PRELIMINARY CHARACTERISATION OF THE SURFACE OF CARTILAGE FOLLOWING EXPOSURE TO SATURATED AND UNSATURATED SYNTHETIC LIPIDS

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Abstract: Articular cartilage is covered by a microscopic structure known as surface amorphous layer. This surface structure is often the first victim of attack during cartilage degeneration, thereby resulting in a gross impairment in cartilage function such as lubrication and load bearing. We hypothesize that incubation of degraded cartilage in solutions of different species of synthetic surface active phospholipids (saturated and unsaturated species) can remodel this lost surface structure. To test this hypothesis, the structural configuration of the surface of articular cartilage was studied and characterised with the lipid filled surface amorphous layer intact using the AFM. The results were then compared with those obtained following a systematic removal (delipidization) and replacement (relipidization) of this layer. Our results show that the unsaturated surfactant partially restored the lost surface amorphous layer while the saturated surfactant specie settled on the surface due to its poor solubility in aqueous solution.

Key words: AFM, articular cartilage, surface amorphous layer, lubrication, load bearing, delipidization, relipidization

1 INTRODUCTION

Surface damage or fissuring has been recognised as one of the indicators of articular cartilage degeneration. Of importance in this paper is the role of the surface amorphous layer (SAL) in determining the integrity of the articular surface. The surface amorphous layer performs both mechanical and biochemical roles that determines the physiological functional integrity of contacting cartilage surfaces relative to lubrication, load spreading and overall contact conditions from very low to high impact loading (McCutchen, 1962; Mow & Ling, 1969; Little, Swanson, & Freeman, 1969; Oloyede, Gudimetla, Crawford, & Hills, 2004a,b). It is therefore imperative to able to characterize this surface layer to facilitate the distinction between normal healthy and degrading tissue especially in the early stages of degradation (Jurvelin et al., 1996; Kumar et al., 2001; Crockett et al., 2005; Grant et al., 2006) and more importantly in the assessment of the effectiveness of biological remedies of ailing joints.

The surface amorphous layer is an organized membrane that overlays the surface of cartilage. It is made up of multi-bilayered phospholipid structure (a two-dimensional network of amphiphilic molecules) that plays a crucial role in the smooth functioning of the surface amorphous layer (Redondo & LeSar, 2004). Attached to this lipid bilayer are proteoglycans, glycoproteins, cholesterol, hyaluronan and water (Hills, 1992). The multi-bilayered phospholipid structure also known as surface active phospholipids (SAPL) has been conceptualized to be capable of forming form giant reverse micelles (GRM) which facilitate joint lubrication (Oloyede, Gudimetla, Chen, & Crawford, 2008; Pawlak & Oloyede, 2008); it has also been demonstrated that they form a semipermeable membrane for the diffusion of polar solutes across the articular surface (Chen, Crawford, & Oloyede, 2007). The transport of proteins and other ions across lipid bilayer membranes has been studied both experimentally and theoretically (Saffman & Delbruck, 1975; Saffman, 1976), with several applications to cell signaling using classical diffusion equations (Chapman, 1984; Lipowsky & Sackmann, 1995) and a combination of Monte Carlo and Brownian dynamics techniques (Im, Seefeld, & Roux, 2000; Redondo & LeSar, 2004). When articular cartilage degenerates, this surface structure is compromised with most of its lipids lost (Sarma, Powell, & LaBerge, 2001; Oloyede et al., 2004a,b). This loss impairs joint lubrication with detrimental effects on mobility and human activities (Vecchio, Thomas, & Hills, 1999; Sarma et al., 2001; Oloyede et al., 2004b).

Analysis of the lipids obtained from the surface of bovine cartilage reveals that the type of surfactants found in the joint of mammals consists of saturated and unsaturated species with the latter being more dominant (Sarma et al., 2001). The work of Chen et al. (2007) revealed that the surface of articular cartilage consist of 23% Dilinoleoyl-phosphatidylcholine (DLPC), 30% Palmitoyl-linoleoylphosphatidylcholine (PLPC), 17.5% Palmitoyl-oleoyl-phosphatidylcholine (POPC) and 16% Stearoyl-linoleoylphosph atidylcholine (SLPC) coexisting in mixture with a small quantity of Dipalmitoyl-phosphatidylcholine (DPPC), which make up only 8% of the entire mixture.

It has been hypothesized that lipid loss in cartilage may be partially or fully recovered by re-introducing synthetic lipids into the degrading joints. It is however not known whether the replacement synthetic lipid mixture must contain all the components identified above in order to ~~"r~~remodel" the surface of a degraded cartilage. To investigate this hypothesis, Vecchio et al. (1999) conducted a clinical trial in which a single injection of saturated synthetic SAPL (DPPC) was delivered into one knee of each of ten patients with bilateral moderate-to-severe osteoarthritis. The lipid injection resulted in gout (an inflammation of the joint), necessitating the question as to how exactly these synthetic lipids act when introduced into the body and in contact with articular cartilage. Also, Oloyede et al. (2008) established through in vitro relipidization that the dominant unsaturated SAPL component of knee surfactant can reverse the load-bearing properties of a degenerating tissue. However, the study did not attempt to characterize the surface of the relipidized tissue. The existing work in the literature has provided knowledge in distinguishing the surface of cartilage with and without lipids, a gap still exist in the literature with respect to the characterization of relipidized cartilage, where relipidization is defined as the deposition of synthetic lipids on the surface cartilage via incubation. The aim of

this work is to characterize the surface of relipidized cartilage with the AFM and then compare the actions of saturated and unsaturated SAPLs in the remodelling of the surface of degraded cartilage.

2 MATERIALS AND METHODS

There are several methods for characterizing the surface structure of soft tissues such as scanning electron microscopy (SEM), transmission election microscopy (TEM), confocal microscopy (COFM) and atomic force microscopy (AFM). Of all these methods, the AFM is the most widely used. This is because of its versatility not just as an imaging tool but also the ability to measure biomechanical properties of soft tissues such as cartilage especially when it is critical to conduct measurements in liquid environment. Most of the work done so far has been on imaging and force analysis of normal intact; lipid depleted and roughened/modified articular cartilage surfaces (Jurvelin et al., 1996; Kumar et al., 2001; Grant et al 2006).

2.1 Sample preparation for AFM

The articular cartilage specimens used in this study were taken from the patellar grooves of 3–4 year old bovine animals (n = 20) harvested from the local abattoir and stored at 20^{o}C until required for testing. The joint samples were thawed out in continuous running water at room temperature and kept in phosphate buffered solution (PBS) solution prior to testing. Osteochondral plugs, full thickness articular cartilage-bone laminate (8 samples per patella) were taken from the thawed joints and trimmed into specimens of about 5 by 5mm. The bony layer underlying the cartilage was dried with a paper towel and immediately glued onto a Petri dish (1.5 cm in diameter) using a two-sided adhesive tape and a fast-drying glue Loctite® 454.

2.2 AFM Imaging

The experimental protocol for the microscopy and imaging of samples is presented in the schematic flow chart (Fig. 01).

A two-sided adhesive tape was used to fix the Petri dish-cartilage specimen onto the piezoscanner of the AFM. During gluing, the articular surface was moistened with drops of PBS to keep the surface intact. The sample was then submerged in PBS by pipetting PBS into the Petri dish containing the sample to create a liquid environment for AFM measurements.

To preserve the integrity of the samples, all experiments were conducted in liquid environment containing PBS using the SMENA® head of the NT-MDT P47 Solver scanning probe microscope (SPM) (NT-MDT, Russia). The imaging was performed with a very soft triangular cantilever (spring constants of between 0.05 - 0.10 kN/m) carrying contact tips (Veeco probes, California, USA). After mounting the specimen and setting up the AFM, the instrument was allowed to undergo thermal relaxation for 30 minutes; and to ensure that the drift of the cantilever deflection angle was minimized before imaging in accordance with (Jurvelin et al., 1996). The triangular cantilevers used in the experiment were calibrated using the method in (Sader, Chon, & Mulvaney, 1999).

AFM imaging was conducted in the error signal mode. Images were obtained along the 2D planes of the articular surfaces of over 150 samples from 20 normal intact joints. Topographical and deflection images were simultaneously captured with height and deflection signals respectively. In order to obtain high resolution image, the deflection signal was minimized by optimizing scanning parameters such as feedback gain, set-point and scanning speed/frequency. Also, the trace and retrace signals were continuously monitored with the oscillograph to ensure that they were tracking each other. Fig. 02 overleaf shows the schematic diagram of a real-time imaging process of normal cartilage with the AFM. The trace and retrace images look similar.

2.3 Surface Lipid removal (Delipidization) Process

After 30 minutes in the PBS to recover from the AFM scanning process, samples were carefully debonded from the Petri dish and then wiped progressively with kimwipe that had been soaked in Folch reagent (i.e. a mixture of chloroform:methanol (2:1)) (Folch, Lees, & Sloane-Stanley, 1957). After the delipidization process, the bony layer underlying the cartilage was dabbed with paper towel and immediately glued onto a Petri dish using a two-sided adhesive tape and fast-drying Loctite® 454 glue as mentioned above.

Subsequent to this delipidization protocol, 40 specimens from 5 joints were subjected to delipidization and imaging. The Petri dish containing the specimen was mounted back onto the AFM. In order to maintain the integrity of the samples used for the experiments, PBS solution was pipetted into the Petri dish to create a liquid environment for AFM measurements. The AFM imaging described above for the normal intact articular cartilage specimens was repeated for the delipidized samples using the same scanning parameters. After the AFM experiments, the delipidized samples were then divided into two sets, ready for relipidization.

2.4 Incubation in lipid filled environment (Relipidization)

Two types of lipids were applied in this component of the experiments, namely dipalmitoyl-phosphatidylcholine (DPPC) and palmitoyl-oleoyl-phosphatidylcholine (POPC). These represent the saturated (DPPC) and unsaturated (POPC); and the least (8 wt. %) and one of the most abundant (17.5 wt. %) components in the lipid content of the joint surfactant respectively.

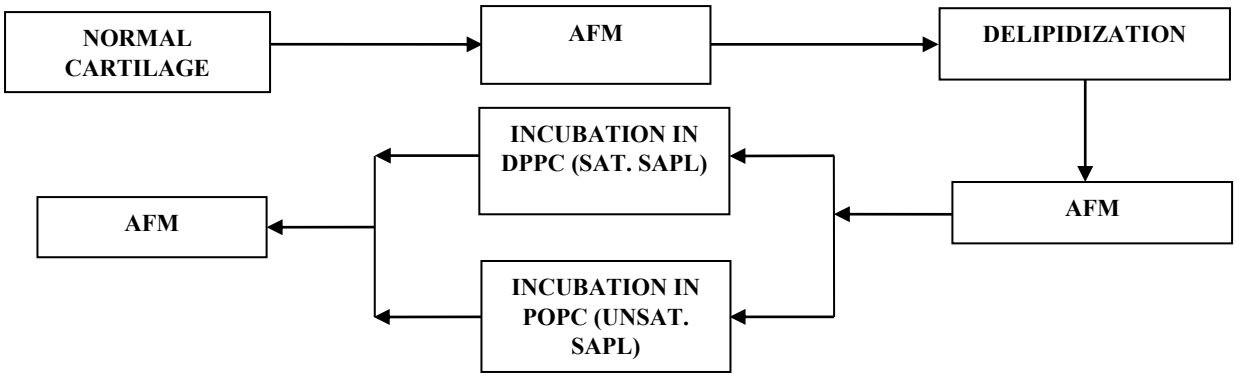


FIGURE 01: Schematic flowchart of the AFM for normal, delipidized and relipidized cartilage

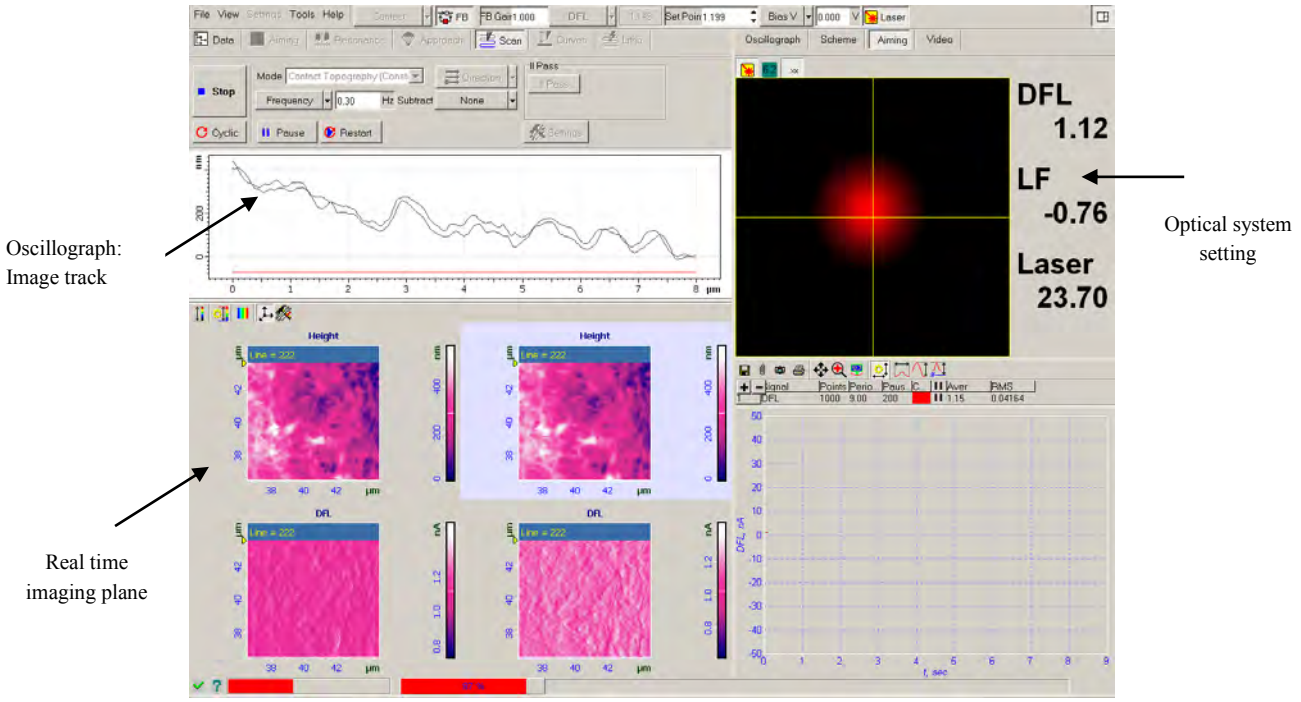


FIGURE 02: Schematic representation of the imaging process of normal cartilage with the AFM

Relipidization is the process of reintroducing lipids either by intra-articular injection of synthetic lipids into joints to repair degraded cartilage or by *in vitro* incubation of lipid-depleted cartilage in solutions of synthetic lipids in controlled environment (Oloyede et al., 2008). After the AFM measurements on the delipidized samples, the first set of samples (n = 20) were debonded from the Petri dishes and transferred into labelled test tubes containing 5ml of 1 wt. % of lipid species in solution. The in vitro relipidization process was conducted in this study. (Avanti Lipids, Alabama, USA). The test tube containing POPC was placed in a radial agitating incubator which was maintained at 37°C, while the test tube containing DPPC was maintained at 43°C. The samples were incubated for 24h. After the relipidization process, each sample was removed from its test tube container and then rinsed in PBS solution. The bony layer underlying the cartilage was dried with a paper towel and immediately glued onto a Petri dish using a two-sided adhesive tape and a fast-drying Loctite® 454 glue. The Petri dish containing the relipidized specimen was mounted back onto the AFM and filled with PBS solution using a pipette. The AFM measurements described above was then repeated for the relipidized specimens.

3 RESULTS

3.1 AFM Imaging

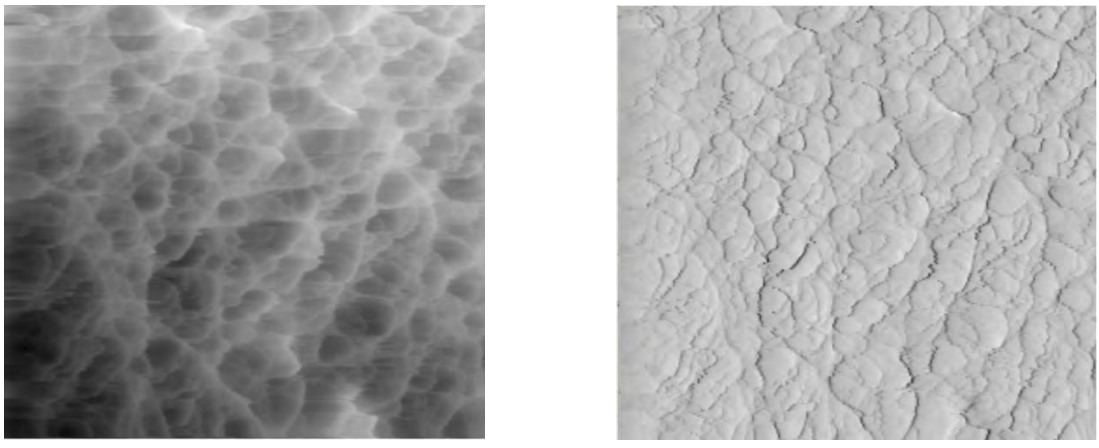
The surface topographical images obtained for normal and delipidized and relipidized cartilage are presented in this section.

3.1.1 For normal cartilage

Fig. 03a & 03b show the topographical and deflection images acquired simultaneously for normal intact articular cartilage. The figures show that a normal cartilage is covered by a non-fibrous 1ayer of organized surface structure with several peaks and troughs. Hills (1990) described this structure as oligolamella layer formed by the SAPL.

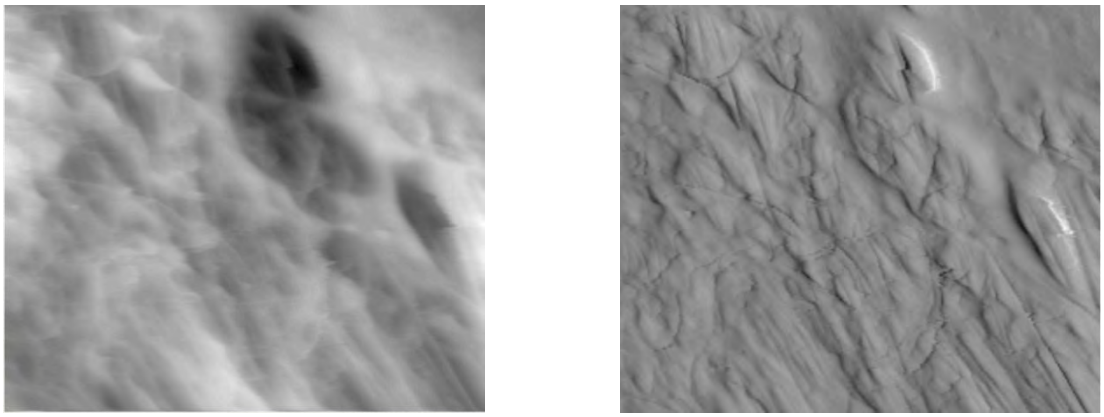
3.1.2 For surface delipidized cartilage

Fig. 04a & 04b overleaf show the topographical and deflection images of the surface of delipidized articular cartilage (obtained after wiping the surface of normal cartilage already imaged with AFM with kimwipe soaked in chloroform:methanol (2:1) as described in section 2.3 above.



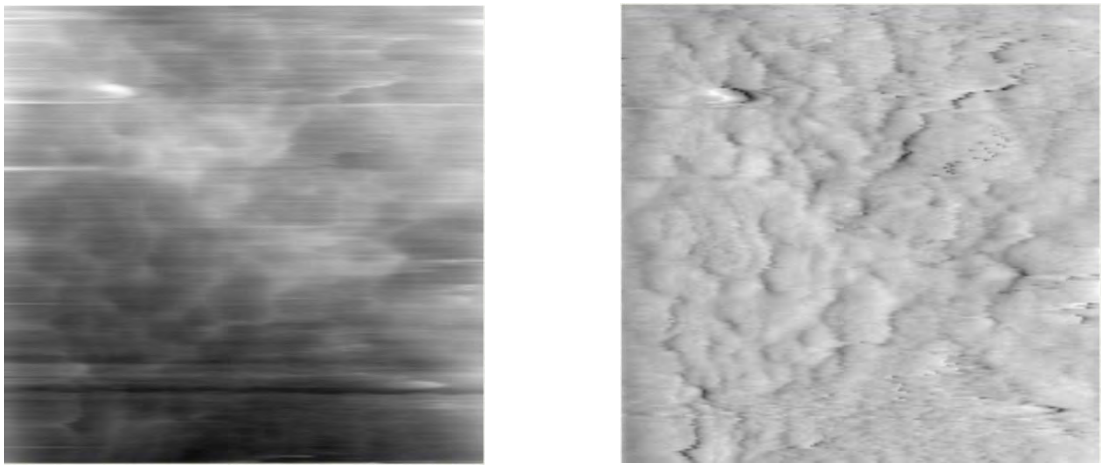
(a) **(b)**

FIGURE 03: 2D Images of Normal articular cartilage surface (Frame size: 5 by 5µm), (a) Topographical, and (b) Deflection



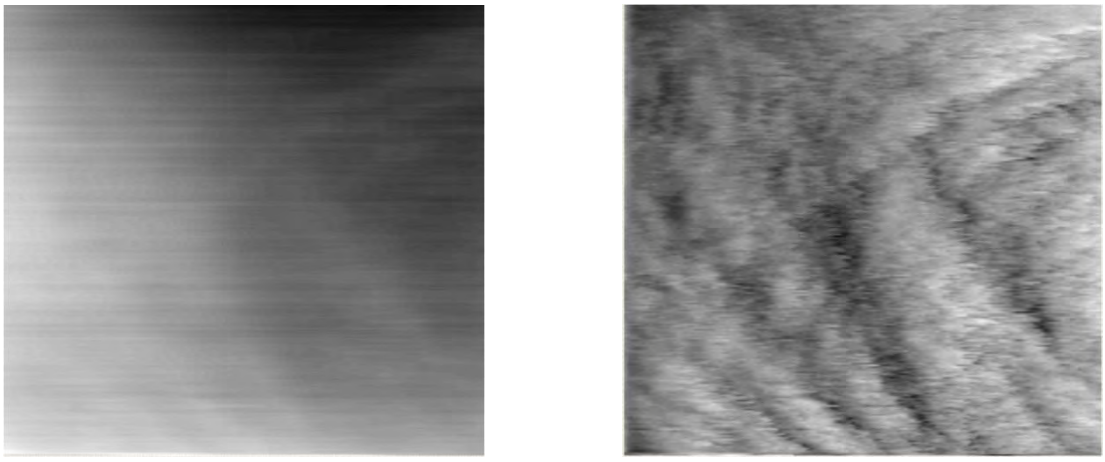
(a) (b)

FIGURE 04: 2D Images of surface delipidized articular cartilage surface (Frame size: 5 by 5µm), (a) Topographical, and (b) Deflection



(a) (b)

FIGURE 05: 2D Images of surface relipidized articular cartilage in unsaturated POPC (Frame size: 5 by 5µm), (a) Topographical, and (b) Deflection



(a) (b)

FIGURE 06: 2D Images of surface relipidized articular cartilage in saturated DPPC (Frame size: 5 by 5µm), (a) Topographical, and (b) Deflection

Wiping of the surface with lipid rinsing agent almost complete removed the organized surface layer but no fibre structure was observed in the subsurface layer in contrast to the work of Jurvelin et al. (1996).

3.1.3 For relipidized cartilage in unsaturated SAPL (POPC)

Fig. 05a & 05b show the topographical and deflection images of the surface of cartilage after 24h incubation in unsaturated POPC at 37^{o}C. After relipidization in unsaturated POPC, there was a significant change in the surface structure of the delipidized cartilage which resulted in the partial restoration of its lost surface lipid structure.

3.1.4 For relipidized cartilage in saturated SAPL (DPPC)

Fig. 06a & 06b show the topographical and deflection images of the surface of cartilage after 24h incubation in saturated DPPC at 43^{o}C. Incubation in saturated DPPC did not restore the lost surface lipid layer but instead the synthetic lipid settled on the articular surface.

4 DISCUSSION

In this study, we examined the effect of wiping the surface of normal healthy cartilage with lipid rinsing agent (delipidization) on the surface structure using the AFM, while studying the nature of cartilage surface that has been incubated (or relipidized) in synthetic surfactants. Alhough the relipidization was done using only two species of the six components of the surface amorphous layer (SAL) in the joint, the model can be argued to be adequate for studying the consequences of of relipidizing or ~~"re~~surfacing" the tissue with saturated DPPC and unsaturated POPC.

Our experimental results reveal that the two surfactants led to very distinct effects on the surface of delipidized cartilage. After 24h incubation in unsaturated POPC (Fig. 05), there was partial restoration of the lost SAL as manifested in the change in surface structure when compared with normal cartilage (Fig. 03). Conversely, the saturated DPPC did not appear to restore the lost SAL but instead settled on the surface. This could be as result as result of its poor solubility in aqueous solution.

Also noting that the joint contains other unsaturated SAPL species such as PLPC, SLPC and DOPC, it is not known at this stage whether or not the entire mixture will be required to completely remodel the surface of degraded cartilage. This will be tested in our future work. Although we did not calculate other characterising parameters such as adhesion and chemical binding forces between the lipids and the cartilage, the outcome of this study has established a basis for the viability and potential of synthetic lipid-based remodelling or resurfacing cartilage following degeneration or disease such as osteoarthritis.

5 CONCLUSION

We have characterised the surface of relipidized cartilage for the first time with the AFM. The outcome of this work contributes further to the existing knowledge on the role of synthetic lipids on the biochemical characteristics of articular cartilage.

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