Biolubricants and Biolubrication

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Doctoral Thesis

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"Human subtlety will never devise an invention more beautiful, more simple or more direct than does Nature, because in her inventions, nothing is lacking and nothing is superfluous."

Leonardo da Vinci (1452-1519)
Abstract

The main objective of this thesis work was to gain understanding of the principles of biolubrication, focusing on synergistic effects between biolubricants. To this end surface force and friction measurements were carried out by means of Atomic Force Microscopy, using hydrophilic and hydrophobic model surfaces in salt solutions of high ionic strength (≈ 150 mM) in presence of different biolubricants. There was also a need to gain information on the adsorbed layers formed by the biolubricants. This was achieved by using a range of methods such as Atomic Force Microscopy PeakForce imaging, Quartz Crystal Microbalance with Dissipation, Dynamic Light Scattering and X-Ray Reflectometry. By combining data from these techniques, detailed information about the adsorbed layers could be obtained.

The biolubricants that were chosen for investigation were a phospholipid, hyaluronan, lubricin, and cartilage oligomeric matrix protein (COMP) that all exist in the synovial joint area. First the lubrication ability of these components alone was investigated, and then focus was turned to two pairs that are known or assumed to associate in the synovial area. Of the biolubricants that were investigated, it was only the phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) that was found to be an efficient lubricant on its own. Deposited DPPC bilayers on silica surfaces were found to be able to provide very low friction coefficients (≈ 0.01) up to high pressures, ≈ 50 MPa. A higher load bearing capacity was found for DPPC in the liquid crystalline state compared to in the gel state.

The first synergy pair that was explored was DPPC and hyaluronan, that is known to associate on the cartilage surface, and we also noticed association between hyaluronan and DPPC vesicles as well as with adsorbed DPPC bilayers. By combining these two components a lubrication performance similar to that of DPPC alone could be achieved, even though the friction coefficient in presence of hyaluronan was found to be slightly higher. The synergy here is thus not in form of an increased performance, but rather that the presence of hyaluronan allows a large amount of the phospholipid lubricant to accumulate where it is needed, i.e. on the sliding surfaces.

The other synergy pair was lubricin and COMP that recently has been shown to be co-localized on the cartilage surface, and thus suggested to associate with each other. Lubricin, as a single component, provided poor lubrication of PMMA surfaces, which we utilized as model hydrophobic surfaces. However, if COMP first was allowed to coat the surface, and then lubricin was added a low friction coefficient (≈ 0.03) was found. In this case the synergy arises from COMP facilitating strong anchoring of lubricin to the surface in conformations that provide good lubrication performance.

Keywords: Hyaluronan, Phospholipid, Lubricin, Cartilage Oligomeric Matrix Protein, COMP, Adsorption, Surface Force, Friction, Biolubrication, Boundary Lubrication, Load Bearing Capacity, Synergistic Effects, DLS, QCM-D, AFM.
Sammanfattning

Huvudsyftet med det här avhandlingsarbetet var att öka förståelsen för den låga friktion som finns i vissa biologiska system, med fokus på synergistiska effekter mellan de smörjande molekylerna. För detta ändamål studerades ytforlängare och friktion med hjälp av atomkraftsmikroskopi. Mätningarna utfördes med hydrofila och hydrofoba modellflytter i lösningar med hög salthalt (≈ 150 mM) i närvaro av smörjande biomolekyler. Det var också nödvändigt att få information om de adsorberade skiktens biologiska system. Det åstadkoms med hjälp av en roller av tekniker så som AFM PeakForce avbildning, kvartskristallmikrovåg, dynamisk ljusspridning och röntgen reflektometri. Genom att kombinera data från dessa tekniker erhölls detaljerad information om de smörjande skiktens biologiska system.

De smörjande biomolekyler som valdes ut för studierna var en fosfolipid, hyaluronan, lubricin, och cartilage oligomeric matrix protein (COMP) vilka alla finns i synovialledsområdet. Först undersökt den smörjande förmågan hos dessa komponenter var för sig, och sedan fokuserade vi på två par av biomolekyler som man vet eller antar bildar associationsstrukturen i synovialleder. Av de enskilda biomolekyler som undersökt var det endast fosfolipiden 1,2-dipalmitoyl-sn-glycero-3-fosfokoline (DPPC) som visade sig vara en effektivt smörjande molekyl. Deponerade biskikt av DPPC på silikaytor gav upphov till mycket låga friktionskoefficienter (≈ 0.01) upp till höga pålagda tryck, ≈ 50 MPa. DPPC bilager i flytande kristallin fas visade sig ha högre lastbärande förmåga än DPPC bilager i geltillstånd.

Det första synergistiska par som undersökt var DPPC och hyaluronan vilka man vet associerar på broskytan, och vi visade att hyaluronan associerar med såväl DPPC vesiklar som med DPPC bilager. Genom att kombinera dessa två komponenter uppställde en synergistisk förmåga som var jämförbar med den som DPPC ensam uppvisar. Även om friktionskoefficienten något högre i närvaro av hyaluronan. Synergieffekten här består inte av en bättre smörjande förmåga, utan istället gör närvaron av hyaluronan att de smörjande fosfolipiderna kan ansamlas i stora mängder där de behövs, dvs. på de glidande ytorna.

Det andra synergiparet var lubricin och COMP vilka nyligen har visats vara lokaliserade på samma platser på broskytan, vilket tyder på att de associerar med varandra. På egen hand var lubricins smörjande förmåga av PMMA, våra hydrofoba modellflytter, dålig. Emellertid, om COMP först adsorberades på PMMA och sedan lubricin tillsattes uppställde en låg friktionskoefficient (≈ 0.03). I det här fallet består synergin av att COMP möjliggör en stark inbindning till ytan av lubricin i konformationer som ger god smörjande förmåga.

Nyckelord: Hyaluronan, Fosfolipid, Lubricin, Cartilage Oligomeric Matrix Protein, COMP, Adsorption, Ytkraft, Friktion, Biologisk smörjning, Gränskiktssmörjning, Lastbärande förmåga, Synergieffekter, DLS, QCM-D, AFM.
List of papers

The papers listed below are included in the thesis. In the following these papers are referred as “Paper I”, etc.

I. Chao Liu, Min Wang, Junxue An, Esben Thormann and Andra Dédinaitė

“Hyaluronan and phospholipids in boundary lubrication”

*Soft Matter, 2012*, 8, 10241-10244

II. Min Wang, Chao Liu, Esben Thormann, and Andra Dédinaitė

“Hyaluronan and Phospholipid Association in Biolubrication”

*Biomacromolecules, 2013*, 14 (12), 4198–4206

III. Min Wang, Matthew Fielden, Per Claesson and Andra Dédinaitė

“Adsorption and Friction Performance of Layers formed from Mixed Hyaluronan - Dipalmitoylphosphatidylcholine (DPPC) Vesicle Solutions”

*Manuscript*

IV. Min Wang, Thomas Zander, Xiaoyan Liu, Chao Liu, D.C. Florian Wieland, Vasil M. Garamus, Regine Willumeit-Römer, Per Claesson, Andra Dédinaitė

“The Effect of Temperature on Supported Dipalmitoylphosphatidylcholine (DPPC) Bilayers: Structure and Lubrication Performance”

*Submitted to Journal of Colloid and Interface Science*

V. Min Wang, Akanksha Raj, Chao Liu, Per Claesson, Liaqat Ali, Niklas Karlsson and Andra Dédinaitė
“Molecular synergies in biolubrication”

Manuscript

Contribution by the respondent:

I. Major part of experimental work except for Ellipsometry measurements. Part of data analysis.

II. Major part of experimental work. All the data analysis and major part of manuscript preparation.

III. Major part of experimental work. All the data analysis and major part of manuscript preparation.

IV. Major part of experimental and analysis work except for XRR experiments. Major part of manuscript preparation.

V. Major part of experimental work except for QCM-D measurements. Part of data analysis.
Summary of papers

The first three papers of my thesis consider mixtures of hyaluronan and the saturated phospholipid DPPC. They deal with different aspects of their association as well as lubrication properties of mixed hyaluronan/DPPC layers adsorbed on silica surfaces.

In Paper I it is reported that hyaluronan and DPPC associates at the silica-water interfaces in 155 mM NaCl solution. A mixed layer was formed by first allowing a DPPC bilayer to coat the silica surface via a vesicle fusion process, and then hyaluronan was added. Hyaluronan adsorbed to this DPPC-coated surface but not to bare silica. Our results showed that the DPPC bilayer alone and with added hyaluronan was able to provide favourable lubricating properties.

The sequential adsorption of hyaluronan and DPPC was explored in more detail in Paper II. It was shown that a thick composite layer of these two components could be built. We found that a very low friction coefficient characterized the sliding when DPPC was added as the last component, and such low friction was maintained up to the pressures significantly above what is encountered in healthy synovial joints. Hyaluronan as the last added component was found to increase the friction coefficient and decrease the load bearing capacity somewhat.

Even though sequential deposition is scientifically interesting, it is not Nature’s way to form adsorbed layers. Rather, the adsorbing molecules are present together in the solution and the adsorbed layer is formed from such a complex mixture. Thus, in Paper III we prepared a solution containing both DPPC and hyaluronan and investigated the adsorbed layers formed. We noted that first a DPPC bilayer was formed on the silica surface, and then a slow deposition of additional material of hyaluronan decorated DPPC vesicles was following. The layers formed from the mixed solution were not as homogeneous as that formed by DPPC alone. However, also these layers offered sliding with a low friction coefficient and high load bearing capacity. In addition, the large amount of material that can accumulate on the surface in contact with a solution containing both hyaluronan and DPPC can be seen as an advantage since it allows the build-up of a reservoir of the lubricating phospholipids on the sliding solid surfaces.
**Paper IV** discusses how the DPPC bilayer morphology and friction properties change with temperature, covering the range from 25 °C to 52 °C. The morphology was investigated with X-ray reflectivity, XRR, and AFM imaging experiments. It was found that the DPPC bilayer was in the gel state at low temperatures and in the liquid crystalline state at high temperature. As shown by the XRR data, the transition from the gel phase to the liquid crystalline state results in decrease in thickness and increase in roughness towards the aqueous interface. The latter being a consequence of increased molecular mobility perpendicular to the surface in the liquid crystalline phase. Interestingly, we noted that structural changes observed with AFM occurred at lower temperature than in XRR measurements. The reason is suggested to be that the AFM tip transfers energy to the bilayer during tapping, which shifts the phase transition temperature downwards. Friction force measurements revealed that intact DPPC bilayers provided low friction in both the gel and the liquid crystalline state. In contrast, the load bearing capacity of the DPPC bilayer was found to be higher in the liquid crystalline phase. We suggest that this is due to the increased fluidity of the bilayer in liquid crystalline state that allows lipids to diffuse into defects and thus provide a certain self-healing ability.

In **Paper V** the synergistic action of two biomacromolecules were considered, cartilage oligomeric matrix protein (COMP) and lubricin, which recently have been shown to be co-localised at the cartilage surface. The results show that COMP and lubricin associate on poly (methyl metacrylate) that I utilized as model hydrophobic surface. The friction force was high in presence of lubricin, and less in presence of COMP. However, by first adsorbing COMP and then allowing lubricin to bind to this surface resulted in very low friction and high load bearing capacity. It appears that COMP facilitates strong anchoring of lubricin in conformations that allow it to promote sliding with a low friction force. This illustrates that the superb natural lubrication found in synovial joints need to be sought in the synergistic actions of biomolecules.
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1. Introduction

1.1 Background of biolubrication

1.1.1 Inspiration source – mammalian synovial joint

The research topic of the present thesis – *Biolubricants and biolubrication* – stems from my great interest and awe beholding how nature works. Nature inspires us by demonstrating “technical” solutions where great simplicity and complexity intertwine to achieve perfect functionality. One example of such a natural construct is the mammalian synovial joint (Figure 1.1).

![Illustration of the synovial joint capsule](image)

*Figure 1.1 Illustration of the synovial joint capsule*

The synovial joint consists of two articulating bones covered by thin strata of cartilage grown together with the underlying bone that protect the bones from direct contact and reduces friction and wear during motion. The thickness of articular cartilage varies throughout different parts of the body. The thickest cartilage is found in lower parts of the human body where the load is the greatest. For instance, the cartilage found on femur in the human knee was measured to be in the range of 1.76 – 2.65 mm.[1] The Young’s modulus of cartilage is in the range of 12 –50 MPa,[2, 3] which is comparable to small strain rubber that is characterised by a Young’s modulus in the range of 10 – 100 MPa. It is notable that Nature has chosen to use a soft material to cushion and protect the relatively hard underlying structures of bone.[4] In fact,
comparison of the cartilage material with simple rubber is not just. While rubber is a random
tangle of macromolecules, a cartilage material has intricate nanostructure, which renders the
material highly functional. Markedly, the main constituent of the cartilage is water. The
content of it amounts to 70 – 85 w% of the total tissue weight.[5] The compression and the
flow of water present in cartilage has been suggested to play an important role in the
mechanical response of this biomaterial.[6, 7] Apart of water, cartilage contains collagen that
amounts to 60 – 70 w% of the dry tissue and proteoglycans (chondroitin and keratan sulphates)
15 – 30 % by weight. The network of these macromolecules creates the structured hierarchical
carcase of the cartilage. The orientation and size of the collagen fibres varies with the depth of
the cartilage (Figure 1.2). Closest to the bone the collagen fibres are large, radially oriented and
“planted” into subchondral bone, whereby providing firm attachment. Further away from the
bone, in the middle zone of the cartilage, smaller arching collagen fibres are found. And finally,
close to the surface, collagen fibres are small and aligned in parallel to the surface whereby the
smooth and shiny hyaline cartilage surface is formed.[5, 8] The only cells found in cartilage
are chondrocytes and they are responsible for producing and leaching out the proteoglycans
that maintain the structure of the tissue.[9] In the articular cartilage superficial zone
chondrocytes synthesize and secret biomacromolecules such as lubricin into the synovial fluid.

Figure 1.2 Schematic representation of the zones of articular cartilage.
The surface of hyaline cartilage is covered by an amorphous layer consisting of aggregated biomaterial – biomacromolecules and phospholipids - called lamina splendens and is efficiently lubricated by synovial fluid. Healthy synovial joints immersed in synovial fluid operate at a very low friction coefficient range of 0.001 – 0.01.[5, 10] This is under a highly broad range of shear rate regimes (from stagnation to shear values of up to $10^6 – 10^7$ s$^{-1}$) and physiological loads of the synovial joints that in hips in gait in vivo have been measured to be up to 5 - 6 MPa and as high as 18 MPa when descending the stairs.[11] The synovial fluid that lubricates the cartilage, is not a simple liquid, and deserves a close look (Figure 1.3). It contains a mixture of albumin, anionic polysaccharide hyaluronan, glycoprotein lubricin and various phospholipids. The structure of synovial fluid is that of synovial gel enclosed in lipidic pockets, with multilamellar phospholipid walls.[12]

![Figure 1.3 Schematic of synovial fluid structure between two articular cartilage surfaces.](image)

It is clear that the synovial joint with all its components displays a great degree of complexity in its structure. This is due to the interaction of these components and synergy between them that the extraordinary and long-lasting performance of the synovial joint is achieved. With the research performed during my PhD time my ambition was to understand some of the synergistic mechanisms acting between the synovial components that pave the way for the superior lubrication in aqueous environment.
**Synovial components**

Below I will discuss major synovial components and their properties, which are important in understanding their associative and interfacial behaviour and that, encouraged me to select these components for my investigations.

**Hyaluronan**

Hyaluronan is a linear anionic polysaccharide consisting of repeating units of D-glucuronic acid and N-acetyl-glucosamine (Figure 1.4) The pKa of D-glucuronic acid is about 3.3,[13] thus the dissociation of the acid makes hyaluronan negatively charged in physiological solution. The average molecular weight of hyaluronan in healthy joints is about 7*10^6 g/mol[14] and its intrinsic persistence length that characterises the inherent stiffness is 9 nm. [15] Hyaluronan’s physiological concentrations in synovial fluid range between 2.5 and 3.6 mg/mL.[16] It is believed that in joints hyaluronan serves both as a lubricant and as a shock absorber. [17]

![Figure 1.4 Structure of the repeat unit in hyaluronan](image)

Pure hyaluronan solutions exhibit peculiar rheological properties - pronounced shear thinning of hyaluronan solutions at high shear rates[18] and ability to stretch significantly under conditions of high flow, resulting in significant increase of extensional viscosity have been reported.[19] The latter is likely an important functional aspect of synovial fluid. It is important to notice that though the hyaluronan molecule seems to be just a simple linear polysaccharide, it possess a secondary structure resulting in hydrophobic domains consisting of about 8 CH units.[20, 21] I mean that this likely is an important feature of hyaluronan that considering its association with large and small molecules as, e.g. phospholipids.
Lubricin

Lubricin is another major component of synovial fluid with concentrations of 0.052 – 0.45 mg/mL,[3] and the illustrate sketch is shown in Figure 1.5. Lubricin is a glycoprotein consisting of a central heavily glycosylated negatively charged (isoelectric point pI = 4 – 7.5) domain flanked by positively charged (pI = 9.49 – 9.89) C and N terminal globular protein domains.[22, 23] The lubricin molecule is about 200 nm long with the molecular weight of ~240 kDa.[24] For the first time lubricin was isolated, named and partly characterized by Swann et al.[25] The mucinous domain of lubricin consists of a polypeptide backbone where the main amino acids are threonine, glutamic acid, proline and lysine.[25] Carbohydrates constitute about 40% (w/w) of lubricin with the main residues being galactosamine, galactose, and N-acetylneuraminic acid.[25, 26] Thus, lubricin molecules carry a considerable number of anionic side chains and the solution structure of the mucinous part of the molecule is that of a charged bottlebrush with two positively charged ends. It is interesting to note that Swann and co-workers failed to characterize about 12 – 14 w% of the lubricin molecule when they first isolated it.[27] It was later shown that lubricin carries 12w% of phospholipid.[28] This detail may be important considering lubricin’s protective and friction reduction role in synovial joints. Other highly important features of a lubricin macromolecule as a purported lubricant in joints are the type of its glycosylation and the amount of charge that it carries. It has been demonstrated that O-linked β(1-3)Gal-GalNAc oligosaccharide removal from lubricin results in considerable reduction of lubricin’s ability to reduce surface friction.[29] Recently, the O-glycosylation of lubricin has been mapped and it became evident that the density of glycosylation on the mucinous part of the lubricin is high. There were found 168 sites of glycosylation concentrated in the central bottle-brush region, rendering every 4\textsuperscript{th} or 5\textsuperscript{th} amino acid residue glycosylated. More to this, O-glycosylated residues were shown to be predominantly sialylated, which makes the domain highly negatively charged.[23]
**Phospholipids**

Phospholipids are important small molecular components of synovial fluid and they have been strongly argued to have a major role in granting synovial surfaces with their amazing lubrication properties.[27] The total amount of phospholipids is healthy synovial fluid is in the range of about 0.1 to 0.2 mg/mL.[23, 30] There is a variety of phospholipids in synovial fluid. The analysis of phospholipids present on the surface of healthy cartilage from bovine femoral condyles showed that phosphatidylcholines comprise a major part, 41%, phosphatidylethanolamines amount to 27% and sphingomyelins comprises 32% of the total phospholipid found.[31] For each lipid type there is present a mixture of fatty acid chains but the unsaturated fatty acids prevail. The most abundant fatty acid was found to be oleic acid (C18:1).[31]

The modern lipidomics analysis of synovial fluid obtained from 9 previously healthy post-mortem donors showed that also in humans phosphatidylcholine was the predominant phospholipid, accounting for approximately 67% of all phospholipids, followed by sphingomyelin and lysophosphatidylcholine (17% and 10%, respectively).[32] In human, as well as in bovine synovial fluid, unsaturated fatty acids predominate in the phospholipid structure. All in all thirty-three phosphatidylcholine species with varying chain lengths and degrees of saturation were identified in human synovial fluid.[32] Thus, it is likely that in multilamellar phospholipid films that have been shown to form on cartilage surfaces[27] phospholipids are present in fluid state.

Analysis of retrieved implant surfaces showed that phosphatidylcholines dominate also on the surfaces. There the average proportion of phosphatidylcholine major types was found on polymeric and metallic implant components, respectively, as follows. Stearoyl linoleoyl phosphatidylcholine 27% and 28%; palmitoyl oleoyl phosphatidylcholine 33% and 27%; palmitoyl linoleoyl phosphatidylcholine 28% and 24% , and dipalmitoyl phosphatidylcholine 11% and 15% .
Based on the above considerations, I have chosen 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC, (Figure 1.6) for my studies of association of this phospholipid with hyaluronan in bulk and for investigating how DPPC mediates friction between surfaces.

![Figure 1.6 Structure of DPPC molecule](image)

DPPC is a saturated (C16:0) zwitterionic phospholipid (and thus not prone to chemical changes) with the bulk transition temperature $T = 41.2$ °C.[33] The surface supported DPPC bilayer due to the influence of the surface exhibits somewhat different melting transition temperature.[34] DPPC is practically insoluble in water and the CMC for DPPC is 0.46 nM.[35]

It is worth noting though that the polar phosphocholine groups of DPPC interact favourably with water, rendering them highly hydrated. I will return to the importance issue of hydration of phospholipids in the context of lubrication later in this work.

**COMP**

Cartilage Oligomeric Matrix Protein (COMP) $M_r=524,000$[36] shown in Figure 1.7 is a non-collagenous anionic[36] protein mainly found in cartilage, ligament and tendon. Native COMP is an oligomer composed of five identical units with coiled-coil chains associating at the N-terminus.[37] Five arms connect the coiled-coil domains to the globular C-terminus domains.[38] COMP is found in healthy synovial fluid at low average levels of 0.047 mg/mL.[39] It has been seen, by immunofluorescence imaging, that COMP is present in the cartilage throughout the tissue and that at the surface of cartilage it is co-localized with lubricin. Biochemically, it was also detected that the C-terminus of COMP associates with the N-terminus of lubricin by forming non-covalent and covalent S-S bonds via reaction of cysteine groups both in bulk and
at the interface. This was utilized in my study for formation of COMP-lubricin surface aggregates possessing good lubricity.

![Figure 1.7 Schematic of the COMP molecule](image)

1.1.2 The build-up of a meaningful model of a tribological system of the synovial joint and the choice of techniques to study it.

The above description of the articular cartilage and synovial fluid components is not an exhaustive account of how the tribological system of a synovial joint is built. There is an inherent complexity to this system with multiple components interacting in synergy to yield a result that enables us to move painlessly through life. Synovial fluid is, on the one hand; a serum that is passively dialysed through the synovial membrane and, on the other hand, it is filled with substances actively secreted by the cells in synovial tissue. Besides the components that I have described above, proteins like albumin, enzymes like proteinases, collagenases, fragments of aggrecan,[40] as well as glucose, uric acid and simple salt, NaCl, can be found in synovial fluid. The pH of healthy synovial fluid is about 7.8.[41] This system is difficult to study in all of its complexity. In order to obtain meaningful and interpretable results we need to
identify and select some key ingredients and study the key aspects of their interaction. Below I will give an account of this procedure.

1.1.3 The scope of this thesis

The ultimate question that I would like to have answered is how biolubricants and biolubrication in joints work. The sub-questions are: What mechanisms govern biolubricant association? Are synergistic associative structures present? If so, what are they and in what way are they synergistic? To achieve some progress in this area it is natural to break down the complex system into parts and in the first place gain understanding of the mode of action of these smaller parts – their associative and frictional properties.

The choice of surfaces

The surface of cartilage, though studied for many years, remains of elusive nature for the physical chemist. Not only the precise composition but also the position of it is a matter of dispute. The uppermost surface is called lamina splendens, however the nature of it has been variously reported in different studies. One reason for this is that as soon as this surface leaves the living body and ends-up on the experimentalists table, it starts to change.[42] Removed from synovial fluid it dehydrates, immersed in buffer solution it leaks the components and, when touched, it quickly loose the adsorbed layer. Thus, in order to study the adsorption of synovial components and their aggregation on surfaces I mean that it is rational to start with well-defined surfaces of both hydrophilic and hydrophobic character. As hydrophilic surface I have employed silica and as hydrophobic surface I have used poly (methylmetacrylate) (PMMA). I have modified these surfaces by spontaneous adsorption of synovial components from solution.
**The choice of adsorbates**

The choice of adsorbates in my work was dictated by what is known to be present in synovial fluid and the questions that I wanted to answer. **Hyaluronan** was chosen because it is, as described above, the major component of synovial fluid and it is known to associate with **phospholipids** to yield a variety of structures.[43] The phospholipid **DPPC** was chosen for its abundance in synovia and its surface activity. The lubrication ability of DPPC alone on silica surface and in association with hyaluronan was investigated. **Lubricin** was selected for its fame of being a main lubricant in synovial joint, and **COMP** because of the new knowledge arising about the interactions of COMP and lubricin. Thus, the choice of the latter couple of substances allowed me to investigate the role of COMP in structuring lubricin on surfaces and thereby providing the synergistic association that yields superior lubrication.

**The choice of techniques**

To study complex systems as above, a battery of techniques was employed. In the table below I list the techniques used and specify what information that I extract about the investigated system by use of these techniques, which will be described further in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Technique used</th>
<th>Information obtained</th>
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<tr>
<td>Dynamic Light Scattering (DLS)</td>
<td>The size of DPPC vesicles and the change in size upon association with hyaluronan.</td>
</tr>
<tr>
<td>Quartz Crystal Microbalance with Dissipation (QCM-D)</td>
<td>Formation of DPPC bilayer on silica surface, sensed mass and layer thickness. Adsorption of hyaluronan on DPPC bilayer. Build-up of multilayers of DPPC and hyaluronan. Adsorption of COMP on PMMA surface. Formation of lubricin layer on COMP-</td>
</tr>
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X-ray reflectometry & DPPC bilayer formation on silica surface at atmospheric and elevated pressures

Atomic Force Microscopy (AFM)
- Peak Force QNM Imaging & Topographical image of DPPC bilayer, lateral homogeneity of adsorption layer
- AFM - colloidal probe force & Normal forces acting between surfaces
- AFM - colloidal probe friction & Lateral forces acting between surfaces

1.2 Self-assembly structures of synovial biomolecules

1.2.1 Phospholipid, DPPC.

DPPC is extremely poorly soluble in aqueous solutions. In fact, DPPC is quoted to be practically insoluble in water, and its critical micellar concentration is 0.46 nM.[35] Simulation studies have shown that small DPPC vesicles can form from disordered solution of DPPC.[44] If phospholipid molecules exist or not in vesicle form in synovial fluid is not known. Nevertheless, in my study I have used small unilamellar DPPC vesicles prepared in 155 mM NaCl solution by the method of sonication and have discovered that the average size distribution of the vesicles in 0.5 mg/mL DPPC dispersions is 110 ± 4 nm.[45] Small unilamellar vesicles usually have a diameter around 100 nm.[46] They consist of a single phospholipid bilayer surrounding an aqueous core.

On silica surfaces, above the phase transition temperatures, in 155 mM NaCl solution DPPC vesicles rupture and fuse to form a 5-6 nm thick self-assembled bilayer.
1.2.2 Phospholipids and hyaluronan

Hyaluronan associates with phospholipid self-assembly structures in bulk and at the surfaces. In bulk, hyaluronan wraps around the vesicle surface thus increasing the vesicle hydrodynamic diameter by $30 \pm 8$ nm (see Paper II). At interfaces hyaluronan and phospholipids also associate. I did not detect any ordered association structures between these molecules, but clearly have seen that the presence of hyaluronan enables accumulation of more than one bilayer of DPPC on silica surfaces.

1.2.3 COMP and lubricin

COMP and lubricin have been shown to associate in bulk and at the cartilage interface. It has been demonstrated that lubricin is co-localized at the surface of cartilage together with COMP and that it is capable of forming both covalent and non-covalent complexes with lubricin via interactions between cysteine residues of C-terminus of COMP and N-terminus of lubricin.

1.3 Surface interactions

Understanding of interactions between surfaces, normal and lateral, are of utmost importance in technology and in interpreting behavior of colloidal systems. Below I will discuss major classes of forces that govern interactions between surfaces. I will accentuate forces that are of greater importance when biomolecules at relatively high inert salt concentrations are involved in interactions.

1.3.1 Electrical double-layer and van der Waals interactions (DLVO – theory)

When surfaces of the same nature are close together in a polar medium that is described by its dielectric properties (i.e. ignoring its molecular structure), it is often the case that their interactions are mainly governed by electrostatic double-layer repulsion and van der Waals attraction. The classical Derjaguin, Landau, Verwey and Overbeek (DLVO) theory proposed and developed in 1941 successfully captures this repulsion – attraction interplay.[47-49] In the DLVO theory, the total free energy of interaction between colloidal particles/charged surfaces is described as:
Electrical double-layer and van der Waals forces are very well understood and explicitly treated in the original work of the theory founders and in several good textbooks.[50-52] Thus, I will not expand this text by explaining these forces in detail. Several facts, though, are of exceptional importance for understanding the behaviour of the systems that I studied, thus I will mention them here.

Van der Waals interactions originate from the motion of electrons around the atomic nucleus, and the thermal motion of molecules with permanent dipole moments. These motions generate a fluctuating electric field that extends beyond any interface. It is the interaction between these fields that is the ever-present van der Waals interaction. Despite that the van der Waals interaction has a clear molecular origin, it is – for macroscopic surfaces – best described by a theory that completely neglects the molecular nature of the interacting bodies and the intervening medium. This theory is known as the Lifshitz theory.[53] It is rather complicated but provides an easy way for calculating the Hamaker constant, \( A \), that describes the van der Waals interaction between two interacting bodies (marked as 1 and 2) across a medium (marked as 3). The only thing one needs to know is the dielectric properties as a function of frequency, and in practise it is often sufficient to know the static dielectric constant and the refractive index. An approximate expression for the non-retarded Hamaker constant is:[54]

\[
A_{total} = A_{v=0} + A_{v>0} 
\approx \frac{3}{4} kT \left( \frac{\varepsilon_1 - \varepsilon_3}{\varepsilon_1 + \varepsilon_3} \right) \left( \frac{\varepsilon_2 - \varepsilon_3}{\varepsilon_2 + \varepsilon_3} \right) + \frac{3\hbar \nu_e}{8\sqrt{2}} \frac{(n_1^2 - n_3^2)(n_2^2 - n_3^2)}{(n_2^2 + n_3^2)^{1/2}(n_1^2 + n_3^2)^{1/2}[(n_1^2 + n_3^2)^{1/2} + (n_2^2 + n_3^2)^{1/2}]} \tag{1-2}
\]

Where \( \varepsilon_1, \varepsilon_2 \) and \( \varepsilon_3 \) are the static dielectric constants of the three media; \( n_1, n_2 \) and \( n_3 \) the refractive index of three media. \( \hbar \) Planck’s constant, \( \nu_e \) the main electronic absorption frequency. The free energy of interaction between a flat surface and a spherical particle, which is my experimental geometry, is given by:[54]

\[
W(d)_{vdW} = -\frac{AR}{6D} \tag{1-3}
\]

Where \( A \) is the Hamaker constant, typical value \( 10^{-20} \) J for SiO\(_2\) surface in water solution. \( R \) is the radius of the sphere and the \( D \) is the distance between the surface and sphere. The double layer interaction arises due to confinement of counterions between the surfaces and is always
repulsive between identical surfaces. When two charged surfaces approach each other the ion concentration in the gap between two surfaces increases. This generates an osmotic repulsion, which at large separations decays exponentially as:

\[ W(d)_{\text{EDL}} = Ce^{-\kappa D} \]  

Where the \( D \) is the distance between the two surfaces, and \( C \) is a constant related to the surface charge density.

The range of the electrical double layer interaction decreases with increasing ionic strength of the solution as described by the Debye length, which in aqueous monovalent electrolyte solutions at room temperature can be expressed as: \[ \kappa^{-1} \approx \frac{0.304}{\sqrt{c}} \]  

Where \( \kappa^{-1} \) is the Debye length, expressed in nm, and \( c \) is the salt concentration expressed in mol/L.

In my experiments, at physiological salt concentrations (155 mM NaCl), the Debye length is 0.77 nm and thus the range of the electrical double-layer interaction was smaller than the range of other contributions to the interaction. I will briefly describe these interactions in the following sections.

1.3.2 Attractive forces

Bridging attraction

When surfaces are coated with polymers, at low coverage and small enough separations, the polymer chains can bind to both surfaces. This phenomenon is termed bridging.\[56\] Upon separation the polymer ends that are adsorbed on opposing surfaces resist detachment, thus giving rise to an attractive force. This explanation, however, does not capture the fact that the bridging force to a large degree is of entropic nature.\[57\] When the two polymers coated surfaces are close together, a polymer can satisfy the requirement of acquiring a low energy
state by many more conformations when binding is possible to both surfaces. This increases the entropy and decreases the free energy of the system. Bridging forces came into play in my study both with hyaluronan adsorbed on phospholipid bilayer and lubricin adsorbed on the surface of PMMA. In none of the abovementioned cases the double-layer forces are expected to play a role.

**Hydrophobic attraction**

Molecules in water are ordered in a short-range highly dynamic three dimensional hydrogen-bonded tetrahedral network.[58] Introducing a hydrophobic entity (e.g. an alkane molecule) requires rearrangement of water molecules around it in so-called clathrate structures, in order to maintain hydrogen bonding. This infers a considerable thermodynamic cost due to reduced entropy of water molecules.[59] To minimise the disturbance, an interaction occurs that causes clustering of hydrophobic units.[60] This force, which acts between non-polar solutes in water, is known as “the hydrophobic interaction”. Its range is only 1-2 water molecules, but it is a very important type of interaction since it drives self-assembly of biologically and technologically important molecules such as phospholipids and surfactants. In my work hydrophobic interactions between non-polar areas of hyaluronan molecules and acyl chains of DPPC may rationalize adsorption of hyaluronan on DPPC vesicles or bilayers. Further, adsorption of lubricin and COMP on PMMA, is, presumably, due to unspecific hydrophobic interaction between the surface and hydrophobic regions present on these proteins.

An attractive force, significantly stronger than the expected van der Waals force, is also observed between non-polar macroscopic surfaces in aqueous environment. This force is long-ranged and is also often referred to as “hydrophobic interaction” even though the mechanism behind this force is far from clear. However, in recent years it has become increasingly evident that what traditionally has been referred to as a “hydrophobic interaction” would in many cases (but not necessarily in all cases) better be called a “capillary attraction” since it is due to formation of a vapour cavity between the non-polar surfaces when their separation is small.[61] In the present work a long range attraction was observed between PMMA surfaces immersed in aqueous solution. The mechanism behind this attraction was not explored, but one possible
mechanism is a “hydrophobic interaction”.

1.3.3 Repulsive forces

Steric repulsion

In the presence of polymers or polyelectrolytes adsorbed on surfaces steric repulsion forces are often observed. In contrast to DLVO forces, the distance dependence of steric forces is not easily captured by analytical theories. For instance, the range and strength of the steric interaction is affected by adsorbed amount, solvent – polymer interactions, polymer-surface interactions, polymer architecture and molecular weight.[62] For instance, in good solvent and with not too strong affinity of the polymer to the surface, as is the case in my study with the COMP adsorbing on the PMMA surface, see Figure 1.8, the polymer will adopt conformations with loops and tails extending into solution, which upon compression, due to restriction of the conformational freedom of the polymer will express itself as a repulsive force, normally termed “steric repulsion”. Replacement of segment-solvent contacts with segment-segment contacts upon compression also provides a repulsive contribution to the steric force in a good solvent. One example of a force curve dominated by steric repulsion is shown in Figure 1.8.

Figure 1.8 The steric force between PMMA surfaces coated with COMP. Filled and unfilled symbols represent forces measured on approach and separation, respectively.
**Protrusion repulsion**

A protrusion repulsion always acts between self-assembled bilayers of amphiphilic molecules. It arises from the perpendicular (relative to the bilayer surface) thermal motion of the molecules in the adsorbed layers. This motion is restricted when two bilayer coated surfaces are in close proximity to each other, which results in a decrease in entropy and thus a repulsive force.[63] It has been observed that the repulsive force between phospholipid bilayers is stronger in the liquid crystalline state than in the gel state, where the molecules are less mobile.[64] This observation suggests that the protrusion force is more important than the hydration force, at least for phospholipid bilayers in the liquid crystalline state.

**Hydration repulsion**

A strong, short-ranged exponentially decaying repulsive force is often observed between two hydrophilic surfaces immersed in water, and this force is called hydration repulsion.[65] Hydration repulsion arises whenever water molecules bind strongly to surfaces containing hydrophilic groups (e.g. zwitterionic phospholipid headgroups). The strength of the hydration force depends on the energy required to dehydrate the two surfaces as they approach each other. In Figure 1.9 the short range interaction between silica surfaces is compared to that between DPPC bilayer coated silica. For silica we note the absence of a clear van der Waals attraction, which is due to the presence of a hydration force. A more long-range interaction is observed between DPPC-coated silica. This repulsive force is due to a combination of hydration and protrusion forces.
1.3.4 Friction forces

Friction is our friend. Due to friction we can hold objects firmly in our grip, run fast, and sit on a chair without slipping of it; On the other hand, thanks to low friction our joints feel comfortable. It is extremely important to have friction control in our daily life. Leonardo da Vinci was the first to formulate the rules of sliding friction, and Amontons in 1699 proposed that the friction force ($F_{friction}$) between two sliding bodies is proportional to the applied load ($F_{load}$). According to Amontons’ first rule:

$$F_{friction} = \mu F_{load}$$  \hspace{1cm} (1-6)

Where $\mu$ is the friction coefficient that only depends on the material properties.[66] Amontons’ second and third rules state that for a given load the friction force is not affected by the contact area and sliding velocity. [66, 67]

Amontons’ first rule does not apply when there is an adhesion between the sliding surfaces. This adhesion can be due to a van der Waals force, a hydrophobic force, or any other attractive interaction. The adhesion can be seen as an internal load that presses the surfaces together.

---

**Figure 1.9** Force normalized by radius as a function of surface separation. The experiments were performed in 150 mM PBS buffer at 25˚C. Filled and unfilled symbols represent interactions between silica surfaces and DPPC coated silica surfaces, respectively.
which results in a friction force when the surfaces slide against each other even when the applied load is zero. The modification of Amontons’ first rule can be written as:

\[ F_{friction} = \mu F_{load} + F(0) \]  \hspace{1cm} (1-7)

Here \( F(0) \) is the friction force at zero load. Amontons’ first rule and the modified Amontons’ first rule apply in many cases. However, when studying friction between surfaces carrying lubricants in a liquid media it is not uncommon that these rules do not hold. In such cases it is appropriate to define a load-dependent effective friction coefficient, \( \mu_{eff} \) as:

\[ \mu_{eff} = \frac{F_{friction}}{F_{load}} \]  \hspace{1cm} (1-8)

This concept has been used in my work to understand friction between PMMA surfaces coated with lubricin and COMP-lubricin complexes.

1.3.4.1 Energy dissipative mechanisms

The friction force, \( F_{friction} \) is related to the energy dissipation, \( W \), as:

\[ F_{friction} = \frac{W}{l} \]  \hspace{1cm} (1-9)

where \( l \) is the sliding length. This means that every process that causes energy dissipation during sliding contributes to the friction force. It worth noticing that for polymer-coated surfaces several energy dissipative mechanisms can be visualized, as discussed in detail by Klein.[68] Among these we can mention disruption and reformation of attractive segments-segments contacts in a poor solvent, the moving of polymer chains through the interpenetration region (the region where polymer chains from both surfaces can be found), and breakage and reformation of segment–surface attachment points. In light of this we can suggest several reasons for why lubricin, when firmly attached to surfaces in a high amount, should provide low friction. First, the carbohydrate side chains are interacting favourably with water, which means that there will hardly be any energy dissipation due to breakage of attractive segment-segment interactions. The bottle-brush structure of lubricin with a large number of
carbohydrate side chains extending from the backbone makes it difficult for two such layers to interpenetrate each other. Thus, the interpenetration region will be small, reducing energy dissipation due to dragging of polymer chains through this layer. As I showed in Paper V lubricin binds strongly to COMP-coated PMMA, and then energy dissipation due to breakage of segment-surface attachment points will also be low.
2. Materials and methods

2.1 Materials

The phospholipid, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), was purchased from Avanti Polar Lipids (Avanti Polar Lipids, Inc., Alabama, US) in powder form (batch No. 850355P), and used as received.

Hyaluronan with a weight average molecular weight, \(M_w\), of \(6.2 \times 10^5\) g/mol, and polydispersity, \(M_w/M_n\)[69] of 1.9 in powder form was kindly gifted by Novozymes (Novozymes, DK), and characterized by means of asymmetric flow flow field fractionation, AFFFF, by Postnova analytics GmbH.

The lubricin used was provided by Dr. Niklas Karlsson, Gothenburg University. His team isolated lubricin from synovial fluid collected from a pool of patients diagnosed with rheumatoid arthritis. Lubricin was collected after anion exchange chromatography, and further purified using high-pressure liquid chromatography. Dialysed and lyophilized protein powder from the above purification process was provided to me.

Recombinant Human Cartilage Oligomeric Matrix protein (COMP)/Thrombospondin-5 (Cat.no. 3134-CP-050) with predicted molecular mass of 81.8 kDa was purchased from R&D Systems (R&D Systems, Inc., Minneapolis, US). This COMP is in monomeric form, while native COMP is found as a pentamer.[70]

Sodium chloride (ACS reagent, assay \(\geq 99.0\%\)), Phosphate Buffer Saline (Sigma P4417) and chloroform (ACS) were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC. US). The water used in all experiments was purified by using a Millipore system consisting of a Milli-Q Integral 15 system with a 0.22 µm Millipak filter. The purified water had a resistivity of 18.2 MΩcm at 25 °C and the total organic carbon content was less than 3 ppb.
Thermally oxidized silicon wafers (Wafer Net, Germany) with a 100 nm thick SiO₂ layers, were used as a hydrophilic surface in all AFM experiments. They were cut into desired sizes and immersed into 2% Hellmanex (Hellma, USA) for 30 minutes. Some wafers were slightly roughened by treating them with a sonicator at this stage (Bandelin Sonorex Digitec, output power 640 W). The roughened surfaces were used in Paper IV to enhance the friction between silica surfaces in aqueous solution.

AT cut quartz crystals QSX303 (fundamental frequency of 5Hz, Västra Frölunda, Sweden) coated with a 50nm silica surface layer were used in QCM-D experiments. Prior to use, the crystals were cleaned by immersion into 2% Hellmanex for 30 minutes.

Poly (methyl methacrylate), PMMA, coated AT cut of quartz crystals, QSX 999 (fundamental frequency of 4.95Hz, Västra Frölunda, Sweden) were used in QCM-D experiments as hydrophobic surfaces. All surfaces mentioned above were rinsed by large amount of Milli-Q water, and then dried under a gentle flow of filtered nitrogen gas prior to use.

The silica (d ≈ 7 µm) and PMMA (d ≈ 10 µm) colloidal probes used in AFM force and friction measurements were provided by Bang Laboratories (Fishers, US) and Kisker (Steinfurt, Germany), respectively. Before use, the silica colloidal probe cantilevers were cleaned by UV irradiation for 15 minutes (output 15 mW/cm², BioForce, US) whereas the PMMA particles were rinsed with pure water.

2.2 Methods

2.2.1 Phospholipid vesicle preparation

DPPC vesicle solutions were prepared by first dissolving DPPC powder in a small amount of chloroform, and then drying it under gentle nitrogen flow using rotary evaporation to allow the lipids to cover the walls of the bottle evenly. The lipids were then placed into a vacuum desiccator overnight to remove eventual traces of chloroform. Next, buffer solution that mimics physiological concentration (0.155Mm NaCl or PBS buffer) was added at 55 °C
(controlled by a water bath) to give a DPPC dispersion of 1 mg/mL. This dispersion was then placed into an ultrasound bath (Bandelin Sonorex Digitec, output 640 W) until the solution became almost clear (∼60 min). The solution was then diluted to 0.5 mg/mL and sonicated for an additional 15–30 min until totally transparent. The temperature was kept at 55 °C ± 2.5 °C during the whole sonication process. The pH of the final vesicle stock solution was measured to be 5.6–6.1. The prepared vesicles were stored at 55 °C for at the most 4 hours before being used in experiments.

2.2.2 Dynamic Light Scattering (DLS)

Dynamic Light Scattering,[71] also known as Photon Correlation Spectroscopy, is one of the most effective methods to analyse particle size and size distributions in solution. Based on the Brownian motion due to collisions between the particles and surrounding solvent molecules, DLS can measure the diffusion of the particles.[72] The translational diffusion coefficient is related to the particle size according to the Einstein equation:[73, 74] \[ d = \frac{kT}{3\pi\eta D} \] (2-1)

Where \( d \) is the hydrodynamic diameter, \( D \) the translational diffusion coefficient, \( k \) the Boltzmann’s constant, \( T \) the absolute temperature and \( \eta \) the viscosity. It is worth noticing that by applying the Einstein equation one determines an equivalent sphere diameter, i.e. the diameter of a sphere that has the same translational diffusion coefficient as the particles under study. The CONTIN algorithm [75, 76] was used for analyzing the autocorrelation function and determining the particles size distribution. The polydispersity was calculated from a cumulants analysis[77] of the DLS-measured intensity autocorrelation function.

2.2.3 Quartz Crystal Microbalance with Dissipation (QCM-D)

The QCM-D instrument is a highly sensitive balance that determines mass changes of an AT cut crystal by measuring changes in frequency and dissipation. By monitoring the frequency
and dissipation shifts for several different overtones, the increased or lost mass can be calculated very accurately even for viscoelastic layers. This mass includes the mass of the adsorbing species and the mass of water associated with the adsorbed layer.[78] Thus, we refer to this mass as the sensed mass to distinguish it from the adsorbed mass that is detected by optical techniques.

The QCM-D can also provide information of the viscoelastic property of the adsorbed film. This can be achieved by measuring the decay of the oscillation amplitude of the crystal when the driving voltage is turned off. The dissipation is defined as:[79]

$$D = \frac{E_{\text{lost}}}{2\pi E_{\text{stored}}}$$

(2-2)

Where $E_{\text{lost}}$ is the energy lost during one oscillation cycle and $E_{\text{stored}}$ is the total energy stored in the oscillator. Formation of thin, rigid films give rise to small dissipation changes compared to those associated with formation of thick and soft ones. Depending on the viscoelastic properties of the layers, different models have to be used to analyse QCM-D data. In my work I used two models, briefly described below.

The Sauerbrey model[80] is applicable when the dissipation change is small (normally less than 1E-6), that is for thin and rigid layers. In this case the frequency change normalized by overtone number will overlap for different overtones, and the sensed mass, $\Delta m$, can be calculated as:[80]

$$\Delta m = -C \frac{\Delta f_n}{n}$$

(2-3)

Where $C$ is the sensitivity factor for the crystal used and equals 17.7ng cm$^{-2}$ Hz$^{-1}$ for the crystals I used, $\Delta f_n$ is the change in the resonance frequency of the $n$th overtone. In this work the Sauerbrey model was used for analyzing the results obtained for the DPPC bilayer attached directly to the silica sensor.

The Sauerbrey model underestimates the sensed mass when a viscoelastic layer is formed.[81] The damping of the crystal increases due to the viscous dissipation in the film, and therefore
the measured frequency change is a consequence of the mass change and the viscoelastic properties of the film.[82, 83] In this case a viscoelastic model should be applied to quantify the sensed mass, as well as the viscoelasticity of the adsorbed film, as illustrated in Figure 2.1.

**Figure 2.1** Schematic of the relation between spring model, dashpot model and Voigt model.

In my work, the Voigt model [84] was used to analyse adsorbed viscoelastic layers. In the Voigt model the total stress acting on the film is modelled as an elastic spring and a viscous dashpot element coupled in parallel:

\[
G^* = G' + jG'' = \mu + j2\pi f\eta
\]  

(2-4)

Where the \( G' \) and \( \mu \), \( G'' \) and \( 2\pi f\eta \) are storage modulus (elastic shear modulus), loss modulus (\( \eta = \) shear viscosity) of the film, respectively.[82] \( j \) is equal to \( \sqrt{-1} \).

**Figure 2.2** Schematic for three layers system that used for Voigt model simulation.

A detailed expression for calculating the frequency change (\( \Delta f \)) and dissipation changed (\( \Delta D \)) is given below:[85-87]

\[
\Delta f = -\frac{1}{2\pi \rho_0 h_0} \left\{ \frac{\eta_2}{\delta_2} + h_1 \rho_1 \omega - 2h_1 \left( \frac{\eta_2}{\delta_2} \right)^2 \frac{\eta_1 \omega^2}{\mu_3 + \omega^2 \eta_1^2} \right\}
\]  

(2-5)

\[
\Delta D = -\frac{1}{\pi f \rho_0 h_0} \left\{ \frac{\eta_2}{\delta_2} + 2h_1 \left( \frac{\eta_2}{\delta_2} \right)^2 \frac{\eta_1 \omega^2}{\mu_1 + \omega^2 \eta_1^2} \right\}
\]  

(2-6)
where \( \rho \) and \( h \) are the density and thickness, \( \eta \) the shear viscosity, \( \delta \) the viscous penetration depth of the shear wave in the bulk liquid, \( \mu \) the shear elasticity and \( \omega \) the angular frequency of the oscillation. Different subscripts correspond to different layers and a schematic for three layers system is illustrated in Figure 2.2: 0 for crystal, 1 for adsorbed layer and 2 for bulk solution. Changes in frequency and dissipation for several overtones need to be used together to calculate the sensed mass, and the 3\(^{rd} \), 5\(^{th} \), 7\(^{th} \) overtones were used in my studies. In practice these calculations were carried out by using the program Q-tools supplied by Q-sense (Gothenburg, Sweden).

### 2.2.4 Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) is an accurate, versatile and widely used instrument for analyzing surface properties. It was invented by Binnig et al. in 1986[88], and after almost three decades it has developed into more than 20 measurement modes that allow local determination of topography, mechanical, electrical and magnetic properties. Below I briefly describe the main parts of the AFM, and the measurement modes that I used in my thesis work.
**Figure 2.3** Schematic illustration of the most important parts of an AFM: laser, photodiode, cantilever and scanner. A cantilever with a sharp tip is used for AFM imaging, while a colloidal probe is often used for force and friction experiments.

Typically an AFM instrument can be divided into three main parts: the force detecting part (cantilever), position controlling part (scanner) and the signal feedback part (photodiode) with associated electronics (see Figure 2.3). The scanner with the sample can be moved with high precision in x, y and z directions. The cantilever is a soft, force sensitive arm with a sharp tip or colloid sphere at one end. When the sample is interacting with the tip, the cantilever will bend or twist, causing the projected laser position to shift on the photodiode. The photodiode voltage signal will send a feedback to the scanner that, in imaging mode, will react to bring the laser beam back to its original position.

### 2.2.4.1 PeakForce QNM Imaging

PeakForce QNM is a relatively new imaging mode designed for collecting simultaneous information about surface topography and surface material properties.[89-93] Briefly, the surface position is modulated by a sine wave where the sample during each period of oscillation is moved into contact with the AFM tip. Feedback electronics adjust the surface position so that the maximum cantilever deflection (the peak force) equals a predetermine set point value. The information about cantilever deflection and piezo position can be converted to force vs. distance curves describing the tip-sample interaction during approach and separation. From such a force curve, as illustrated in Figure 2.4, one can read the surface deformation due to the tip-sample interaction, the maximum adhesion force between the tip as well as the amount of energy that is dissipated during the interaction. Due to the ability to scan the sample with a low and controlled (peak) force – 500 pN in most of my work – and limited shear forces between the tip and the sample, this mode is highly suitable for imaging soft delicate samples as the ones I investigated.
2.2.4.2 Surface force measurements

Forces acting in the normal direction – surface force – is determined by bring two surfaces together and then separating them again, as illustrated in Figure 2.4. For investigating the nature of the surface forces the AFM colloidal probe technique is preferred since the large size of the probe allows determination of weak forces.

Some important regions can be defined in the force curves. The zero force region is found at large separation when there is no interaction between the two surfaces. At separations smaller than the zero force region, the interesting surface force region is found. It is the distance dependence of the forces operating in this region that one aims to determine. In AFM colloidal force studies, the thickness of adsorbed layer cannot be known from the force measurements. Thus, one needs to define a zero separation. In my case I defined it as the position of the surfaces at the highest load applied. When the surfaces are pressed hard together, the constant compliance region can be found. Here the cantilever deflects as much as the scanner moves.

Figure 2.4 Schematic illustration of a force curve determined on approach and separation indicating which part of the force curve that provides information about the nano-mechanical properties.
From the slope of the constant compliance region we can determine the normal detector sensitivity, $\alpha$:

$$\alpha = \frac{\Delta V}{\Delta x} \quad (2-7)$$

Where $\Delta V$ is the deflection distance of the cantilever, and $\Delta x$ the displacement of the surface. When calculating the force a transformation from photo detector voltage change $\delta V$, the measured quantity, to cantilever deflection, $\delta m$, is made:

$$\delta (m) = \delta (V) \cdot \frac{1}{\alpha} \quad (2-8)$$

$$F_{normal} = \delta (m) \cdot k_z \quad (2-9)$$

Where $k_z$ is the spring constant.

The Derjaguin approximation is very useful as it relates the force $F(h)$ between a sphere and a flat surface, in my experimental geometry, to the free energy of interaction per unit area between identical flat surfaces, $W(h)$:

$$F(h) = 2\pi R W(h) \quad (2-10)$$

Here $h$ is the surface separation, and $R$ the sphere radius. This shows that if we normalize the measured force with sphere radius, $F(h)/R$, then we can directly compare data obtained with spheres of different size.

**2.2.4.3 Friction force measurements**

A friction force measurement can be achieved by sliding the surface forwards and backwards in contact mode.[96] The sliding direction should be perpendicular to the cantilever. The twist of the cantilever is recorded by the photo-detector as a voltage change. Figure 2.5 shows at different stages. The reported friction force is averaged from several friction loops at each load.
Figure 2.5 A schematic illustration of 10 typical friction loops in one friction force measurement. ΔV is the detector signal voltage difference between friction forces in trace direction (black) and retrace direction (red), and can be recalculated to force by equation 2-11. Schematic probe cantilever images are also shown in the figure to give a clear description of different stages.

Conversion of the photo-detector signal into friction force versus applied load is done by means of the following equation:

\[
F_{Friction} = \frac{ΔV \ k_t \ 1}{2 \ α \ h} \tag{2-11}
\]

Where the ΔV is the average voltage difference between trace and retrace (see Figure 2.5). \(k_t\) the torsional spring constant, \(α\) the lateral deflection sensitivity and \(h\) the effective height (diameter of the probe plus half the thickness of the cantilever). If the friction force as a function of applied load follows Amontons’ rule, then the value of the friction coefficient, \(μ\), can be calculated by equation 1-8.
Conversion of applied force to pressure

When discussing friction results the applied pressure is more relevant than the applied load. The reason that the pressure can be compared for different sized spheres and for materials with different elastic modulus. To convert the applied load to pressure one needs to use a contact mechanics model. The PMMA samples are soft and deformable, and then the JKR model is appropriate.[97] In this model, the contact area is given as:

\[ A = \pi \left[ \frac{R}{E^*} \left( F_n + 6\pi R \gamma + \sqrt{12\pi R \gamma F_n + (6\pi R \gamma)^2} \right) \right]^{2/3} \] (2-12)

Where \( R \) is the colloidal particle radius, \( F_n \) the normal load and \( \gamma \) the interfacial tension calculated from the measured adhesion force, \( F_{adh} \), as:

\[ \gamma = \frac{F_{adh}}{3\pi R} \] (2-13)

\( E^* \) is the effective elastic modulus of the surface, calculated from the Young’s modulus and the Poisson ratio of the flat surfaces and particle materials as:

\[ E^* = \left\{ 2 \left\{ \frac{1-\gamma^2}{E} \right\} \right\}^{-1} \] (2-14)

The pressure, \( P \), is calculated as:

\[ P = \frac{F_{load}}{A} \] (2-15)

2.2.5 X-ray reflectivity, XRR, measurements

X-ray reflectivity measurements on the silica-liquid interface were performed at the beamline BL9, Delta, Germany and the X04SA, SLS, Switzerland in order to characterize adsorbed phospholipid bilayers at different temperatures. The contrast in X-ray reflectivity measurements arises due to differences in electron density, and thus a heavy element scatters more than a light one. In X-ray reflectivity measurements the specular reflected intensity \( I \) is measured as function of the incident angle \( \theta \). The scattered intensity is modulated by the
sample’s electron density perpendicular to the surface, and thus by modeling one can extract information on structural variations normal to the surface. In order to model our DPPC bilayers on silicon surface with a thin silicon oxide layer required the use of a 6-layer model as described in Paper IV.
3. Key results and discussion

As briefly mentioned in earlier sections of this thesis, historically the scientific community has split into two teams with the distinct views as to what is the main lubricant in joints. On one side of the barricades scientist were defending the view that it is phospholipids that provide lubrication.[98] On the other side the adepts of biomacromolecules were defending “honour” of lubricin as the main lubricating molecule in joins.[99] In my thesis work I take on clothes of a “peacemaker” and advocate that it is the synergy of the components that does the work.

3.1 Biolubricants on surfaces

Four different biolubricants were investigated in my work – individually and in combination with each other. Three biopolymers - hyaluronan, lubricin and COMP, and one small molecular weight compound – DPPC were employed. The investigated surfaces were both hydrophilic (silica) and hydrophobic (PMMA). The behaviour of the biolubricants on both types of surfaces is physiologically relevant since both hydrophilic and hydrophobic elements are present on the cartilage surface.[24]

3.1.1 Hyaluronan at the negatively charged silica surface

Hyaluronan, which is an anionic polyelectrolyte, does not have any affinity to the negatively charged silica surface (Figure 3.1). This result agrees well with the results reported by Israelachvili et al.,[100] who using a Surface Forces Apparatus observed that at physiological conditions hyaluronan does not naturally adsorbs on the hydrophilic and negatively charged mica surface, and only slips away from the gap between the surfaces upon compression.
3.1.2 Lubricin

Lubricin was found to loosely adsorb from buffered (pH = 7.5) physiological solution on a weakly hydrophobic (water contact angle 68 °) PMMA surface. The adsorption of lubricin was presumably driven by a nonspecific hydrophobic interaction between non-glycosylated protein parts of lubricin and PMMA. It was easy to expel weakly adsorbed lubricin from between the surfaces by applying a force as low as ~ 0.15 mN/m, and this can be seen from the surface jump in (Figure 3.2). As can be expected, friction between two PMMA surfaces coated with lubricin was relatively high. For any given load the friction force is higher on unloading, suggesting that lubricin is removed from the surface due to the combined action of load and shear. As seen from the results, lubricin alone is not a superior lubricant on moderately hydrophobic surfaces. Also in this case, my results are in good agreement with observations of Zauscher et.al.[24] who studied friction mediated by lubricin adsorbed on CH$_3$ terminated surfaces and detected relatively high friction coefficients of 0.1 – 0.2, depending on lubricin concentration.

**Figure 3.1** Adsorption from a 0.5 mg/mL hyaluronan solution in 155mM NaCl on a silica surface solution at 55 °C (frequency (○) and dissipation (●) change). Rinsing was done with 155mM NaCl solution at 55 °C. For clarity, only every 46th point is plotted. The pH of the hyaluronan solution was about 6.5.
Figure 3.2 (a): Force normalised by radius as a function of surface separation between PMMA surfaces coated with lubricin measured across 150 mM PBS (pH = 7.5) (b): Friction force as a function of load. Solid and open circles represent loading and unloading, respectively.

3.1.3 Cartilage Oligomeric Matrix Protein (COMP)

The QCM-D studies showed that monomeric COMP readily adsorbs on PMMA surfaces (Figure 3.3), and an about 23 nm thick layer with a sensed mass of 26.5 mg/m² was formed. The forces measured between such layers are completely repulsive both on approach and separation (see Figure1.8). The COMP layer adsorbed on PMMA reduces the friction markedly, and the friction coefficient is ~0.3. The effect of COMP as a protein that reduces friction between surfaces can be compared to that of serum albumin, the most abundant protein in periprosthetic fluid that often provide a friction coefficient of about 0.2.[101] Albumin has been recognized as critical in boundary lubrication. However, close attention has to be paid on adsorbed amount and conformation of the albumin[101] as depending on the conditions it may either favor lubrication or affect it adversely (e.g. when coagulation takes place).
3.1.4 Synergy between COMP and lubricin

As seen from the above discussion, despite that lubricin and COMP clearly are able to markedly reduce the friction between hydrophobic surfaces sheared in aqueous solution, in none of the cases described thus far friction even approaches that found in synovial joints. Thus there is a reason to suggest that in joint the biolubricants operate in synergy and thus exhibit cooperative effects that cannot be seen when they act individually in our experiments. Indeed, the work of my collaborators from the Gothenburg University on mapping the occurrence of synovial molecules clearly shows that COMP and lubricin are both enriched and co-localised on the surface of cartilage. I carry this further by adsorbing lubricin on COMP-covered PMMA by exposing it to a solution containing 100 μg/mL human lubricin dissolved in 150 mM PBS. This results in formation of a lubricin layer that is about 20 nm thick and has a sensed mass of about 23 mg/m², as determined from the QCM-D data shown in Figure 3.4 a. When normal forces are measured between surfaces carrying lubricin adsorbed on COMP, completely repulsive forces are measured both on approach and separation. And further,
specific association of lubricin with COMP on surfaces leads to marked reduction of friction, and now the coefficient of friction is only about 0.06 up to the load corresponding to a pressure of at least 7 MPa with no hysteresis between data obtained during loading and unloading, signifying that the COMP-lubricin layer has a high resistance to wear (Figure 3.4 b). Thus, we have clearly demonstrated synergy between COMP and lubricin on PMMA. COMP acts as an anchor for lubricin, binding it strongly to the surface in a conformation that is suitable for generating low friction. Neither COMP nor lubricin alone perform equally well as lubricants for PMMA.

![Figure 3.4](image)

**Figure 3.4** (a): Adsorption of lubricin on COMP-coated PMMA at 25°C from 100 μg/mL solution in 150 mM PBS, pH 7.5. Rinsing was done with 150 mM PBS. Solid and open symbols are frequency change and dissipation change, respectively. For clarity, only every 35th point is plotted. (b): Friction force as a function of applied load between PMMA surfaces coated with COMP (open circles) and COMP-lubricin (solid circles).

### 3.1.5 Phospholipids as lubricants

One of the important phospholipids found in synovial fluid is DPPC, which in free bilayers in contact with aqueous solution undergoes a main phase transition temperature at 41.5 °C. It is unlikely that the mixed phospholipid films on bodily surfaces are in frozen state, thus I have investigated DPPC as lubricant at a range of temperatures covering the gel and liquid crystalline states.
3.1.5.1 DPPC bilayer surface morphology at different temperatures.

In order to characterize the DPPC bilayer structure on the silica surface at different temperatures, we utilized X-ray reflectivity, XRR, measurements and AFM imaging. The XRR measurements allow us to obtain a detailed picture of how the electron density varies with the distance from the supporting silica surface, and whereby define the location of the headgroups and tailsgroups of DPPC. The results are shown below in Figure 3.5.

![Figure 3.5](image)

**Figure 3.5** Electron density profiles of DPPC bilayers at the silica liquid interface at different temperatures. \( z \) denotes the distance from the silicon surface. A small sketch of the bilayer structure is shown as inset.

The XRR data show that the DPPC bilayer at 25°C has a total thickness of 5.6 nm, consistent with AFM data reported in Paper IV, and a similar thickness is found at 39 °C. However, when the gel layer melts at higher temperatures the bilayer thickness decreases to 4.8 nm, and the interface towards water becomes rougher due to increased thermal motion of the molecules.

Imaging of the DPPC bilayer at different temperatures in pure 150 mM PBS solution revealed clear morphological changes taking place with temperature (Figure 3.6). The images recorded at 52 °C and on cooling to 47 °C and 37 °C are similar, only showing gentle height variations. A further cooling to 32 °C renders the surface more coarse and small grainy structures are clearly seen in the image recorded at 25 °C. Heating the bilayer again to 32 °C does not change the morphology back to that observed at the same temperature as on cooling, suggesting a hysteresis during the time scale of the measurement (the time between recording images at the
different temperatures is about 30 min). Heating to 37 °C and above changes the morphology towards that observed at these temperatures prior to cooling. At temperatures of 32 °C or below, the bilayer imaged with AFM remains in the gel phase and small domains are visible. The height variation across the surface is somewhat larger when the grainy structure is observed, see Figure 3.6 a.

**Figure 3.6** AFM PeakForce height images of a DPPC bilayer on a silica surface, taken at different temperatures. The images were recorded in 150 mM PBS buffer solution, and flattened to remove tilt. The image size is 1x1 µm², and the height scale bar is 3 nm in each case. The arrows show the different order at which the images were recorded, and the temperature is provided below each image. (a): Scan lines over the regions marked with correspondence colors in height images at 52 °C and 25 °C, respectively.

It is interesting to note that the phase transition observed with XRR occurs at higher temperature than the morphological change noted during AFM imaging. The reason for this is
that XRR determines the structure of the unperturbed bilayer whereas AFM imaging disturbs the bilayer. Most importantly the tapping AFM tip transfers kinetic energy to the bilayer that in our case shifts the phase transition temperature downward by several degrees. This is consistent with the recent observation that aggregate structures of cationic surfactants on mica surfaces reported by AFM imaging are inconsistent with XRR data for the same systems\cite{102} due to energy transfer between the tip and the imaged soft matter structure.

3.1.5.2 Surface forces

The force acting between DPPC bilayer covered silica surfaces were measured in 150 mM PBS solution at different temperatures. In Figure 3.7, force curves obtained at 25 and 47 °C are shown. Clearly, there is no long-range interaction between the surfaces at any of the temperatures. At short separations, 1~2 nm from a hard wall contact, a repulsion is observed that is due to the combination of protrusion and hydration forces.\cite{103, 104} This force increases with increasing temperature, suggesting predominance of the protrusion force contribution.

![Force curves](image)

**Figure 3.7** Force normalised by radius as a function of surface separation, measured between a silica surface and a silica sphere (r= ~3.5μm) coated by a DPPC bilayer immersed in 150 mM PBS. Symbols (●) and (○) represent forces measured on approach and separation, respectively. The force curves determined at 25 °C and at 47 °C are shown.
### 3.1.5.3 Friction forces

The friction force as a function of load between DPPC bilayer coated silica surfaces measured at different temperatures are shown in Figure 3.8. At all temperatures the friction forces are low and remain such even at the highest loads applied. Thus, DPPC bilayers provide excellent lubrication under the combined action of high load and shear with a friction coefficient of less than 0.03 at temperatures of 47 °C and below. The maximum load, converted to average pressure using the JKR model,[97] is found to be around 42 MPa, which is close to twice as high as the axial load that the cartilage can sustain before breaking.[105] Thus, the load bearing capacity of the deposited bilayers of DPPC is very large. The data presented in the inset in Figure 3.8 shows that the DPPC bilayer is good lubricant in both 150 mM PBS and 155 mM NaCl solutions at 47°C.

![Friction force graph](image)

**Figure 3.8** Friction force as a function of load between DPPC bilayer coated silica surfaces immersed in 150 mM PBS at different temperatures. Inset: Comparison of friction forces between DPPC bilayers coated silica surfaces measured across 150 mM PBS (open circles) and across 155 mM NaCl at 47 °C (filled circles).

In some cases we note that the bilayer structure can be destroyed during shearing. An example of surface failure at 25 °C was adopted to illustrate the phenomenon in Figure 3.9. The friction
force remained low up to a load of about 18 nN, indicating that the DPPC bilayers are intact and bear the load. However, as the load was increased further the friction grew significantly, suggesting that the integrity of DPPC bilayers have been compromised by the combined action of load and shear. The failure of the bilayer activates a new energy dissipative process that increases the friction force, for example attractive hydrocarbon-hydrocarbon contacts between phospholipid acyl chains may develop between the sliding surfaces, and thus contribute to high friction force. As can be seen in the Figure 3.9, the friction force gradually decreases while the load is decreased again. Still, it remains significantly higher than that observed on loading, suggesting that once the DPPC bilayer is destroyed, it does not heal again at 25 °C.

Figure 3.9 An example of a friction vs. load cycle measured between DPPC bilayers at 25 °C in 150 mM PBS. In this case the bilayer structure was compromised at a load of about 18 nN. Friction forces measured on loading and unloading are shown with filled (●) and unfilled (○) symbols, respectively.

The load bearing capacity of the bilayer in an individual experiment can be determined accurately from curves of the type shown in Figure 3.9. However, the load bearing capacity found at a given temperature differs between different experiments and one has to use a statistical evaluation as shown in Figure 3.10.
The percentage of friction experiments performed at a given temperature where the load bearing capacity was found to be less than 20 nN.

At 25 °C, where the acyl chains are in the frozen state, 50% of the experiments showed bilayer failure in total 8 experiments. However, no DPPC bilayers were compromised at 52 °C and only 1 was compromised at 47 °C, where the acyl chains are in fluid state. Thus, it appears that the fluidity of the bilayer above the chain melting temperature improves the load bearing capacity by reducing the brittleness of the layer and increasing its healing capacity. This also explains why bilayers remain intact during the AFM imaging at these temperatures. The good lubrication performance is due to the short-range but strong protrusion/hydration force that allows an easy sheared water layer to remain between the bilayers even under high load.

3.2 Is there a lubrication synergy between DPPC and hyaluronan?

As we have seen from the discussion above, DPPC is a truly superb lubricant, capable to reduce friction to very low levels and simultaneously to withstand very high applied loads. Hyaluronan, on the other hand, did not give much promise as a lubricant. So why then does Nature pack synovial fluid full with hyaluronan? [14, 106] Is there a lubrication synergy between DPPC and hyaluronan? With these questions in mind I formulated my research that I
will summarize below. In this part of the work, the association and the lubrication properties of DPPC and hyaluronan are analysed and discussed.

### 3.2.1 Association of phospholipid and hyaluronan in bulk solution

DPPC vesicles prepared by the sonication method (see Paper II) with a hydrodynamic diameter of around 110 nm were characterized by dynamic light scattering (See table 3.1). Addition of hyaluronan (hydrodynamic diameter ~70 nm), to the DPPC vesicle dispersion lead to increase in mean diameter of the scattering units in the mixture to 140 nm, indicating DPPC and hyaluronan association. The suggested structure of DPPC-hyaluronan is that of a vesicle wrapped by hyaluronan with the estimated hyaluronan hydrodynamic layer thickness being about 15 nm.

**Table 3.1** Hydrodynamic diameter and polydispersity as determined from DLS data evaluated by the CONTIN method.

<table>
<thead>
<tr>
<th></th>
<th>0.5mg/mL hyaluronan</th>
<th>0.5mg/mL DPPC</th>
<th>0.5mg/mL mixture of DPPC and hyaluronan (1:1 ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrodynamic diameter (nm)</td>
<td>70</td>
<td>110±4</td>
<td>140±8</td>
</tr>
<tr>
<td>Polydispersity</td>
<td>0.20</td>
<td>0.24±0.01</td>
<td>0.27±0.04</td>
</tr>
</tbody>
</table>

### 3.2.2 Association of phospholipid and hyaluronan on silica surface

DPPC adsorbs significantly on the QCM-D silica crystal as shown in Figure 3.11 a. As seen from Peak Force QNM height images, the DPPC bilayer that forms is smooth and featureless. (Figure 3.11 b). The initial peak in dissipation and minimum in frequency indicates vesicle adsorption followed by vesicle rupture. After this transition region, the frequency change stabilizes at a value of about – 25 Hz, whereas the dissipation increases slowly. No changes are observed upon rinsing. The QCM-D data are consistent with what has been reported for POPC and DOPC, and mixed DOPC/DOPS bilayers. [107-110] As can be seen in Figure 3.11 c, a significant adsorption of hyaluronan on the DPPC bilayer occurs and only a little amount of
hyaluronan can be removed by rinsing with 155 mM NaCl solution. Again, PeakForce QNM imaging shows a clear morphological change in the adsorbed layer (Figure 3.11 d), induced by hyaluronan adsorption.

![Figure 3.11](image)

**Figure 3.11** (a): DPPC adsorption on a silica surface from 0.5 mg/mL DPPC solution in 155 mM NaCl at 55 °C monitored by QCM-D (frequency (○) and dissipation (●) change). Rinsing was done with 155 mM NaCl solution at 55 °C. For clarity, only every 46th point is plotted. (b): Peak Force QNM height image of DPPC bilayer deposited on silica surface. (c): Adsorption of hyaluronan on DPPC bilayer (frequency (○) and dissipation (●) change). For clarity, only every 20th point is plotted. (d): PeakForce QNM image of DPPC bilayer with hyaluronan adsorbed on it. The pH of the hyaluronan solution was about 6.5, and that of the DPPC vesicle solution was 5.6~6.1.
3.2.3 Sequential DPPC and hyaluronan adsorption

Sequential adsorption of DPPC and hyaluronan on silica from 155 mM NaCl solution is shown in Figure 3.12. A total of 7 DPPC injections and 6 hyaluronan injections were executed. It is thus clear that sequential addition of DPPC and hyaluronan can be used for building a composite layer of the two components, even when using hyaluronan with somewhat lower molecular weight than that found in healthy synovial fluid. This is in agreement with the observation of Steitz et al. that low molecular weight hyaluronan stabilizes multiple phospholipid bilayers on silicon surfaces. [111] Thus, phospholipid – hyaluronan multilayers have been shown to be stable on both hydrophilic silica and hydrophobic silicon surfaces. This suggests, but does not prove, that such association structures may also spontaneously form and be used for lubrication of cartilage surfaces in vivo.

![Figure 3.12](image.png)

**Figure 3.12** Sequential DPPC and hyaluronan adsorption on silica surface from 0.5 mg/mL DPPC and 0.5 mg/mL hyaluronan in 155mM NaCl solution. The adsorption was carried out at 55 °C. Downward arrows indicate rinsing by 155 mM NaCl solution. Arrows marked “D” show DPPC injections and arrows marked “H” show hyaluronan injections. For clarity, only every 91th data point is plotted. Data captured from the third overtone. Filled and unfilled symbols represent dissipation and frequency changes, respectively.
3.2.4 Adsorption from mixed hyaluronan-DPPC vesicle solutions

It is unlikely that Nature would engage in layer-by-layer deposition of synovial components on cartilage surfaces. Thus it is of interest to investigate how the growth of adsorption layers would appear from the mixtures of purported biolubricants. The adsorption form mixture of DPPC and hyaluronan is shown in Figure 3.13. Initially, after injection of the DPPC/hyaluronan mixture the frequency rapidly changes by ~25Hz. This indicates that a DPPC bilayer initially forms (cf. Figure 3.11 a). However, adsorption does not stop here, but both the magnitude of $\Delta f$ and $\Delta D$ continue to increase with time in a close to linear manner. The adsorption was allowed to continue for about 100 minutes, but no tendency of surface saturation was observed. During the rinsing procedure, which lasted for 20 minutes, the frequency and dissipation changes were small, suggesting limited desorption. AFM tapping mode images show that the layer that forms due to adsorption of DPPC/hyaluronan mixture is less homogenous than that formed by DPPC alone (Figure 3.13 b). We judge that DPPC/hyaluronan aggregates were formed on top of the DPPC bilayer, and the lateral size of these aggregates was measured to be in the range 150 – 250 nm, consistent with the expected size of flattened DPPC/hyaluronan vesicles. A second adsorption step was then initiated by injecting the DPPC/hyaluronan mixture again. This resulted in a close to linear change in $\Delta f$ and $\Delta D$ with time, demonstrating continued adsorption.

![Figure 3.13](image)

Figure 3.13 (a) Adsorption from a 155 mM NaCl solution containing 0.5 mg/mL DPPC and 0.5 mg/mL hyaluronan (marked as ↑) on a silica surface at 55 °C, monitored by QCM-D.
(frequency (●) and dissipation (○) change). Rinsing (marked as ↓) was done with 155 mM NaCl. For clarity every 30 points are shown. (b) AFM tapping mode image of a DPPC/hyaluronan mixed layer adsorbed on silica.

3.2.5 Friction between surfaces coated with DPPC and DPPC/hyaluronan complexes

Friction forces between DPPC bilayer coated silica are low with high load bearing capacity (Figures 3.8 and 3.14 a). The friction coefficient, μ, is around 0.01, and the load bearing capacity is at least 68 MPa. The friction force between hyaluronan coated DPPC bilayers on silica surfaces (Figure 3.14b) is still low, 0.03, and the load bearing capacity remains high. However, the friction coefficient increases to 0.1 when the load is about 20 nN, indicating that a new energy dissipative mechanism become operative. We suggest that this is caused by disruption and reformation of the DPPC-hyaluronan attachment sites during shearing.[112, 113] If we take 20 nN as the load bearing capacity, then it corresponds to a pressure of 56 MPa.

![Figure 3.14](image)

**Figure 3.14** (a) Friction force as a function of load between two silica surfaces coated by DPPC bilayers (■), and DPPC adsorbed on previously formed DPPC-hyaluronan layers (□). (b) Friction force between surfaces coated by hyaluronan adsorbed on previous formed DPPC bilayers (●) and hyaluronan layer coated on previous DPPC–hyaluronan–DPPC layers (○).

Adsorption of additional DPPC on already formed DPPC–hyaluronan layer brings the friction force back to low values and high load bearing capacity (Figure 3.14a). Adsorption of further hyaluronan on the DPPC-hyaluronan-DPPC layer leads to a new friction increase (Figure
3.14b), which is consistent with the adhesive force that we observed during measurement of surface forces.

Friction forces between layers formed from solutions containing 0.5mg/mL DPPC and 0.5mg/mL hyaluronan on silica at 47 °C are shown in Figure 3.15. The friction coefficient was found to be around 0.006, up to the maximum load of 25 nN. This result is similar to what we reported for DPPC bilayers (µ = 0.01) in Figure 3.14 a. The maximum load during the experiment, converted to pressure, is around 45 MPa.

**Figure 3.15** Friction force as a function of load between two silica surfaces carrying an adsorbed layer formed by adsorption from a 155mM NaCl solution containing 0.5mg/mL DPPC and 0.5mg/mL hyaluronan in 155mM NaCl. The temperature was 47 °C. Insert: Illustration for mixture absorbed onto the silica surface during the friction test.
4. Summary and concluding remarks

In this thesis work the focus was on exploring lubrication performance of several biomolecules found in synovial joint, such as phospholipids, hyaluronan, lubricin and COMP. The main question addressed was if there is a synergy between the synovial components that result in lubrication that is far better that that granted to surfaces by the individual components. A battery of techniques was employed to tackle these questions - Dynamic Light Scattering (DLS), Quartz Crystal Microbalance with Dissipation monitoring (QCM-D), AFM scanning probe technique for surface imaging and AFM colloidal probe technique for surface force and friction studies.

My main finding – somewhat unexpected - was that phospholipid, DPPC, alone is perfectly capable of lubricating surfaces with very low friction coefficients and high load bearing capacities. Interestingly, the load bearing capacity of DPPC bilayer adsorbed on silica surface increases with increasing temperature, i.e. with increasing ability of the bilayer molecules to rearrange and heal small imperfections caused by the scanning colloidal probe (Paper IV).

The presence of one of the major synovial components, hyaluronan, does not improve the lubrication by phospholipids but it does not destroy it either. However, it does enable large phospholipid quantities to accumulate on surfaces (Papers I and II) whereby an essential lubricant is collected in the critical area. Thus my conclusion is that the synergy between hyaluronan and phospholipids is in hyaluronan facilitating phospholipid accumulation on the surface. In Paper III we can see that the preformed mixture of DPPC/hyaluronan lubricates as well as DPPC alone.

Glycoproteins also play an important role in synovial lubrication. I have investigated two of them – lubricin and COMP – and showed that COMP is essential in structuring lubricin on the moderately hydrophobic PMMA surface that I used. In fact, lubricin, which by many is regarded as the main lubricant in synovial joints, did not bind strongly to PMMA and thus it was a very poor lubricant on its own. However, if COMP first was adsorbed and then lubricin was allowed to bind to the COMP-coated surface, then excellent lubrication performance was
observed. Thus, there is a clear synergy between COMP and lubricin, where COMP facilitates surface anchoring of lubricin in conformations that allow efficient lubrication.
5. List of abbreviations

DPPC  \(1,2\text{-dihexadecanoyl-sn-glycero-3-phosphocholine}\)
COMP  Cartilage Oligomeric Matrix Protein
PBS   Phosphate buffered saline
PMMA  Poly (methyl methacrylate)
\(M_w\)  weight average molecular weight
\(M_n\)  number average molecular weight
\(R_g\)  Radius of gyration
QCM-D  Quartz Crystal Microbalance with Dissipation
DLS   Dynamic Light Scattering
AFM   Atomic Force Microscopy
AFM PeakForce QNM  AFM PeakForce Quantitative Nanomechanical Property Mapping
XRR   X-ray reflectivity
6. Acknowledgments

First and foremost, I would like to thank Assoc. Porf. Andra Dédinaitė and Porf. Per Claesson for providing me with the precious opportunity to study at KTH. I still clearly remember that exciting telephone interview 4 years ago, and your offer let me receive more than what I expected during the 4 years study.

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Finally, to my 4 months old son, Ruizhi, You are my pride! You know it.
7. References


