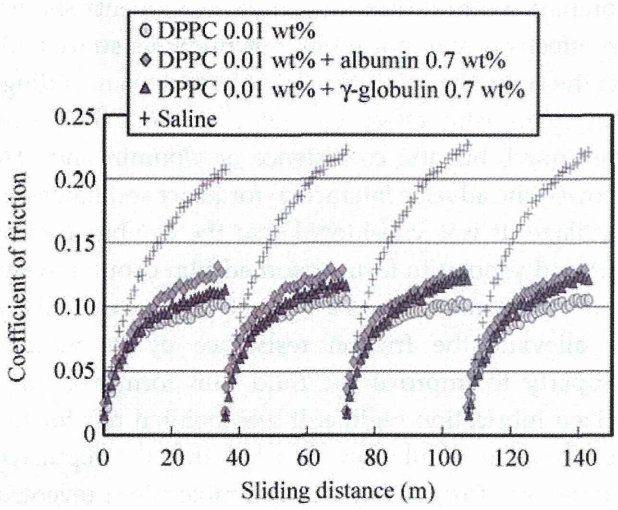

Fig. 6 Influence of DPPC and proteins on frictional behaviors for damaged articular cartilage.

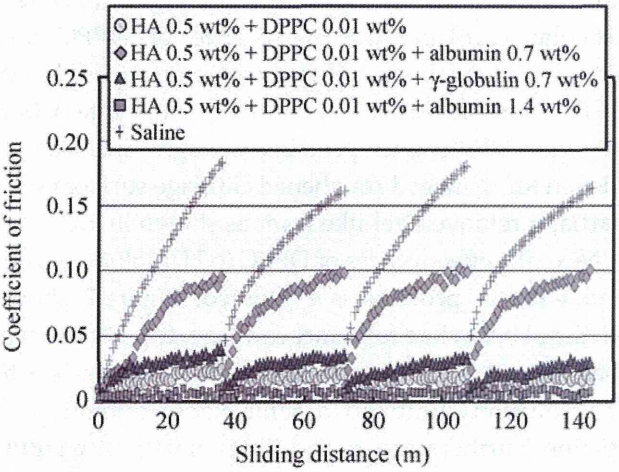

Fig. 7 Influence of DPPC, proteins and HA on frictional behaviors for intact articular cartilage.

in HA solution demonstrated the lowest frictional behaviors as about 0.01 without gradual increase until each 36 m sliding. In this lubricant composition, it is confirmed that the friction does not increase but maintains a very low steady level. On the contrary, the addition of DPPC with albumin alone or γ-globulin alone in HA solution shows higher friction than DPPC alone in HA solution but lower than saline solution. In Fig. 8 the frictional behaviors of these combinations for damaged cartilage are shown. Compared with intact cartilage, the friction levels are generally increased and the order of friction level is partly changed, i.e., HA solution containing DPPC

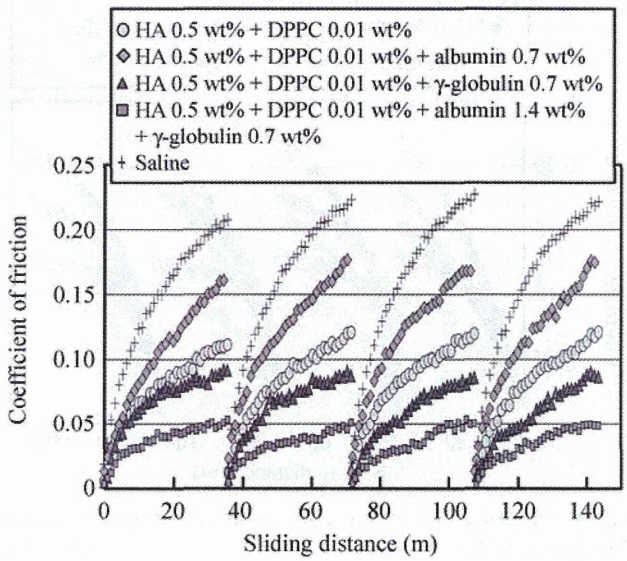

Fig. 8 Influence of DPPC, proteins and HA on frictional behaviors for damaged articular cartilage.

becomes higher than HA solution containing DPPC and γ-globulin. HA solution with 0.01 wt% DPPC, 1.4 wt% albumin and 0.7 wt% γ-globulin maintains minimum friction but friction gradually increases until the level of 0.05 as coefficient of friction at each 36 m sliding for damaged cartilage.

The comparison of friction at restart and at steady state for both intact and damaged cartilage is summarized in Fig. 9. It is noteworthy for intact articular cartilage that the lubricant of HA solution with 0.01 wt% DPPC, 0.14 wt% albumin and 0.7 wt% γ-globulin showed the minimum coefficient of friction 0.003 and 0.01 at restart and at steady state, respectively. For damaged cartilage, these values showed 0.004 and 0.05, respectively. It was confirmed that the optimum combination of DPPC, albumin and γ-globulin with HA for minimum friction is common (No. 13 in Table 1) for intact and damaged cartilage specimens.

To sustain superior tribological properties of articular cartilage, not only low friction but minimum wear are required in various daily activities. Therefore, wear on cartilage surfaces was evaluated. The articular cartilage contains plenty of water, therefore, it is difficult to measure actual changes due to wear in weight. In this study, the changes in surface photographs were compared with a surface before testing. Representative photographs are shown in Fig. 10. Intact

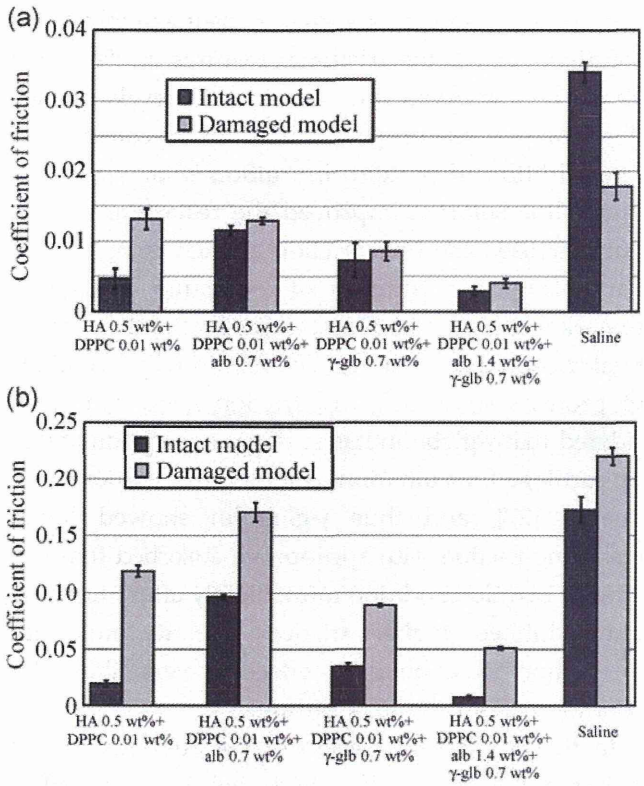

Fig. 9 Friction levels at restart and steady state for intact and damaged cartilage (error bars indicate standard deviation): (a) friction at restart and (b) friction at steady state.

articular cartilage has smooth surface with some irregularity (left picture). Tests lubricated with No. 10 lubricant (HA solution containing 0.01 wt% DPPC) and No. 12 (HA solution containing 0.01 wt% DPPC and 0.7 wt% γ-globulin) exhibited the low friction and mild wear with scratches on surfaces. On the contrary, minimum friction as 0.01 and little wear were confirmed for lubricant No. 13 (HA solution containing 0.01 wt% DPPC, 1.4 wt% albumin and 0.7 wt% γ-globulin), where superficial gel-like layer may have been slightly removed without scratching during rubbing.

4 Discussion

For natural synovial joint systems, the synergistic action between articular cartilage and synovial fluid appears to play an important role in minimizing friction and wear. In this study, the repeated reciprocating tests including interrupting-unloading periods for 5 min for ellipsoidal cartilage specimen against flat glass plate were conducted, where the contact zone of articular cartilage was not migrated and thus the effect of the interstitial fluid pressurization in articular cartilage

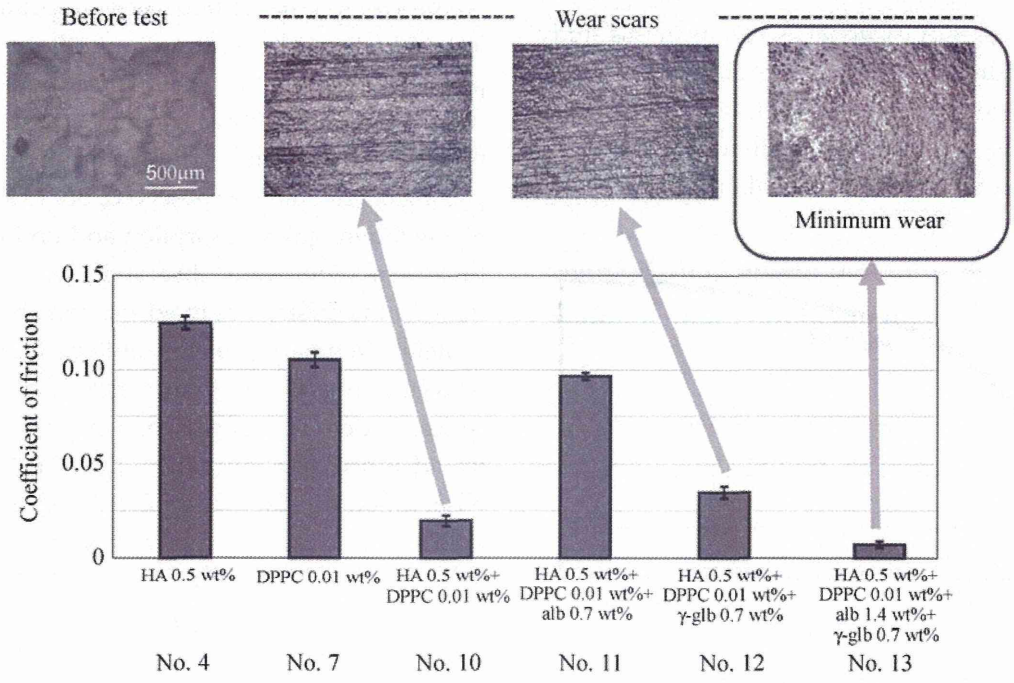

Fig. 10 Average friction levels at steady state and cartilage surface photographs for intact cartilage (error bars indicate standard deviation).

was gradually diminished. Under such severe rubbing conditions as model B in Fig. 1, the effectiveness of lubricant constituents and the influence of cartilage surface conditions on tribological behaviors were evaluated.

The common features in frictional behaviors of articular cartilage in the reciprocating tests are as follows.

(1) Initial low friction is established by biphasic/hydration and/or mixed lubrication for cartilage surface with sufficient adsorbed films.

(2) Time-dependent gradual increase in friction during rubbing process is controlled by biphasic property of cartilage, interaction of adsorbed molecules and/or slight removal of cartilage surfaces.

(3) Reduction in restarting friction is brought by the recovery of hydration and biphasic property with recovery of deformation accompanied with adsorbed film formation after unloading for 5 min.

As indicated by the Eq. (1) in FE analysis, we can estimate the frictional behaviors of various cartilage surfaces different in adsorbed film formation, i.e., coefficient of friction for solid-to-solid contact μ_{eq}. In Fig. 11, the changes in friction estimated from total traction force in biphasic FE analyses during rubbing process under constant load are shown for μ_{eq} = 0.01 and 0.2. Most cases of frictional behaviors in this study except for addition of a single protein seem to be located between the upper (high friction) and lower (low friction) curves in Fig. 11, although FE analysis was conducted for two dimensional model.

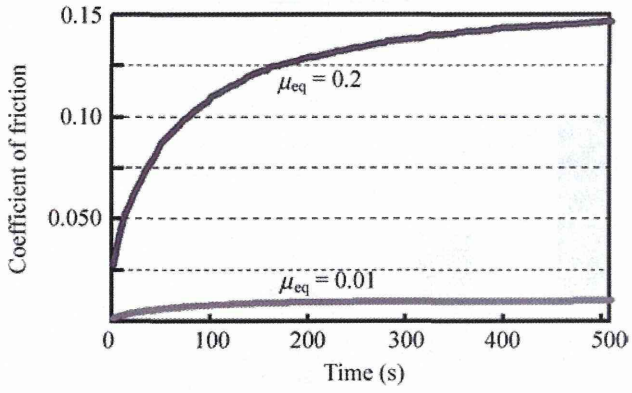

Fig. 11 Influence of μ_{eq} on time-depending frictional behaviors estimated by biphasic theory for cartilage.

In cases of addition of a single constituent into saline solution (Fig. 4), the frictional features as described above are observed, but the friction levels change depending on the properties of lubricant constituents. The addition of protein, i.e., albumin or γ-globulin into saline solution improved the restarting friction but increased the final friction at each 36 m sliding. Particularly, the addition of γ-globulin brought a remarkable lowering in restarting friction but higher final friction than albumin. The reason why two kinds of proteins show different friction levels was considered that γ-globulin has stronger adsorption ability on cartilage than albumin as indicated by fluorescent images [27], and thus γ-globulin showed lower restarting friction with appropriate adsorbed film formation in mild condition immediately after reloading, but exhibited higher friction due to molecular interaction as a bonding effect in very thin film condition after each 36 m sliding.

In the *in situ* observation of the rubbing pair of poly(vinyl-alcohol) (PVA) hydrogel and glass plate by Yarimitsu et al. [28], the fluorescent images for proteins adsorbed on glass plate, protein aggregates between rubbing surfaces and proteins on PVA hydrogel surface were discriminately observed in reciprocating tests for boundary lubrication regime at low sliding speed of 0.2 mm/s and the average contact pressure of 0.104 MPa. This reciprocating apparatus was constructed on the stage of the inverted fluorescent microscope. In saline solution of albumin, the easy peeling of albumin was observed, but in saline solution of γ-globulin, quick adsorption and uniform adsorbed film formation were observed. These phenomena indicate the differences in adsorption abilities for both proteins. In binary protein solutions with coexistence of albumin and γ-globulin, the relative ratio and concentration of proteins had an intense influence on adsorbed film formation [29]. Furthermore, the observation of adsorbed molecules in the evanescent field within about 200 nm from surface by using the total internal reflection fluorescence (TIRF) microscopy indicated in binary protein solutions that the bottom layer of stable protein adsorbed film is mainly composed of γ-globulin and the friction-induced enhancement of forming protein adsorbed film occurs

in lubricant with appropriate protein composition [30, 31]. The competitive adsorption of albumin and γ-globulin appears to affect these behaviors as indicated in study of adsorption and desorption of both proteins with TIRF spectroscopy by Tremsina et al. [32]. Furthermore, the differences in adsorption behaviors of serum proteins depend on the changes in conformation, molecular weight, charge condition, hydrophobic/hydrophilic properties of proteins and solid surfaces, pH of lubricant, and so on. Particularly under rubbing, denatured proteins change their conformations and adsorption properties, and thus affect the tribological behaviors [33–35]. Therefore, overall viewpoints are required to elucidate the actual adsorption behaviors of serum proteins.

The addition of HA with viscous property in lubricants was expected to improve the fluid film thickness, and subsequently improved friction level compared with saline [26]. The addition of DPPC alone is the most effective in reduction of friction but the final coefficient of friction is not so low (about 0.1) in Fig. 4.

Therefore, the effect of combination of different constituents was evaluated. The influences of coexistence of protein with HA on friction were examined in our previous study [26]. The coexistence of γ-globulin and HA showed the lowering of both the restarting and final or steady friction compared with HA solution. However, albumin exhibited higher final friction than HA solution although it showed a little lower restarting friction than HA solution. These facts suggest the synergistic effect of γ-globulin and HA, but indicate the adverse interaction of albumin and HA for intact cartilage. It is reported that albumin and HA show repulsive interaction [36] and the HA-protein complexes in natural synovial fluid contain globulin but almost no albumin at pH 7–8 [37]. These frictional trends for both proteins are similar for damaged cartilage with partially removed surface proteoglycan gel layer. The suppressive action between negatively charged albumin and negatively charged HA molecules was observed in fluorescent images of sparsely distributed adsorbed films, compared with intimate adsorbed films for γ-globulin and HA [26].

In this study, the effect of addition of neutral phospholipid DPPC with and without protein was examined. It should be noted that the coexistence of DPPC with protein is effective for intact cartilage (Fig. 5), but increases friction for damaged cartilage (Fig. 6). This difference appears to be brought about by changes in adsorbed film formation on damaged cartilage surface. For reciprocating tests of PVA hydrogel and glass plate lubricated with saline solution of DPPC alone, the Janus-faced property for high or low friction was affirmed in accord to either irregular adsorbed film or uniform DPPC adsorbed film formation in AFM images [38]. It is pointed out by Hills [10] that even only the oligolamellar phospholipid plays an effective lubricating role in natural synovial joints. By *in situ* fluorescent observation of forming adsorbed films for sliding pair of PVA hydrogel and glass plate in coexistence of DPPC and albumin [38], it was clarified that the formation of albumin-DPPC sheet-like composite film was found and therefore the friction was reduced. It is pointed out that DPPC with a neutral charge is likely to bind to albumin [39].

Next, the influence of addition of DPPC in HA solution with and without proteins was examined. The addition of DPPC alone in HA solution was considerably effective in reduction of friction for intact cartilage compared with coexistence of DPPC and either albumin or γ-globulin in HA solution (Fig. 7). This fact may suggest the formation of lubricating complex materials as membrane-like and roller structures composed of DPPC and HA [40]. Mirea et al. [41] indicated that HA has high affinity to phospholipid bilayer in the force-distance curve in AFM study. The detailed structure of HA-DPPC complex has not yet been clarified but the coexistence of DPPC and HA is likely to act synergistically as lamellar lubrication or related mechanism. Furthermore, for coexistence of DPPC and HA, HA-DPPC composite boundary film was visually confirmed [38] and friction was remarkably lowered, where the lubricating ability by HA-DPPC complex as gel-like film is supposed to become effective with high water retention ability of HA. However, HA solution containing DPPC showed an effective but limited protective property with local scratching as shown in Fig. 10.

On the contrary, albumin-DPPC composite was not

found in coexistence of three constituents, i.e., DPPC, HA and albumin [38], probably due to repulsive interaction between albumin and HA. This fact corresponds to the phenomenon in which the friction for HA solution with DPPC and albumin (Fig. 7) is higher than saline solution with DPPC and albumin (Fig. 5).

However, the supply of both albumin and γ-globulin as definite ratio into HA solution containing DPPC (lubricant No. 13) could remarkably improve the friction at very low level of 0.01 as final coefficient of friction (Fig. 7) and high wear resistance (Fig. 10). For damaged cartilage, the friction level increased in general but No.13 lubricant showed the minimum friction (Fig. 8).

In natural synovial joints, various lubricating constituents such as HA, proteins, glycoproteins and phospholipds different in molecular properties and sizes play different roles. Therefore, the interaction and/or synergistic action between phospholipids and other constituents seem to control the adsorbed film formation and tribological behavior. The influences of lubricants as HA solutions containing DPPC with or without proteins on the friction at restart and at steady state are summarized in Fig. 9 for intact and damaged cartilage specimens. The effectiveness of adsorbed film on reduction in restarting friction and steady friction is clearly demonstrated compared with saline solution. Particularly, it is noticed that the lubricant No.13 (HA solution with 1.4 wt% albumin, 0.7 wt% γ-globulin and 0.01 wt% DPPC) provided very low restarting friction for both intact and damaged cartilage specimens (Fig. 9(a)). This lubricant maintained very low friction until each 36 m sliding for intact cartilage, but the friction gradually increased until 0.05 as coefficient of friction for damaged cartilage (Fig. 9(b)). In the study by Nakashima et al. [29], HA solution with 1.4 wt% albumin and 0.7 wt% γ-globulin (albumin/globulin = A/G ratio of 2:1) or 0.7 wt% albumin and 1.4 wt% γ-globulin (A/G ratio of 1:2) showed very low wear for rubbing of PVA hydrogel against itself. For low wear condition in the latter, the layered adsorbed film formation was observed by the fluorescent method. In these cases, it is suggested that the γ-globulin forms protective adsorbed layer on cartilage surface and albumin plays as low shearing layers. On the contrary, HA solution with 1.4 wt% albumin and 1.4 wt% γ-globulin (A/G ratio of 1:1) formed the heterogeneous adsorbed film and showed higher wear.

The lubricant No.13 has similar composition to that in natural synovial fluid as hyaluronate solution containing lubricating constituents such as 1.25 wt% albumin, 0.75 wt% globulin (including α-, β-, and γ-globulins) as medium values [42], 1.1 wt% albumin and 0.7 wt% globulin [36], or 1.9 wt% albumin, 1.1 wt% globulin and 0.01 wt% DPPC [12]. In this lubricant, the lubricating layered structure in adsorbed film is expected for low friction and minimal wear, but the detailed elucidation of this mechanism is required in the future study. As exhibited in Figs. 7 and 9, lubricant No. 13 showed very low and steady friction in repeated reciprocating test at 20 mm/s. *In situ* fluorescent observation at very slow speed with this lubricant [38] showed the stable mixed adsorbed film containing albumin and γ-globulin but friction is not so low probably due to very thin film condition at 0.2 mm/s condition. Therefore, we plan to observe *in situ* the actual adsorbed film formation and frictional behavior at 20 mm/s or so. In various daily activities, synovia constituents appear to play their appropriate roles depending on the severity of operating conditions. DPPC and albumin are likely to act as low shearing layer, and γ-globulin acts as the protective film as strongly adsorbed on cartilage surface. HA has ability to thicken the lubricating fluid film and form some lubricating gel-like layer. Although some of synergistic mechanisms between lubricating constituents were shown in this study, the overall mechanisms are expected to be clarified from the viewpoint of multiscale level in future. On the role of lubricin as another lubricating constituent, Mirea et al. [41] suggested that it anchors lipid layers on the cartilage. We confirmed that the addition of lubricin in HA solution could reduce friction for intact cartilage in the preliminary test. In future study, we plan to evaluate the effective roles of all influential synovia constituents.

For damaged cartilage specimens with partially removed proteoglycan brush-like layer, the best composition in lubricant for low friction is the same lubricant No. 13 which is the best for intact cartilage, but the second one was changed to the HA solution

containing DPPC with γ-globulin from the HA containing DPPC solution without protein as the second one for intact cartilage. It is suggested for damaged cartilage that the protective role of γ-globulin with strong adsorption ability becomes important.

As discussed above, the effectiveness of lubricant constituents changes depending on rubbing cartilage properties in reciprocating tests of cartilage-on-glass. To evaluate rigorously the influence of synovia constituents on tribological behaviors of articular cartilage in natural synovial joints, the rubbing pair of cartilage-on-cartilage [8, 43] or cartilage-on-meniscus [44] should be used, and therefore the influence of glass plate on tribological behaviors in this study should be discussed. As mentioned in Section 1, the glass plate surface possesses hydrophilic characteristics with negatively charged property similar to proteoglycan on superficial cartilage layer in wet condition, whilst it is hard, smooth and nonporous/impermeable material. The adsorption of synovia constituents on glass plate is expected to be considerably similar to boundary film formation on intact cartilage but the interaction to the smooth, hard and nonporous/impermeable glass surface may be different. HA and albumin (at pH > 4.7) are negatively charged but γ-globulin is positively charged (at pH < 7.5). These electrostatic properties of adsorbed molecules have an influence on adsorption. On the contrary, the ploughing friction may be minimized for smooth surface, but adsorbed proteins on very smooth surface may induce high friction by their intense adhesive effect as hydrophobic bonding in watery system in very thin film condition. However, the effectiveness of lubricant constituents on tribological behaviors of compliant and biphasic articular cartilage appear to be reflected appropriately even in sliding pair of articular cartilage and glass plate. In pendulum friction tests for cartilage-on-cartilage of porcine shoulder joints composed of humerus head and glenoid cavity (cup) [8], the effectiveness in friction reduction by addition of 0.01 wt% DPPC or 1.0 wt% γ-globulin to HA solution for cartilage treated with detergent had been confirmed as similar effect to cartilage-on-glass combination. In contrast, the addition of 1.0 wt% or 3.0 wt% albumin to HA solution did not improve friction of cartilage-on-cartilage, which corresponds to adverse interaction of albumin and HA for cartilage-on-glass [26]. In contrast, the sliding pair of cartilage and clean glass plate showed higher friction in HA solution than that of cartilage and glass plate treated with Langmuir–Blodgett (LB) film as 5 to 10 bilayer of DPPC alone or mixed LB film of DPPC and γ-globulin [12]. As mentioned above, common features and/or some differences seem to occur in frictional behavior for cartilage-glass combination compared with cartilage-cartilage. In the next stage, therefore, further studies for cartilage-on-cartilage or cartilage-on-hydrogel (artificial cartilage) are required to elucidate strictly the influence of synovia constituents on tribological behaviors of articular cartilage in natural synovial joints. The sustaining of the synergistic mechanism of various synovia constituents on matched cartilage surfaces in natural synovial joints is expected to maintain the healthy condition.

5 Conclusions

In this study, at repeated reciprocating tests including restarting after interrupting-unloading process, the changes in friction were observed for intact and damaged articular cartilage specimens against glass plate lubricated with lubricants containing phospholipid, protein and hyaluronic acid as synovia constituents. The optimum composition in lubricants for low friction and minimum wear of both intact and damaged cartilage specimens was exhibited to be similar composition to natural synovial fluid. Furthermore, it was shown that the effectiveness of lubricant constituents changes depending on the surface conditions of articular cartilage.

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References

[1] Dowson D. Modes of lubrication in human joints. *Proc Inst Mech Engrs* **181**: 45–54 (1966)

[2] Unsworth A, Dowson D, Wright V. Some new evidence on human joint lubrication. *Ann Rheum Dis* **34**(4): 277–285 (1975)

[3] McCutchen CW. The frictional properties of animal joints. *Wear* **5**: 1–17 (1962)

[4] Walker P S, Dowson D, Longfield M D, Wright V. Boosted lubrication in synovial joints by fluid entrapment and enrichment. *Ann Rheum Dis* **27**(6): 512–520 (1968)

[5] Mansour J M, Mow V C. On the natural lubrication of synovial joints: Normal and degenerate. *J Tribol* **99**: 163–173 (1977)

[6] Dowson D, Jin Z M. Micro-elastohydrodynamic lubrication of synovial joints. *Eng Med* **15**: 65–67 (1986)

[7] Murakami T. The lubrication in natural synovial joints and joint prostheses. *JSME International Journal Ser III* **33**(4): 465–474 (1990)

[8] Murakami T, Higaki H, Sawae Y, Ohtsuki N, Moriyama S, Nakanishi Y. Adaptive multimode lubricaion in natural synovial joints and artificial joints. *Proc Inst Mech Eng Part H* **212**: 23–35 (1998)

[9] Swann D A, Hendren R B, Radin E L, Sotman S L, Duda E A. The lubricating activity of synovial fluid glycoproteins. *Arthritis Rheum-US* **24**: 22–30 (1981)

[10] Hills B A. Oligolamellar lubrication of joints by surface active phospholipids. *J Rheum* **16**(1): 82–91 (1989)

[11] Higaki H, Murakami T. Role of constituents in synovial fluid and surface layer of articular cartilage in joint lubrication (part 2) the boundary lubricating ability of proteins. *Jpn J Tribol* **40**(7): 691–699 (1996)

[12] Higaki H, Murakami T, Nakanishi Y, Miura H, Mawatari T, Iwamoto Y. The Lubricating ability of biomembrane models with dipalmitoyle phosphatidylcholine and γ-globulin. *Proc Inst Mech Eng Part H* **212**: 337–346. (1998)

[13] Murakami T, Sawae Y, Horimoto M, Noda M. Role of surface layers of natural and artificial cartilage in thin film lubrication. In *Lubrication at Frontier*. A msterdam: Elsevier, 1999: 737–747.

[14] Ikeuchi K. Origin and future of hydration lubrication. *Proc Inst Mech Eng Part J* **221**: 301–305 (2007)

[15] Klein J. Molecular mechanisms of synovial joint lubrication. *Proc Inst Mech Eng Part J* **220**: 691–710 (2006)

[16] Zappone B, Ruths M, Greene W G, Jay G D, Israelachvili J N. Adsorption, lubrication, and wear of lubricin on model surfaces: Polymer brush-like behavior of a glycoprotein. *Biophysical J* **92**: 1693–1708 (2007)

[17] Forster H, Fisher J. The influence of loading time and lubricant on the friction of articular cartilage. *Proc Inst Mech Eng Part H* **210**: 109–119 (1996)

[18] Ateshian G A. Theoretical formulation for boundary friction in articular cartilage. *J Biomech Eng* **119**(1): 81–86 (1997)

[19] Krishnan R, Kopacz M, Ateshian G A. Experimental verification of the role of interstitial fluid pressurization in cartilage lubrication. *J Orthop Res* **22**: 565–570 (2004)

[20] Ateshian G A. The role of interstitial fluid pressurization in articular cartilage lubrication. *J Biomech* **42**: 1163–1176 (2009)

[21] Mow V C, Kuei S C, Lai W M, Armstrong C G. Biphasic creep and stress relaxaion of articular cartilage in compression: Theory and experiments. *ASME J Biomech Eng* **102**: 73–84 (1980)

[22] Pawaskar S S, Jin Z M, Fisher J. Modelling of fluid support inside articular cartilage during sliding. *Proc Inst Mech Eng Part J* **221**: 165–174 (2007)

[23] Sakai N, Hagihara Y, Furusawa T, Hosoda N, Sawae Y, Murakami T. Analysis of biphasic lubrication of articular cartilage loaded by cylindrical indenter. *Tribol Int* **45**: 225–236 (2012)

[24] Murakami T. Importance of adaptive multimode lubrication mechanism in natural and artificial Joints. *Proc Inst Mech Eng Part J* **216**: 827–837 (2012)

[25] Ateshian G A, Wang H, Lai W M. The role of interstitial fluid pressurization and surface porosities on the boundary friction of articular cartilage. *ASME J Tribol* **120**: 241–248 (1998)

[26] Murakami T, Nakashima K, Yarimitsu S, Sawae Y, Sakai N. Effectiveness of adsorbed film and gel layer in hydration lubrication as adaptive multimode lubrication mechanism for articular cartilage. *Proc Instn Mech Eng Part J* **225**: 1174–1185 (2011)

[27] Murakami T, Nakashima K, Sawae Y, Sakai N, Hosoda N. Roles of adsorbed film and gel layer in hydration lubrication for articular cartilage. *Proc Inst Mech Eng Part J* **223**: 287–295 (2009)

[28] Yarimitsu S, Nakashima K, Sawae Y, Murakami T. Study on mechanisms of wear reduction of artificial cartilage through *in situ* observation on forming protein boundary film. *Tribol Online* **2**(4): 114–119 (2007)

[29] Nakashima K, Sawae Y, Murakami T. Study on wear reduction mechanisms of artificial cartilage by synergistic protein boundary film formation. *JSME Int J* **48**(4): 555–561 (2005)

[30] Yarimitsu S, Nakashima K, Sawae Y, Murakami T. Effect of lubricant composition on adsorption behavior of proteins on rubbing surface and stability of protein boundary film. *Tribol Online* **3**(4): 238–242 (2008)

[31] Yarimitsu S, Nakashima K, Sawae Y, Murakami T. Influences of lubricant composition on forming boundary film composed of synovia constituents. *Tribol Int* **42**: 1615–1623 (2009)

[32] Tremsina Y S, Sevastianov V I, Petrash S, Dando W, Foster M D. Competitive adsorption of human serum albumin and gamma-globulin from a binary protein mixture onto hexadecyltrichlorosilane coated glass. *J Biomater Sci Polym Ed* **9**(2): 151–162 (1998)

[33] Heuberger M P, Widmer M R, Zobeley E, Glockshuber R, Spencer N D. Protein-mediated boundary lubrication in arthroplasty. *Biometarials* **26**: 1165–1173 (2005)

[34] Nakashima K, Sawae Y, Murakami T. Influence of protein conformation on frictional properties of poly)vinyl alcohol) hydrogel for artificial cartilage. *Tribol Lett* **26**: 145–151 (2007)

[35] Nakashima K, Sawae Y, Murakami T. Effect of conformational changes and differences of proteins on frictional properties of poly(vinyl alcohol) hydrogel. *Tribol Int* **40**: 1423–1427 (2007)

[36] Oates K M N, Krause W E, Jones R L, Colby R H. Rheopexy of synovial fluid and protein aggregation. *J R Soc Interface* **3**: 167–174 (2006)

[37] Curtain C C. The nature of protein in the hyaluronic complex of bovine synovial fluid. *Biochem J* **61**(4): 688–697 (1955)

[38] Yarimitsu S, Nakashima K, Sawae Y, Murakami T. Influences of synovia constituents on frictional behavior of artificial cartilage material and formation of boundary lubricating film (in Japanese). *Tribologist* **55**(7): 489–498 (2010)

[39] Hernández-Caselles T, Villalaín J, Gómez-Fernáindez J C. Influence of liposome charge and composition on their interaction with human blood serum proteins. *Mol Cell Biochem* **120**: 119–126 (1993)

[40] Pasquali-Ronchetti I, Quaglino D, Mori G, Bacchell B. Hyaluronan-phospholipid interactions. *J Struct Biol* **120**: 1–10 (1997)

[41] Mirea D A, Trunfio-Sfarghiu A-M, Matei C I, Munteanu B, Piednoir A, Rieu J P, Blanchin M G, Berthie Y. Role of the biomolecular interactions in the structure and tribological properties of synovial fluid. *Tribol Int* **59**: 302–311 (2013)

[42] Sasada T, Tsukamoto Y, Mabuchi K. *Biotribology* (in Japanese). Sangyo Tosho, 1988.

[43] Roberts B J, Unsworth A, Mian N. Modes of lubrication in human hip joints. *Ann Rheum Dis* **41**: 217–224 (1982)

[44] McCann L, Ingham E, Jin Z, Fisher J. Influence of the meniscus on friction and degradation of cartilage in natural knee joint. *Osteoarthr Cartilage* **17**: 995–1000 (2009)

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Direct observation of selective protein capturing on molecular imprinting substrates

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Photoreaction

ABSTRACT

A sensing interface for specific protein capture was fabricated using a novel molecular imprinting (MIP) process. Bovine serum albumin (BSA) and ovalbumin (OVA) were imprinted on a quartz substrate with modified alkyl groups, and target protein capture was detected using a deep-UV fluorescence image microscope (UVFLIM). The imprinted protein was immobilized to silica beads (diameter: 15 μm) using a phospholipid polymer containing both active ester groups and silane coupling groups, which were used as protein stamps to prepare the imprinting surface. Protein recognition sites were constructed by integrating sodium dodecyl sulfate (SDS) as the ligand, which was immobilized with a biocompatible photoreactive phospholipid polymer. When BSA solution was added to the BSA-based MIP substrate, strong fluorescence was observed from the tryptophan residue of BSA. In contrast, for the OVA-based MIP substrate and non-MIP substrate, no fluorescence was observed. The surface showed good selectivity of BSA against OVA. The phospholipid polymer layer prevented non-specific protein adsorption, resulting in highly selective protein recognition. Further, when the protein-imprinted substrate was constructed without ligands, neither protein was captured on the substrate. We demonstrated the importance of ligand integration for capturing target proteins at specific positions. UVFLIM can be used to detect biomolecules at the single-molecule level by using intrinsic fluorescence without molecular labeling. Our new protein-imprinted surface used with UVFLIM is a versatile tool for capturing biomolecules.

1. Introduction

Biosensors have been developed for use in biomedical diagnosis, environmental monitoring, food safety, and agriculture. The affinity of target molecules for an interface is a critical factor in biosensor performance (Berney et al., 1997; Ibii et al., 2010; Hall et al., 2011). In many cases, to recognize target molecule selectively, natural recognition biomolecules such as antibodies, enzymes and nucleic acids are installed on the sensing substrate. Although they can capture to the target molecules with strong biological affinity, they are unstable chemically and physically and their initial property is disappeared with time. Therefore, robust sensing substrates are necessary for reliable analysis.

Molecular imprinting (MIP), which involves shape and chemical recognition of target molecules by using synthetic polymer systems, is attracting attention, with artificial receptors being replaced by biomolecules (Fu et al., 2007; Hua et al., 2008; Ye and Mosbach, 2008; Wang et al., 2010; Soares da Silva et al., 2012). In the normal MIP process, the functional monomers and cross-linkers are complexed with imprinted molecules via weak interactions and then polymerized in situ to prepare a matrix. Imprinted molecules are extracted from the matrix to provide capturing sites for target molecules. Many successful studies examining the use of MIP for capturing small organic molecules (e.g., amino acids, peptides, nucleotides, and pharmaceuticals) have been reported (Ansell and Mosbach, 1997; Osawa et al., 2006; Yaqub et al., 2011). However, the use of MIP for protein capture is difficult because of the fragility, complexity, and flexible conformation of proteins. To success of protein MIP, it is important to produce the multiple binding sites with high mobility and the denaturation of target proteins should be suppressed during MIP process including polymerization. Additionally, the matrix surface must inhibit non-specific protein adsorption of the protein to enhance selectivity.

Previously, we proposed an MIP procedure for protein capture based on molecular integration (Fukazawa and Ishihara, 2009). Briefly, silica beads, which form a single layer of imprinted protein, were used for surface MIP. Sodium dodecyl sulfate (SDS), an anionic

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surfactant, interacted with the imprinted protein via electrostatic interactions, and the surfactant was fixed using a water-soluble photoreactive phospholipid polymer. After photoreaction, the imprinted protein was removed together with silica beads to form molecular recognition sites. SDS bound to the protein functioned as both a ligand for capturing target protein and reactive points for the photoreactive polymer. However, complete removal of imprinted proteins from the matrix with silica beads was difficult. Complete removal of imprinted proteins from the surface leads to increased capturing efficiency of target protein. Therefore, we designed a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer with both active ester groups and triethoxysilane groups to immobilize the imprinted protein on silica beads (Mieda et al., 2012). It is well known that MPC polymers effectively prevent protein adsorption (Ishihara et al., 1991; Ishihara et al., 1998; Murphy et al., 1999). Additionally, biomolecules at the MPC polymer surface can maintain and stabilize protein function (Sakaki et al., 1999; Nishizawa et al., 2008; Goto et al., 2008; Tajima et al., 2011). We expect that imprinted proteins can be removed from the matrix with the silica beads and that denaturation can be suppressed during the MIP process.

Protein MIP approaches include bulk, surface, particle, and epitope imprinting (Kryscio and Peppas, 2012). Among these, the surface MIP approach can be combined with a sensor device (Belmont et al., 2007; Sunayama et al., 2010; Zhou et al., 2011). Imprinted protein can be removed more easily from the substrate surface than with the bulk MIP approach, which involves extraction of imprinted proteins from the matrix after polymerization. Only a small amount of protein is imprinted during surface MIP; therefore, a highly sensitive detection system is necessary for determining the concentration of captured target proteins.

In this study, we fabricated a protein MIP as a sensing interface on quartz glass. Captured proteins were detected using a deep-UV fluorescence imaging microscope (UVFLIM). UVFLIM can be used to detect biomolecules at the single-molecule level by using intrinsic fluorescence without molecular labeling (Li et al., 2004; Li and Seeger, 2006, 2009, 2011). Bovine serum albumin (BSA) and ovalbumin (OVA) were selected as target proteins. The effectiveness of the ligand surfactant and selectivity against BSA and OVA were evaluated.

2. Experimental

2.1. Materials

MPC was synthesized according to a previously reported method (Ishihara et al., 1990). Poly(ethylene glycol) monomethacrylate (Blenmer PE-200) were obtained from NOF Co., Ltd. (Tokyo, Japan). Thionyl chloride and 4-azidobenzoic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Triethylamine (TEA), 2-hydroxyethyl methacrylate (HEMA), and ligroin were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). HEMA and TEA were purified by distillation and fractions collected at the boiling points (bp) (70 °C/0.2 kPa and 87 °C, respectively), were used. 3-Methacryloxy propyl trimethoxysilane (MPTS) was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). 2-(Carbomethoxy)ethyltrichlorosilane was purchased from Gelest, Inc. Quartz slide glass ($24 \times 24 \times 0.15$–$0.18\ mm^3$) was purchased from Tosoh Quartz Corp. (Yamagata, Japan). Porous silica beads (average particle diameter: 15 μm; average pore diameter: 7 nm) were obtained from Fuji Silysia Chemical (Tokyo, Japan). Bovine serum albumin (BSA, A-8022), ovalbumin (OVA, A-5503), *n*-butyltrichlorosilane, and *p*-nitrophenylchloroformate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents and solvents were commercially available in extra-pure grade and used without further purification.

2.2. Synthesis of PMSiN for protein stamp bead preparation

p-Nitrophenyloxycarbonyl polyethyleneglycol methacrylate (MEONP) was synthesized according to a previously reported method (Konno et al., 2004). The chemical structure of MEONP was confirmed using ^1H NMR (α-300, JEOL, Tokyo, Japan) in $CDCl_3$. Poly(MPC-*co*-MPTS-*co*-MEONP) (PMSiN) was synthesized by a conventional radical polymerization method in ethanol using 2,2′-azobisisobutyronitrile (AIBN) as a radical polymerization initiator (Ueda et al., 1992). Desired amounts of MPC, MPTS, and MEONP (monomer molar fractions were 0.70, 0.10, and 0.20, respectively, and total monomer concentration was 0.50 M) and AIBN (5.0 mM) were used for polymerization. Polymerization was carried out at 60 °C for 7.0 h. The formed polymer was purified by pouring the reaction mixture into an excess volume of diethylether/chloroform (80/20 v/v) for precipitation. After precipitation, the PMSiN was dissolved again in ethanol. The solution was evaporated under reduced pressure to remove residual diethyl ether and chloroform. The final concentration of the PMSiN–ethanol solution was adjusted to 5.0 wt% and stored in a freezer. The chemical structure of the PMSiN was confirmed using ^1H NMR (α-300, JEOL, Tokyo, Japan) in CD_3CD_2OD. Molecular weight of the polymers was evaluated using gel permeation chromatography (GPC, Jasco, Tokyo, Japan) in 1,1,1,3,3,3-hexafluoroisopropenol, and retention time was compared with that of the poly(methyl methacrylate) standard (Showa Denko, Tokyo, Japan). The chemical structure of PMSiN is shown in Fig. 1.

2.3. Synthesis of PMPAz for matrix preparation

The synthetic procedure for the photoreactive monomer unit, MPAz, was improved from that reported previously (Fukazawa and Ishihara, 2009). 4-Azidobenzoyl chloride (9.0 g, 50 mmol), synthesized using previously described method, was added to a 300-mL three-necked round-bottomed flask equipped with a dropping funnel, thermometer, and magnetic stirrer, and was dissolved in 80 mL of diethyl ether. After the solution was cooled at −10 °C, HEMA (50 mmol) and TEA (50 mmol) were dissolved in 50 mL of diethyl ether and were added drop-wise to the stirred solution over a period of 1 h. The reaction was carried out for 12 h. After filtering the resulting triethylamine hydrochloride (TEAC), the solvent was evaporated under reduced pressure. The residue was dissolved in diethyl ether to precipitate the residual TEAC, which was then filtered out of the solution. Following addition of diethyl ether, unreacted HEMA was extracted using

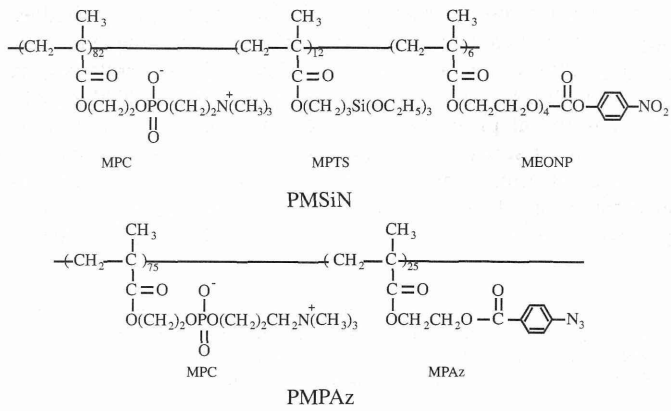

Fig. 1. Chemical structures of MPC polymers.