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DOI:
10.1016/j.micron.2012.06.007

Document Version
Peer reviewed version

Citation for published version (Harvard):
Ghosh, S, Bowen, J, Jiang, K, Espino, DM & Shepherd, DET 2013, 'Investigation of techniques for the measurement of articular cartilage surface roughness', Micron, vol. 44, pp. 179-184. https://doi.org/10.1016/j.micron.2012.06.007

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Investigation of techniques for the measurement of articular cartilage surface roughness

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Author generated final version. Published as:

Ghosh S, Bowen J, Jiang K, Espino DM, Shepherd DET (2013). Investigation of techniques for the measurement of articular cartilage surface roughness. *Micron* 44: 179–184

Abstract

Articular cartilage is the bearing surface of synovial joints and plays a crucial role in the tribology to enable low friction joint movement. A detailed understanding of the surface roughness of articular cartilage is important to understand how natural joints behave and the parameters required for future joint replacement materials. Bovine articular cartilage on bone samples were prepared and the surface roughness was measured using scanning electron microscopy stereoscopic imaging at magnifications in the range 500 × to 2000 ×. The surface roughness (two-dimensional, R_a, and three-dimensional, S_a) of each sample was then measured using atomic force microscopy (AFM). For stereoscopic imaging the surface roughness was found to linearly increase with increasing magnification. At a magnification of 500 × the mean surface roughness, R_a, was in the range 165.4 ± 5.2 nm to 174 ± 39.3 nm; total surface roughness S_a was in the range 183 nm to 261 nm. The surface roughness measurements made using AFM showed R_a in the range 82.6 ± 4.6 nm to 114.4 ± 44.9 nm and S_a in the range 86 nm to 136 nm. Values obtained using SEM stereo imaging were always larger than those obtained using AFM. Stereoscopic imaging can be used to investigate the surface roughness of articular cartilage. The variations seen between measurement techniques show that when making comparisons between the surface roughness of articular cartilage it is important that the same technique is used.

Keywords: Articular cartilage; Atomic Force Microscopy; Scanning Electron Microscopy; Stereoscopic imaging; Surface roughness.

1. Introduction

Articular cartilage is the bearing surface of synovial joints. It comprises a relatively small number of cells (chondrocytes) and an abundant extra cellular matrix of collagen, proteoglycan and water (McNary et al., 2012; Shepherd and Seedhom, 1997). This structure makes articular cartilage a viscoelastic material (Fulcher et al. 2009). Articular cartilage plays a crucial role in load transmission in joints and in the tribology to enable low friction joint movement (Dowson and Jin, 1986; Yao and Unsworth, 1993). Therefore, a detailed understanding of the surface roughness of articular cartilage is important to understand how natural joints behave. Knowledge of surface roughness will also be important for the development of new synthetic or tissue engineered materials to replace diseased or damaged articular cartilage (Ateshian, 2007; Danisovic et al., 2012; Ma et al. 2010). Articular cartilage in need of replacement is typically damaged (Fick and Espino, 2011; Fick and Espino 2012).

The surface roughness of a material can be characterised by a variety of roughness parameters. The most widely used measure of surface roughness is the arithmetic average, R_a, which is determined from a two-dimensional profile. Surface roughness measurements of an area can also be made and S_a is the arithmetic average of a three-dimensional roughness (Al-Nawas et al., 2001). A variety of techniques have been used to measure the surface roughness of articular cartilage. Contacting methods include the use of talysurf measurements (Forster and Fisher, 1999; Jones and Walker, 1968; Sayles et al., 1979) or Atomic Force Microscopy (Moa-Anderson et al., 2003). Non-contacting methods have included optical coherence tomography (Saarakkala et al., 2009), ultrasound (Saarakkala et al. 2004), laser profilometry (Forster and Fisher, 1999) and scanning electron microscopy stereoscopic imaging (Bloebaum and Radley, 1995). Contact methods may not be appropriate because articular cartilage is a soft tissue that can deform during measurements; non-contact methods have not been investigated across a range of scales/magnifications which could affect the measurements.

This paper investigates the surface roughness of articular cartilage using scanning electron microscopy stereoscopic imaging and atomic force microscopy. The aim was to investigate the effect of magnification on surface roughness using stereoscopic imaging and to compare with results obtained by atomic force microscopy. As articular cartilage is a natural material with a high variability in surface properties, a standard roughness specimen was also used to quantify the two surface roughness techniques.

2. Materials and methods

2.1 Standard sample preparation

A standard surface roughness sample made from electroformed nickel (specimen type 543E sinusoidal, Rubert + Co Ltd, Cheadle, UK), with R_a = 35.48 ± 1.2 nm was used to produce a polydimethylsiloxane (PDMS) mold so that surface roughness values from scanning electron microscopy (SEM) stereoscopic imaging and atomic force microscopy could be compared. The PDMS was prepared with 10:1 w/v of Sylgard 184 silicone elastomer and its curing agent (Dow Corning Corporation, Midland, USA), followed by rigorous mixing of them in a teflon beaker using a spatula. The mixture was degassed by keeping the viscous fluid in open air for 20 minutes. After that the liquid PDMS was poured onto the standard surface roughness sample. It was then allowed to cure overnight, which produced a transparent and solid peelable material at room temperature. Six PDMS samples were produced. Since PDMS is a non-conductor, for SEM the surface was sputter coated with ~10 nm of platinum (Emscope SC-650, Quorum Technologies Ltd, Ashford, UK).

2.2 Articular cartilage preparation

A bovine joint, aged approximately three years, was obtained from a local butchers shop (Johnstans Butchers, Kings Heath, Birmingham, UK). On arrival in the laboratory the cartilage surfaces were inspected with the naked eye and found to be free from damage. Samples of cartilage on subchondral bone were then cut from the lateral humeral condyle. Four samples, of approximate dimensions 10 mm × 10 mm × 10 mm were produced. Ringer's solution was used to keep the specimens hydrated; sodium azide (Sigma Aldrich,

Gillingham, UK) was added to the Ringer's solution to prevent the growth of bacteria on the cartilage surface.

The specimens were then fixed with 2.5% glutaraldehyde (Agar Scientific Ltd, Essex, UK)) and 8% paraformaldehyde (Sigma Aldrich, Missouri, USA) solution in 0.1 M physiological equivalent phosphate buffer solution for 20 minutes at room temperature prior to dehydration (ap Gwynn et al., 2000). Dehydration involved using graded ethanol solutions of 50%, 70%, 90%, 100% and 100% dry ethanol, each being applied for 10 minutes (Kääb et al., 1999). Supercritical drying was then undertaken with CO_2 using a Critical Point Dryer (E3000, Quorum Technologies Ltd, Ashford, UK) to remove the internal fluid from the samples. The cartilage samples were then sputter coated with ~10 nm of platinum (Emscope SC-650, Quorum Technologies Ltd, Ashford, UK) for SEM imaging.

2.3 Surface roughness from scanning electron microscopy stereoscopic imaging

The surface roughness of each PDMS and cartilage specimen was measured using scanning electron microscopy (SEM) stereoscopic imaging. Imaging was performed using a Philips XL30 ESEM-FEG (FEI Company, Hillsboro, Oregon, USA), operating in a conventional high vacuum mode at a pressure less than 1×10^{-7} mbar. All the images were scanned at 10 kV accelerating voltage of the electron beam, with the working distance approximately 10 mm. The surfaces were scanned at magnifications of 500 ×, 800 ×, 1200 × and 2000 ×. In order to produce a stereoscopic image it was necessary to take images of the surface at 0°, 5° and -5° normal to the beam. These images were then reconstructed and surface characterisation performed using the MeX software package, version 5.0.1 (Alicona Imaging GmbH, Graz, Austria). Further details of the technique are provided by Ostadi et al. (2010). Four surface roughness measurements (R_a) were made on each specimen of PDMS and cartilage, as well as the total surface roughness (S_a).

2.4 Surface roughness from atomic force microscopy

After the SEM images were obtained for each PDMS and cartilage specimen, the surface roughness was then measured using atomic force microscopy (AFM). Measurements were made using a Nanowizard II AFM (JPK Instruments AG, Berlin, Germany), operating in intermittent contact mode. CSC17 silicon cantilevers were employed, exhibiting ~10 nm diameter pyramidal tips (MikroMasch, Tallinn, Estonia). An area of 25 μm × 25 μm was scanned. Four surface roughness measurements (R_a) were made on each specimen of PDMS and cartilage, as well as the total surface roughness (S_a).

3. Results

3.1 PDMS

The surface roughness measurements for each PDMS sample using SEM stereoscopic imaging and AFM are shown in Table 1. Figure 1 shows images of a PDMS surface from SEM stereoscopic imaging and AFM. It was observed the surface roughness, R_a, for each PDMS sample was found to linearly increase with increasing magnification, as shown in Figure 2 for two of the PDMS sample. The same trend was seen for the other four samples. All linear regressions were found to be significant ($p < 0.05$). Surface roughness, S_a, was also found to linearly increase with increasing magnification. The lowest values of surface roughness were found at a magnification of 500 ×. At this magnification the mean surface roughness R_a for each PDMS sample was in the range 52.3 ± 8.8 nm to 72 ± 8.2 nm; total surface roughness S_a was in the range 72 nm to 99 nm. The surface roughness measurements made using AFM showed R_a to be in the range 24.7 ± 1.4 nm to 29.9 ± 9.7 nm and S_a in the range 28 nm to 29 nm. Values obtained using SEM stereoscopic imaging were always larger than those obtained using AFM.

3.2 Articular cartilage

The surface roughness measurements for each articular cartilage sample using SEM stereoscopic imaging and AFM are shown in Table 2. Figure 3 shows images of a cartilage surface from SEM stereoscopic imaging and AFM. It was observed that for samples 2, 3 and 4 the surface roughness, R_a was found to linearly increase with increasing magnification, as

shown in Figure 4; all linear regressions were found to be significant ($p < 0.05$). The surface roughness for sample 1 increased between magnifications of 500 × and 800 ×, but then decreased (Figure 4). There was no significant linear regression found for this sample as $p > 0.05$. Surface roughness, S_a, was also found to follow the same trend as R_a.

Excluding sample 1, the lowest values of surface roughness were found at a magnification of 500 ×. At this magnification the mean surface roughness R_a for each cartilage sample was in the range 165.4 ± 5.2 nm to 174 ± 39.3 nm; total surface roughness S_a was in the range 183 nm to 261 nm. The surface roughness measurements made using AFM showed R_a in the range 82.6 ± 4.6 nm to 114.4 ± 44.9 nm and S_a in the range 86 nm to 136 nm. Values obtained using SEM stereo imaging were always larger than those obtained using AFM (Figure 5).

4. Discussion

Surface roughness values obtained using SEM stereoscopic imaging for the PDMS specimens were found to increase with increasing magnification. This shows that in order to compare the surface roughness of specimens using this technique, it is vital that details of the magnification used are included so that suitable comparisons can be made.

The authors are unaware that any other study has been undertaken to investigate the relationship between magnification and surface roughness using scanning electron microscopy stereoscopic imaging. As magnification increases, the images show finer and finer features of a surface. This may then show deeper troughs in the surface, which leads to a higher surface roughness. At lower magnifications the deeper troughs will not be visible.

For the PDMS samples, which has been molded from a standard roughness sample, the AFM results gave roughness values in the range 24.7 to 29.9 nm. This manufacturer gave the surface roughness as 35.48 nm. The difference may be due to small deformations of the

PDMS using AFM. At the lowest magnification of 500 × the surface roughness values were in the range 52.3 nm to 72 nm, showing roughness values higher than those made with AFM. These experiments just used a single tip diameter of 10 nm for the AFM, but variations in roughness values with AFM will occur if different tip sizes are used. Sedin et al. (2001) showed that increasing the tip size caused a decrease in surface roughness for an image size of 500 nm, but an increase in surface roughness for a image size of 5000 nm.

Surface roughness values for articular cartilage obtained using SEM stereoscopic imaging were also found to increase with increasing magnification, except for sample 1, where it is possible that errors occurred in the image reconstruction. The reconstruction errors come from matching the SEM images together to form a single three-dimensional image; a peak and valley in the surface may get reconstructed the wrong way round. The technique uses Baysien statistics and the explanation of these errors is beyond the scope of this paper, but the reader is directed to Belhumeur (1996). For stereoscopic images the surface roughness values, R_a, were found to vary between 165.4 nm to 174 nm at a magnification of 500 × and 313.7 nm to 418.1 nm at a magnification of 2000 ×. A previous study that used SEM stereoscopic imaging at a magnification of 100 × found features on the cartilage surface to have a mean height of 7700 nm (Bloebaum and Radley, 1995). This mean value is much higher than the measurements found in the current study and are likely to be caused by contamination seen on the cartilage surface in the SEM images, which Bloebaum and Radley (1995) acknowledge. In our study we used sodium azide to prevent the problem of contamination.

The AFM surface roughness values obtained in this study were in the range 82.6 nm to 114.4 nm. A previous study that used AFM has shown bovine surface roughness values to be 72 ± 23 nm and 65 ± 24 nm for fresh and frozen samples, respectively (Moa-Anderson et al., 2003).

Values of articular cartilage surface roughness have also been determined previously using other techniques. Forster and Fisher (1999) found cartilage to have a surface roughness of around 800 nm, when measured using laser profilometry and 1600 nm when using stylus profilometry. Other stylus profilometry measurements have shown have found human articular cartilage to have surface roughness values of 2700 nm (Jones and Walker, 1968) and up to 7000 nm (Sayles et al., 1979). Bovine articular cartilage has been found with a roughness of around 8000 nm measured with Optical Coherence Tomography (Saarakkala et al., 2009) and between 6800 nm and 12,300 nm using ultrasound (Saarakkala et al., 2004).

The fixing of specimens with glutaraldehyde is an established technique (e.g. Kääb *et al.*, 1999) that prevents further degradation of tissue *post mortem*. The technique will increase the stiffness of specimens (Elber *et al.*, 2011), however, the authors are unaware that it causes any changes to surface roughness. Fixing and dehydration of the cartilage is required for conventional scanning electron microscopy. Unfixed specimens can be imaged using cryo-electron microscopy, but this can lead to cracks in the specimens. Unpublished work by the authors has compared the surface of articular cartilage using scanning electron microscopy (fixed specimens) and cryo-electron microscopy (unfixed specimens) and the surface morphology looks similar.

SEM stereoscopic imaging has been shown to be a suitable technique for measuring the surface roughness of articular cartilage. While AFM is also suitable, the time required for accurate scanning of surfaces with features such as those displayed by the cartilage make it a slow process; imaging a 25 µm × 25 µm area takes approximately 12 hours for a pixel density of 1024 x 1024.

The results show that the measurement of surface roughness of articular cartilage can be technique specific and that magnification is an important consideration for SEM stereoscopic

imaging. The compressive modulus and thickness of articular cartilage have been found to vary between individuals and joints (Shepherd and Seedhom 1999a,b). Articular cartilage surface roughness is also likely to vary between individuals and joints. This study shows that where comparisons are being made between cartilage samples, it is important that the same technique is used so that comparisons are valid.

5. Conclusions

The following conclusions can be drawn from the results:

1) Using SEM stereoscopic imaging, the measured surface roughness of PDMS and articular cartilage increases with increasing magnification.

2) The surface roughness, R_a, of bovine articular cartilage was in the range 165.4 ± 5.2 nm to 174 ± 39.3 nm, when measured using SEM stereoscopic imaging.

3) The surface roughness, R_a, of bovine articular cartilage was in the range 82.6 ± 4.6 nm to 114.4 ± 44.9 nm, when measured using AFM.

4) When making comparisons between the surface roughness of articular cartilage it is important that the same technique is used.

Acknowledgements

The authors would like to thank Mr Paul Stanley and Mrs Theresa Morris for technical assistance with the Scanning Electron Microscopy and Mr Hossein Ostadi for help with the MeX Alicona Software. Daniel Espino is supported by a Marie Curie Intra-European Fellowship within the 7th European Community Framework Programme (Programme number: *FP7/2007-2013;* under *grant agreement* n°*252278*). We thank the Spitzer Family Foundation for financial support for the MeX Alicona Software. The atomic force microscope (JPK Instruments AG, Berlin, Germany) used in this research was obtained, through Birmingham Science City: Innovative Uses for Advanced Materials in the Modern World (West Midlands Centre for Advanced Materials Project 2), with support from Advantage West Midlands (AWM) and part funded by the European Regional Development Fund (ERDF).

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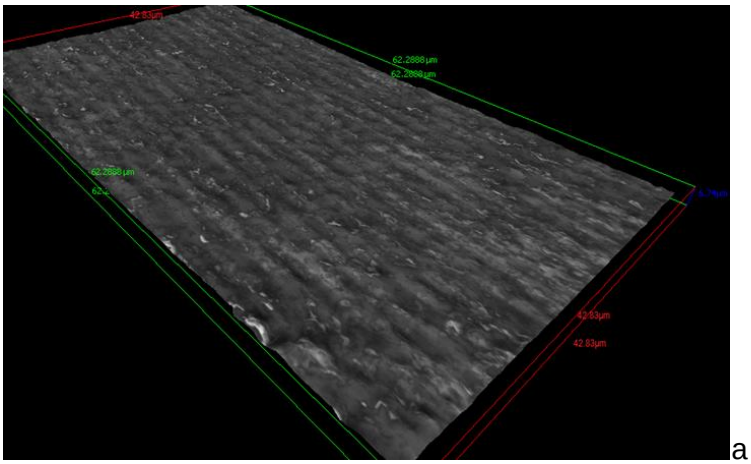
Table 1. Surface roughness values for the six PDMS samples measured using SEM stereo imaging (at magnifications of 500 ×, 800 ×, 1200 × and 2000 ×) and AFM.

Sample	SEM or AFM	Mean surface roughness, R_a (nm)	Standard Deviation (nm)	Total surface roughness, S_a (nm)
1	SEM 500×	64.0	11.5	87
	SEM 800×	134.8	6.9	183
	SEM 1200×	278.0	42.8	278
	SEM 2000×	563.8	94.8	1126
	AFM	25.7	0.8	29
2	SEM 500×	52.3	8.8	72
	SEM 800×	101.3	18.2	135
	SEM 1200×	222.0	40.4	283
	SEM 2000×	490.8	29.1	837
	AFM	24.7	1.4	29
3	SEM 500×	67.0	10.7	91
	SEM 800×	143.0	23.2	181
	SEM 1200×	301.5	49.0	292
	SEM 2000×	648.0	89.5	962
	AFM	25.3	0.6	29
4	SEM 500×	69.0	12.9	99
	SEM 800×	144.5	24.6	185
	SEM 1200×	280.3	53.8	351
	SEM 2000×	648.5	116.9	829
	AFM	29.9	9.7	28
5	SEM 500×	63.5	13.6	84
	SEM 800×	148.5	29.6	185
	SEM 1200×	276.3	14.2	298
	SEM 2000×	699.8	136.4	862
	AFM	25.9	1.0	29
6	SEM 500×	72.0	8.2	87
	SEM 800×	151.3	16.0	191
	SEM 1200×	278.5	33.3	281
	SEM 2000×	611.5	127.8	819
	AFM	25.0	0.5	30

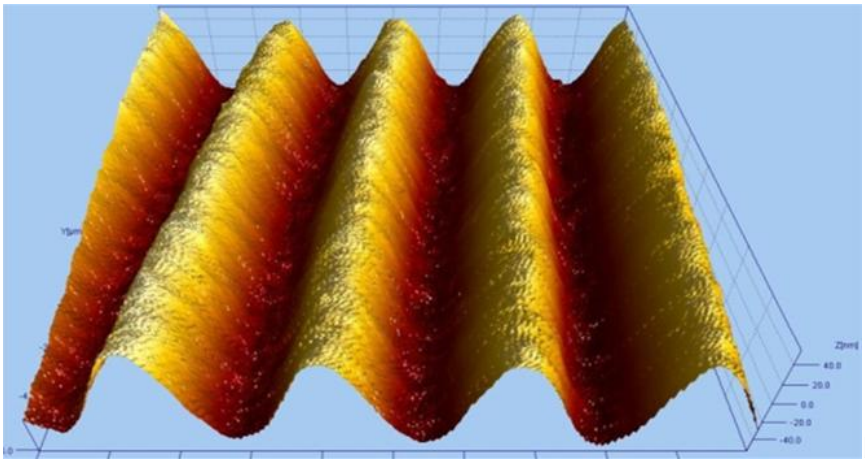
Table 2. Surface roughness values for the four articular cartilage samples measured using SEM stereo imaging (at magnifications of 500 ×, 800 ×, 1200 × and 2000 ×) and AFM.

Sample	SEM or AFM	Mean surface roughness, R_a (nm)	Standard Deviation (nm)	Total surface roughness, S_a (nm)
1	SEM 500×	172.5	5.9	195
	SEM 800×	190.0	3.9	198
	SEM 1200×	122.6	10.2	147
	SEM 2000×	71.3	10.2	86
	AFM	77.9	10.9	90
2	SEM 500×	213.6	52.7	231
	SEM 800×	232.3	51.8	207
	SEM 1200×	283.1	8.9	296
	SEM 2000×	313.7	29.7	316
	AFM	114.4	44.9	136
3	SEM 500×	174.0	39.3	261
	SEM 800×	192.0	39.8	232
	SEM 1200×	216.2	37.4	283
	SEM 2000×	418.1	31.6	398
	AFM	84.1	20.9	86
4	SEM 500×	165.4	5.2	183
	SEM 800×	186.3	5.9	199
	SEM 1200×	216.8	16.2	258
	SEM 2000×	317.8	10.1	319
	AFM	82.6	4.6	89

Figure

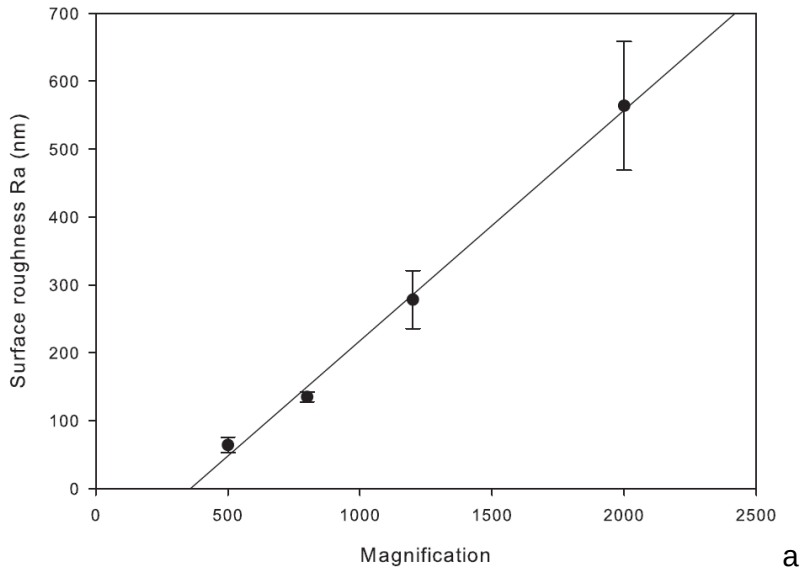


a



b

Figure 1. Images of the PDMS surface roughness. a) SEM stereoscopic image at a magnification of 2000×.; b) AFM image.



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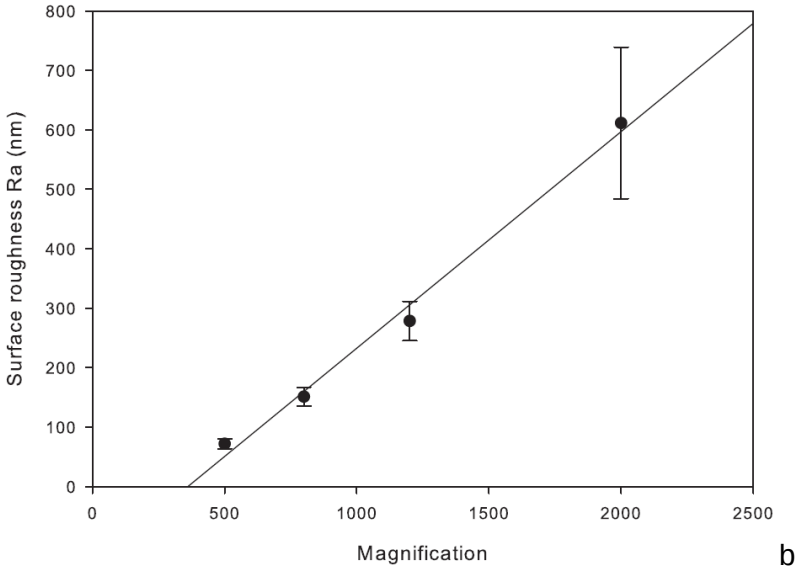
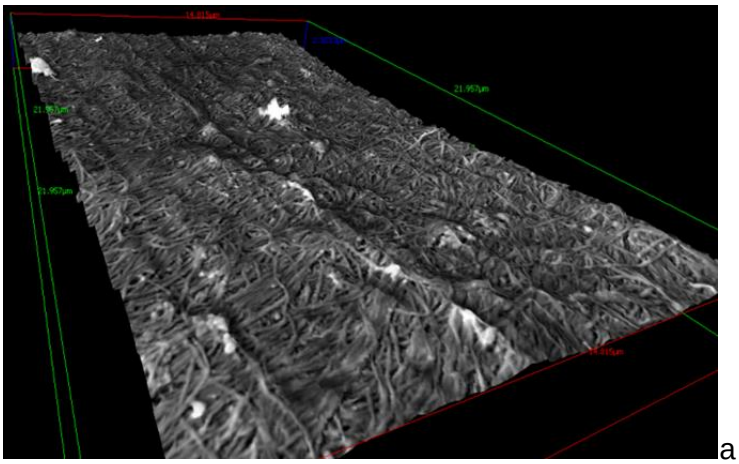


b

Figure 2. Mean surface roughness, R_a, plotted against magnification for PDMS. Line indicates a linear correlation. Error bars represent the standard deviations. a) Sample 1, y = 0.34x-121.8; R^2 = 0.996; p = 0.002; b) Sample 6 y = 0.36x-131.6; R^2 = 0.991; p = 0.004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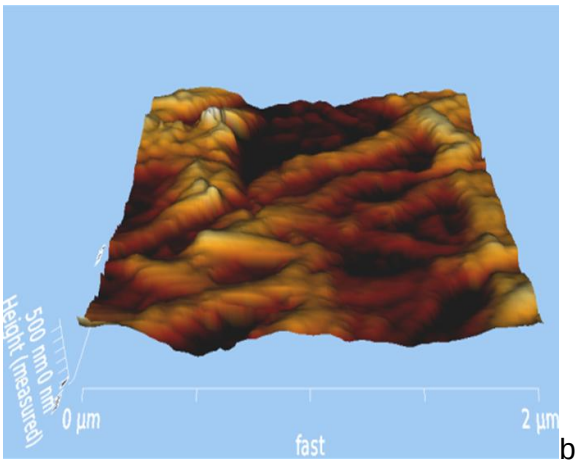
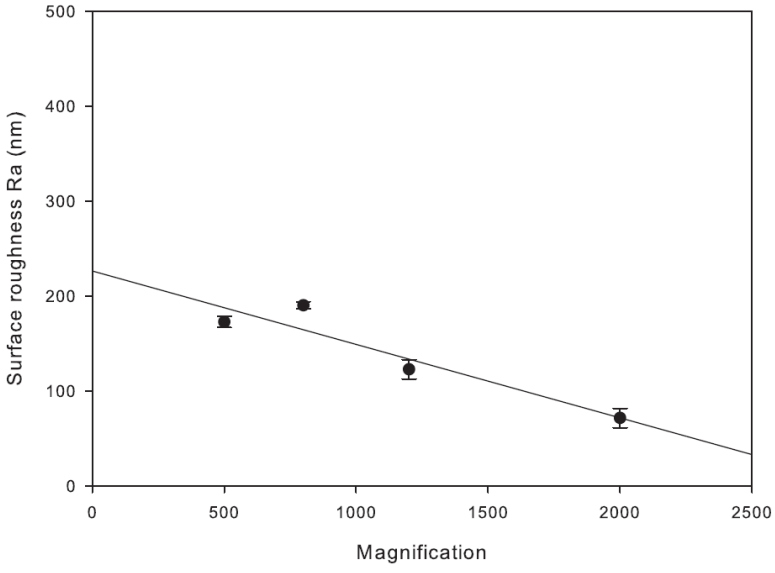
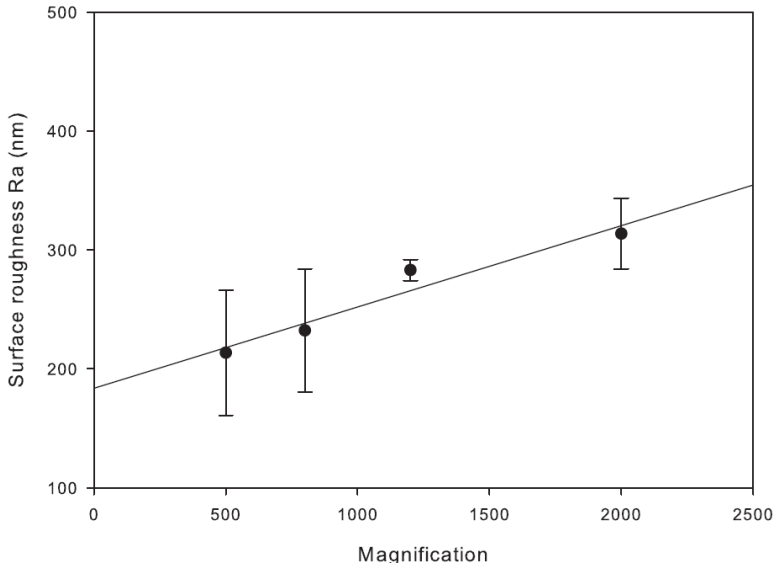


b

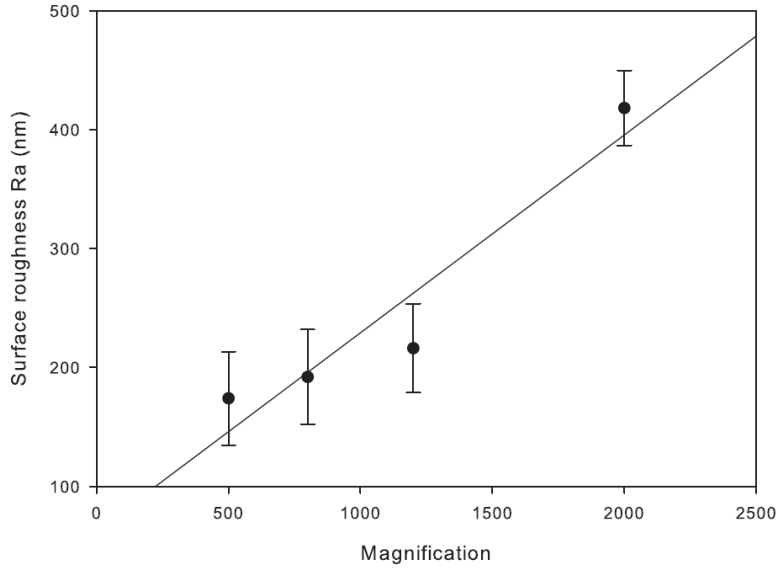
Figure 3. Images of the articular cartilage surface roughness of sample 3. a) SEM stereoscopic image of sample 3 at a magnification of 2000×.; b) AFM image.


Surface roughness Ra (nm)
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Magnification

a


Surface roughness Ra (nm)
500
400
300
200
100
0
500
1000
1500
2000
2500
Magnification

b


Surface roughness Ra (nm)
500
400
300
200
100
0
500
1000
1500
2000
2500
Magnification

c

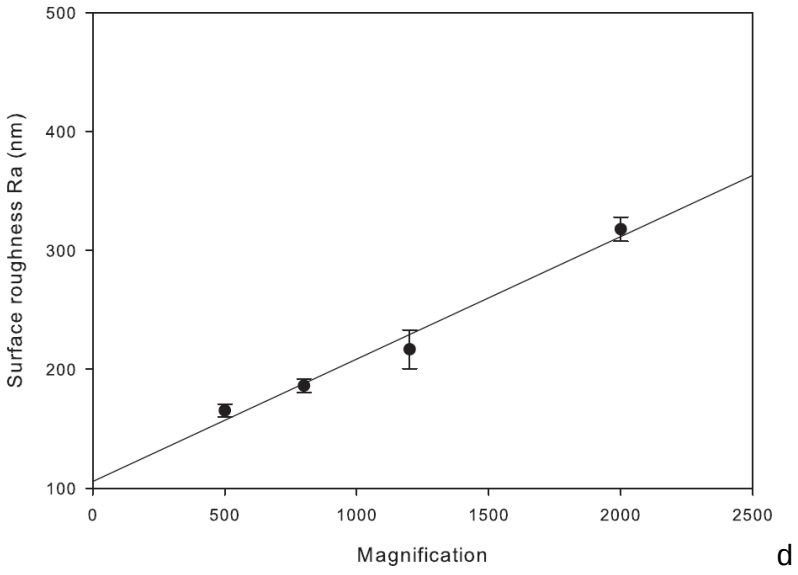


Figure 4. Mean surface roughness, R_a, plotted against magnification for articular cartilage. Line indicates a linear correlation. Error bars represent the standard deviations. a) Sample 1, y = -0.077x+226.1; R^2 = 0.883; p = 0.06; b) Sample 2 y = 0.068x+183.6; R^2 = 0.937; p = 0.032; c) Sample 3 y = 0.166x+62.8; R^2 = 0.91; p = 0.046; d) Sample 4 y = 0.102x+105.7; R^2 = 0.98; p = 0.01.

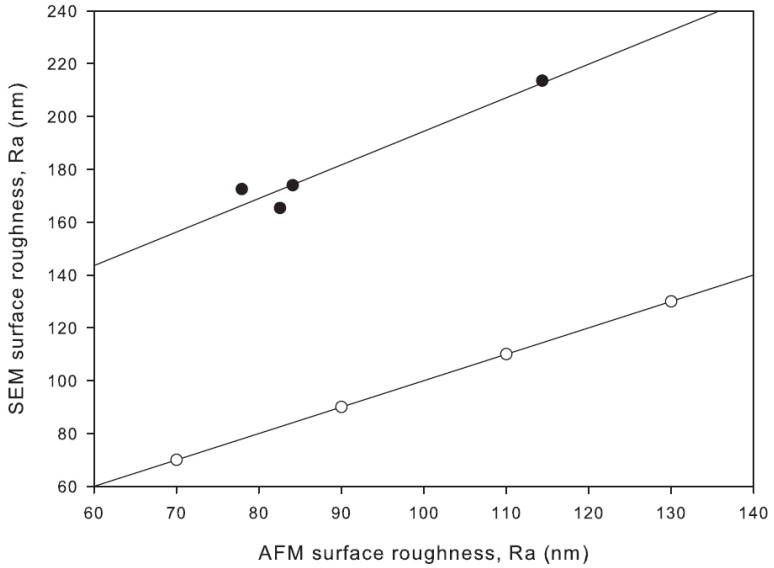


Figure 5. Articular cartilage mean SEM surface roughness plotted against mean AFM surface roughness. The magnification of the SEM was 500 ×. Upper line indicates a linear correlation $y = 1.27x+67.3$; $R^2 = 0.94$; $p = 0.03$. Lower line is $y = x$. Error bars have been removed for clarity.