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Liposomes as potential biolubricant additives for wear reduction in human synovial joints

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ABSTRACT

Wear processes occurring in synovial joints, mainly known as osteoarthritis (OA), affect many people worldwide. One of the symptoms of OA is wear of articular cartilage; it is thought that among other factors this may be due to failure of lubrication. Injections of biolubricants into human joints can be used in order to maintain the proper functioning of the joint. Phosphatidylcholines, being major constituents of synovial fluid surface active phospholipids, are natural candidates for investigation as additives for cartilage lubricants. Wear tests are described using human cartilage-on-cartilage scheme, *in vitro*, in the presence of different phospholipid-based liposomal bio-lubricating fluids. It is shown that most liposome additive-based lubricants induce less wear in comparison to inflamed synovial fluid.

1. Introduction

Osteoarthritis (OA), also known as degenerative joint disease, is the most common form of arthritis, affecting over 20 million adults in the United States alone [1]. One of the symptoms of OA is wear of the articular cartilage, which may eventually require total joint replacement. Current treatments of OA, such as injection of hyaluronic acid (HA) into joints of OA patients, may reduce that wear and postpone the need for joint replacement [2]. Components of the synovial fluid (SF) are the natural lubricants of cartilage surfaces [3]. HA, a major component of the SF was considered for many years to be responsible for the SF lubricating abilities [4,5] but this became recently questionable [6,7]. Several studies indicate that lubricin, and more specifically its surface active phospholipids (SAPL), is the component accountable for the good lubrication properties of SF [6,8–10]. The SAPL consist of about 41% phosphatidylcholines (PCs), in addition to smaller percentages of other phospholipids [9,11,12].

While many studies on the effect of SAPL on articular cartilage friction can be found in the literature, only a few report on cartilage wear [13] using mostly animal-sourced cartilage rather than human cartilage [14–19]. In all these studies it was concluded that SAPL are critically important for proper functioning of synovial joints. Wear studies with artificially damaged sheep [18] and rabbit [19] knee joints, simulating OA, showed that injecting a solution of the phospholipid DPPC (see Table 1) directly into the joint is effective in reducing cartilage wear, and that HA + DPPC liposomes may be more beneficial than HA alone [19]. Cartilage surfaces of OA patients who were operated on for total joint replacement [20] showed deficiency of the outermost lubricating layer of SAPL, suggesting that administration of exogenous SAPL may delay joint degradation.

This paper reports a wear study using a human cartilage-on-cartilage scheme, *in vitro*, in the presence of different phospholipid-based liposome dispersions. The amount of cartilage wear was evaluated by quantifying increase in the glycosaminoglycan (GAG) content in the lubricating solutions used in the tests [21]. It is shown that liposomes in the form of multilamellar vesicles (MLV) composed of specific PCs or of PC mixtures are highly efficient in wear reduction and can induce less wear in comparison to cartilage lubricated with inflamed synovial fluid (ISF) or with ISF plus HA.

2. Experimental details

2.1. Specimen preparation

Human articular cartilage was obtained from femoral head fracture operations with the consent of donors. Donors' age ranged between 70 and 85 years old, both male and female. Tissue was classified as normal or pathological according to the visual diagnosis. Only femoral heads with normal tissue were selected for

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Table 1
List of liposomes used in this study. (All the PCs form a lamellar phase and are vesicle-forming lipids. T_m is the temperature at which the maximal change in heat capacity occurs during the SO-to-LD phase transition [26].).

Lipid	Chemical name (source)	Phase transition temperature T_m, °C
HSPC	Hydrogenated soybean phosphatidylcholine (Lipoid, Ludwigshafen, Germany)	52.5
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (Avanti, Alabaster, AL, USA)	41.4
DMPC	1,2-Dimyristoyl-sn-glycero-3-phosphocholine (Lipoid or Avanti)	23.2
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine (Lipoid or Avanti)	−21
Mixture of DMPC/DPPC (0.6/0.1)		34

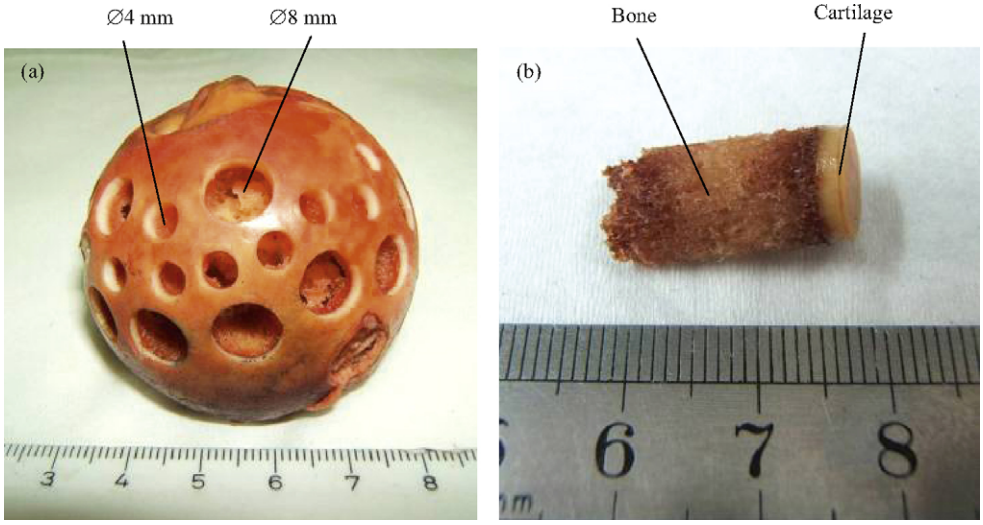

Fig. 1. (a) Human femoral head after plugs removal and (b) a plug sample.

specimen preparation due to inconsistency and possible multiple grades present within OA specimens, which might result in large error with no sound conclusions. Theses healthy femoral heads were frozen at −20 °C until sample preparation in order to keep the mechanical properties close to those of live tissue [1]. Pairs of cylindrical plugs, having 4 and 8 mm in diameter, each pair from the same region of the joint, were prepared (see Fig. 1). These cylindrical plugs, consisting of about 2 mm thick cartilage on top of about 8 mm long bone, were removed from the femoral head using a cork borer. 12–15 pairs of plugs were harvested from a femoral head. The plugs were glued to holders through the bone part, using cyanoacrylate-based adhesive glue, leaving the cartilage projecting out of the holders. Thereafter, these plugs were refrozen at −20 °C until tested. Only cartilage with completely intact and smooth surface was used. More details can be found in Ref. [21].

2.2. Liposome preparation

MLV composed of pure (>98%) PCs (DMPC, DPPC, HSPC, or DOPC or mixture of DMPC/DPPC, see Table 1 for detailed description), were dissolved in ethanol (being less than 5 vol% of the final dispersion) and then hydrated in low ionic-strength histidine buffer (HB) of 5 mM and pH 6.7, at temperatures of at least 5 °C above the membrane phase transition temperature (T_m). Ethanol was then removed from the dispersion by dialysis against HB, to reach levels of <0.5 vol% (determined by osmotic pressure measurements). Liposomes were characterized for size distribution by light diffraction using a Beckman Coulter LS Particle Size Analyzer 13-320 (Fullerton, CA) equipped with polarization intensity differential scattering (PIDS) to provide a detection range up to 2000 μm. Liposome mean sizes were 3.086 ± 0.529 (±SD), 3.212 ± 0.045, and 2.909 ± 0.667 μm for DMPC-MLV, DPPC-MLV and DMPC/DPPC-MLV, respectively.

2.3. Test apparatus description

Fig. 2 shows a schematic diagram of the cartilage-on-cartilage wear test rig. The apparatus is designed to provide a reciprocating sliding motion between two samples of cartilage subjected to an applied constant normal load, and immersed in a given lubricating fluid [21]. The upper specimen, 4 mm in diameter, is attached by the upper holder to a normal loading mechanism, which can only move vertically and is stationary in the horizontal plane. The lower specimen, 8 mm in diameter, is fixed by the lower holder in a bath containing the lubricating fluid. The bath is attached to a linear reciprocating moving table. Both holders and bath are made of acrylic glass. During the wear test both cartilage specimens are immersed in the lubricating fluid to ensure lubricant presence at their contact interface. A controlled flow of distilled water from a reservoir is provided to compensate for evaporation of water

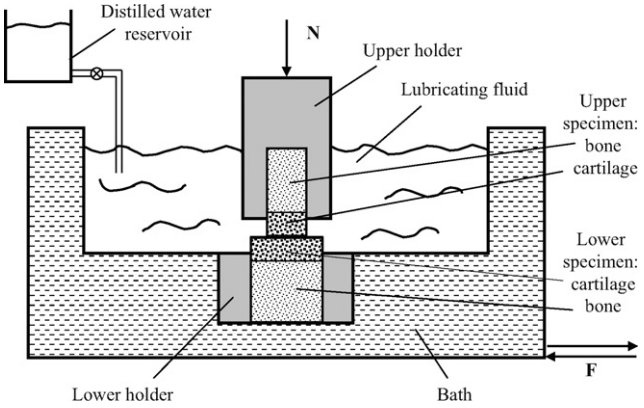

Fig. 2. Schematic diagram of the wear test apparatus.

Table 2
List of lubricating fluids used in this study.

Lubricating fluid	Chemical name (source)	Concentration (pH)
Saline	NaCl (Teva medical, Israel)	150 mM (pH 7.5)
HB	Histidine buffer (Merck, Germany)	5 mM (pH 6.7)
HA	Hyaluronic acid (Sigma, USA)	10 mg/ml
ISF	Inflamed synovial fluid (pooled from several inflamed joints with the consent of donors)	

content of the lubricant and keep its volume and concentration constant during the test. A heat source was employed in order to perform experiments at body temperature. The temperature of the lubricant and specimens was kept at 37 °C and measured by UIL 6681 infrared thermometer (UIL, Israel). More details on the test apparatus can be found in Ref. [21].

2.4. Test parameters and procedure

Physiological saline and HB, see Table 2, were chosen as potential carrier media for liposome additives. Liposomes composed of different PCs, varying in their T_m from solid-ordered (SO) to liquid-disordered (LD) [22], were screened as candidate additives for cartilage lubricants and wear reducers. In a previous work [23], T_m was shown to have a great effect on the friction between cartilage surfaces. A list of the liposomes used in this study is given in Table 1.

Human ISF, see Table 2, was retrieved from several inflamed joints with the consent of the donors. It was pooled in order to obtain a large quantity of uniform ISF for different tests with and without liposome additives. Commercially available HA, see Table 2, which is used for intra-articular administration into OA joints, was tested as another additive to ISF in order to investigate its effect on articular cartilage wear in comparison to liposome additives.

The reciprocating sliding amplitude was set to 1 mm, assuring full contact between the two cartilage samples during the wear test. Each test was carried out for 7.5 h with a reciprocating speed of 1000 RPM (an average reciprocating velocity of 4 m/min). This resulted in a maximum of 450,000 reciprocating cycles for each test (amounting to a total sliding distance of 1800 m). Normal load of 60 N corresponding to contact pressure of 4.8 MPa, which is well in the range of physiological pressures in joints, was used. In order to compensate for any possible loss of cartilage components (which can occur, for example, from loose ends at the circumferential cylindrical cut surface of the plugs during agitation in the lubricating fluid), experiments with identical test conditions but with no load and no contact between the cartilage specimens were also conducted. The actual wear was calculated by subtracting the results at no load from these obtained when load was applied [21]. The current test procedure was identical to that described in Ref. [21] where detailed careful consideration to various issues related to comparing cartilage wear were given.

A pair of frozen specimens was thawed, and the upper and lower specimens were fixed to the loading mechanism and the bath, respectively. A volume of 1.5 ml of the lubricating fluid was placed in the bath and the reciprocating sliding motion started after applying the normal load (compensation at 0 N, and wear test at 60 N).

2.5. Wear determining method

One of the main components of cartilage is proteoglycan (PG) (about 10–20% of dry weight). PGs are macromolecules with a protein backbone and GAG side chains. More than 95% of GAGs in articular cartilage is sulphated [4] (mainly chondroitin sulphate and keratan sulphate). As the cartilage specimens rub against each other, wear debris are released from both cartilage surfaces into the lubricating fluid. Analyzing the solution containing the wear particles for GAG content can be used to assess the wear of the two cartilage specimens [21].

3. Results and discussion

A total of 10 different biolubricants were tested during the present study. Accordingly, 10 pairs of plugs having similar initial PG concentration (see Ref. [21]), were selected from any given femoral head. Each pair was tested in the presence of only one of the different biolubricants. This was repeated with similar groups of 10 pairs from different femoral heads. Each group had a similar initial PG concentration but this concentration could vary from one femoral head to another. This procedure ensured that each repeated test of a given biolubricant was performed with a pair of plugs from different femoral heads. In the following figures, each data point for every biolubricant represents the mean value of at least 3 repeated tests with three different pairs of cartilage specimens, and the vertical error bars represent the standard errors corresponding to each of these mean values.

3.1. Screening potential carrier media

Physiological saline and HB were tested first as potential carrier media for liposomes MLV as lubricant wear reducer additives. Fig. 3 shows the amount of cartilage wear found in the saline or HB as a function of number of cycles. In both cases a run-in type behavior of the cartilage wear with higher wear rate at the beginning of each test can be seen. The initial wear rate decreased monotonically during the first 120,000 cycles (480 m of sliding distance), after which it became constant and remained so during the rest of the test. The constant wear rate following the run-in period is represented by the slope of the linear portion of each curve. A similar run-in behavior, but with obviously higher wear values, was reported for animal cartilage that was rubbed against a stainless steel plate [24].

Substantially lower wear values were observed during the run-in period when using HB in comparison to saline. Also, the constant wear rate following the run-in period was lower in the case of HB, indicating that HB is a better lubricant than saline for cartilage surfaces. Consequently, HB was selected as the carrier media for all the liposome additives screened in this study for their cartilage-lubricating abilities.

3.2. Screening potential liposome additives

Various liposomes composed of different PCs that cover a wide range of T_m, from −21 to 52.5 °C (Table 1) were screened in this

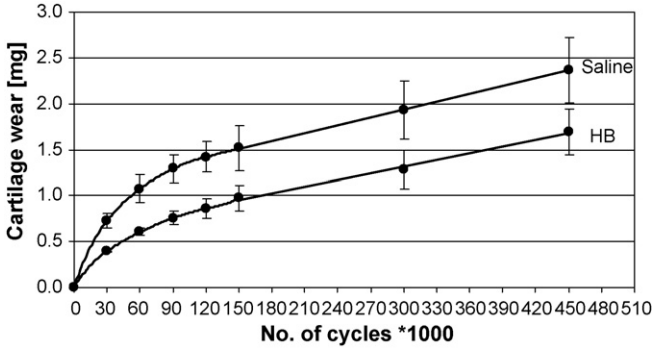

Fig. 3. Cartilage wear as a function of number of cycles in the presence of two different potential carrier media.

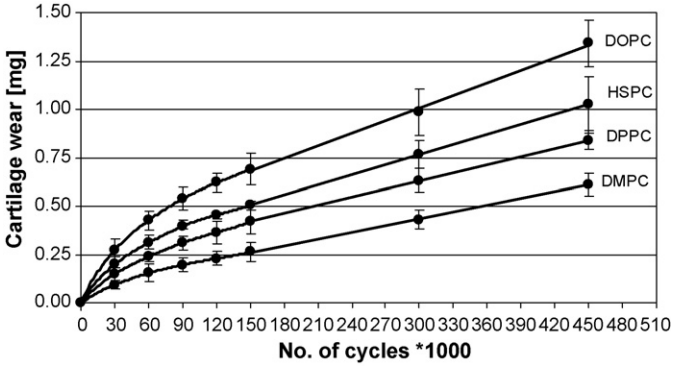

Fig. 4. Cartilage wear as a function of number of cycles in the presence of DOPC, HSPC, DPPC and DMPC, all dispersed in HB.

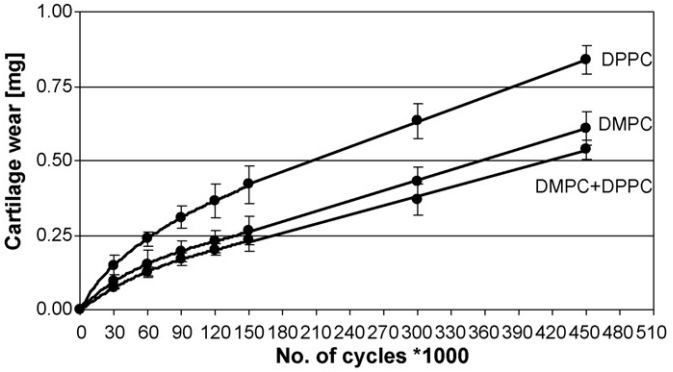

Fig. 5. Cartilage wear as a function of number of cycles in the presence of DPPC, DMPC and a mixture of DMPC/DPPC, all dispersed in HB.

study. All liposomes were dispersed in HB at a phospholipid concentration of 150 mM. This concentration value was selected based on previously obtained low friction results [23]. The phospholipid concentration of liposome dispersions was determined using the modified Bartlett assay [25]. PC bilayers can be either in a solid-ordered (SO) phase (occasionally referred to as gel or solid phase) as exemplified in this study by DPPC, and HSPC, or in a liquid-disordered (LD) phase (also referred to as liquid crystalline or fluid phase) as for DMPC and DOPC [26,27]. The transition from SO-to-LD phase is an endothermic, first-order process [22]. The transition temperature T_m is dependent mainly on the PC hydrocarbon chain composition [28–30]. In the LD phase (but not in the SO phase) the zwitterionic PC headgroups are highly hydrated [26,27]. This study focused on PCs as cartilage lubricants for three main reasons: (i) PCs are major components of synovial fluid, (ii) upon hydration, PCs spontaneously form bilayers which assemble into vesicles, and (iii) PC headgroups in the LD phase are highly hydrated [26,27], and therefore are expected to facilitate "hydrophilic" lubrication, which was proposed by Klein and coworkers to be the main mechanism of joint lubrication [31,32]. MLV were selected due to their much higher retention on the cartilage surface compared to small unilamellar vesicles (SUV) [23].

Fig. 4 shows the amount of cartilage wear found in the presence of DOPC, HSPC, DPPC and DMPC-MLV as a function of the number of reciprocating sliding cycles. The cartilage wear results show that DMPC (T_m = 23.2 °C) is a superior lubricant additive, both in lower wear values during the run-in period and in lower constant wear rate thereafter. Increasing wear is observed in Fig. 4 with DPPC and HSPC in this order (T_m = 41.4 and 52.5 °C, respectively).

The fact that MLV of HSPC and DPPC (T_m = 52.5 and 41.4 °C, respectively) show poorer lubricating abilities at the test temperature of 37 °C compared to DMPC-MLV (T_m = 23.2 °C) suggests that liposomes having a T_m above the test temperature are less effective lubricant additives. The fact that DOPC (T_m = −21 °C) was the least efficacious wear reducer of all other MLV compositions implies that liposomes having T_m much lower than the test temperature would not be effective lubricants. This may be explained by the observation that DOPC bilayers are too soft at the test temperature (that is 58 °C above their T_m) to remain adsorbed to the cartilage under the loading condition during the wear test. Hence, it was hypothesized that efficient lubrication would be achieved by using MLV with T_m only slightly below the test temperature. For this purpose MLV made of a mixture of DMPC/DPPC (0.6/1.0, mole/mole) having a T_m of 34 °C (which is only 3 °C below the test temperature) was also tested. This mixture was selected due to the nearly ideal miscibility of DMPC with DPPC in the liposome bilayer(s). Indeed, cartilage wear in the presence of the DMPC/DPPC-MLV was somewhat lower than that in the presence of each of the unmixed DMPC or DPPC liposomes (see Fig. 5) supporting the above mentioned hypothesis. Using liposomes composed of a mixture of miscible PCs enables fitting their T_m to suit a wide range of temperatures. For example, the ratio of DMPC/DPPC in the MLV can be adjusted so that the phase transition will take place at all physiological temperatures occurring in different conditions of OA.

3.3. Comparing ISF, ISF + DMPC/DPPC, and ISF + HA

In order to determine whether liposome additives in ISF, as would most likely be the case in OA practice, can reduce cartilage wear, tests were performed in the presence of ISF alone and with ISF plus DMPC/DPPC-MLV (150 mM PC) that was prepared by adding a mixture of DMPC/DPPC-MLV in HB with a concentration of 300 mM to ISF with a ratio of 1:1. In addition, ISF + HA was also tested to evaluate the effectiveness of HA on cartilage wear reduction compared to that of the ISF alone and ISF + DMPC/DPPC. A fixed ratio of 1:1 ISF and HA was selected for the current tests in accordance with the same ratio of ISF and DMPC/DPPC. The behavior of cartilage wear found in the presence of ISF, ISF + HA and ISF + DMPC/DPPC as a function of number of sliding cycles is shown in Fig. 6. The behavior is similar to that shown in Figs. 3–5, where a relatively short run-in period is followed by a long constant wear rate period. Comparing the cartilage wear in the presence of the three different lubricants shows that although the addition of HA to ISF reduces wear better than ISF alone, the addition of DMPC/DPPC-MLV to ISF is much more efficacious in terms of reducing cartilage wear.

A summary of the constant wear rates following the run-in period, that were obtained with all the different lubricating fluids and additives is presented in Fig. 7. Screening the different liposomes shows that the lowest constant wear rate is achieved in the presence of the DMPC/DPPC-MLV. Moreover, adding DMPC/DPPC-

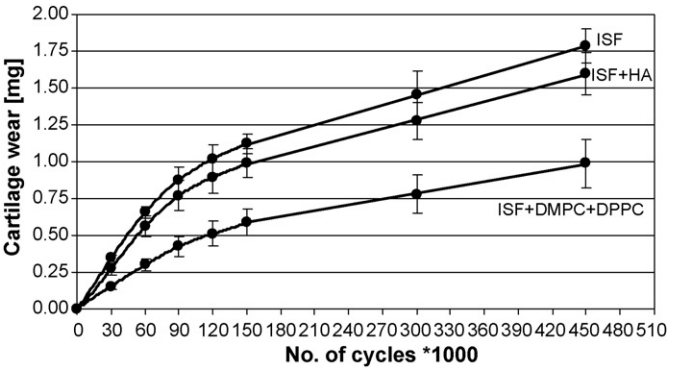

Fig. 6. Cartilage wear as a function of number of cycles in the presence of ISF, ISF + HA and ISF + DMPC/DPPC.

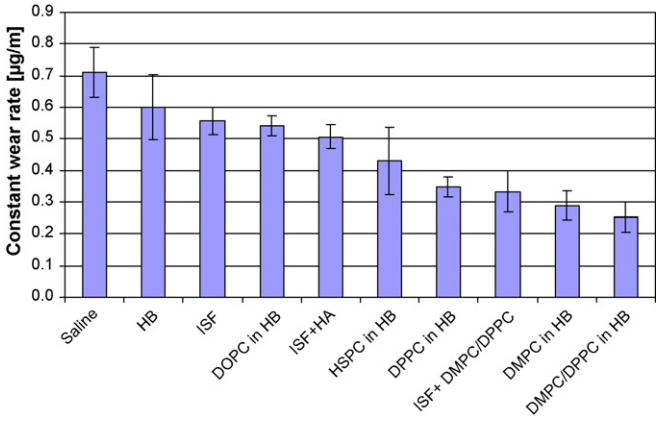

Fig. 7. A summary of the constant wear rates obtained with all the different lubricating fluids and additives following the run-in period.

MLV to ISF reduces the constant wear rate by about 40% compared with ISF alone, while adding HA to ISF reduces the constant wear rate only by about 10%. Reduction of cartilage wear by liposomes as lubricants was demonstrated qualitatively in earlier works [18,19], however, no quantified comparison as that shown in Fig. 7 could be made in those studies.

Liposome physical and chemical integrity was tested after 40 h of incubation at 37 °C in SF, ISF or HB, or after conducting exhaustive wear tests (load 60 N, and 450,000 reciprocating sliding cycles during 7.5 h at 37 °C). In all these cases the level of degradation products such as lyso-PC and fatty acids (PC hydrolysis products) was below detection limits (<2%) and liposome size distribution remained unaltered. Based on the present results it can be proposes that intra-articular injections of DMPC/DPPC-MLV may be used to reduce cartilage wear in OA patients. The superior efficiency of those MLV as wear reducers may be related to the high level of hydration of the phosphocholine head groups of the PC at the LD phase [33], and to high compressibility, and softness [34] (related to being in the LD phase only slightly above their T_m).

Finally, it should be noted that the current study was conducted with a single concentration value of the different liposomes (150 mM PC). Moreover, a single form of commercially available HA was tested. In this regard the present work can be considered a preliminary study. Further evaluation using different forms of HA and various PC concentrations values should be conducted in the future in order to establish the optimal form of HA and optimal PC concentration for wear reduction, which may be different from that for minimum friction. It may also be useful to test a combination of HA and liposomes.

4. Conclusion

Wear tests, using a specially designed test apparatus, of human cartilage-on-cartilage scheme, *in vitro*, in the presence of different phospholipid-based liposomal bio-lubricating fluids were described.

It was shown that most liposome additive-based lubricants induce less wear in comparison to inflamed synovial fluid. MLV made of a mixture of DMPC/DPPC (0.6/1.0, mole/mole) or of DMPC alone protected the cartilage from wear much better than any of the other single component liposomes. Moreover, it was found that the phase transition temperature T_m has an important role, and efficient lubrication is achieved by using liposomes with T_m only slightly below physiological temperature. The present preliminary results are promising but further extensive testing both *in vitro* and *in vivo* is required to support the present findings.

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