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RESEARCH

Visualization of Lubrication Film in Model of Synovial Joint

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A B S T R A C T

Synovial joint is one of the most important parts for human movement system and the right function of it is necessary. When the synovial joint is damaged by illnesses, destroyed natural joint is exchanged for artificial joint. They are commonly used in nowadays, but there are problems with their limited lifetime. Alternative treatment procedures in surgery start appearing in order to postpone acute operation of total endoprosthesis. For proper operation of the alternative treatments lubrication processes have to be understood. The understanding of the lubrication processes can assist in the development of new suitable medical treatments. This study is focused on the visualization of the synovial joint contact and simultaneous measurement of the force effects. Experimental device represents model of synovial joint, which allows pin-on-plate reciprocating tribometer. The goal of this study is to describe the contact area behaviour and to relate it to force effects in the contact.

1. INTRODUCTION

The human movement system is based on joints and muscles. The synovial joints are composed of two bones whose surfaces covers cartilage tissue [1]. The cartilage surfaces are in close contact and the space between them is filled with the synovial fluid. This arrangement, special cartilage structure and synovial fluid, allows movement with very low friction coefficient. Tissue of cartilage is porous material with heterogeneous structure containing very few cells [2]. These specific features cause the unique tribological behaviour. Extracellular matrix is the basis of cartilage structure (ECM). ECM includes type II collagen fibres and proteoglycan [3]. Hyaluronic acid (HA), proteins, decorins, chondrocytes, etc. are also included in the ECM [4]. Cartilaginous bone coating includes three zones. The first one, surface zone, has parallely orientated collagen fibres with respect to the surface. The second one, the middle zone, has randomly orientated fibres and the last one, the deep zone, has fibres perpendicularly orientated to the surface [1]. Each of them has specific composition and properties [5]. Water volume in cartilage tissue is also very important attribute regarding lubricating properties [6,7]. Special lubricating properties are caused by very low elastic module (1 – 20 MPa) of cartilage tissue regarding the position of the cartilage surface and type of the joint [4]. All of the

mentioned specific properties together allow specific lubrication processes in synovial joint with very low coefficient of friction and wear.
Obviously, there is very limited knowledge of lubrication in natural joints in terms of experimental investigation. Only a few studies were published. Topics of these works are frequently focused on visualization of hydrogels, nevertheless, complex visualization of natural cartilage has not been published yet. The majority of the studies is aimed to friction measurement, but studies dealing with visualization of cartilage contact also appear. One of the first works dealing with visualization of hydrogel contact area by fluorescence microscopy [8] was focused on determination of the amount of the fluorescently marked particles contained in the lubricant contact area. Fluorescently marked proteins were used, in each experiment, different type of protein was marked. γ globulin showed the biggest impact on the lubrication processes, therefore, the concentration of it in synovial fluid is very important. The fluorescence microscopy was used for the observation and description of the gel-like layer formation on the cartilage surface. Forsey et al. [9] dealt with impact of the HA in creation of the gel-like layer which is the main component for the formation of the film. The study revealed dependency on size of the HA. The penetration of the cartilage structure by HA was also demonstrated by these experiments. Molecules of HA are bound in the cartilage surface because they are attracted by chondrocytes contained in cartilage structure. Wu et al. [10] showed dependency between the flow of the synovial fluid through cartilage structure and compression of cartilage tissue. The results implied dependency on size of HA molecules, specifically the large molecules of HA were caught on the cartilage surface, while the smaller particles penetrated the cartilage structure. The surface of cartilage is covered by the HA with large molecules, which creates the gel-like layer. This surface layer protects the raw cartilage surface against a damage.

Visualization of joint replacement contact was also carried out with fluorescent microscopy. Number of studies were published at our department. A lot of experience with the use of optical methods was obtained within the mentioned studies. Nečas et al. [11,12] published the papers dealing with visualization of joint replacement contact and soft contact among others.

The vast majority of previous studies mostly dealt with visualization of contact area or with the friction measurements separately. It has never been measured simultaneously yet. This study combinates this two branches of biotribology science. The usage of optical methods used at workplace is described together with classical friction measurements. Specially tailored tribometer was designed for this application. It allows simultaneous visualization of soft contact and friction measurements. Concept like this new designed tribometer, which allows combination of optical methods and friction measurements have never been used yet. The goals of this study are to design the new tribometer, to develop the sampling process and experimental methodology and finally, to perform the pilot experiments.

2. MATERIAL AND METHODS

2.1 Experimental device

The tailored new tribometer allows the measurement of friction forces and insight into the contact area, both simultaneously in real time. The pin on plate configuration of tribometer was used for compliance of this requirements. As an observation method fluorescent microscopy was chosen, therefore the concept of tribometer was adapted to be able to use it. This new design is close to the concept of tribometer which was used in study [13]. The schema of newly designed experimental device is shown in Fig. 1. To allow the visualization of contact area, the cartilage sample is placed under the glass desk. The lubricant flowing through the contact is observed by the fluorescent microscope. Due to the fluorescent microscopy method the high speed camera can record fluorescently marked particles contained in lubricant, which flow through the contact. The mercury lamp was used as a light source. The contact area was flooded by a fluorescently marked lubricant. The floated bath is heated to a human body temperature to achieve comparable conditions to human body joints. The glass desk is mounted to a carriage, which was designed as a moveable part. It performs the reciprocating motion whereas the specimen is stationary.

The new design of the tribometer is outlined in Fig. 1. The essential units of the device are shown in Fig. 2.

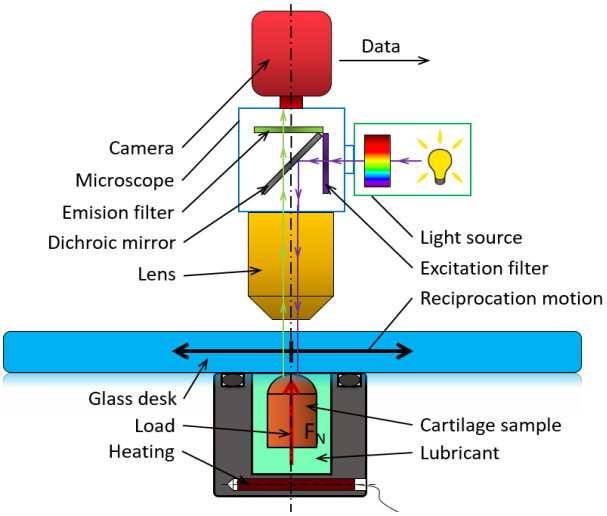

Fig. 1. Schema of the apparatus.

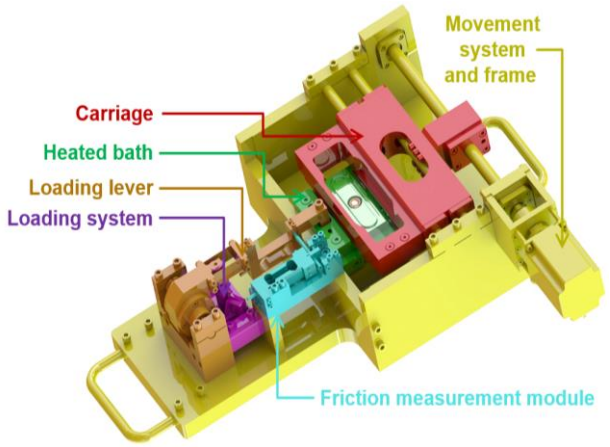

Fig. 2. Real arrangement of apparatus.

Tough frame is a basis of the tribometer, all other components are mounted on it. The carriage as a moveable part, where the glass plate is placed and which performs the reciprocating motion on the guide rods. The accurate guide rods in combination with ball screw and ball bearings in carriage body provide very accurate motion without radial and axial clearances. The heated bath enables flooding and refiling of the contact by a lubricant. The sealing is attached to the glass plate and the bath to avoid the lubricant leakage. Cartilage specimen is mounted on the end of a lever, by which the load is applied. The other end of the lever is placed in two preloaded ball bearings. This arrangement allows rotation around the axis without clearance and radial clearance is precluded too. A strain gauge is connected to the deformation member providing sufficient deformation caused by the low frictional force and great rigidity in the vertical direction (loading direction) at the same time. The first strain gauge is used for measurement of loading, the second one for measurement of friction force. It is connected parallelly to the lever behind the first gauge. Thanks to the deformation member, very low friction forces can be measured. The parallel connection of the second gauge allows its preload to the half of the measuring range, which allows measurement of friction forces during movement of carriage back and forth. The whole tribometer is situated below the fluorescent microscope on an adjustable table. The tailored tribometer is shown in Fig. 3.

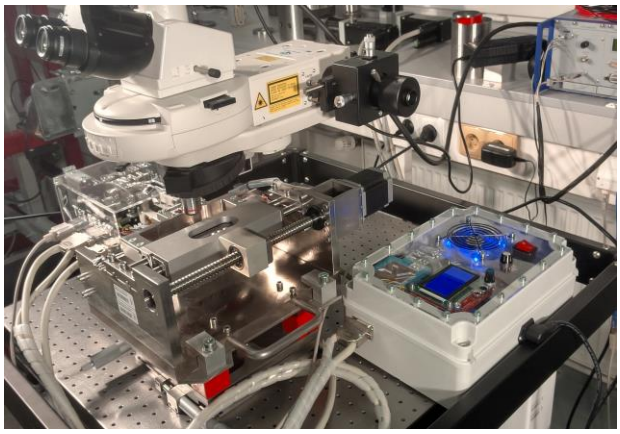

Fig. 3. Complete experimental apparatus.

National Instruments measuring card is a basis of the measuring system. Signal from the gauges is adjusted by the signal amplifier before the measuring card handles them. Data is real-time processed by LabVIEW script through connected PC. Control system is based on Arduino. The motion is ensured by the stepper motor and the load is provided by another linear stepper motor. LCD display and encoder is used as input interface. Both systems (measuring and control) work separately.

Verification and calibration was performed using standard pairs of samples (materials), in order to ensure measurement repeatability among samples. The mentioned material combination was used because the cartilage specimens show variance in results. The pin was made from PTFE-G400 and the plate was made from optical glass B270. The commercial

tribometer Bruker UMT TriboLAB was used to compare with the new tribometer. The compliance of the results obtained using the two simulators was very good.

2.2 Specimens

Specimens from mature pigs were used in the present study. The samples were removed from canopy of the femoral head as soon as possible after the slaughter of animal. The hip joint was chosen for the samples, since it is the most loaded joint, which leads to the best mechanical properties of cartilage tissue [4]. The sampling position was precisely defined through all sample bones. Strict definition assures minimization of deviation in mechanical properties through all samples. Pins of 6 mm diameter were made by the hollow drill bit and the specimens were deeply frozen (-20 ˚C) in PBS immediately after sampling. This sampling process was used in some studies before, e.g. in [14, 15] and the procedure was verified again in [16, 17]. It was proved that the tribological properties did not change. The samples were unfrozen just before testing, otherwise degradation of samples may occur. The sampling process is shown in Fig. 4.

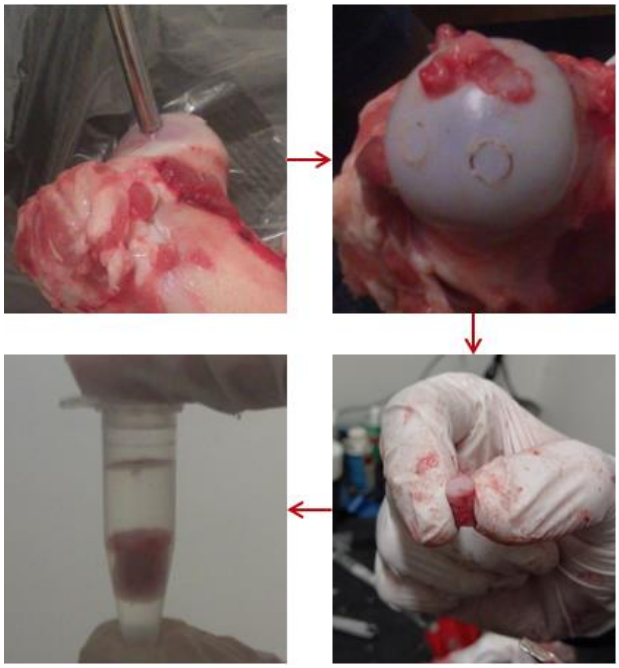

Fig. 4. Specimen preparation process.

Three variants of lubricant were used. The composition of all the used lubricants is shown in Tab. 1.

Table 1. Composition of the used lubricants.

	Concentration [mg/ml]		
	γ-globulin	**HA**	**Albumin**
Lubricant 1	-	-	20
Lubricant 2	3,6	-	-
Lubricant 3	3,6	2,5	20

In the experiments focused on visualization, lubricant 3 was used. The composition of lubricant 3 was constant for all the visualization experiments, despite the fluorescently stained component varied. In the first case, albumin was marked by Rhodamine-B-isothiocyanate (283924, Sigma-Aldrich) and in the second case, γ globulin was marked by Fluorescein-isothiocyanate (F7250, Sigma-Aldrich).

2.3 Experimental methodology

All of the experiments in this study were measured with one sample to be secured comparability of all individual measurements. Although the samples are removed from one rigorously defined place of cartilage surface, there are significant deviations between friction trends of each cartilage samples. This deviation is caused by different mechanical and structural properties among individual animal bones and therefore measurements were performed with one single sample. The validation of one sample measurements was set in this study. 5% deviation in maximum was found comparing the measurements.

The procedure was strictly specified in order to minimize the results deviation and to compare the measurements. Before each measurement, the sample was stored in PBS to rehydrate the cartilage tissue. First, the run-in procedure (20 reciprocating cycles at 10 N load) was performed before each measurement. This procedure suppresses the effect of previous experiments, especially effect of any previously used lubricants.

In the first section, lubricants 1 and 2 were used considering the friction measurements only. In the second section, the fluorescent microscopy was simultaneously combined with friction measurements. The experiments were focused on finding a correlation between visualization of contact area and friction trends. Model synovial fluid with lubricant 3 was used for all measurements in the second section. It had two configurations; the first with fluorescently

marked albumin and the second with stained γ-globulin. The composition was the same in the both cases. The measurements were performed in identic conditions and procedure.

2.4 Experimental condition

The scope of chosen conditions was to simulate human hip joint, therefore the conditions were defined with respects to it. The contact stress was set to1 MPa, which was achieved by 10 N of load. This conditions provides the medium stress comparable with hip joint. Speed of the movement was se to 10 mm/s, which corresponds to slow walking. Stroke of reciprocating motion was taken from the previous studies and it was chosen to be 20 mm. Lubricant bath was heated to 37 ˚C.

3. RESULTS AND DISCUSION

The results of friction measurements are shown in Fig. 5.

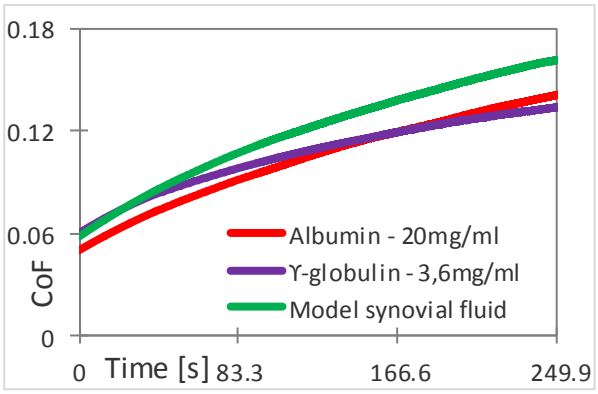

Fig. 5. Friction trends.

Lubricant 1 (albumin 20 mg/ml) exhibits steeper increase of friction than lubricant 2. It can be explained by the presence of higher concentration of proteins and larger size of albumin molecules. This explanation is also supported by images from contact visualization, which show higher light image intensity in the case of measurement with marked albumin. There is only one curve labelled as lubricant 3, which represents both of the performed measurements. In the first case, albumin protein was marked, while in the second case ϒ-globulin protein was visualised. Model synovial lubricant (lubricant 3) shows higher friction than any of the simple protein solutions. The growing global volume of proteins in lubricant leads to apparently higher friction. Very similar lubricant solutions were studied by Murakami et al. [18] who observed higher friction for γ-globulin proteins than for complex synovial fluid. Nevertheless, in the mentioned reference, the authors used different concentration of proteins and different specimens. This can explain the disagreement of the achieved results.

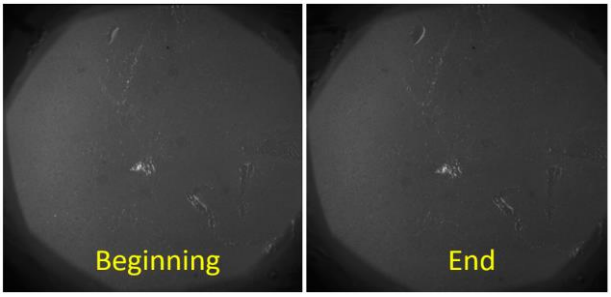

Fig. 6. Contact area visualization – Lubricant 3 with stained albumin at the beginning and at the end of the measurement.

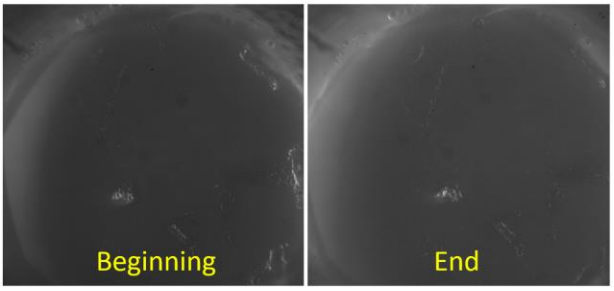

Fig. 7. Contact area visualization – Lubricant 3 with stained ϒ-globulin at the beginning and at the end of the measurement.

In order to visualize the contact area, lubricant 3 was used. The friction measurements were performed simultaneously with the visualization. The visualized contact area shown in Figs. 6 and 7 corresponds to friction curve of lubricant 3 shown in Fig. 5. White spots in in Figs. 6 and 7 are the proteins entrapped within the contact area.

Compared to the Fig. 6, Fig. 7 shows higher global intensity of emitted light. It means, that the thickness of lubricant film formed by lubricant 3 with labelled albumin is higher. Fig. 6 shows more and bigger aggregations of albumin particles. Apparently, the albumin protein is more represented constituent in the contact. The concentration of γ-globulin proteins in lubricant 3 is lower and its molecules are of smaller size at the same time. Comparing the impact of both

marked proteins, we can see that the contribution of albumin is more important in terms of cartilage lubrication.

The proteins are captured in the same location in both experiments (Fig. 6, Fig. 7). It can be assumed, that there are small local damages of the cartilage.

4. CONCLUSION

New specialized reciprocating tribometer for biotribological research was designed. It includes controlling and measuring systems, which allow friction force measurement and contact area visualisation at the same time.

Fluorescence microscopy was chosen as a suitable optical method for visualization of cartilage contact area. It was successfully used for soft contact visualization before and proved again its benefits for such type of visualisations.

The methodology for preparation of the specimens was also demonstrated in the present study.

Calibration and data validation was performed using commercial tribometer Bruker TriboLAB. The capabilities of new device were shown on the set of pilot experiments.

It reveals new unexplored fields in biotribology. Future research may bring a significant contribution, which could be eventually applied in treatment of human joint diseases.

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materials

MDPI

Article

Biotribology of Synovial Cartilage: A New Method for Visualization of Lubricating Film and Simultaneous Measurement of the Friction Coefficient

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Abstract: A healthy natural synovial joint is very important for painless active movement of the natural musculoskeletal system. The right functioning of natural synovial joints ensures well lubricated contact surfaces with a very low friction coefficient and wear of cartilage tissue. The present paper deals with a new method for visualization of lubricating film with simultaneous measurements of the friction coefficient. This can contribute to better understanding of lubricating film formation in a natural synovial joint. A newly developed device, a reciprocating tribometer, is used to allow for simultaneous measurement of friction forces with contact visualization by fluorescence microscopy. The software allowing for snaps processing and subsequent evaluation of fluorescence records is developed. The evaluation software and the follow-up evaluation procedure are also described. The experiments with cartilage samples and model synovial fluid are carried out, and the new software is applied to provide their evaluation. The primary results explaining a connection between lubrication and friction are presented. The results show a more significant impact of albumin proteins on the lubrication process, whereas its clusters create a more stable lubrication layer. A decreasing trend of protein cluster count, which corresponds to a decrease in the thickness of the lubrication film, is found in all experiments. The results highlight a deeper connection between the cartilage friction and the lubrication film formation, which allows for better understanding of the cartilage lubrication mechanism.

Keywords: biotribology; cartilage; reciprocating tribometer; friction; lubrication; fluorescence microscopy

1. Introduction

Painless movement, i.e., the proper function of our joints, is very important in the active life of a human. Despite advanced medicine, there are many diseases of natural joints (osteoarthritis, arthrosis, arthritis, etc.) that lead to degradation and soreness of these joints [1,2]. The extent of the disease depends on many factors and may result in incurable, irreversible damage of natural joints. In this case, a natural joint has to be replaced by an artificial one. However, these replacements have a limited lifetime, which is a problem especially for young active people [3]. When the artificial joint is worn, it has to be replaced by a new one. This process cannot be repeated many times because of the adverse impacts on human health (bone degradation due to a thorn replacement, mental intensity of the operation for the patient, etc.) [4–6].

Some of the methods of the treatment of damaged natural joints are non-invasive methods, e.g., viscosupplementation (the supplement (a gel-like fluid called hyaluronic acid) is injected into the

joint gap) [7]. The general effort of non-invasive treatments is to postpone the operation of total endoprosthesis as long as possible. Viscosupplementation is a treatment method allowing to restart the lubrication process in a damaged synovial joint, which temporarily stops or at least stabilizes the degradation of natural cartilage [7–9]. So far, the function of the supplements has not been completely understood, and their effects are not fully guaranteed for individual patients [10–13]. To better understand these issues, it is necessary to describe the cartilage lubrication. The cartilage and synovial fluid properties are the basis of articular joint lubrication [14,15].

The cartilage together with a natural lubricant (synovial fluid) are very important parts of the synovial joint for tribology investigation. The cartilage surface along with the synovial fluid distribute load between the opposite bones [16]. Due to the very low elastic modulus of cartilage tissue and unique synovial fluid properties, the contact pressure is distributed over a large area of the cartilage surface; therefore, the friction coefficient (CoF) is very low [17]. The cartilage tissue is formed largely by water, type II collagen fibers, hyaluronic acid (HA), lubricin, etc. [16,18–20]. It is a sectionporous fabric with a varying structure through the thickness of the cartilage divided into three zones [16]. The structure varies especially in the composition, shape, and orientation of fibers [21–24]. Due to a low cell density, the cartilage tissue is nourished through the synovial fluid. The synovial fluid of natural joints normally functions as a natural biological lubricant, as well as a distributor of nutrients to the cartilage tissue [25]. The synovial fluid mainly consists of HA, phospholipids, proteoglycans, etc. [26–29]. The structure of cartilage contains negatively charged particles, which ensure sucking of water (synovial fluid) to the structure pores [30]. The accumulation of water in the structure of cartilage is the principle of the unique lubrication system, and many explanatory theories are based on it. In general, these properties are the basis of the lubrication processes in the synovial joint.

The research exploring the tribology of natural joints is focused on various techniques and methods. Some studies are concerned with artificial joints (joint replacement) and observation of their lubrication [31,32]; others deal with biochemical analysis of materials for the manufacturing of joint replacements and their impact on the lubricating processes [33]. The study experimentally tests the chitosan coating of the artificial prothesis, and the results show that this method causes a decrease in CoF. This is caused by the adsorbed protein layer formed on the surface because the chitosan coating binds the proteins. The publications [34,35] were focused on friction testing of artificial materials: hydrogels. Publication [35] tested the artificial cartilage from hydrogel, and publication [34] carried out friction tests with a lens from the hydrogel. The similar values of CoF as in natural cartilage were reported in both studies; however, the work in [34] showed that the friction forces were composed of three components (viscoelastic dissipation, interfacial shear, and viscous shearing). Other studies also dealt with the articular cartilage; however, their investigation was not directly focused on tribology. The work [36] carried out an extensive study focused on the treatment of mature cartilage, and the growth factor-induced therapy was evaluated using gradually carried out analyses.

There are two main types of works describing the cartilage tribological performance. The first group of works deals with cartilage lubrication, while the second one is focused on the friction between the cartilage tissues.

The first group is concerned with several mechanisms for the lubrication of articular cartilage including hydrodynamic lubrication [37], boundary lubrication [38–41], weeping lubrication [42,43], and boosted lubrication [40]. Other works of this group, which presented complementary information for already published results or describing new lubrication mechanisms, appeared in later years: hydration lubrication [44,45] and adaptive multimode [46–48]. These studies were focused on visualization of natural cartilage or hydrogels by fluorescent microscopy; their aim was to support and verify the above lubrication theories. A great influence of ϒ-globulin in the lubrication of hydrogel has been shown, but it depends on its concentration and the concentration of other lubricant components [35]. HA also seems to be very beneficial for the lubrication of cartilage [49]. It creates a gel-like layer on the cartilage surface and binds with chondrocytes contained in the cartilage structure. The penetration of the cartilage surface depends on the size of the particles; small particles penetrate the cartilage, while larger ones adhere on the cartilage surface and create a gel-like layer

[50]. The fluid leakage from the cartilage structure has also been proven, which supports the presented lubrication theories.

The second group of works is focused on cartilage friction. The number of "friction" studies is higher because the friction measurement methodology is better developed, and these works indicate well the understanding of the cartilage friction behavior. The behavior of cartilage lubricated by synovial fluid was presented in [51–55] showing that the synovial fluid reports a very low CoF. HA helps to lower CoF [54]; CoF grows with time [51,52,54,55–58]; CoF decreases with a rising load [51,57,58]; CoF is influenced by the type of movement depending on the cartilage sampling [52,59]; and the cartilage rehydration has a positive impact on CoF [60].

Obviously, there are many friction studies and also studies focused on the visualization of cartilage contact with a well indicated examined area, but there are no works allowing for simultaneous measurement of friction and visualization of cartilage contact. Fluorescence microscopy is a suitable experimental method for visualization of the cartilage contact owing to a very compliant material (cartilage) and the non-reflective surface of cartilage, which is not possible by traditional methods, e.g., optical interferometry. Another limitation of cartilage contact visualization is the non-conductivity of samples (cartilage-glass); therefore, the electrical methods cannot be used. Fluorescent microscopy is the only applicable method that allows for visualization of non-reflective, non-conductive, and compliant materials. The studies focused on the visualization of cartilage lubrication film are very limited, and the relationship with friction measurements is missing. Moreover, there is no work describing the connection between the friction in synovial joints and the visualization of cartilage contact in order to provide a better description of lubrication processes in the synovial joint. The present study explains the missing relationship to allow for a better understanding of lubrication in the synovial joint. The aim of this study is to carry out the visualization of cartilage contact by fluorescence microscopy simultaneously with the friction measurement and to explain the new methodology for the description of lubrication film in the model of the synovial joint. A similar approach to the processing of results has never been published. In this connection, a designed reciprocating tribometer and evaluating software are presented. The research study submitted offers a description of lubricating film formation in the model of the synovial joint, which can help to develop new treatment supplements along with understanding of their function.

2. Materials and Methods

2.1. Experimental Device

A unique design of the reciprocating tribometer was used; the scheme of the device is shown in Figure 1. This new design allowed for the in situ contact observation simultaneously with friction measurement. The design created was inspired by the concept in [60], where a similar design was used. A detailed description was published in the previous studies [61,62]. The use of the reciprocating tribometer simulates the compliant contact between the cartilage sample and the glass plate to create a simplified model of the synovial joint. The cartilage sample was situated under the glass plate in the static position. This arrangement allowed for the contact area visualization by the fluorescence optical system combined with a high-speed camera. The contact area was flooded with lubricant, and the lubricating bath was heated to the temperature of the human body. The glass plate was a moveable part with a reciprocating motion. Load was applied through the cartilage sample, and the lever measured friction forces. The operating conditions (stroke, sliding velocity, and load) could be modified. The tribometer was situated under the optical microscope based on fluorescence microscopy (Nikon, Eclipse NI, Minato, Tokyo, Japan).

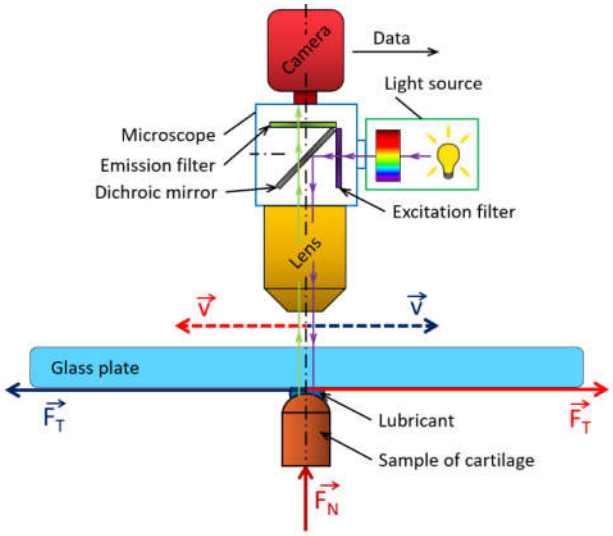

Figure 1. Schema of the experimental apparatus.

The experimental device is shown in Figures 2 and 3. The basis of this concept was a rigid frame where the other components were mounted. The glass plate was fixed to the carriage, which was actuated using a combination of a ball screw and a stepper motor. A clearance-free and accurate reciprocating motion of the carriage was ensured by guide bars and the ball sleeve. The sealing between the glass plate and the heated bath prevented the leakage of lubricant from the bath. The sample was mounted at the end of the lever through which the load was applied and distributed by a linear stepper actuator. This actuator was placed under the lever, at the opposite end to where the sample was mounted. The load sensor was a part of the lever, and the friction sensor was connected in parallel to the lever. The c control system was based on Arduino, which controlled the movement and the loading system. The input parameters were entered through an LCD interface. The measuring system worked separately and was fitted with two single-point tensometric sensors to allow for recording of loading and friction forces. Force curves were saved to the data files; CoF was calculated therefrom.

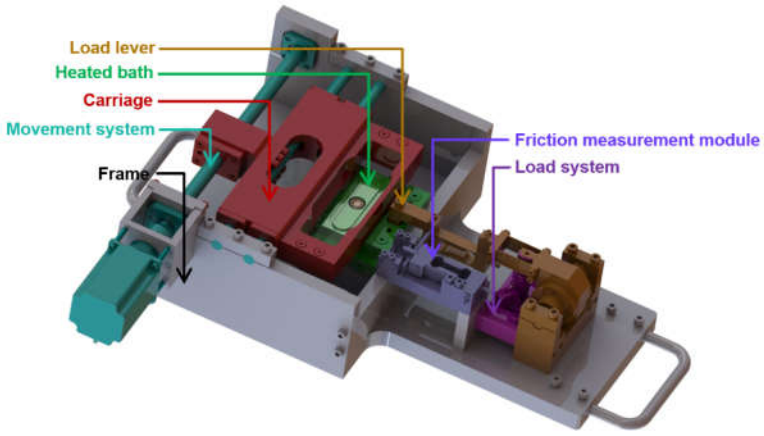

Figure 2. Digital model of the reciprocating tribometer.

Figure 3. Experimental apparatus.

2.2. Experimental Method: Fluorescence Microscopy

In this paper, the optical method based on the principle of mercury lamp inducted fluorescence was used. Fluorescence is the light emission of a substance; it is excited by light or other electromagnetic radiation. The fluorescence phenomenon can be described in three steps: excitation (the excitation photon emitted by the excitation light source is absorbed by the fluorophore contained in the fluorescent dye), excitation state period (dissipation of energy to ensure the emission of fluorescence), and emission (due to the dissipation of energy in the excitation state period, the photon emitted by the dye has a lower energy; therefore, it emits radiation at longer wavelengths). A full description of the fluorescence method was given in [63]. The mercury lamp emitting white light was used as an excitation source. The carousel with various excitation and emission filters were placed in front of the light source. For the purpose of this study, Fluorescein Isothiocyanate (FITC) and Tetramethylrhodamine (TRITC) filters were used; they allowed for a change in the wavelength of excitation and emission light, FITC (excitation on 490 nm, emission on 525 nm) and TRITC (excitation on 557 nm, emission on 576 nm). The double magnification lens was used for the observation of contact. The analysis of the apparatus was carried out, and the axial resolution was approximately 290 μm for the FITC filter and 320 μm for the TRITC filter. The field depth of the apparatus was approximately 200 μm, which was sufficient with respect to the sliding speed and estimated film thickness. Focusing of the image (contact) was carried out by the field diaphragm and slight re-focusing by the z-feed of the microscope. The focusing procedure was realized before each experiment, and it was immutable during the experiments. The fluorescence method was introduced and described in previous studies visualizing joint replacements for different material combinations [31,32]. The glass plate, through which the contact was visualized, was made from optical glass B270; therefore, the light excitation and emission were not affected. The scheme of the apparatus is shown in Figure 1.

2.3. Specimens and Lubricants

Samples were removed from the femoral hip head of mature pigs. The sampling process was realized as early as possible after the slaughter of the animal. The samples were removed from the canopy of the most loaded site of the femoral head, which ensured the best mechanical properties. This site was selected for all sample bones to achieve a low deviation of mechanical and tribological properties. The samples were removed by a hollow punch with an internal diameter of 9.7 mm and stored in phosphate-buffered saline (PBS), deeply frozen (−20 °C), and defrosted immediately before the experiments. The same sampling procedure was used in [55,58] and verified in [64,65]. The sampling process is shown in Figure 4. The opposite sample to the cartilage was the glass plate, which fulfilled the immediate requirement for insight into the contact area. The dimension of the glass

sample was 154 mm long, 43 mm wide, and 4 mm thick, which allowed for a sufficient stroke without leakage of testing fluid.

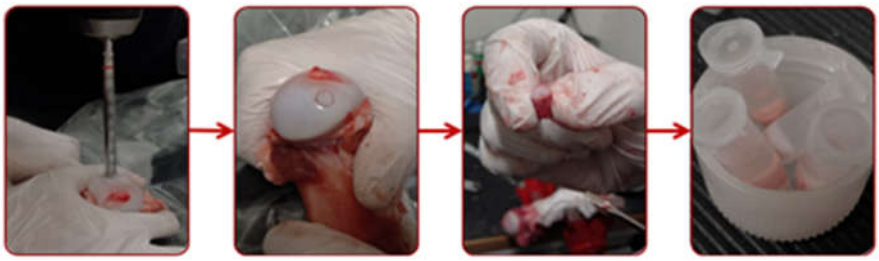

Figure 4. Sampling process.

As an experimental lubricant, a model synovial fluid was used. The composition of the model synovial fluid corresponded to the physiological synovial fluid; see Table 1. Bovine serum (BS) albumin (powder, ≥96%; A2153, Sigma-Aldrich, St. Louis, MO, USA) was labelled by Rhodamine-B-isothiocyanate (283924, Sigma-Aldrich, St. Louis, MO, USA) in this case, and the other components were mixed without dye. The protein solution was further comprised of ϒ-globulin from bovine blood (powder, ≥99%; G5009, Sigma-Aldrich, St. Louis, MO, USA) and HA with a molecular weight of 1000 kDa. All these components were mixed in PBS solution. The lubricant degraded in air; therefore, the lubricant specimens were stored in a frozen state at −20 °C and defrosted immediately before the experiments. A single lubricant was used for each experiment, and after that, the lubricant sample was discarded. The duration of each experiment (see Table 2) was too short to degrade the lubricant sample. The other conditions of the experiments (especially contact pressure and temperature) did not speed up the degradation.

Table 1. Lubricants' composition.

Lubricant	Albumin (mg/mL)	ϒ-globulin (mg/mL)	HA (mg/mL)	Labelled Component
Model Synovial Fluid 1	20	3.6	2.5	Albumin
Model Synovial Fluid 2	20	3.6	2.5	ϒ-globulin
Calibration Fluid 1	20	-	-	Albumin

2.4. Methodology and Conditions

A strictly-defined procedure of the preparation of each experiment was defined for adherence to the repeatability of the results as described in the previous study [61]. All experiments were carried out under the same experimental conditions; see Table 2. These conditions were defined based on the previous studies and inspired by natural synovial joints [20,66,67]. The contact pressure was approximately 0.8 MPa. The value of the contact pressure was determined by the Hertz theory. This value was strongly dependent on the modulus of elasticity of removed cartilage, which varied through all specimens. The contact pressure was applied as 10 N of load. Similar conditions were used for example in [54,60]. The tribometer allowed for a constant majority of the velocity trend, as shown in Figure 5, where one cycle of reciprocating motion can be seen. The constant part of the velocity trend was 96.5% of every cycle.

Table 2. Experimental conditions.

Load	Contact Pressure	Velocity	Stroke	Total Distance	Number of Cycles	Duration	Temperature
10 N	0.8 MPa	10 mm/s	20 mm	1200 mm	60	approximately 4.5 min	37 °C

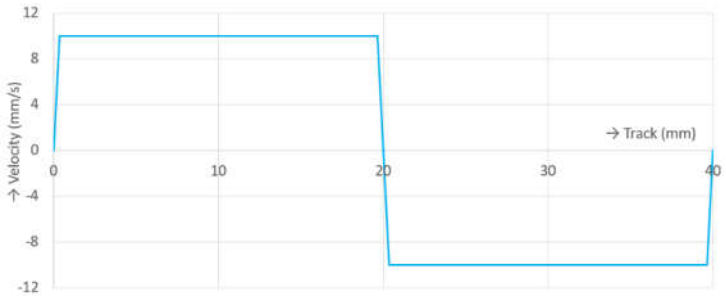

Figure 5. Sliding velocity trend.

The output of each experiment was a force record and recorded snaps of the contact area. Each experiment had to be post-processed in two ways: the effect of the forces and the visualization (snaps of contact); however, both processed results were linked at the end of post-processing. The schema of the experiments' evaluation is shown in Figure 6. The raw data from the effects of forces needed to be edited and filtered for further processing. Due to the preloaded friction sensor, the raw signal had to be offset in the first step (Figure 6A), and after that, CoF could be calculated (Figure 6B) (ratio of friction force and normal force). The complete procedure for evaluation of the effect of forces was published in [52]. The CoF trend was determined from each measurement, and the percentage difference of the CoF value from the beginning to the end was evaluated for each experiment; see Figure 6C. The percentage difference was determined from the average of the last 1000 values of CoF and subtracted from the average of the first 1000 values; this was the first evaluation parameter for the final evaluation.

The record of contact from the camera was the second parallel way of input from the experiments. The raw record from the camera was exported to the particular snaps in the first step (Figure 6D). The snaps were in a resolution of 2560 × 2140 pixels, and the pixel size while using the double magnification lens was 3.75 micrometers. The individual snaps are processed by specially designed software, which filtered out the background of each snap and highlighted the lighter points; see Figure 6E. A description of the software is given in the paragraph below. The lighter points show the labelled proteins in the lubricant; this was a significant evaluation parameter. The background noise was caused by the lubricant, which was sucked in the cartilage pores. The particle count in the contact was an output of each snap. The dependence of particle count on time was an output of the entire record; see Figure 6F. The software processed every snap in every recording according to the input parameters. The difference between the initial and final particle counts in the contact was the second evaluation parameter for final evaluation; see Figure 6G. The dependence between the particles' difference and the friction difference led to a lubrication description, especially the determination of the influence of synovial fluid individual components on the lubrication of cartilage.

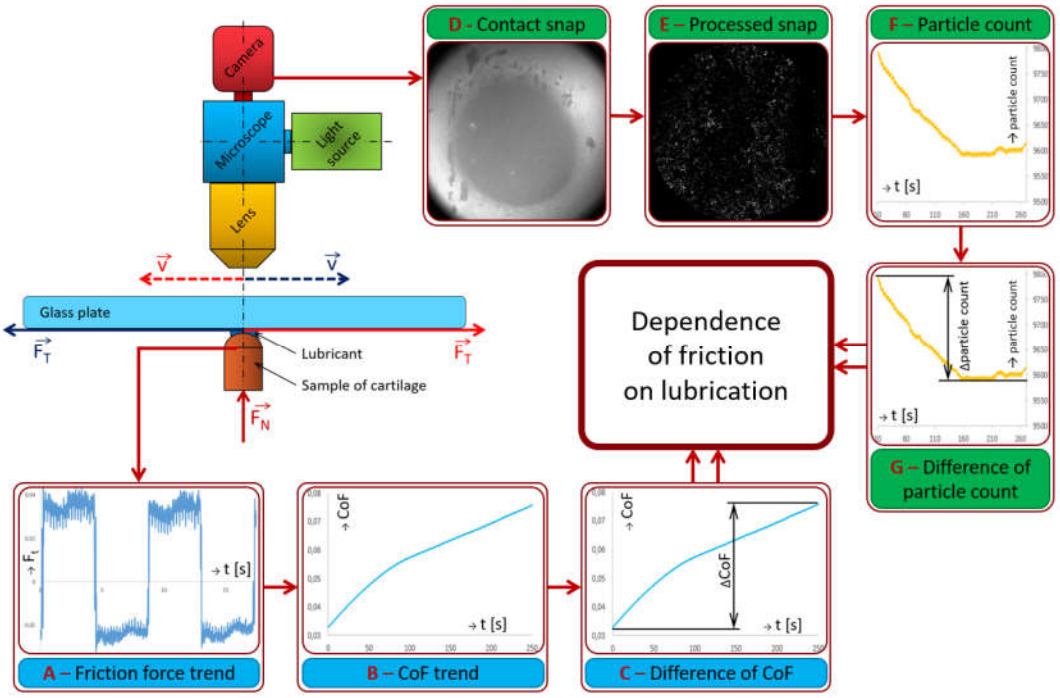

Figure 6. Evaluation schema. (**A**) Friction force trend (**B**) CoF trend (**C**) Difference of CoF (**D**): Contact snap (**E**) Processed snap (**F**) Particle count (**G**) Difference of particle count.

The processing of snaps was based on the unique software design (Figure 8) using the segmentation principle of image processing. Segmentation is the process of transforming the individual parts of the snap into meaningful regions or objects [68,69]. This software removed the background and highlighted the labelled proteins from snaps in several steps. The whole procedure of snap processing is shown in the diagram in Figure 7. First, the contact area was defined by a circle, Steps 7B and 7C, and the surrounding area was suppressed, i.e., the surrounding area did not enter the future processing. The contact area was defined based on visual observation; the contact area was clearly visible in each snap. In the second step, dual operations, erosion and dilation, were carried out (this combination is called morphological opening, Step 7D); therefore, at first, structuring element (SE) was searched in the examined area, and if SE was detected, the pixel was added into the center of SE (i.e., the examined area was reduced by the SE radius). After that, the overlap of SE in the examined area was determined. If SE, at least partially, overlapped the examined area, the center of the resulting area was added (i.e., the examined area was magnified by the SE radius) [69]. The opened snap was subtracted from the original snap (Steps 7D–E). Finally, thresholding was carried out; see Steps 7F and 7G in Figure 7; according to the threshold value, all points that were below the threshold were suppressed. This principle was used in [70]. The author used the morphological opening for processing of microscope snaps of a metallic alloy to highlight some parts of the snap and remove the background.

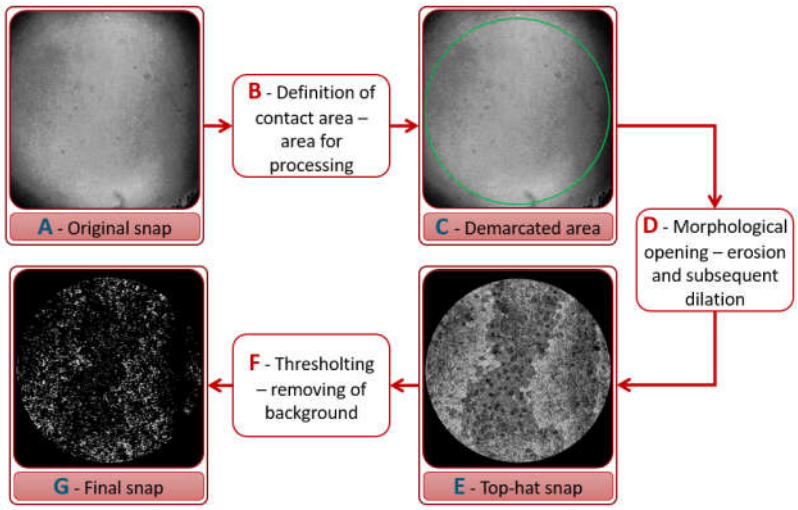

Figure 7. Snap processing diagram. (**A**) Original Snap (**B**) Definition of contact area-area for processing (**C**) Demarcated area (**D**) Morphological opening-erosion and subsequent dilation (**E**) Top-hat snap (**F**) Thresholting-removing of background (**G**) Final snap.

A snapshot of the software with its description is shown in Figure 8. In the top right corner, there are fields for input parameters. Functions "Mask width" and "Mask center" define the circular area by which the size and position of the contact are defined. The "TopHat width" value defines sensitivity to the local snap maximum, and consequently, the areas for morphological opening are defined. The last box "Threshold" defines the threshold to determine the background. The processing steps of snaps are shown under the boxes with input parameters. The software determined the count of detected proteins and their average size; this is shown under the images with processing steps. The graph in the lower right corner describes the count and the size of particles found. The final processed snap is shown on the left side of the software window.

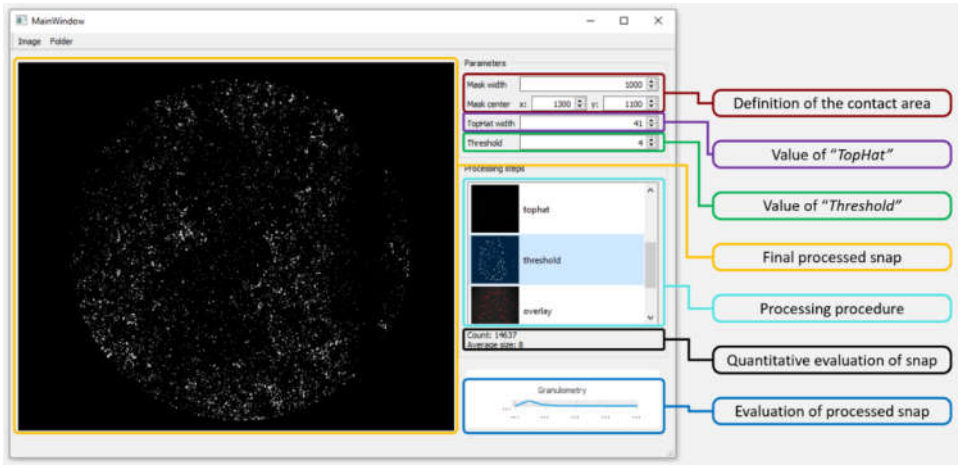

Figure 8. Evaluation software.

3. Results and Discussion

3.1. Verification and Calibration of the Method

The cartilage contact was verified by a spectrometer. The individual lubricants and their combinations with the cartilage were tested by spectral analysis. They were radiated using TRITC

and FITC filters, and their emissions were monitored. The results are shown in Figure 9. Obviously, no emission was detected if the cartilage alone was excited using both filters, as shown by Curves E and F in Figure 9. The cartilage in combination with labelled lubricants always emitted only at wavelengths of the filter used, which was obvious from Curves B and D. The labelled lubricant alone also emitted only at wavelengths of the filter used (see Curves A and C); however, the emission was stronger than in the case of lubricant with cartilage. The chart in Figure 9 shows that the individual lubricants and cartilage combinations were not mutually affected throughout measurements because their emissions were offset relative to each other.

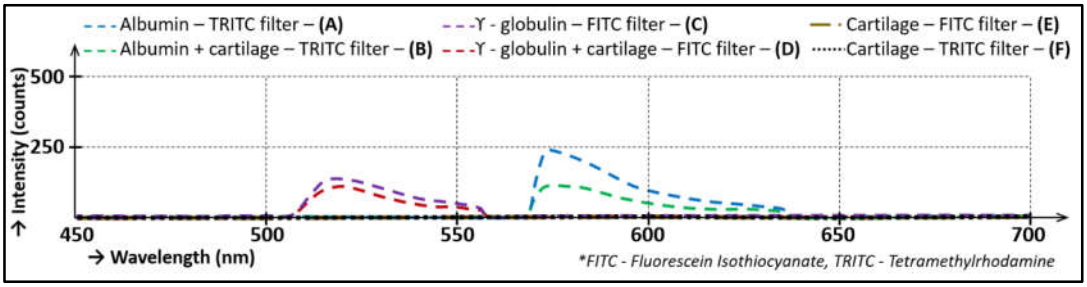

Figure 9. Lubricant emission.

The software was calibrated based on the fluorescence intensity trend, which was directly exported from camera records. The recording device was a high-speed camera allowing for recording with a high framerate. The originally delivered camera software was used to export the intensity and to convert the video record into individual snaps. These snaps must then be processed by the newly designed software. A wide spectrum of combinations of input parameters for processing by this software was compiled; however, only a small section of the spectrum was selected using the knockout chart in Figure 10. This chart shows the entire spectrum of different combinations of software input settings. Each individual point represents one input combination, and the whole chart shows a percentage decrease of particle counts throughout the experiments. The total percentage decrease was determined similarly to the CoF difference (Section 2.4), specifically from the average of the last six cycles of particle counts, and it was subtracted from the average of the first six cycles. The value from the middle of each cycle was used for average calculation. The points, which report the opposite trends to fluorescent microscopy, were eliminated (points in Figure 10 under the red line). From the remaining trends, only the points reporting similar trends as the fluorescent intensity trend were used for the following step.

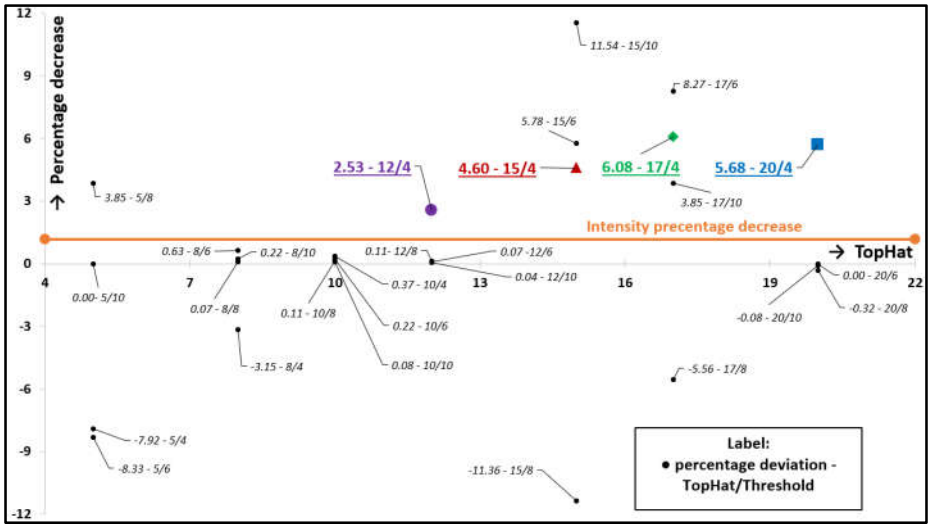

Figure 10. Knockout chart.

In this step, the similarity of the percentage decrease of particle count trends and fluorescence intensity was compared. For the next step, only the results closest to the fluorescence percentage decrease trend were used; therefore, the two y axis graph was compiled. The y axis on the right represents fluorescence intensity (Fluorescence microscopy (FLM) in Figure 11). The y axis on the left represents the particle count. The x axis is time. This dependency is shown in Figure 11. These trends were interpolated by a linear curve, and the tangent directions of these curves were compared. The most similar settings were Quantification Monitoring software (QM) 15/4 and QM 17/4; both of them well described the intensity trend, and their tangent directions were the most similar ones. As the final setting of software, setting 15/4 was defined, which meant TopHat 15 and Threshold 4. However, this setting was valid only for measurement with labelled albumin. A new calibration was necessary for each measurement to validate the data from the new software. The independent calibrations of evaluation software were carried out for both model synovial fluids, and the determined calibration inputs are shown in Table 3.

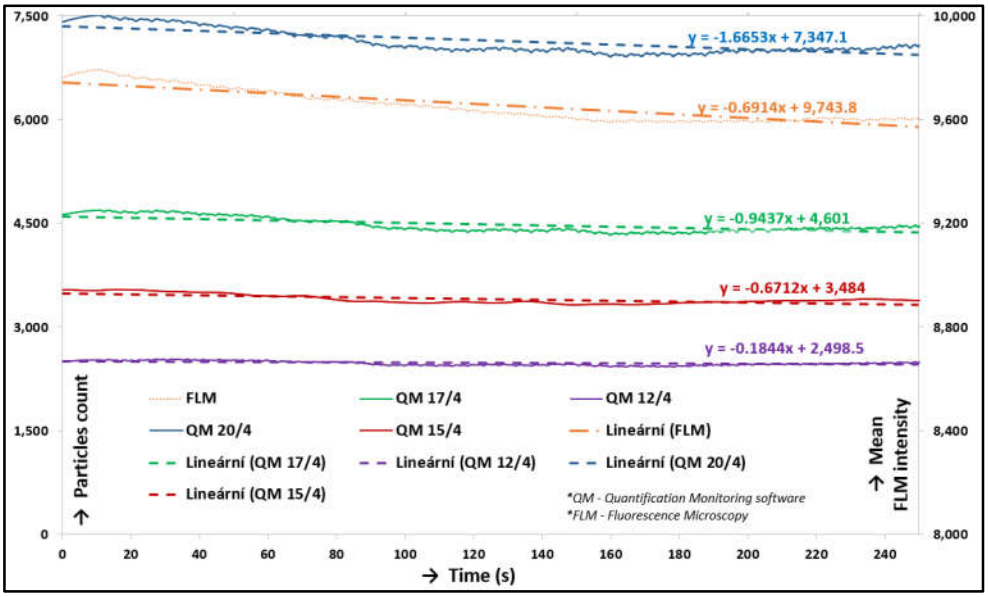

Figure 11. Comparison chart of individual input parameters.

Table 3. Percentage deviation of CoF.

Lubricant	Mask Radius (pixel)	Contact Center x/y (pixel)	Top Hat	Threshold	Labelled Component	Fluorescence Filter
Model Synovial Fluid 1	1000	1300/1100	15	4	Albumin	TRITC
Model Synovial Fluid 2	1000	1300/1100	13	4	ϒ-globulin	FITC

3.2. Friction and Lubrication in Cartilage Contact

At first, prior to all experiments focused on lubrication analysis, the static experiment without reciprocating motion was carried out. The cartilage specimen was gradually loaded from the unloaded state to the load of 10 N by a linearly rising loading process. The contact was recorded simultaneously. This experiment was carried out only with the solution of albumin and PBS (Calibration Fluid 1; see Table 1). Figure 12 shows the states immediately before the contact was loaded (12A), immediately after the contact was loaded (12B), and after the contact was fully loaded (12C). The red circle in the snaps represents a cartilage contact area, and the white points represent clusters of labelled proteins; in this case, albumin. The lower snaps in Figure 12 show the original snaps, and the clusters of proteins are obstructed by thin red curves. The decrease of the count of particles in contact was obvious throughout loading. The particle count curve (Figure 13) showed a

strong fall during the loading process; nevertheless, the following particle count trend did not vary much and reported only a slight decrease. A decreasing trend of particle count corresponded to a decreasing trend of fluorescence intensity (Figure 14). However, the size of protein clusters sharply rose during the loading process, i.e., the size of protein clusters increased with rising load. This trend corresponded to the contact snaps in Figure 12. Conclusions from these experiments confirmed the presence of protein clusters in cartilage contact and their squeezing out from the cartilage pore structure, and subsequently from the contact. This confirmed a decreasing trend of particle count and fluorescence intensity. A similar theory, the escape of proteins from the contact and synovial fluid from the cartilage tissue during loading, was suggested and described in [40–43]; nevertheless, this phenomenon was described only at the theoretical level in connection with lubrication theories. The study [71] described similar results, the extrusion of lubricant during load, but based on different experiments.

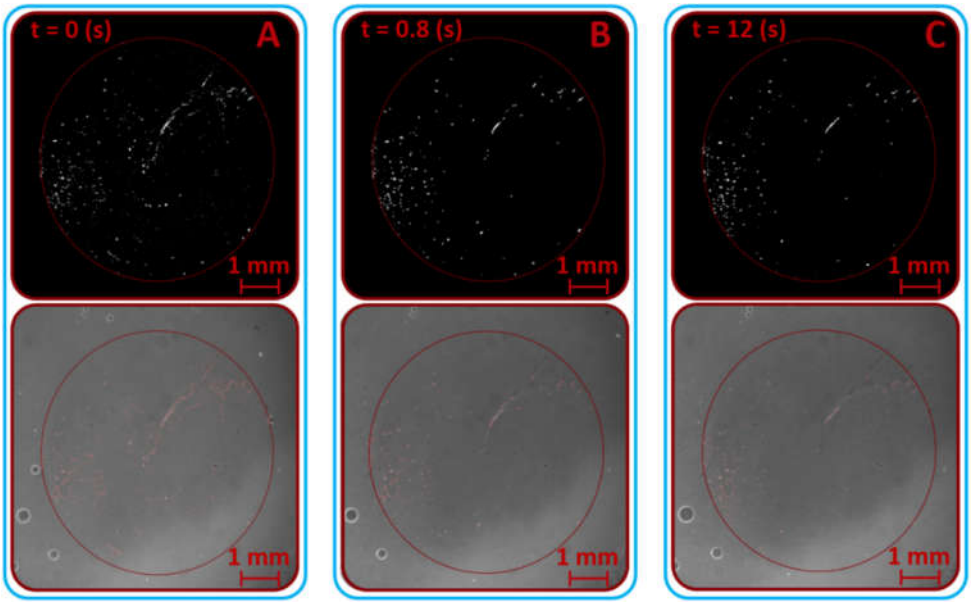

Figure 12. Loading of contact snaps – highlighted protein clusters by the software. (**A**)—protein clusters at the beginning of the experiment (in the time 0 s), (**B**)—protein clusters in the time 0.8 s, (**C**)—protein clusters at the end of experiment (in the time 12 s).

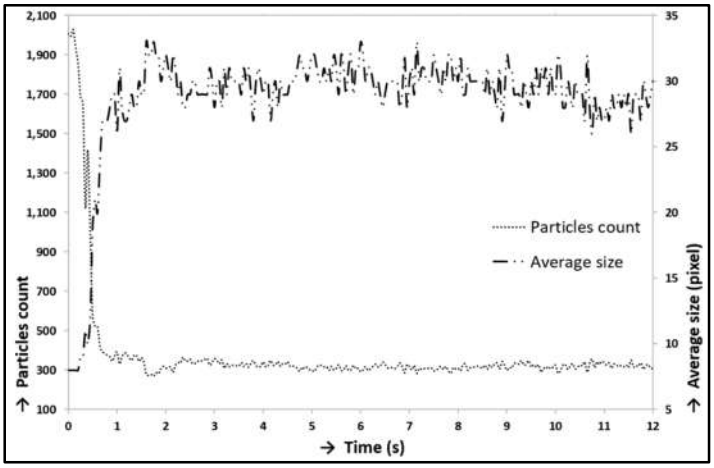

Figure 13 Loading of contact–particles count/average size.

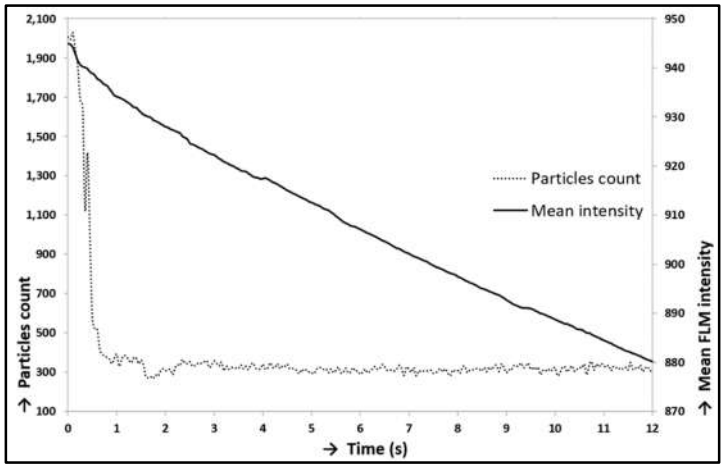

Figure 14. Loading of contact–particles count/FLM (fluorescence microscopy) intensity.

Two different measurements were carried out. The variation of experiments lied on two model synovial fluid variants with the same composition; nevertheless, a different labelled component was used (see Table 1). Both measurements were carried out under the conditions in Table 2 and focused on simultaneous recording of friction forces and visualization. The particle count trends and the trends of average size of protein clusters were evaluated and shown in Figure 15. Decreasing trends of particle counts were obvious from both curve trends. The percentage deviation of the decreases is given in Table 4. Model Synovial Fluid 1 showed a more significant decrease of particle count than Fluid 2 (see Figure 15) and also on deviation (Table 4). The average size of ϒ-globulin clusters (Model Synovial Fluid 2) decreased simultaneously with the decrease of the ϒ-globulin particle count; however, the average size of albumin protein clusters (Model Synovial Fluid 1) was unchangeable. The opposite deviations were shown by the simultaneous friction measurements. The deviation (percentage increase) of CoF trends was evaluated (see Table 4). This parameter was the second main output from the experiments. The correlation between CoF and particle count trends, measurement with Model Synovial Fluid 1, is shown in Figure 16. These trends implied the dependency between the initial increase in CoF and the decrease in particle count; i.e., the trends did not change much, and their changes were gradual. In the second measurement, a similar dependency was not distinct (measurement with Synovial Fluid 2, labelled ϒ-globulin).

Table 4. evaluation outputs from experiments: percentage deviations of measured magnitude.

Lubricant	Deviation of CoF	Deviation of Particle Count
Model Synovial Fluid 1	61.55%-Increase	3.73%-Decrease
Model Synovial Fluid 2	56.96%-Increase	23.33%-Decrease

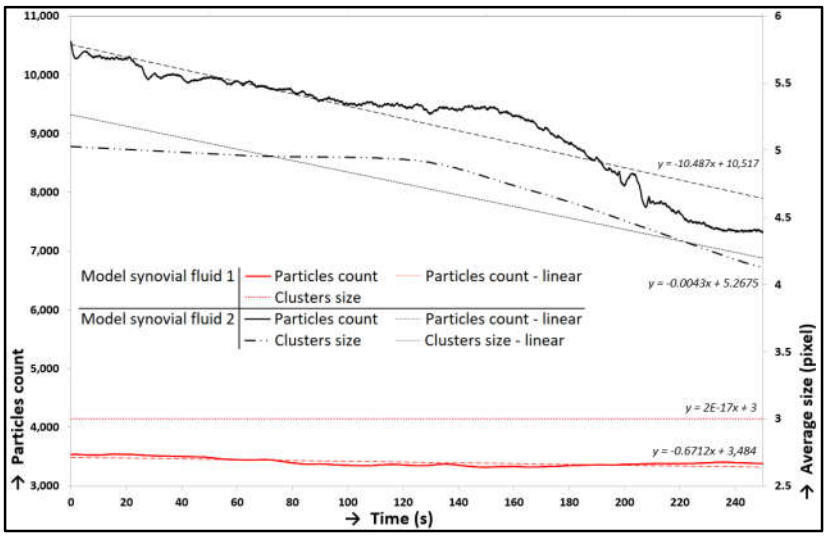

Figure 15. Particle count/average size/time-dependence: Model Synovial Fluids 1 and 2.

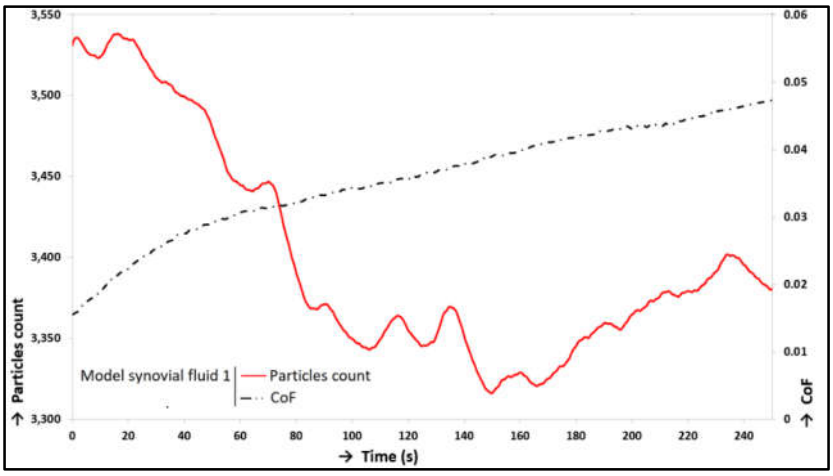

Figure 16. CoF trend: Model Synovial Fluid 1.

A comparison of particle count trends showed that the Υ-globulin protein clusters were wiped off faster from the contact than the albumin; thus, the lubrication film was formed especially by albumin clusters. Υ-globulin helped with the forming of the lubrication film at the beginning of the experiments, and the size of its clusters was much larger than that of the clusters formed by albumin; nevertheless, the lubrication film formed by albumin was more stable. The results suggested a greater influence of albumin proteins on lubrication because of their higher stability in contact, but the clusters formed by Υ-globulin were more numerous. Similar conclusions were also presented in [35], but different lubrication compositions of the fluids were used with the same labelled components. The study suggested more Υ-globulin clusters in the contact, but the trends of particle counts with both labelled components were different. This could be caused by the different composition of the experimental fluids, experimental conditions, and in particular different specimens (PVA hydrogel was used instead of cartilage samples). The decreasing trends of particle counts were shown in both measurements, although the trend of CoF was increasing. The measurement with Model Synovial Fluid 2 showed a steeper decline of particle counts, which indicated a connection with the growing trend of CoF (see Figure 15), but with a smaller impact on lubrication film forming. However, measurement with Fluid 1 showed a very gradual decrease of particle counts, which indicated a greater impact on lubrication, but a smaller impact on the increasing trend of CoF. These results showed that, in this case, the albumin proteins represented the main component responsible for

lubrication film formation. In general, a decrease in particle counts in both measurements indicated squeezing out of lubricant from the contact area, i.e., weeping and squeezing out of protein clusters from the contact, which weakened the adsorbed film formed by the proteins. In general, the connection between the rising CoF trend and the decreasing trend of particle count pointed out that the adsorbed lubricating film on the cartilage surface, which was created by the protein clusters, was a key factor for the low friction in the contact and protected the cartilage surface from damage [50]. When the adsorbed lubricating film covered the cartilage surface, the motion (friction) took place inside or between the protein layers, i.e., lower CoF. The results showed that the albumin clusters were the basis of the lubricating film; they were adsorbed at first on the cartilage surface, and the Ɣ-globulin clusters were bonded to them. The protein clusters were gradually wiped off from the contact; at first, the Ɣ-globulin clusters and after that, the albumin clusters. This caused, in the extreme case, the contact of raw cartilage tissue, which damaged the cartilage tissue and increases the CoF. The presence of protein clusters was partly due to weeping of lubricant from cartilage pores, which was proven by the static loading experiment in correspondence with the lubrication theories published in [42,43]. These theories describe the cartilage lubrication based on the weeping of lubricant from cartilage pores during motion. The pores serve as a lubrication stack, and this is exhausted during motion, which causes a CoF increasing trend. In general, the decreasing trends of particle counts caused a decreasing thickness of lubrication film until the proteins were completely removed from the contact. According to the indications, it was expected that the carried-out experiment worked at the transition of the boundary and mixed lubrication regime depending on the value of CoF. A decrease in lubrication film led to an increasing CoF trend. Albumin had a greater effect on the CoF change from this perspective, but Ɣ-globulin showed a greater change of particle count. Albumin showed a greater impact on lubrication in both cases (lubrication and friction). The values of CoF started approximately between 0.01 and 0.02 and ended at approximately 0.05 after 250 s of testing. Other publications showed slightly different values and also in comparison with these findings above. Publication [60] showed similar values of CoF with a similar configuration of the tribometer; nevertheless, different operating conditions and lubricants were tested. Publications [46–48] showed experiments with a similar model of synovial fluids providing similar results of CoF. Different cartilages were also tested: a cartilage removed from guinea pigs [38], bovine bones [57], and human specimens [51]. Though the samples were removed from different animals, the values and trends of CoF were comparable; nevertheless, the deviation of CoF depended on the combination of friction samples, testing conditions, etc. A rise in CoF trends, which was shown in this study, has been known from other publications [46–60, 38], and the values of CoF were comparable depending on the processing conditions, configuration of specimens, and lubricants.

3.3. Methodology and its limits

All measurements were carried out with one cartilage specimen. The reason is a great variation of tribological and mechanical properties across cartilage samples removed from different bones. Mechanical properties, especially elasticity modulus, depend on the site of sample removal, which is connected with the deviation of tribological properties [72,73]. Mechanical and tribological properties are better when the specimens are removed from the most loaded site of the bone surface. Specimen properties also deviate due to the type of joint because each joint is differently loaded [17,66,74]. Tribological properties also depend on the age of the animal from which the specimen is removed [38]. The use of only one specimen declared the comparability of the percentage deviations of measurement values. The cartilage as a biological material tissue is also sensitive to degradation in air; therefore, all experiments were carried out consecutively in one day [75]. The lubricant was also a biological specimen so there was a risk of degradation. Moreover, there was also a risk of gradual loss of the fluorescence properties of the dye contained in the lubricant specimens. When the lubricant was excited by the light source, the emitted light from the dye gradually decreased. It could be assumed that the measured fluorescence intensity, which was the basis for the evaluation of the particle count by the new software, was thus affected.

Real natural synovial joints operate under various conditions (load, sliding speed between cartilages) depending on the type of movement (walk, trot, run, etc.) [73]. The direction and type of movement between the surfaces of cartilages vary depending on the type of joint (knee, hip, etc.); each joint operates under special kinematic conditions [73,76,77]. The present study used a simplified model of the synovial joint. The kinematic operating conditions were simplified to the reciprocating motion with a constant velocity (see Figure 5); i.e., the kinematic operating conditions were simplified from the multi-axis motion with various velocity and load to the single-axis motion with constant velocity and load. This simplified model of the synovial joint with a reciprocating tribometer used the cartilage as a testing specimen and the glass plate as the friction specimen. The glass plate ensured insight into the contact; however, the modulus of elasticity of glass is of a larger order than that of cartilage tissue. The performed experiments used model synovial fluids that did not contain all of the components of physiological synovial fluid. The compositions of the fluids were adapted to the possibilities of fluorescence labelling of synovial fluid components. Previous research studies used different fluid compositions; moreover, each animal has a unique synovial fluid composition.

4. Conclusions

The present research showed a new approach to cartilage friction and lubrication evaluation. Friction measurement was connected with simultaneous visualization of cartilage contact, which helped to better understand the cartilage lubrication processes. This opened a new look at the evaluation of individual components of the lubricant and the correlation with the friction coefficient. The newly designed evaluation software and experimental device were presented. The evaluation of lubrication was based on the processing of the contact record provided by this software, which evaluated records (snaps) from the fluorescence microscope. Friction measurements simultaneously with visualization were carried out for on-off loaded contact and the reciprocating test with the model synovial fluid. The conclusions from the measurements were as follows:

The on-off loaded contact showed a decreasing trend of particle count in the contact, which pointed to weeping of lubricant out of the contact.

The albumin protein played a major role in lubrication and created a stable lubrication film in the contact.

The connection between the rising trend of friction and the trend of albumin particle count was indicated.

The Ɣ-globulin protein showed a significant decrease of particle count in the contact, which pointed to its smaller role in the cartilage lubrication.

This methodology represented great potential for understanding the lubrication system in human synovial joints, which will help to treat joint diseases. Our research assumed the future experiments to be focused on the analysis of the impact of individual components of synovial fluid on lubrication and friction, and also the rehydration of cartilage will be examined. The newly designed experimental apparatus together with the newly developed evaluation methodology could open new possibilities for testing of other soft contacts, such as contact lenses, rubbers, or soft polymers.

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Biotribology of synovial cartilage: Role of albumin in adsorbed film formation

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ABSTRACT

A properly lubricated natural synovial joint is the basis of the proper function of the natural musculoskeletal system to lead an active and painless life. A properly lubricated natural synovial joint is the basis of the proper function of the natural movement system to lead an active and painless life. Well lubricated synovial joints are expressed, in particular, by an extremely low coefficient of friction and wear between cartilage surfaces. The presented manuscript is focused on the impact of albumin protein on the formation of adsorbed boundary layer in the contact of cartilage – a simplified model of synovial joint. This can contribute to better understanding of the lubrication in synovial joints. All presented experimental tasks were performed using a reciprocating tribometer along with fluorescence microscopy – friction forces were measured simultaneously with fluorescence records of contact. This unique experimental approach used a newly designed evaluating procedure based on image processing. The experimental results show a great impact of hyaluronic acid; adding of hyaluronic acid leads to a reduction in friction and a larger area of albumin adsorbed boundary layer; however, the phospholipids show the opposite effect. A combination of the individual protein solutions, albumin and γ-globulin, has no significant effect on the particles count of albumin clusters adsorbed in the contact; however, the area of albumin adsorbed boundary layer with simple albumin solution was much larger than the solution combining both proteins. The conclusions and discussion of this study describe the role of albumin protein in the lubricating process prevailing in a simplified model of synovial joint under conditions corresponding to slow human gait.

1. Introduction

The comfortable and active human life needs a proper functioning of the musculoskeletal system; however, despite a high level of current state of healthcare, we suffer from a number of joint diseases [1]. The progression of joint diseases may vary depending on many factors and can reach the state when the joint is incurable. So far, the most common solution for the incurable natural joint is its removal and replacement by an artificial one [2]. Although the advanced joint prosthesis has a long lifetime, sometimes it has to be replaced repeatedly. A reoperation of the prosthesis is a considerable burden for the human body. It has the impact on bone degradation, mental health of patients, etc. [3–5]. This is enormously stressful for people who prefer an active life but are afraid of reoperation; therefore, the general effort is to postpone the necessity of operation of prosthetics as long as possible.

One of more contemporary ways of how to treat or, at least, delay or stabilize the disease of the natural joints which are not completely destroyed, is a non-invasive treatment (the intervention does not require surgery) using viscosuplementation (supplement – a gel-like fluid consisting mostly of hyaluronic acid (HA) is injected into the joint gap) [6]. Due to the limited lifetime of the prosthesis, the common endeavor of viscosuplementation is to defer the urgency of operation of prosthetics as long as possible. The supplements should restart the lubrication processes in degraded natural joints, which ideally stops future damaging of joints or, at least, slows down the process of degradation of natural joints (natural cartilage) [6,7]. The supplement therapy is usually effective, but the same therapeutic effect is not guaranteed for all patients, and how exactly the supplement works has not yet been proven [8–10]. To better understand this issue, the full principle of lubrication in natural synovial joints needs to be described, i.e., it is necessary to describe the adsorbed boundary layer formation in

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the contact realized by the articular cartilage and lubricated by synovial fluid (SF) [11].

The basis of the unique tribological and mechanical properties of natural synovial joints is the contact of two bones, whose surfaces are covered by the articular cartilage, and the lubrication is realized by SF [12]. Due to the specific mechanical and tribological properties of cartilage, the contact pressure is dispersed over a large area on the surface of cartilage where the SF ensures a very low friction coefficient (CoF) [13]. An alternative to a natural cartilage is a hydrogel, which represents an artificial model of cartilage with similar tribological properties [14]. Most of natural cartilage tissue consists of water and of type II collagen fibres as a matrix, HA, and lubricin [12,15]. The tissue of a fabric structure is filled with water and its thickness is divided into three zones, varying especially in orientation, shape, and amount of collagen fibres [12,16]. The cartilage tissue is characterized by low cell density; therefore, the tissue of cartilage is nourished through the SF [17]. The SF is mainly formed by proteins, HA, phospholipids and proteoglycans [18–20]. Based on the study [20], the albumin protein is the most abundant in the SF. The porous cartilage structure contains negatively charged components which provide the attraction and detention of water (SF) to the pores [21]. The lubrication system in natural synovial joints works on the principle of porous structure and absorption of water, on which is based the synovial joints lubrication system. There are many theories which seek to explain this principle, but so far there is no work that would give a comprehensive overview of the lubrication function in the synovial joint.

The research focused on tribology of cartilage can be divided into two main groups. The first one is focused on cartilage lubrication and the other one describes the friction performance of cartilage. The main discussed topic in the lubrication studies is the lubrication regime, where there are several theories attempting to explain the lubrication mechanism including hydrodynamic lubrication [22], boundary lubrication [23,24], weeping lubrication [25,24], and boosted lubrication [26]. Some studies published in later years show more advanced lubrication theories or present new theories of lubrication mechanisms – hydration lubrication [27], and adaptive multimode [28,29]. Although the studies on visualization are less common, the published ones mostly describe the use of hydrogel instead of cartilage. These studies seek to support and verify the mentioned theories, and the authors try to classify the influence of individual components of SF. The γ-globulin protein was shown as a significant component in the lubrication process of hydrogel; nevertheless, a degree of influence depends on the γ-globulin protein concentration and the concentrations of remaining components of the lubricant [30]. The gel–like layer on the surface of cartilage is also essential for lubrication processes, because it can protect the surface from wear. HA is the main component of gel-like layer. This layer is formed on the cartilage surface due to bonding of HA with chondrocytes contained in the structure of cartilage [31]. The thickness and composition of gel-like layers depend on the size of individual molecules and the pore sizes. Large molecules cling to the surface of cartilage and the cartilage structure is penetrated only by smaller particles. [32]. All presented studies reflect the fluid leakage from the cartilage structure, which is the basis of all theories.

Another topic discussed by the researchers is focused on the friction properties of cartilage. The "friction" issues of cartilage are better understood due to a simpler but more developed methodology related to the experiments and evaluation of data. These "friction" studies indicate the understanding of cartilage behaviour due to friction. They deal with the behaviour of cartilage contact lubricated by SF and indicate very good friction properties [33,34,35], which is expressed by very low CoF. The positive impact of friction (lower CoF) may be expressed by a larger volume of HA in SF [34], rehydration [36], higher load [33,37,36]. There is the dependence between the level of CoF and the type of movement [37]. The change of the sampling point on the cartilage surface has a significant impact on CoF; this depends on the mechanical properties of cartilage samples [38]. The general CoF trend has a growing character [33,34,37].

As is obvious, many studies are focused only on friction of cartilage contacts while the studies dealing with visualization are less common. Moreover, there are no studies connecting these two issues into one experimental task; i.e., the experimental device and methodology, which allow for simultaneous measurement of friction forces and visualization of cartilage contact (a simplified model of synovial joint). Some authors used a fluorescence microscopy for visualization of cartilage or hydrogel contacts, from which it follows that this method is the most appropriate for the visualization of compliant (cartilage) contacts on non-reflective surfaces. Studies dealing with visualization of cartilage contact are not frequent and the link with measurement of frictional forces is missing. The present study supplements the relationship between the cartilage visualization and friction measurement in the simplified model of synovial joint; therefore, a better description of cartilage lubrication processes will be replenishing, which can help to better understand the cartilage lubricating processes. The previous study [39] presents the methodology for the evaluation of simultaneous visualization of cartilage contact together with friction measurement. This study is a follow-up one and extends the research dealing with the experimental task focused on the behaviour of albumin protein in the formation of adsorbed boundary layer in the cartilage contact and the methodology presented in [39] is used. This study aims to clarify the link between the influence of albumin protein (as the protein with the highest concentration in SF [20]) in the formation of adsorbed boundary layer and the change of CoF trends. An explanation of the lubricating processes and the adsorbed boundary layer formation in the synovial joint contact can contribute to understanding of viscosupplements function and ensuring the right effect on all patients.

2. Materials and methods

2.1. Experimental device

A pin-on-plate tribometer of a unique own design was used as described in detail in [39–41]. The basis of the experimental device (schema is shown in Fig. 1A) is a cartilage sample in contact with a reciprocating glass plate (material B270). The contact is loaded using a lever through the cartilage sample and this is placed under the glass plate. The optical system is located above the contact and the observation record is obtained by camera. The contact is flooded with lubricant – in this case, the SF model. The lubricating bath is heated to the human body temperature (37C) by temperature controller Hotset C448 together with heating cartridges. These are placed under the bath and the temperature sensor is placed as close to the lubricant as possible. This arrangement prevents the lubricant from overheating (overheating of lubricant would cause rapid degradation of used components). A rigid frame of tribometer is the basis of the whole device and, together with the ball screw, allows for the reciprocating motion without clearance. The loading mechanism is based on the principle of a lever mounted on two preloaded bearings. The loading lever is equipped with a deformation member allowing for a minimum deformation in the loading direction and a high deformation in the friction direction, which permits the measurement of very low friction forces. Both forces (normal and friction) are measured by tensometric sensors. The reciprocating motion and loading are ensured by the stepper motors - rotational in the reciprocation case, and linear in the other

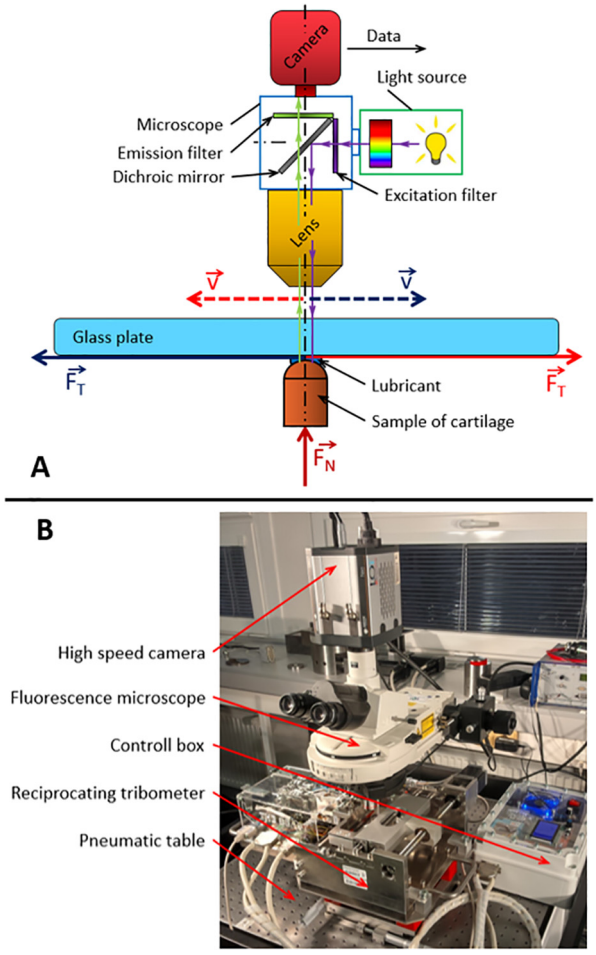

Fig. 1. Experimental apparatus. (A) schema of experimental apparatus, (B) experimental apparatus in laboratory.

case. The reciprocating tribometer is placed on the pneumatically balanced table, where the fluorescence microscope is also anchored. The entire experimental device is shown in Fig. 1B. The outputs of each experiment are the record of contact area and the friction and load forces trends.

2.2. Fluorescence microscopy

The fluorescence microscopy was used as an optical (observation) method for the experiments presented in this study. The basis of this method is a light emission of a substance excited by radiation. The fluorescence principle can be divided into three steps. First, it is excitation, when the excitation photon is absorbed by the fluorophore contained in the fluorescent dye. It is followed by the excitation state period; the absorbed energy is dissipated to ensure the emission of fluorescence. The last step is emission – the dye emits radiation; however, the level of energy is lower due to dissipation of energy in the excitation state period, which is the reason why the emitted radiation has a longer wavelength. A detailed description of fluorescence principle is shown in [42]. The mercury lamp allowing for the emission of a white light was used as a light source. The FITC and TRITC filters placed behind the mercury lamp were used to achieve the required wavelength of emitted and excited light for the dyes. The FITC filter has an excitation wavelength of 490 nm and the emission wavelength of 525 nm, the TRITC filter has an excitation wavelength of 557 nm and the emission wavelength of 576 nm. The optical method and the fluorescence microscope were described in detail in previous studies, where the methodology of visualization of joint replacement for different material combinations was published [43,42]. The visualization (recording of the contact area) is performed through the glass plate, the material of which ensures that the excitation and emission of the contact are not affected. The schema of the optical system is shown in Fig. 1A.

2.3. Specimens and lubricants

The femoral hip heads of mature pigs were utilized for samples removal. The area with the highest contact pressure on the surface of femoral head of hip joint was defined as a sampling area; this definition provides the most mechanical properties of cartilage sample. Due to a compliance of the same placement of removed samples (for all removed bones), the deviation between all samples was minimized, and the mechanical properties were comparable. The samples were removed by the ejector with internal diameter of 9.7 mm. The sampling was performed without delay after the slaughter of the animal. The samples were inspected after sampling with a focus on the preservation of the cartilage surface and on the cartilage edges damage (not to be frayed) to avoid influencing the experiments. The samples were deep frozen (−20 ℃) in phosphate buffered saline (PBS) immediately after sampling. Samples were defrosted immediately before the experiments to avoid degradation. Defrosting of cartilage samples was performed without heating at laboratory temperature, after that the sample is removed from the test tube and placed to the tribometer and flooded by lubricant. The same sampling process was verified in [44]. The sampling process used in this study is shown in Fig. 2. Due to the variability of cartilage samples, the samples were preselected before the experiments. The used cartilage sample was selected using a strict laboratory protocol. The second sample from the contact pair was the glass plate, which fulfils the important premise of transparency in order to observe the contact. The glass plate is 154 mm long, 43 mm wide, and 4 mm thick.

The model of physiological SF and its partial solutions were used as an experimental lubricant. The composition of synovial fluid was inspired by the native synovial fluid. The analysis of native synovial fluid was performed in [20] and the used composition of lubricants was inspired by this article. The variation and composition of all experimental lubricants are shown in Table 1. As is obvious from Table 1, the most represented component of synovial fluid is the albumin protein. Due to this fact, the albumin protein was defined as one of the most important components of synovial fluid; therefore, this article is focused on visualization and behaviour of albumin protein - the albumin protein was examined across all experiments. A bovine serum (BS) albumin (Sigma-Aldrich, A7030) was labelled by Rhodamine B isothiocyanate (283924, Sigma-Aldrich) in this case. The magnetic stirrer was used to stir the other components without dye with the labelled component. The other component of the experimental solutions was γ-globulin from bovine blood (Sigma-Aldrich, G5009), HA = Sodium Hyaluronate HySilk (powder, quality class-cosmetic; molecular weight = 820–1020 kDa, Contipro, Dolní Dobrouč, Czech Republic) and phospholipids = L - α - Phosphatidylcholine (powder, Type XVI-E, lyophilized powder; ≥ 99%; vesicles form; P3556, Sigma-Aldrich, St. Louis, MO, USA). The final solution was prepared by mixing all components with PBS solution. Solutions were mixed using a magnetic stirrer at a maintained laboratory temperature – without heating. The mixing process performs without air access (to avoid degradation) – the laboratory vessel is covered with a nontoxic parafilm foil. The process takes approximately 2 h, until all lubricant components are complete dispersion. The prepared protein solutions were kept in a deep-frozen state (−20 ℃) in opaque and darkened test tubes to prevent degradation and ordination

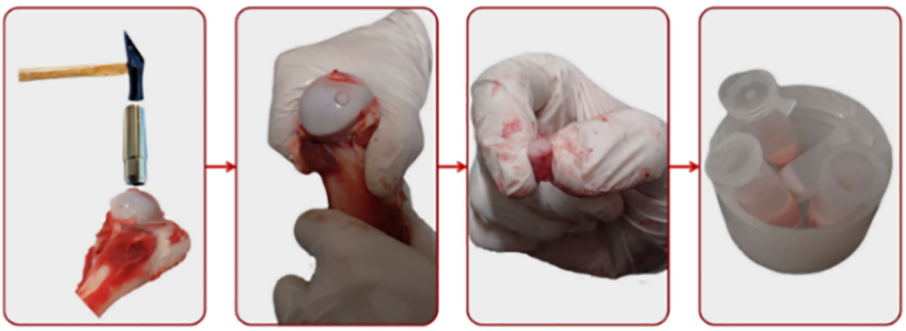

Fig. 2. Sampling process.

Table 1
Lubricants composition, labelled component - albumin.

Lubricant label	Albumin (mg/ml)	γ-globulin (mg/ml)	HA (mg/ml)	Phospholipids (mg/ml)
Lubricant 1	20	–	–	–
Lubricant 2	20	3.6	–	–
Lubricant 3	20	3.6	2.5	–
Lubricant 4	20	3.6	2.5	0.15

of lubricants. Defrosting was performed immediately before the experiments.

2.4. Methodology and conditions

The performed experiments followed a strictly defined procedure for preventing the undesired errors in the results and to help a sufficient repeatability of results. The established procedure of each experiment was described in detail in previous works [41,39]. The same experimental conditions were used for all performed experiments to make the results comparable, see Table 2. The conditions laid down are based on the presented studies in this field regarding the prevailing conditions in the natural synovial joint [45,46]. The load was set at 10 N, which corresponds approximately to 0.8 MPa of contact pressure and 10 mm/s of sliding speed. These conditions correspond to a very slow human gait and average joint pressure [45,46], which is a joint regime corresponding to a large part of human joint life. The experimental temperature was maintained at 37C. A similar condition was used for experiments in previously published studies; it allows to compare the results between this study and the studies that have already been published [34, [36]. Each experiment, i.e., the experiment with one type of lubricant, was carried out 9 times (3 experiments, where one experiment counts 3 repetitive experiments with an in - between hydration cycle, together with 9 individual experiments). The schema of one set of experiments is shown in Fig. 3. The selected count of experimental tasks allows to determine the repeatability of the experiments and the impact of rehydration. All experiments were performed on one sample so that the results of the individual experimental sections can be compared; especially, the recordings of the contact area. To ensure the same state of the cartilage sample before each experimental set, the run – in a cycle was carried out before each set of experiments, which helps to bring the cartilage structure and surface to the same state before each experimental section.

Output procedures are described in detail in the previous work [39]. The process to evaluate the results is shown in Fig. 4. As is obvious, the evaluation process is divided into two parts – CoF processing (Fig. 4A – C) and processing of the record of contact area snaps (Fig. 4D – G), which finally shows the dependency between friction and lubrication. The raw main data output from each experiment are friction forces measured in the contact and the record of the contact area through the fluorescence microscope. This data was further processed. The friction trend is transformed to CoF and is fitted by the straight line. The tangent slope is determined for each CoF trend and it is the evaluation value for the connection with lubrication.

The record of the contact area from each experiment whose output are the snaps, is an input for the processing by the specially designed software described in detail in [39]. This software is based on the principle of image segmentation, allowing for the removal of the background and highlighting particles that have an order of magnitude higher intensity. This processing ensures that only the marked particles to be monitored are left in the snap. The software calculates the particle count and the average size of particles in each snap. These values are determined for all snaps; therefore, the particle count trend was defined for each experiment. This trend is fitted by a straight line and the tangent slope was determined, whereby it is expressed as the relative difference of particle count and it is the output evaluation value from visualization, the second evaluation parameter.

The culmination of the evaluation process is the linking of both relative differences which form the final output from each experiment – dependency between the particle count and CoF. Both differences are relative to allow for a comparison between both output parameters and also between all performed experiments. The final CoF and the particle count dependency allows to determine the impact of the individual compositions of the lubricant on the lubrication process.

Table 2
Experimental conditions.

Load	Velocity	Stroke	Number of cycles	Duration	T
10 N	10 mm/s	20 mm	25	2 min	37 C

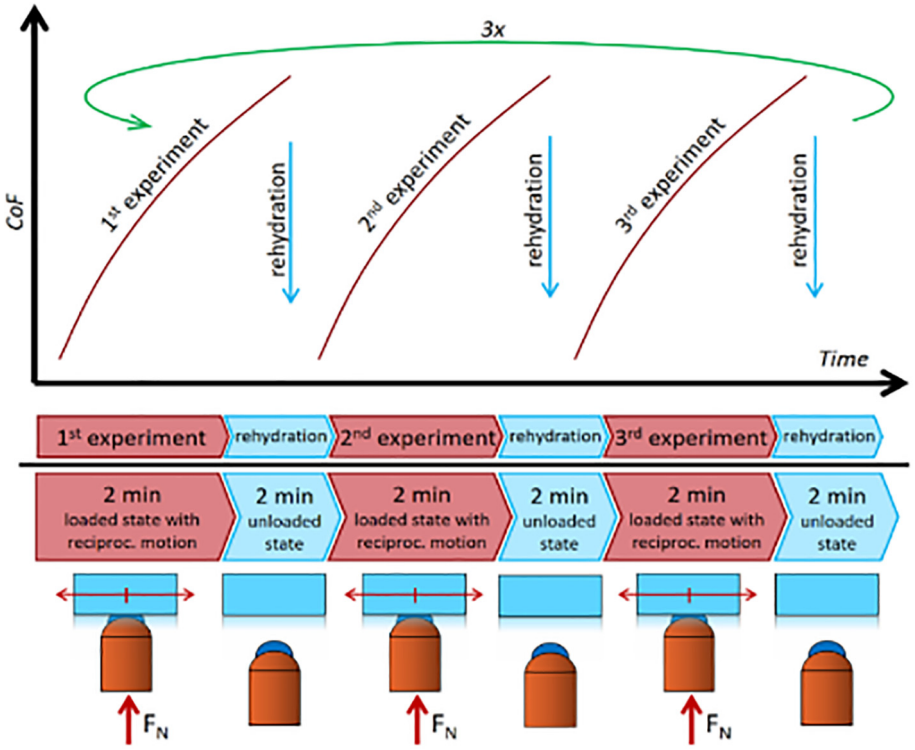

Fig. 3. Schema of each experimental set.

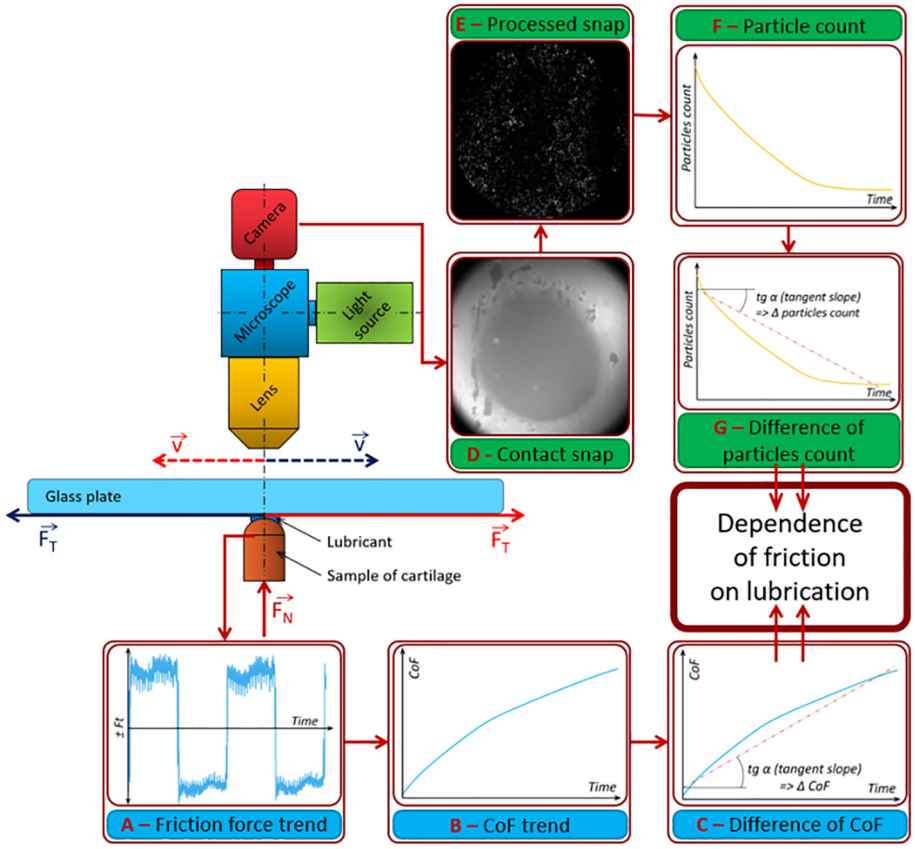

Fig. 4. Evaluation schema.

3. Results

3.1. The toolbar and its menus

Four sets of experimental tasks were carried out and each set was performed with one modification of lubricant (lubricant 1 – 4, see Table 1). Each lubricant was used for 3 replicate measurements consisting of 3 consecutive measurements with rehydration between each experiment, 9 individual experimental tasks in total (see Fig. 3). The friction trends for all modifications of lubricant are shown in Fig. 5. One curve in this graph represents 3 averaged measurements, and each type of curves represents one set of experiment (1,2,3) with in–between rehydration (see Fig. 3). The lowest fiction is reported by lubricant 3, although it is not the complex SF. However, the lubricant 4 (complex SF model - adding of phospholipids to the lubricant unlike a lubricant 3) causes deterioration of friction properties; nevertheless, this lubricant reports a lower CoF than lubricants 1 and 2, which represent the proteins solutions (lubricant 1 – simple albumin and lubricant 2 - albumin + γ-globulin solution). The worst CoF is reported by lubricant 1 – simple albumin solution. The rehydration has an expected impact on CoF trend, the CoF value is restarted after each rehydration.

3.2. Visualization of contact

The albumin protein was labelled with all modifications of the lubricant (see Table 1); therefore, all records of the contact area show only albumin dependencies. Snaps were processed by the evaluation software [39] and correspond to one value of CoF; consequently, each snap shows the count of albumin protein clusters and their average size. The evaluation software was calibrated by comparing the trend (tangent slope) of emission intensity (raw output from the experiment – measured by a fluorescent microscope) and the trend (tangent slope) of particle count [39]. The sensitivity of the calibration process to the setting of input parameters is analysed in chapter 4.3. The examples of processed snaps (the protein clusters are highlighted) are shown in Fig. 6; they were taken with lubricant 3 (albumin + γ-globulin + HA – measuring number 5/9). Fig. 6A shows the beginning of the experiment (particles – protein clusters, nearly 1600 clusters of albumin proteins), Fig. 6B represents the state in time t = 25 s (the particle count has increased, nearly 1700 clusters of albumin proteins) and the last

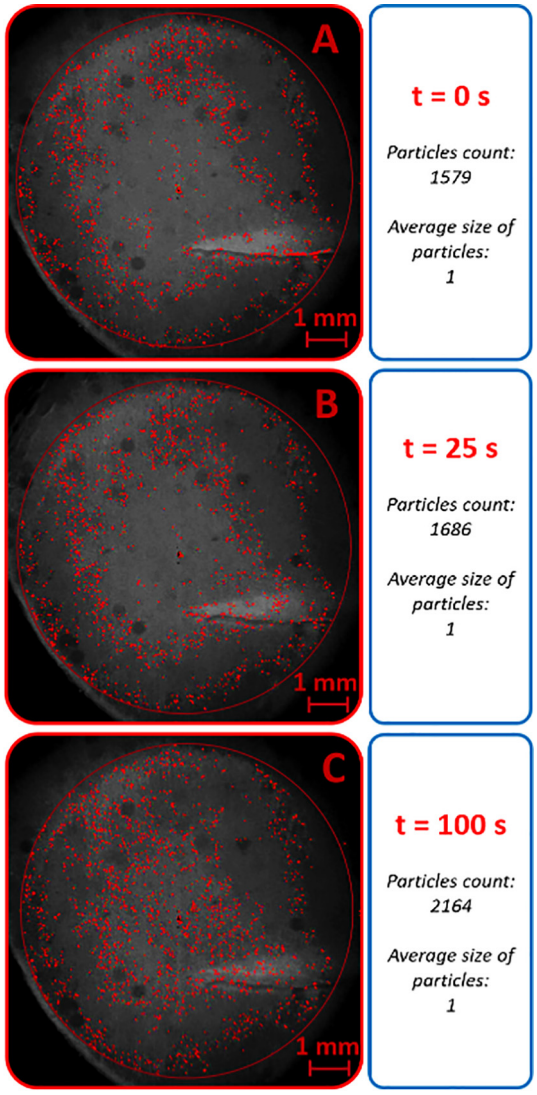

Fig. 6. Snaps of contact area of cartilage – highlighted protein clusters. Experiment with lubricant 3 (albumin + γ-globulin + HA). (A) the start of the experiment – time 0 s, (B) time 25 s, (C) the end of experiment –time 100 s.

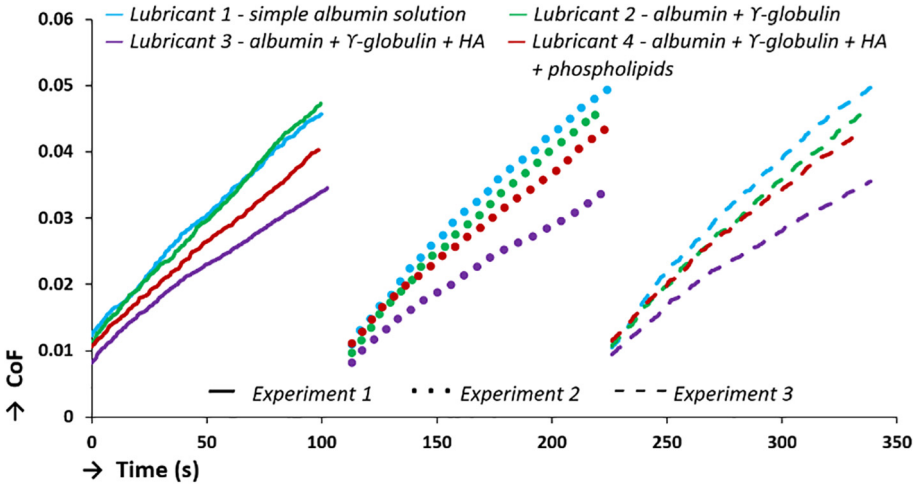

Fig. 5. CoF trends - comparison of all experiments.

snap (Fig. 6C) represents the state at the end of the experiment (the count of protein cluster is the largest, nearly 2200 clusters of albumin proteins). Each of these values belong to one snap. The average size of the detected particles does not change during this experiment. The increase in the particle count is clear from Fig. 6 (the difference between Fig. 6A and Fig. 6B).

The output of evaluation software was determined for each snap in each experimental task so that the particle count trend for each experimental task can be depicted – see Fig. 7. As with CoF trends, one curve in this graph represents 3 averaged measurements, and each type of curve represents one set of experiment (1,2,3) with an in - between rehydration (see Fig. 3). The trends representing protein solutions (lubricant 1 and lubricant 2) show a lower total count of albumin protein clusters than the more complex modification of lubricant 3 and complex SF model – lubricant 4. The trends of lubricant 1 and lubricant 2 mostly show a declining character; however, trends representing more complex modifications of lubricants (lubricant 3 and lubricant 4) show a rising trend of particle count of albumin clusters. Lubricant 3 and lubricant 4 (representing a more complex SF model) show a higher total count of albumin protein clusters than simpler modifications of lubricants (lubricant 1 and lubricant 2). The largest count of albumin protein clusters is shown by lubricant 3 (partial SF – albumin + γ -globulin + HA), although the complex SF model (lubricant 4) reports lower values of protein cluster count.

The particle count does not always have to be an authoritative benchmark for evaluation, quality and quantification of the adsorbed boundary layer; nevertheless, the area of adsorbed boundary layer formed by the labelled component of the lubricant (in this case by albumin protein) has a higher strength of value. The area of the adsorbed boundary layer in the contact is calculated by the multiplication of the particle count and the average size of particles in the contact and is then specified in pixel units. Trends of the albumin adsorbed boundary layer area are shown in Fig. 8. As with CoF trends, one curve in this graph represents 3 averaged measurements, and each type of curve represents one set of experiments (1,2,3) with an in - between rehydration (see Fig. 3). Lubricant 3 (partial SF - albumin + γ-globulin + HA) shows the largest area of the adsorbed boundary layer, although it is not a complex SF model which reports the adsorbed boundary layer mostly with the smallest area. The albumin solution (lubricant 1) shows only a slight reduction in the area of the adsorbed boundary layer. Lubricant 2 (albumin + γ-globulin solution) shows slightly higher values in the area of the adsorbed boundary layer. The adsorbed boundary layer area is further used as an evaluation value for linking the friction in the cartilage contact and its lubrication.

3.3. Connection between friction and lubrication

This study joints two approaches to the evaluation of tribological properties of cartilage - friction evaluation and visualization of cartilage contact. The final output is shown in Fig. 9, where a dependency of friction on lubrication can be seen. The graph shows the impact of friction and also the impact of adsorbed boundary layer; nevertheless, the quality and quantity of adsorbed boundary layer is shown. The larger points in the graph represent the arithmetic mean from one set of measurements (3 individual measurements) and the smaller points represent all experiments carried out. The friction property is represented by *the arithmetic mean of CoF* (x–axis), and the lubrication impact is represented by *the albumin adsorbed boundary layer area* (y-axis). Lubrication is represented by the area of albumin adsorbed boundary layer (count of albumin protein clusters × average size of clusters), which is a representative value expressing formed adsorbed boundary layer. Four shapes can be seen in Fig. 9; each of them represents one lubricant. When the shape is moved closer to the left (closer to the y–axis), the lubricant reports better friction properties and when the shape is moved up (further from the x–axis), the labelled part of the lubricant reports better lubricating properties. The goal is to move the shape as close as possible to the y–axis and as far as possible from the x-axis - as shown by the dashed arrow. Therefore, the best lubricant is number 3 – it is moved further in the direction of the dashed arrow. This lubricant shows the best friction properties while the larger albumin adsorbed boundary layer is formed. Lubricant 4 (complex SF model) shows good tribological properties; however, the adsorbed boundary layer formed by albumin is smaller. Partial SFs (lubricant 1 and lubricant 2) show both inferior frictional and lubricating properties, although the lubricant 1 turns to be slightly better in the area of adsorbed boundary layer. The graph also suggests that the lubricant 3 forms the most stable adsorbed boundary layer because the area of its shape is the smallest; therefore, the measured points are closest to each other. On the contrary, the least stable adsorbed boundary layer is formed by simple albumin solution.

4. Discussion

4.1. Global discussion

A healthy natural synovial joint ensures painless human movement with the phenomenal low friction. The proper function of natural joints is based on the unique properties of cartilage tissue in connection with natural SF [12]. Unfortunately, many diseases

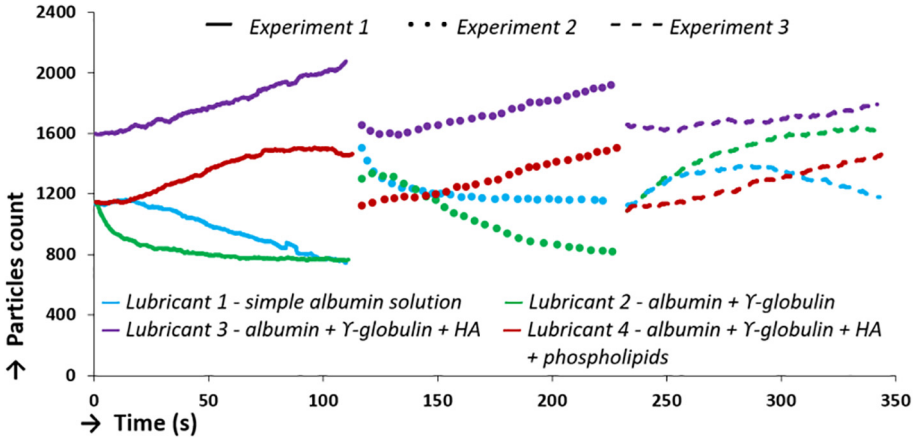

Fig. 7. Particles count trend - comparison of all experiments.

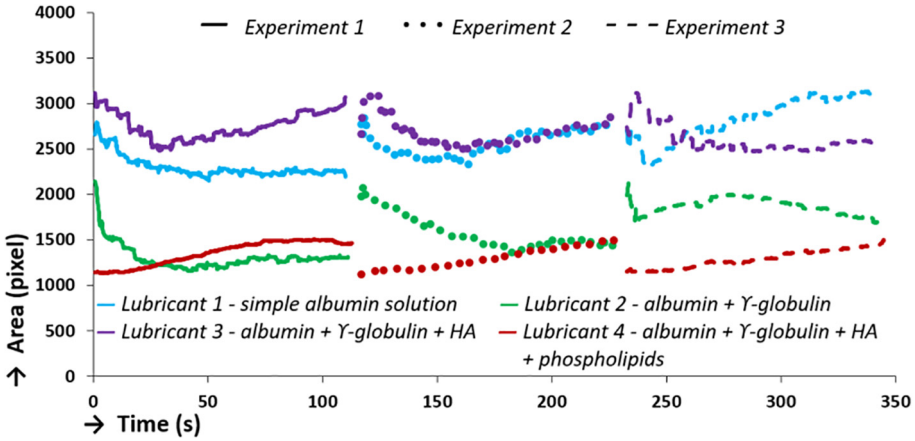

Fig. 8. Trend of adsorbed boundary layer area of albumin - comparison of all experiments.

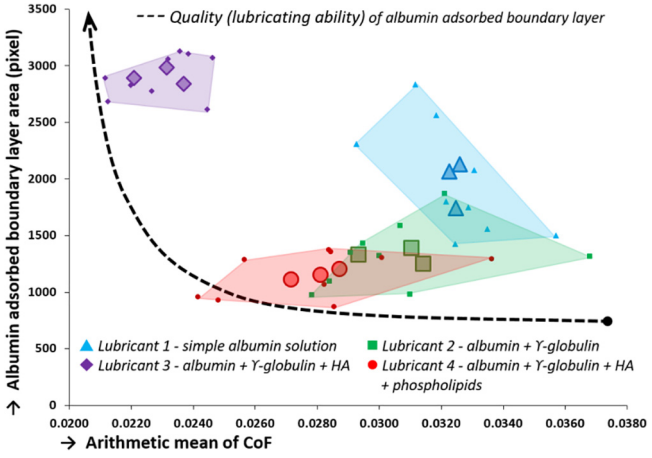

Fig. 9. Dependence of friction on lubrication - arithmetic mean of CoF represent impact of friction and albumin adsorbed boundary layer area represents impact of adsorbed boundary layer.

Table 3
Albumin impact on the lubrication process.

CoF		
Lubricant label deviation	Arithmetic mean	Standard deviation
Lubricant 1	0.0324	0.0016
Lubricant 2	0.0306	0.0025
Lubricant 3	0.0230	0.0012
Lubricant 4	0.0280	0.0027
Particles count in the contact		
Lubricant label	Arithmetic mean	Standard deviation
Lubricant 1	656	485
Lubricant 2	772	291
Lubricant 3	1908	138
Lubricant 4	1155	188
Lubrication film area		
Lubricant label	Arithmetic mean	Standard deviation
Lubricant 1	1980	471
Lubricant 2	1321	276
Lubricant 3	2907	183
Lubricant 4	1158	190

cause degradation and painfulness of joints [1]. The diseases affecting the joints can degrade up to the state when the human movement is not possible without pain. In this case, it is necessary to replace the degraded joint by the artificial one using surgery [2]. The artificial joints do not have an unlimited lifetime; the reoperation is possible but not indefinitely [3]. This is the reason why it is beneficial to postpone the necessity of surgery and stabilize the disease as early as possible. The explanation of cartilage lubricating system is exceedingly substantial for description of lubricating system in natural synovial joint; this knowledge helps to find effective drugs to cure in the best case or, at least, to stabilize or slowdown the disease of natural joints. This study deals with the adsorbed boundary layer formation in the simplified model of synovial joint and describe the role of albumin protein in the SF model.

The global impact of albumin on the lubricating process can be seen in Fig. 9; it is obvious from the shape size that the adsorbed boundary layer formed by the albumin protein is not stable when a simple protein solution is used as lubricant (lubricants 1 and 2). These lubricants show a high standard deviation (see Fig. 9 and Table 3); however, it drops when the lubricants are more complex (lubricants 3 and 4), which is also evident from the area size of individual shapes in Fig. 9. The complex SF model (lubricant 4) and lubricant 3 show lower values of average CoF than simple protein solutions representing lubricants 1 and 2. When the simple albumin solution was used (lubricant 1), the number of protein clusters is declining during the experiment (see in Fig. 7); nevertheless, the average size of its clusters is rising (see in Fig. 8), which causes a slightly increasing area of albumin adsorbed boundary layer in the contact. Simple albumin creates an adsorbed film on hydrophilic surfaces [47]. The cartilage tissue is of a porous structure, whose polarity attracts and absorbs water solutions [48]; therefore, the cartilage surface is suitable for albumin protein adsorption. One part of the lubricant flows through the contact, where the proteins adsorb on the cartilage surface and the other parts flow through the cartilage pores [49]. The albumin proteins create larger clusters when the pressure gradient is higher (in the centre of the contact). It is the reason why the protein clusters on the cartilage contact are smaller than in the case of artificial joints [42]. The increase in the size of albumin protein clusters, and thus also in the area of albumin film, causes, in our opinion, the CoF trend to grow faster and due to the average value of CoF, it is the highest (Fig. 5 and Table 3). The schema of adsorbed boundary layer formation in the case of lubrication by a simple albumin solution (lubricant 1) is shown in Fig. 10-A.

The second variant of the lubricant (lubricant 2) with an added γ–globulin component (see Table 1) shows lower values of CoF than lubricant 1 with albumin only. The particle count also always declines during the experiment (Fig. 7), but the decline is slightly steeper. However, this lubricant shows smaller protein clusters, which causes a lower area of albumin protein film in the contact (Fig. 8). The γ-globulin proteins are much larger than albumin proteins and bind with albumin, as Nečas published in [42]. The albu-

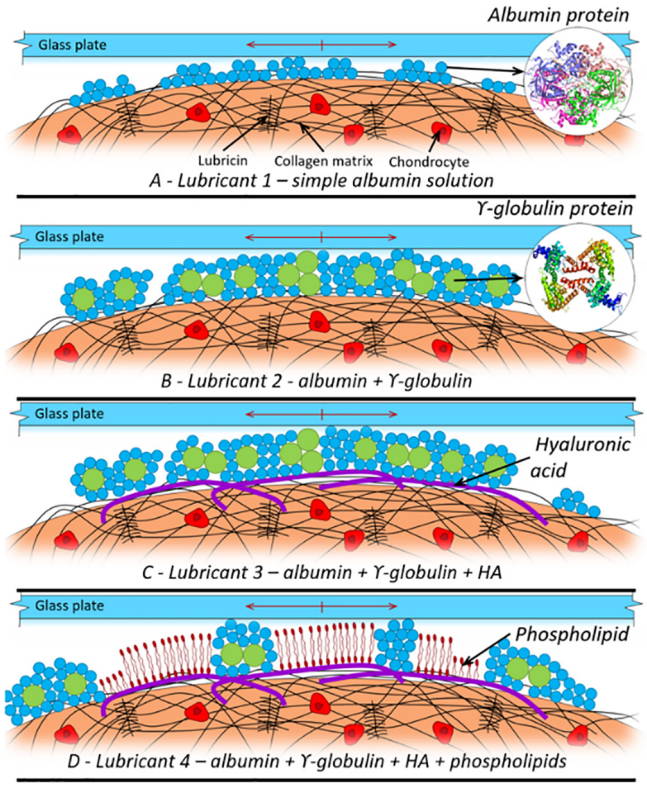

Fig. 10. Schema of adsorbed boundary layer formation. (A) lubricant 1, (B) lubricant 2, (C) lubricant 3, (D) lubricant 4.

min and γ-globulin are proteins characterized by a "string-like" structure. The albumin protein is predominantly characterised by α-helix structure and γ-globulin predominantly by β-sheet [50,51]. These proteins bond together due to their structure - "the string structure becomes entangled in itself" [42,50,51]; therefore, the albumin proteins can bind to the γ-globulin proteins and also the γ-globulin proteins can bond to each other. The adsorbed boundary layer is formed by the albumin cluster, which is divided by γ–globulins; therefore, the cluster formed by albumin is smaller (see Fig. 10-B), where the schema of adsorbed boundary layer formed by lubricant 2 is shown. In our opinion, the combination of albumin clusters and larger γ–globulins causes a greater thickness of the adsorbed boundary layer, leading to lower values of CoF. This deduction is supported also by [35], where the simple γ-globulin shows lower values of CoF.

The lowest values of CoF are produced by lubricant 3, where HA was added. The HA plays an extremely important role in the lubricating system of cartilage [35,49,10] and forms the gel-like layer on the cartilage surface [29]. The surface layers formed by HA protect the cartilage surface against damage [32] and, due to strong hydrophilicity, create an attracting environment for albumin proteins, which adsorb on the surface with better and stronger hydrophilicity. The lubricant 3 (albumin + γ-globulin + HA) shows the best values of CoF (Fig. 5), but also the best values of the number of albumin clusters in the contact (Fig. 7) and the best area of the adsorbed boundary layer formed by albumin (Fig. 8). The number of albumin protein clusters increases during each experiment with lubricant 3; this indicates that the albumin clusters are trapped on the contact mode during experiments. The area of the albumin film formed in the contact decreases in all experiments, though it seems to increase slightly in the other section. The albumin protein clusters are better bonded to the hyaluronic surface layer, which causes a more appropriate fastening of albumin in the contact. The interaction with protein is most present in high molecular weight HA, which also forms the gel-like layer on the cartilage surface. The HA fraction with lower molecular weight penetrated the cartilage structure, in particular the collagen fibres contained in the cartilage structure [31,32,52]. The connection of constitution units of HA causes a firm grip of HA gel–like layer on the cartilage surface. The highest number of albumin clusters in the contact causes a higher thickness of adsorbed boundary layer; therefore, the hyaluronic protecting surface layer is not present in the contact with the raw cartilage surface on the glass plate. In our opinion, it is the reason of the rapid decline of CoF values. The schema of the adsorbed boundary layer provided by lubricant 3 is shown in Fig. 10-C. As is obvious from Fig. 8, the area of lubricant 3 shape is the smallest, i.e., the HA acts as a stabilizer of the adsorbed boundary layer to achieve the same quality of adsorbed boundary layer in each experiment.

The experiments with complex SF (lubricant 4) showed slightly higher values of CoF and lower values of adsorbed boundary layer area formed by albumin, protein, and particle count. In general, it seems that the complex SF has worse tribological properties than the partial complex SF (lubricant 3); nevertheless, the complex SF model is the only one which shows an increasing trend in the number of albumin protein clusters in the contact and an increasing trend of the area of albumin adsorbed boundary layer. This indicates the increasing amount of adsorbed boundary layer during the experiment, which provides a complete protection against cartilage wear. The phospholipids added to the lubricant bind to the HA surface layer on the cartilage. The phospholipids also interbond by lipid tails [27], which allows for the hydration lubrication. However, this is based on the lipid bilayer on each side of the contact, which assumes the same or, at least, similar hydrophilic surface on the other side of the contact. The experimental model used in our study allows for the visualization of the contact of the glass transparent plate; nevertheless, the glass does not meet the requirement for hydrophilicity of surface. The phospholipids bind only to the cartilage surface, or to HA surface layer; therefore, the hydration lubrication is not applied in this case. The schema of the adsorbed boundary layer formation by lubricant 4 is shown in Fig. 10-D. In our opinion, the albumin clusters bind to the cartilage surface and they are imprisoned between a bilayer of phospholipids, which cause a gradual attachment of albumin proteins in the contact. The interaction between proteins and phospholipids is minimal because the phospholipids are strongly bound to the high polar hydration gel-like HA layer; therefore, the phospholipids do not attract low polar albumin and Y-globulin proteins. The phospholipids contain a negative charge phosphate residue of phosphatidic acid and nonpolar lipids residue. In polar solutions (SF), phospholipids are oriented in bilayers or have a micellar orientation, while the phosphate residue is oriented outside of the layer [11,27,53]. The presence of phospholipids causes the increase in CoF [35]; nevertheless, it depends on the amount of all components of the lubricant. In complex SF model (lubricant 4), the presence of phospholipids causes a higher CoF value but the number of protein clusters and the area of albumin film increases during the experiment. The phosphate nuclei bind to water [27,53], and this, without the presence of the second bilayer, together with the structure of phospholipids, causes the lubricant to flow through the contact with higher resistance. The increasing amount of albumin in the contact seems to be the reason of higher value of CoF, as confirmed in [35], where the result shows the same conclusion. The lubrication provided by the complex SF model (lubricant 4) is the only one that allows for long-term operation while avoiding the stable adsorbed boundary layer in the contact.

One of the unique properties of cartilage is the ability of rehydration. As is obvious from Fig. 5, the rehydration between the individual steps of experiment causes the CoF trend to return to

the initial value after each rehydration. Trends showing the adsorbed boundary layer quality (Fig. 7 and Fig. 8) are obviously also affected by rehydration; the trend of adsorbed boundary layer area formed by albumin protein returns to the initial value after each rehydration unlike in the case of particles count in the contact. The regularity of behaviour is better when the lubricant composition is more complex (lubricant 3 and 4). In our opinion, the irregular influence of albumin particle counts in the contact area by the rehydration after each experiment is caused by the count of albumin particles that are nearby the contact area when the experiment is started. The reason is probably that the albumin and γ-globulin proteins in the simpler protein solutions (lubricants 1 and 2) are not as hydrophilic as HA, which causes less ability of individual proteins to bind to the cartilage surface. The regular behaviour of the CoF trends (the value of CoF after rehydration restores to the initial value) in connection with not entirely regular behaviour of trends of particles count through experiments (the value of particles count after rehydration does not always restore to the initial value) point out that the regularity of CoF is not caused only by albumin proteins but it is also affected by other components of synovial fluid. Future research will offer a complete study of each component of synovial fluid. This can contribute to determination of complete dependency of individual components of synovial fluid on the lubricating behaviour and CoF.

4.2. Methodology limitation

The main aim of this study is measuring of friction effects while visualizing the cartilage contact area. The main premiss of this measurement is that one of the contact pairs has to be transparent (the glass plate). The glass plate allows for the reciprocating tribometer to create the model of the synovial joint and allows for the in-situ view of the lubricating processes in the contact. This study admits that the simplified model of synovial joints represents only a very simplified natural synovial joint; however, neither friction measurement nor visualization in-situ can be carried out. In this case, only half of the real joint is preserved – the cartilage sample. The limitation is the elasticity modulus of the glass plate, which is many times higher than the modulus of the second real joint pair, and of different structural, tribological, and hydrophilic properties. Another limitation is the operating condition, which respects the actual pressure in the human joints and the average sliding speed between bones; nevertheless, the variable load cycle, as in the natural joint, is not applied. The values of contact pressure were determined on the basis of maximum values prevailing in the human hip joint; however, the cartilage samples were removed from pigs' joint. To sum up, considering all limitations, the results can only zoom the real adsorbed boundary layer formed in the synovial joint.

As with all experimental bio-tribological tasks, there is a problem with repeatability of measurements performed with the same lubricant but another cartilage sample because each cartilage has a different modulus of elasticity, geometry, and other properties [38]. It is the reason why the measurements were performed on cartilage samples. The aim of this study is to compare the performed measurements and evaluate the data obtained in experiments. The use of a unique sample of cartilage for each measurement could cause the difference between the data from each experiment, especially in visualization, which makes the comparison very difficult. The risk of using one sample is that it can affect consecutive measurements due to the clinging of individual components of the lubricant in the porous structure of cartilage. The components can have a chemical bond to its structure which is very difficult to remove. This study tries to prevent this influence by the initial run-in cycle before each experiment in order to remove all undesired residues from the cartilage structure.

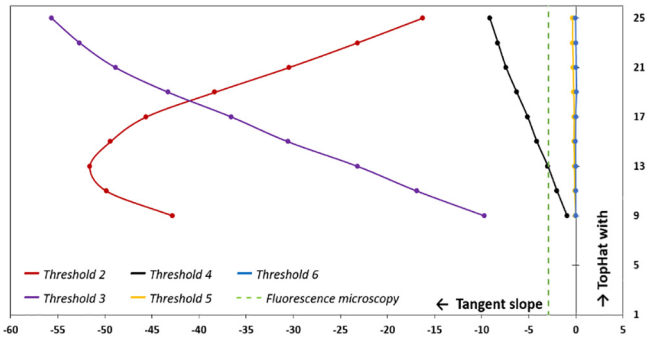

Fig. 11. Sensitivity of processing software settings.

Another provision how to protect the experiment from being influenced by the previous experiment is to determine the lubricant with gradual addition of individual components. Thus the acceptable repeatability can be achieved, see Table 3. The standard deviations of frictional measurements – expected by the CoF, report a magnitude smaller value than the CoF average value – in units of percent. The corresponding value from the assessment of friction/lubrication properties of the lubricant is the CoF; in both cases, it is the area of albumin adsorbed boundary layer calculated from the particle count of albumin protein clusters in the contact and the average size. The arithmetic average and its standard deviation are shown in Table 3.

A part of the experimental apparatus is a specially designed software for processing the snaps from the visualization by fluorescence microscope; a detailed description was published in [39]. For the processed snaps, the software results (particle count and average size of protein clusters) depend on the setting of the input parameters, especially "*TopHat width*" and "*Threshold*". The important input parameters affect the processed snaps; nevertheless, a degree of influence depends on the settings of software. It is calibrated before each experimental set based on the fluorescence trend; however, the calibration may vary depending on the quality of records from the camera, etc. To clarify the magnitude of the effect of variation of input parameters on the software, the sensitivity analysis was performed. The graph in Fig. 11 shows the dependency of the software input parameters in correlation with fluorescence microscopy (dashed green line in Fig. 11). The setting determined for this study is *Threshold* 13, *TopHat width* 4 and the calibration parameter is the tangent slope of intensity trend gained from the fluorescence record; therefore, the settings for these experiments are almost ideal. The set input parameters respond very well; nevertheless, it is not possible to set absolutely the same input parameters as has the intensity trend. Although the input parameters are set to "error", the dependency of other sets is linear; at least in the area (*Threshold* 4). If different input parameters were set (the processing error is linear), the processing error would be deducted because the whole set is processed by one set of input parameters of software.

5. Conclusions

The presented results used the new evaluation procedure introduced in the previous study [39]. This evaluation method allows for simultaneous friction measurement and visualization of contact. The connection of these two previously unconnected tribological approaches contributes to a deeper understanding of tribological behaviour of natural synovial joint including the impact of lubrication on the friction. This research presents a complex study of the impact of albumin protein on the lubrication process of natural cartilage. Fluorescence microscopy, a specially

designed tribometer and evaluation software allow to determine the amount of albumin clusters in the active contact and their average size. The output of visualization is connected to simultaneously measured friction forces, which allows to determine the impact of individual components of the lubricant (albumin in the SF) on the CoF and adsorbed boundary layer formation. Four sets of experiments were carried out, each with one lubricant. The SF model was gradually built from a simple albumin solution to a complex SF model. The concentration corresponds to physiological SF.

The basis of the results are trends of CoF and their relationship with trends represent a lubrication behaviour – the particles count of albumin in the contact and the area of adsorbed boundary layer formed by the albumin protein. The best tribological behaviour was found with lubricant 4, which represents a complex model of synovial fluid. This is the only lubricant which shows low values of friction and a stable adsorbed boundary layer – a permanently rising area of adsorbed boundary layer created by albumin protein. The lubricating behaviour of complex synovial fluid is apparently caused by the presence of HA in combination with phospholipids. In our opinion, HA, due to high hydrophilicity, binds to phospholipids, which causes detention of proteins in the contact. Although the fully complex lubricant represents a good long - term protection of cartilage surface, better friction properties were shown by the lubricant without phospholipids (albumin + γ-globulin + HA). However, a long - term protection of raw cartilage surface is not guaranteed. Although the trends of particles count of albumin in the contact rise quite steeply, the area of albumin adsorbed boundary layer is not always rising over time; the adsorbed boundary layer is likely to break and the raw cartilage surface comes into contact with the glass – the cartilage tissue can be damaged. In the case of protein solutions (simple albumin on one hand and a combination of albumin + γ-globulin on the other hand), a stable adsorbed boundary layer was not observed. In the both cases, the trends of albumin particle counts were decreasing; however, relatively high values of albumin adsorbed boundary layer area were observed, especially for simple albumin lubrication. The trends representing the lubricating quantity in these two cases are not guaranteed. This behaviour seems to be caused by absence of HA, which allows for stronger bonding of proteins with the cartilage surface in the contact. The particles count of albumin and also the area of albumin adsorbed boundary layer show low values for the lubricant combining albumin and γ-globulin. The adsorbed boundary layer consists of a smaller number of particles with a smaller size. The γ–globulin protein is compared to the albumin protein much larger and, based on the previous studies, the γ-globulin binds with albumin. This indicates γ–globulin as a separator of albumin adsorbed boundary layer. The impact of rehydration was also evaluated. The CoF trends return to the initial values after each experiment but the trends representing the adsorbed boundary layer do not show the same trend. This indicates that the restart of CoF values is not only affected by the albumin protein lubrication, but another component is also involved.

The authors presented the first study where the visualization of albumin protein in cartilage contact was performed simultaneously with friction measurement and a newly developed method [39] was used for evaluation of adsorbed boundary layer in a simplified synovial joint model. These methodologies and experimental devices allow for certain limitations and represent not only a simplified model of synovial joints; regardless of this knowledge, this can contribute to the understanding of the lubrication system prevalent in the human synovial joint. In order to approximate the real situation on the nature synovial joint, the future experiments will focus on the evaluation of all components contained in SF. Furthermore, our research assumes the improvement of the experimental equipment using a hydrogel instead of glass, which bring the experimental device closer to the real synovial joint. Newly acquired knowledge gained through a special evaluation method and experimental equipment also allows for a new opportunity in the field of soft contact research (tribology of the eyes, fascia or tissue).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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