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Case Report

Atomic force microscope observation of the surfaces of natural articular cartilage

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Abstract

Hydrophilic and negatively charged natural cartilage surface is covered by phospholipids bilayers. These phospholipids have been demonstrated to exert highly desirable characteristics on the surface articular cartilage such as efficient lubrication, load processing, and semipermeability for nutrient transport. We examined a bovine cartilage (BC) surface using atomic force microscope. The study was performed using cartilage samples with healthy surfaces and completely depleted surface phospholipids. The artificially degraded cartilage surfaces (lipid depleted) were resurfaced with different species and combinations of synthetic bilayer of phospholipids found in human joints. Our results demonstrated that it is possible to recreate a potentially viable layer of phospholipids on the surface of degenerated cartilage. However, further studies will be required to advance the resurfacing idea developed in this paper for the potential treatment of osteoarthritis and other related orthopedic joint conditions.

Keywords: atomic force; bovine cartilage; articular cartilage

Introduction

The surface-active phospholipids (SAPL) coating on the articular surface possess highly desirable lubricating properties for efficient joint function. The basic hypothesis of this research is that the surface-based degradation of articular cartilage can be corrected, or "resurfaced". The resurfacing process involved the deposition of single lipid components (palmitoyl-oleoyl-phosphatidylcholine, POPC and dipalmitoyl-phosphatidylcholine, DPPC) and complete joint SAPL mixture on the surfaces of lipid-depleted cartilage. The analyses of the composition of the SAPLs in the human knee joints reveal that they mostly contain unsaturated phospholipids; 30% palmitoyl-linoleoylphosphatidylcholine (PLPC), 23% dilinoleoyl-phosphatidylcholine (DLPC), 17.5% palmitoyl-oleoyl-phosphatidylcholine (POPC) and 16% stearoyl-linoleoylphosphatidylcholine (SLPC), 8% saturated dipalmitoyl-phosphatidylcholine (DPPC) [1]. The uppermost lipid bilayer (i.e. surface amorphous layer (SAL)) of articular cartilage surface is degraded during osteoarthritic disease [2-3]. The surface amorphous layer is of utmost importance to the effective load-spreading, lubrication, and semipermeability of articular cartilage in the mammalian joints [4-7]. The outcome will potentially contribute to knowledge that will facilitate the repair of the articular surface of cartilage where degradation is limited to the loss of the lipids of the SAL only.

The surface amorphous layer (SAL) is the topmost layer of articular cartilage often in contact during physiological function, thereby making it prone to wear and tear. The site of repeated contact can lead to early stages of joint degeneration like osteoarthritis and discomfort leading to low quality of life in affected patients. In order to simulate the loss of cartilage surface lipids, an artificial lipid extraction process was used (delipidization). It is hypothesized that restoration of the surface amorphous layer can be achieved by re-introducing synthetic surface-

active phospholipids (SAPL) into the joint space. This hypothesis of restoration of the surface amorphous layer was tested in this work by re-introducing synthetic saturated and unsaturated phospholipids into the joint space. By exposing cartilage samples whose surface lipids had been depleted to individual and mixtures of synthetic saturated and unsaturated phospholipid solutions. In this paper, laboratory procedure was developed for re-introducing lipids onto the surface of lipid-depleted articular cartilage. The process was termed lipid "resurfacing".

Material and Methods

The articular cartilage samples used in this study were obtained from the patellae of 3-4 year old bovine animals harvested from the local abattoir and stored at -20ºC until required for testing. The samples were thawed out in continuous running water at room temperature and kept in saline solution (0.15M sodium chloride) prior to testing. A stainless-steel punch was used to cut osteochondral plugs (n = 20), containing full thickness articular cartilage-bone laminate and trimmed into specimen of 5 mm by 5 mm**.** The bony layer underlying the cartilage was dabbed with a paper towel and immediately glued onto a Petri dish using fast-drying Loctite® 454 glue (Henkel Australia PTY Ltd, Victoria, Australia). The Petri dish was mounted onto the AFM sample holder, ready for AFM measurements. During gluing, the articular surface was moistened repeatedly with drops of saline solution to keep surface intact. The glued sample was submerged in saline solution ready for AFM imaging using the SMENA® head of the NT-MDT P47 Solver scanning probe microscope (SPM) (NT-MDT, Moscow, Russia). The surface imaging was done using methods described elsewhere in the literature [8].

After imaging, lipids were selectively removed from the articular surface in accordance with the delipidization procedure described elsewhere in the literature [9] using Folch reagent (i.e. a mixture of chloroform/methanol, in the ratio 2:1 v/v). The delipidized samples were placed in saline solution for 30 min for rehydration and to remove the lipid rinsing agent and any organic solvent left on the surface of the tissue. Each sample was then mounted on the AFM for imaging. The delipidized specimens were divided into three groups (A, B and C), ready for relipidization with each group comprising of 5 samples.

Relipidization is the process of resurfacing delipidized cartilage with synthetic phospholipids. Group A samples were incubated in DPPC solution (a saturated phospholipid), group B in POPC solution (an unsaturated phospholipid), and group C in complete joint SAPL mixture (containing both saturated and unsaturated lipids). The protocol for the relipidization process is described in our previous work [9]. After relipidization, the incubated samples were removed from the test tube container, rinsed saline solution and then mounted on the AFM for imaging using the scanning parameters as previously described for normal and delipidized cartilage samples.

Results and Discussion

The surfaces of normal, delipidized, and relipidized samples of cartilage were characterized for their structural integrity and functionality using atomic force microscope (AFM). The results from AFM imaging revealed a successful deposition of a new surface layer on the lipid depleted cartilage when incubated in synthetic phospholipid solutions. The relipidization resulted in a noticeable improvement in the surface nanostructure of the artificially degraded cartilage, with the complete SAPL mixture providing better outcomes in comparison to those created with the single synthetic phospholipid components (palmitoyl-oleoyl-phosphatidylcholine, POPC and dipalmitoyl-phosphatidylcholine, DPPC).

Phospholipids as boundary lubricants are highly self-organized biomolecules in aqueous media, and their structure allows them to form spontaneously vesicles, lamellar phases, and surface membranes. The multilamellar structure of phospholipids, namely the surface amorphous layer (SAL), covers the natural surface of articular cartilage found in diarthrodial joints. A very high porosity (75 to 80 %) is concluded to be a critical factor in providing excellent lubrication, more specifically hydration lubrication properties displayed by articular cartilage [10]. This is made possible by the amphoteric characteristics presented by the articular surface under physiological joint conditions [11]. Thus, making the articular surface highly efficient and extremely frictionless.

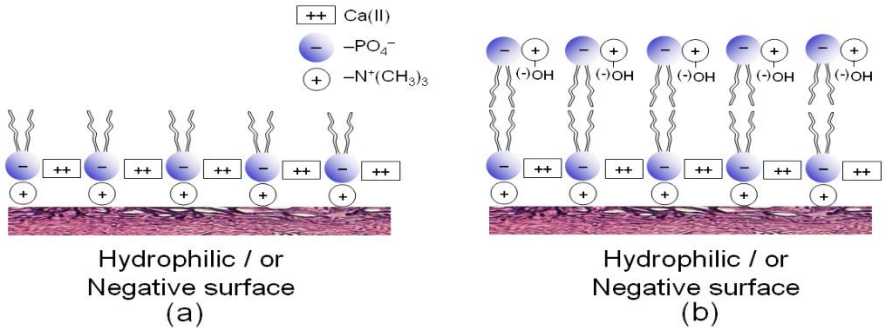


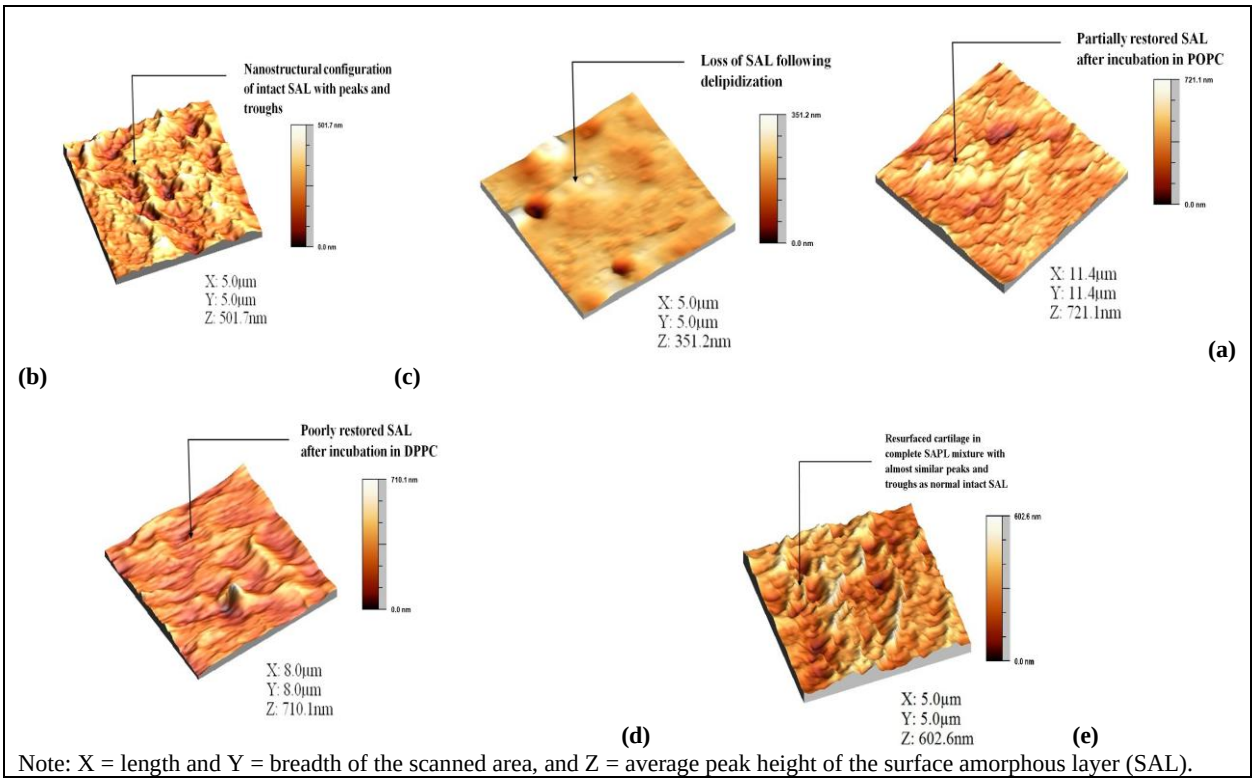
Figure 1. *The hydrophobic (a) and hydrophilic cartilage surface (b) model with negatively charged phosphate ions on its surface.*

The lipid bilayers on the surface of the healthy joint cartilage comprises mostly unsaturated phospholipid species with phosphatidyl-choline, PC (41%), phosphatidylethanolamine, PE (27%), and sphingomyelin, SP (32%) being the major components [12]. Liposomes and lamellar phases are composite structures made of phospholipids in synovial fluid and bilayers on cartilage surface (Figure 1). The strong adsorption of PLs molecules by their quaternary ammonium positive ion (Me_3N^+-) to hydrophilic cartilage surface (a proteoglycan) is [12] hydrophilic model of the cartilage surface. The strong cohesion between phosphate ions and calcium (II) (-PO_4^--Ca-PO_4^--), make the close-packed hydrophobic solid layer (Figure 1a).

However, considering pH 7.4 condition and properties of phospholipids (PLs) of being highly self-organized bilayer structure, and the surface is negatively charged (Figure 1b). The multilamellar structure of phospholipids, namely the surface amorphous layer (SAL), covers the surface of diarthrodial joints bearing the articular cartilage. It can be concluded that a very high porosity (75%) is a critical factor in providing an excellent hydration lubrication of the articular cartilage. The phospholipid molecules in the SAPL exhibit a unique amphiphilic

behavior possessing positive quaternary ammonium ions and negative phosphate ions. The quaternary ammonium (QA) ions has strong electrostatic bond strength and thus able to bind to surfaces with excess negative charges [4-13]. Since the proteoglycan molecules in the articular surface have excess carboxyl and sulphate ions, the surface of cartilage is attractive to the QA ions, thereby leaving the excess phosphate ions accessible for the positive mobile ions (Na^+, Ca^{2+}, H^+) present in the synovial fluid [4,13].

The surface ionic interactions keep the articular cartilage electrically neutral, and an excellent boundary lubricant. It is hypothesized in paper that this exceptional surface chemical property-nanostructure possessed by the articular surface due its surface amorphous phospholipid layer is disrupted or lost when the surface lipids are eroded following early stages of cartilage degeneration (onset of osteoarthritis). In order to selectively remove lipids from cartilage surface, an artificial lipid removal process was employed (delipidization) [14].



Note: X = length and Y = breadth of the scanned area, and Z = average peak height of the surface amorphous layer (SAL).

Figure 2. *3D topographical image of articular cartilage: (a) normal healthy; (b) delipidized articular cartilage; (c) relipidized articular cartilage in palmitoyl-oleoyl-phosphatidylcholine, POPC; (d) relipidized articular cartilage in dipalmitoyl-phosphatidylcholine, DPPC; (e) relipidized articular cartilage in complete SAPL mixture.*

The topographical images of articular cartilage specimens with normal intact, delipidized and relipidized (in aqueous solutions of POPC, DPPC and complete joint SAPL mixture) surfaces were captured with the AFM (Figure 2a to 2e). A study of the 3D resolved AFM image of the unaltered cartilage surface (Figure 2a) showed a unique organization of the SAL in a lamella-like arrangement as previously described by Hills et al. (1990) [15]. The nanostructural arrangement of the surface amorphous layer with several peaks and troughs are clearly apparent. Wiping of the normal intact surface with lipid rinsing reagent resulted in a noticeable change in the surface topographical structure. Figure 2b however, shows the loss of the lipid membranous overlay (surface amorphous layer) of the articular surface. Thus, confirming that lipid extraction process is effective [14].

The exposure of the delipidized of the second group of samples in unsaturated POPC partially restored the lost surface phospholipid nanostructural surface patterns. Figure 2c shows a moderately restored lamella layer of phospholipids slightly similar to intact articular surface. The relipidization in saturated DPPC did not yield any noticeable improvement in the surface configuration relative to normal intact articular surface sample. Figure 2d shows almost featureless structure of the articular surface when compared with an intact cartilage surface. Interestingly, the incubation in synthetic aqueous solution containing the complete joint SAPL species provided a much better resurfacing outcome, with the newly deposited lipid layer exhibiting almost similar SAL configuration observed in normal intact articular surface. Figure 2e shows similar peaks and troughs seen on the surface of normal cartilage surface (Figure 2a). Although, there is a close similarity in the surface nanostructural configuration of cartilage samples resurfaced with synthetic phospholipids, with complete SAPL showing more promising signs than other unsaturated SAPL species (POPC), further studies are required to determine the functionality of the newly laid surface under physiological conditions.

The artificially degraded cartilage surface (lipid depleted) is only partially comparable with a natural joint under inflammation where participation of β2-Glycoprotein I, (β2-GPI) is most important. Molecules of β2-Glycoprotein I, (β2-GPI) (MW of 50 kDa) circulate in the body and autoimmune disease transforms β2-GPI in an antibody [16-17]. The β2-GPI participates in antiphospholipid antibody syndrome (APS) through binding of β2-GPI to the anionic charged phospholipid ($-PO_4^-$) group. At a pH around 7, β2-GPI - amino acids (arginine, lysine and tryptophan) are positively charged ($-NH_3^+$): an acid-base interaction occurs between the protonated amino acid group ($-NH_3^+$) and the phosphate ($–PO_4^-$) membrane group: (β_2-GPI-NH_3^+) + (PLs–PO_4^-) → ($-NH_3^+$ PO_4^-) interaction and electrostatic attractions is strong enough to destroy the PLs bilayer on the articular surface and deactivate all phospholipids in the

synovial fluid (SF). Under the conditions of our experiment, the phospholipids in the solutions can be deactivated by β2-GPI as well [18-19]. It is important to consider the effect of β2-Glycoprotein I in future studies on articular cartilage degeneration.

Conclusion

It has been demonstrated, using individual components and complete mixture of surface-active phospholipids found in the normal surface amorphous layer overlaying the articular cartilage, that it is possible to amend the structural configuration of a delipidized articular surface, recreating it to a form approaching the normal intact surface. This result adds further support to the hypothetical position that it is possible to artificially "resurface" a degenerating articular cartilage, especially in the early stage condition such as osteoarthritis, by introducing synthetic phospholipid mixtures into the joint space. It should be noted that this work is still in its early stages and still requires more detailed research, in which more case scenarios will be considered with various combinations and concentrations of unsaturated phospholipid constituents such as SLPC, PLPC, and DLPC, and other components of the joint fluid, such as lubricin and hyaluronic acid. In conclusion, this study has established that it is possible to deposit a potentially viable layer on the surface of cartilage following degradation through incubation in synthetic phospholipid solutions. However, further studies will be required to advance the ideas developed for treatment of osteoarthritis and other joint conditions. The surface restoration in osteoarthritic conditions considering the effect of deactivation of phospholipids requires additional experiments.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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