm, as was earlier seen for HA-DPPC[26].

The avidin-bHA-DMPC- and avidin-bHA-POPC-bearing surfaces lubricate quite efficiently on initial compression and sliding, but are less efficient at high P ($P = 14.3 \pm 7.5$ atm) compared to the avidin-bHA-HSPC-bearing surfaces, as shown in Figures 8 and 9, which show typical shear traces and summaries of the F_s vs. F_n data for these lipids

respectively. The friction coefficient for both these lower T_M lipids is $\sim 0.005 - 0.01$ only up to relatively low pressures – $P = 14.3 \pm 7.5$ atm for DMPC and $P = 9.9 \pm 1.5$ atm for POPC.

At higher pressures the friction increases abruptly; that is, the frictional force F_s exceeds the maximal applied shear force $K_s \Delta x_{0, \max}$ so that the surfaces no longer slide relative to each other (there is no plateau regime in the F_s traces) so that F_s can not be measured. This is indicated by the vertical arrows in Figure 9.

4. Discussion

The main findings of this study concern the lubrication provided by boundary layers consisting of HSPC, DMPC or POPC lipids complexed with HA (designated HA/PC) extending from a substrate to which the polysaccharide is attached. While the HA/HSPC layer can provide extremely efficient lubrication when compressed and made to slide against a similarly-coated surface at physiological pressures (μ down to 10^{-3} at the highest pressures, $P > 100$ atm), the more fluid lipids DMPC and POPC can provide significant friction reduction only up to much lower pressures. What underlies this behavior, and what light – if any – may it shed on boundary lubrication of articular cartilage?

As noted in the previous section, and in our earlier study[26], we attribute the friction reduction by the HA-PC complexes - relative to surface-attached HA alone which is known to lead to much higher μ values[15, 26, 38] ($\mu \approx 0.3$) – to hydration lubrication by the highly hydrated phosphocholine groups exposed at the surfaces of these complexes. These and other experimental models developed to study cartilage lubrication are described in more detail in ref. [10]. Indications for the nature of the surface structures are provided by the cryo-SEM and AFM images, though both of these methods, for the case of the HA-PC layers, suffer to

some extent from artifacts intrinsic to the techniques themselves, as described in detail in the Results section. The HSPC case is of greatest interest, but we consider first the cryo-SEM images of the DMPC and POPC cases, figs. 1B, C; these show surfaces from which any liposomes adsorbed to the surface-attached appear to have been ruptured and/or removed by the preparation protocol, as remarked earlier. We note a similarity with cryo-SEM imaging of vesicles of HSPC, DMPC and POPC attached to chitosan-alginate bilayers on mica: for the HSPC case a clear presence of vesicles was noted while the POPC and DMPC liposomes were mostly removed from the surface[44]. In the present study, for the HSPC case, fig. 1A, the cryo-SEM images show that liposomes are indeed present at the surface, attached to the linear HA polymer which is itself attached (via avidin) to the mica (as revealed by their bead-on-thread structure extending from the surface). The more robust nature of the HSPC-SUVs (with $T_M(\text{HSPC}) = 53^\circ\text{C}$) relative to the DMPC and POPC with much lower T_M values, is responsible for preventing the rupture/removal of the HSPC during the preparation procedure for the cryo-SEM, in contrast to what happens to the more fluid PCs. Even for the HSPC-SUVs, however, the hollowed out (or in some cases ruptured) appearance of the vesicles (fig. 1A) is likely an artifact introduced at the sublimation stage of the cryo-SEM preparation. The AFM micrographs of the HA-PC surface complexes, in contrast, figs. 2B-D, are rather similar to each other but all show a very different surface structure to the corresponding cryo-SEM images. Since the AFM was carried out (in tapping mode) under water, and without the samples having crossed a liquid/air interface, the AFM micrographs may be interpreted as follows. We know from the cryo-SEM image of fig. 1A that the PC vesicles adsorb to the surface-attached HA; thus we interpret the AFM images to mean that the AC mode measurements, where the sharp AFM tip (tip radius 2 nm) scans across the

vesicles, result in the rupture of the liposomes and partial removal of the lipids from the region being imaged. The residual surface structure is thus likely to reflect complexes of lipids on bHA extended from the surface and which is attached to avidin molecules coating the mica. Because of the loose, mobile nature of the HA-PC complexes extending from the surface it is difficult to visualize them directly, but this picture is consistent with the width of the quasi-linear beaded structures seen in figs. 2B-D, which is ca. $(10-11) \pm 1.0$ nm (this value appears to be significantly wider than the characteristic width of an avidin-bHA complex alone, which is around $(7-8) \pm 1.0$ nm (unpublished data), which may indicate lipids complexed with the HA). Such a structure, schematically shown in fig. 10, was also deduced from similar AFM micrographs of HA-DPPC complexes in our earlier study[26]. We attribute a similar structure of the boundary layers once the two mica surfaces, bearing the avidin-HA-PC-SUV complexes, are strongly compressed and sheared as they slide past each other in the SFB.

The normal force profiles in figs. 3 – 5 are consistent with this picture. The long-ranged repulsion seen for all three lipids in pure water is attributed to a combination of steric forces, and double-layer interactions arising from residual charging of the surface layers. The latter (in water with no added salt) have a typical range of ca. 200 nm (when forces exceed the scatter range of 0.05 – 0.1 mN/m), with a decay length of ca. 50 – 100 nm, and reaching characteristic values of ca. 1 mN/m; the precise values vary with residual surface charge and bulk ion concentration. At closer separations, on first approach, steric repulsion sets on as the lipid-bearing HA molecules extending from the surfaces interact with each other. The range at which steric interaction exceeds $F_n/R \gtrsim 1$ mN/m (the characteristic electrostatic repulsion) corresponds to $D \approx 120$ nm for the HSPC case and to $D \approx 40 - 60$ nm

for the DMPC and POPC lipids. We attribute this difference to the more robust nature of the HSPC vesicles which retain their integrity on initial shear as the surfaces approach and slide past each other; the onset of strong steric interaction thus reflects the presence of the HSPC-SUVs in the surface complexes. In the case of the DMPC and POPC lipids, the force profiles suggest that their HA-attached vesicles are ruptured/sheared-off already on initial overlap and shear. Thus the much shorter range of steric interaction observed reflects a surface structure that consists of an HA-bilayer/monolayer complex alone (as in the schematic of fig. 10).

At the highest loading, corresponding to $P \approx 150$ atm, the HA-HSPC complexes are compressed to a thickness $D_f = 32.5 \pm 3.2$ nm (15 – 17 nm/surface-layer, fig. 3). This is somewhat larger than the value $D_f = 22.0 \pm 3.0$ nm (ca. 11 nm/surface-layer) seen for the earlier-studied case of avidin-bHA-DPPC-SUV layers[26], which was interpreted as a complex of HA with monolayers and bilayers of the lipid[10]. The thicker avidin-bHA-HSPC complexes suggest that following high loading and shear these surface layers may still have some residual HSPC vesicles attached in addition to being complexed with HSPC monolayers and bilayers[10, 26], consistent with the more robust nature of HSPC relative to DPPC vesicles. For the case of DMPC and POPC, the vesicles are removed by the shear, resulting in much smaller D_f values already at lower loads and pressures: on first approaches ($D_{f1DMPC} = 11.9 \pm 1.7$ nm, $D_{f1POPC} = 11.1 \pm 1.0$ nm) and on second approaches ($D_{f2DMPC} = 28.4 \pm 4.9$ nm, $D_{f2POPC} = 29.2 \pm 4.8$ nm). This suggest that most of the lipids were removed by shear, and the residual avidin-HA-PC complexes were damaged by the consequent high friction following the first approach/shear cycle, with the resulting debris leading to a higher interaction range and D_f – and much higher friction on a subsequent approach.

The most interesting feature of our results concerns the lubrication. Whatever the detailed structure of the HA-PC surface complexes is, we see unambiguously that, relative to mica-avidin-HA layers alone for which $\mu \approx 0.3$ [10, 15, 26], the addition of the lipids results in reduction in the friction by up to 2 orders of magnitude. This is attributed to the highly-hydrated phosphocholine groups terminating the PC lipids, which are exposed at the slip-plane between the mica surfaces bearing the HA-PC boundary layers, as detailed earlier[26] for DPPC lipids in terms of an HA-PC complex similar to that schematically shown in fig. 10. For the HSPC lipids, the high-pressure lubrication is similar to that of DPPC, as seen in fig. 7 where the range of DPPC data from reference[26] is superposed (shaded region). However, from the normal force profiles – which suggest an HA-HSPC layer thickness of ca. 15-17 nm at the highest compressions – it may be that, in addition to HA-bilayer complexes, there are some residual HSPC vesicles attached to the HA molecules in the surface complexes. This scenario is consistent with the relatively large friction coefficient in the HA-HSPC case at lower loads, inset to fig. 7, as well as to the substantially higher friction (at lower loads) for the first approach (black data) relative to the second approach at the same contact point (blue data). This is attributed to the larger viscoelastic dissipation as the extended HA-HSPC layers first interpenetrate and slide past each other, while at the higher loads (and pressures) the hydration lubrication mode prevails. Likewise, removal of the looser HSPC vesicles on a first approach decreases the subsequent dissipation on sliding at low loads (second approach, blue data points in fig. 7), as the layers become somewhat less extended.

The DMPC and POPC data, fig. 9, show that at low pressures there is quite efficient lubrication (down to $\mu \approx 4 \cdot 10^{-3}$ for the POPC), attributed to the exposed, highly-hydrated

phosphocholine groups in the respective HA-PC complexes. At pressures higher than ca. 10 – 20 atm the friction diverges and the boundary layers are damaged, with subsequent debris between the surfaces resulting in high friction (as well as the longer-ranged steric repulsions noted above). This is attributed to the less robust nature of these lipids, which at the temperature of the measurements are either in their liquid phase (POPC) or just a few degrees below it (DMPC), whereas at ambient conditions the HSPC is some 30⁰C below its transition temperature. Similar indications for DMPC layer damage have been seen when it is adsorbed on bare mica[30] or on mica coated with a chitosan-alginate bilayer[44]. We remark that the HA-DMPC layers appear to withstand about a 2-fold larger pressure than the POPC ones, before damage and divergence of the pressure, likely because the DMPC is slightly into its gel phase while the POPC is in its liquid phase at the temperature of the measurements. We note that, unexpectedly, at lower loads (and local pressures $P < 10 - 20$ atm) the friction (and the friction coefficient) is substantially lower for both the DMPC and POPC on first approach of the surfaces than in the case of the HSPC. This better lubrication at low P may be due to two factors. Firstly, the rupture/removal of the DMPC or POPC vesicles on approach of the surfaces, noted earlier, results in the HA-DMPC or HA-POPC layers being less extended from the surface, as indicated also by the lower onset separation for strong steric forces (40 – 60 nm for DMPC, POPC, vs. 100 – 120 nm for HSPC). These more compact layers (as long as the pressure is sufficiently low for them not to be damaged) entail, in turn, less viscoelastic dissipation through interpenetration on sliding than the thicker, vesicle-coated HA-HSPC complexes (fig. 1A). Secondly, one expects a somewhat higher extent of hydration of the phosphocholine groups for the more fluid, or less closely-packed, bilayers (DMPC, close to its T_M , and POPC in particular) relative to the HSPC, which would reduce the friction

dissipation via hydration lubrication mechanism.

It is of interest to consider the implication of our results for boundary friction of articular cartilage (other frictional modes for articular cartilage, such as thin-film lubrication, have been considered elsewhere[1, 10] and will not be elaborated here). Before we do this, it is appropriate to consider the relation between our measurements of friction between boundary layers on a model smooth and hard substrate (mica), and friction between much softer and rougher substrates such as cartilage. The friction measured in the SFB in our study arises from the energy dissipation *at the slip-plane* between the two boundary layers (in this case, surface-attached HA complexed with the respective PC lipids) as they slide past each other. As such, it depends only on the nature of the molecular dissipation processes at that slip plane, which in turn depend on the local molecular interactions and dissipative processes[14]. These include rupture and recovery of van der Waals bonds, breaking and reforming of charge-charge or charge-dipole interactions and irreversible energy loss on hopping past molecular energy barriers on sliding[45], as well as viscous losses if the opposing layers interpenetrate and drag past each other; these processes at the slip-plane do not depend on the substrate (we emphasize that different substrates may result in different structures of the boundary layer, in which case the boundary friction will differ; but for *given* boundary layers with the same structure sliding past each other, the frictional dissipation at their slip plane – which results in the boundary friction – does not depend on the substrate to which the layers are attached). In the case of the same or similar boundary layers on a softer and less smooth surface (such as articular cartilage), one would expect the same sliding boundary friction at the slip plane, as well as additional frictional dissipation pathways. These additional modes include, for example, ploughing or viscoelastic losses upon asperity

deformation/recovery as the softer surfaces slide past each other, or viscous losses associated with squeezing water through the microporous network comprising the softer substrate. These latter losses would depend on the extent to which the shear stress deforms the substrates upon sliding, and so in turn also depend on the boundary friction (as measured in the SFB experiments). Thus the boundary friction measured in the SFB, as in the present study, and the frictional dissipation between softer, rougher substrates exposing the same boundary layers, are closely related (though the latter is expected to be larger due to the additional dissipation pathways available).

Phospholipids have long been implicated in cartilage lubrication[23, 46], while more recently (as elaborated in the Introduction) the lubricating boundary layer on articular cartilage has been proposed to consist of HA molecules, anchored by lubricin to the cartilage at its surface, and complexed with PC lipids that lubricate via their exposed, highly-hydrated, phosphocholine groups. Here we consider, in the light of the present study, the role that different PC lipids in synovial joints may play in this boundary layer. Our earlier work and this study show that DPPC ($(C_{16})_2$ acyl tails) and HSPC ($(C_{16})_{15\%}(C_{18})_{85\%}$) lipids can, when complexed with surface-attached HA, provide efficient boundary lubrication (μ down to 10^{-3}) at physiologically-high pressures ($P \gtrsim 100$ atm), while DMPC ($(C_{14})_2$ acyl tails) and POPC ($(C_{16})(C_{18:1})$), similarly complexed, provide good lubrication only up to some 10 – 20 atm. Recently the phospholipid composition of synovial joints has been examined by Sarma et al.[34] and by Kosinska et al.[33], revealing more than 100 distinct lipids belonging to 8 lipid classes. Among them, PC lipids constitute the most abundant form[34]. The study by Sarma et al.[34] points to the $(C_{18:1})$ acyl tails as being most common among the PC lipids, as well as indicating a substantial amount of (C_{16}) and (C_{18}) acyl tails, while the study by Kosinska et

al. [33] indicates PC(C_{34:1}), which corresponds to POPC in terms of acyl tails, to be among the most abundant of the PC species. This suggests that POPC may play a role in cartilage lubrication by complexing at its surface, despite being less robust to damage under high compression and shear. A possible scenario for the cartilage boundary lubricating layers, may involve structures formed of surface-attached HA (e.g., attached via lubricin as suggested earlier[10, 26]) complexed with mixtures of PC lipids. More fluid ones such as POPC may be more exposed at the outer surfaces to provide efficient low-pressure lubrication, and when they degrade at higher contact pressures (as suggested in this study) the more robust high T_M lipids (e.g. DSPC, which has very similar lubricating properties to HSPC) can provide low μ to the highest physiological pressures. A recent study showing the propensity of POPC bilayers to heal rapidly when sheared in the presence of a POPC reservoir in the surrounding fluid[31] (as might be expected in the synovial environment) suggests the means by which damaged POPC (or other fluid) bilayers may be healed following their degradation at high pressure and shear. We remark that neither of these two studies of lipids in synovial joints[33, 34] indicates the presence of C₁₄ acyl tails, which suggests that DMPC may not be present.

5. Conclusions

Phosphatidylcholine (PC) lipids POPC, DMPC and HSPC, covering a wide range of solid-ordered-to-liquid-disordered transition temperatures, were shown to complex with surface-attached hyaluronic acid (HA), and the layers formed by such HA-PC complexes provided (via the hydration lubrication paradigm) very efficient reduction in boundary friction up to high pressures (> 100 atm). The PCs with the shorter or un-saturated acyl tails (POPC, DMPC), and thus with the less robust or less-rigid bilayer structure, provided good lubrication (μ down to $10^{-2} - 4 \times 10^{-3}$) up to pressures ca. 10 - 20 atm, while the longer-tailed,

more robust HSPC complexes could reduce the sliding friction to $\mu \approx 10^{-3}$ even at the highest physiologically relevant pressures (ca. 150 atm). This behavior, together with the proposed similar structure of boundary layers at articular cartilage surfaces, suggests a possible synergy of different synovial joint PCs in creating lubricating boundary layers that reduce sliding friction efficiently over the entire physiological pressure range. This insight may have relevance to treatment modalities for joint diseases (e.g. osteoarthritis) which involve intra-articular administration of PC lipids.

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Figure Captions

Figure 1. Cryo-SEM images of freshly cleaved mica coated sequentially with avidin, bHA and PC-SUVs. (A) HSPC-SUV, where inset 1 is for HSPC-SUVs on bare mica from ref. [29], and inset 2 is from ref. ⁴². (B) DMPC-SUV. (C) POPC-SUV. All scale bars are 100 nm.

Figure 2. AFM images of HSPC SUVs and HA-PC bearing mica layer in water. All of the AFM images are $250 \times 250 \text{ nm}^2$ in size. (A) HSPC-SUVs adsorbed on freshly-cleaved bare mica. (B) – (D): AFM images of freshly cleaved mica coated sequentially with avidin, bHA and PC-SUVs. (B) HSPC (C) DMPC. (D) POPC. Lower left insets in (B), (C) and (D) are cross sections taken at locations marked with red lines in the images. The upper left inset to (D) is taken from the AFM image of DPPC on freshly cleaved mica coated sequentially with avidin and bHA, taken from ref. [26].

Figure 3. Normal force $F_n(D)$ versus distance D profiles between two avidin-bHA-HSPC bearing surfaces: black filled symbols are first approaches, empty symbols are receding profiles, and crossed blue symbols are second approaches. Red symbols refer to measurements in 0.15 M KNO_3 salt solution. Hard wall separation is $D_f = 32.5 \pm 3.2 \text{ nm}$ for both first and second approaches. The inset is a schematic of the configuration of the surfaces in the SFB: K_n and K_s are the normal and shear springs respectively, D is the surface separation.

Figure 4. Force $F_n(D)$ versus distance D profiles between two avidin-bHA-DMPC bearing surfaces: black filled symbols are first approaches, empty symbols are receding profiles, and crossed blue symbols are second approaches. All the measurements were performed at $T =$

20°C. Hard wall separation for first (D_{f1}) and second approaches (D_{f2}) are 11.9 ± 1.7 nm and 28.4 ± 4.9 nm, respectively.

Figure 5. Force $F_n(D)$ versus distance D profiles between two avidin-bHA-POPC bearing surfaces: black filled symbols are first approaches, empty symbols are receding profiles, and crossed blue symbols are second approaches. Hard wall separation for first (D_{f1}) and second approaches (D_{f2}) are 11.1 ± 1.0 nm and 29.2 ± 4.8 nm, respectively.

Figure 6. Typical shear force versus time traces for avidin-bHA-HSPC bearing surfaces, at a given contact point on a first approach. Pressures were calculated using $P = F_n/A = F_n/(\pi a^2)$, where a is the radius of the contact area measured directly from the flattening of the interference fringes. The upper set of curves is at lower pressures ($P < 50$ atm) while the lower set is for higher P values, where the top trace in each set is the applied lateral motion. Plateaus in the traces indicate sliding (including the stick-slip sliding); where no plateau is clearly seen, fast fourier transform of the data yields F_s at the lateral drive frequency[47].

Figure 7. Shear forces F_s as a function of applied loads F_n between two avidin-bHA-HSPC bearing surfaces. Filled black symbols: first approaches; crossed blue symbols: second approaches at the same contact position; filled red symbols: measurements in 0.15 M KNO_3 solution. Some limiting values of pressure P and corresponding friction coefficient ($\mu = F_s/F_n$) are shown for the arrowed points. The blue and green hatched regions correspond to the data for DPPC in water and in 0.1M KNO_3 , respectively, from reference [26]. The inset shows the friction coefficient μ versus pressure P , with symbols corresponding to those in the main figure.

Figure 8. Typical shear force $F_s(t)$ versus time t traces for avidin-bHA-DMPC and avidin-bHA-POPC bearing surfaces. Pressures were calculated using the Hertzian expression rather than from the flattening (see text).

Figure 9. Typical shear force F_s as a function of applied loads F_n between avidin-bHA-DMPC bearing surfaces, and avidin-bHA-POPC bearing surfaces. Curves a-d show the variation $F_s = \mu F_n$, with friction coefficient μ values as indicated in the insets. Arrows indicate the points at which further increase in the load leads to an abrupt increase in friction such that $F_s > K_s \Delta x_{0, \max}$, so that the surfaces no longer slide relative to each other.

Figure 10: Schematic of the HA-PC complexes on top of the avidin layer, adapted from ref. [26]. The lipids may be in the form of bilayers (blue) attached to the negatively-charged HA via a dipole-charge interaction with the dipolar phosphocholine PC headgroups[26, 48, 49], or monolayers (red) attached via the acyl chains of the PCs to the hydrophobic patches along the HA chain[50], or a combination of both, or indeed PC multilayers.

Figure 1:

Figure 2

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

A

B

Figure 10.

Graphical abstract