Relation between surface properties of thin composite films and osteoblast behaviour *in vitro*

2007 J. Phys.: Condens. Matter 19 285216

(http://iopscience.iop.org/0953-8984/19/28/285216)

IOP PUBLISHING JOURNAL OF PHYSICS: CONDENSED MATTER

J. Phys.: Condens. Matter **19** (2007) 285216 (10pp) doi:10.1088/0953-8984/19/28/285216

Relation between surface properties of thin composite films and osteoblast behaviour *in vitro*

B Polak[1], R Olkowski[2], T Kobiela[3,4], M Lewandowska-Szumieł[2] and W Fabianowski[1]

[1] Department of Chemistry, Warsaw University of Technology, Noakowskiego 3, 00 664, Warsaw, Poland
[2] Department of Biophysics and Human Physiology, Medical University of Warsaw, Chałubińskiego 5, 02 004 Warsaw, Poland
[3] Institute of Physics, Polish Academy of Sciences, Aleja Lotników 32/46, 02-668 Warsaw, Poland
[4] Institute of Physical and Theoretical Chemistry, University of Bonn, Wegelerstrasse 12, D-53115 Bonn, Germany

Received 30 September 2006, in final form 13 March 2007
Published 25 June 2007
Online at stacks.iop.org/JPhysCM/19/285216

Abstract

Si supports for cell culture were modified using poly(acrylic acid) (PAA) and bentonite in order to obtain 'sandwich'-like structures. A layer of PAA cast from water solution was followed with a bentonite layer also cast from water dispersion, then another PAA layer and so on up to six layers. The prepared surfaces had different physical and chemical properties like thickness, topography and elasticity. Chemical composition was characterized by Raman spectroscopy. The elastic properties and topography of modified sandwich-like surfaces were evaluated using nanoindentation and atomic force microscopy measurements. In the next step bone cells were cultured on such modified surfaces composed of one to six layers. The influence of the substrate surface properties on the growth and behaviour of human bone derived cells (HBDC) was studied. The influence of surface topography, elasticity and chemical composition on cells is discussed.

1. Introduction

The aim of bone tissue engineering is to create a 3D scaffold in which bone cells can adhere, grow and proliferate in order to regenerate damaged tissue. It is commonly known that there are many important aspects of the surface properties of biomaterials that can influence cell growth and behaviour [1–5]. In particular, surface modifications of biomaterials may improve cell adhesion, growth and differentiation. This study was undertaken in order to establish a correlation between chosen surface properties and cell response. Several hybrid thin films with different surface modifications made from poly(acrylic acid) (PAA; layer A) and bentonite (magnesium silicate; layer B) were prepared. The concept is based on the ability of these

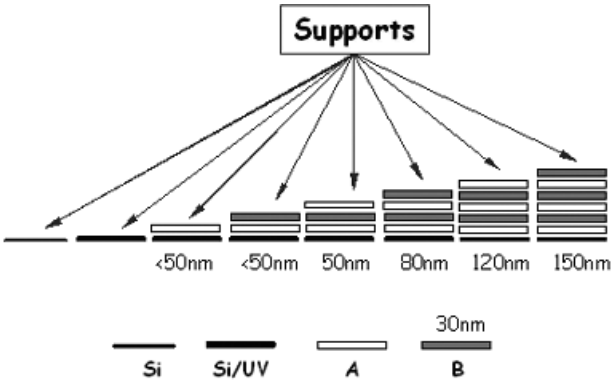

Figure 1. Scheme of prepared alternating modifications made from poly(acrylic acid) (A), and bentonite (B).

materials, which differ in both in elasticity and microtopography, to offer surface support for cells. It is expected that the increasing number of layers should result in increasing elasticity of the investigated cell support. PAA layers are used for this purpose, as they are well tolerated by cells [6]. PAA has been particularly used for grafting to substrates to enhance their wettability [7] or for introducing carboxyl groups onto the surface [8]. Bentonite (containing 90% of montmorillonite) is a bioinert material [9, 10] that strongly influences the surface topography. It was confirmed in preliminary experiments that it does not cause any adverse effect in cell culture *in vitro*. Surface physical and chemical properties as well as the behaviour of cells in direct contact with surfaces were investigated as a continuation of a previously reported preliminary study [11]. In particular the effect of wettability, chemical composition, topography and mechanical properties on the behaviour of osteoblasts *in vitro* were considered.

2. Experimental details

2.1. Sample preparation

The first set of round silicon supports of diameter 6.1 mm etched from Si wafer was placed into 96-well standard tissue culture polystyrene dishes (TCPS). In the next step thin films were cast in order to obtain 'sandwich'-like structures on the Si supports. The following structures were prepared:

#1—Si round support, uncoated (control),
#2—as above, additionally with UV cleaning (365 nm, 10 min),
#3—as #2 + poly(acrylic acid)—50 μl of 0.05% poly(acrylic acid) water solution—layer A,
#4—as #3 + bentonite—50 μl of bentonite, 0.05% water dispersion—layer AB,
#5—as #4 + poly(acrylic acid)—layer ABA,
#6—as #5 + bentonite—layer ABAB,
#7—as #6 + poly(acrylic acid)—layer ABABA,
#8—as #7 + bentonite—layer ABABAB.

The whole experiment was repeated three times and the most representative results were selected for this paper. In each experiment for biological testing all modifications were repeated seven times. The scheme of sample preparation is given in figure 1. The thickness of deposited layers was measured using a Tencor Alphastep 200 Profilometer. We also prepared a second set

rectangular silicon supports (1 cm × 3 cm) for contact angle (CA) measurements and silicon squares (1 cm × 1 cm) for AFM, nanoindentation and Raman spectroscopy measurements. All samples were prepared under the same conditions and dried at 37 °C for 24 h. Samples were divided into two groups. The first set (round Si supports modified with alternate AB layers placed into TCPS) was used for osteoblast culture. The second set (rectangular and square Si supports also modified with alternate AB layers as depicted at figure 1) was used for chemical and physical surface characterizations. Both prepared samples for cell culture as well as samples for surface chemical and physical characterization were sterilized (dose 25 kGy, Institute of Nuclear Chemistry and Technology, Warsaw, Poland).

2.2. Cell culture and viability

After sterilization by irradiation (25 kGy), specimens were seeded with human bone derived cells (HBDCs). The cells were harvested from pieces of tissue removed at surgery, which would otherwise have been discarded. The procedure for isolation of HDBCs was based on the protocols described by Gallagher *et al* [12] with modifications [13]. A suspension of HBDCs in culture medium (DMEM, Dulbecco's modified Eagle's medium, supplied with 100 mM L-ascorbic acid 2-phosphate) was seeded at a density of 15 000 cells per specimen (50 000 cells cm^{-2}). Cell culture was set up under standard conditions (37 °C, 5% CO_2, humidity 90%). After 7 days of culture morphological observations of cells as well as XTT tests were performed. The XTT test is used in toxicology and is based on the capacity of mitochondrial dehydrogenase enzymes present in living cells to convert the XTT substrate (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyloamino) carboxyl]-2H-tetrazolium hydroxide) into a water-soluble formazan product [14]. The final product of the reaction was measured by an ELISA reader at 450 nm. The results of the XTT assay are regarded as being proportional to the number of living cells [15–17], although they strictly reflect the mitochondrial activity of cells. The non-parametric ANOVA (Kruskal–Wallis, followed by the Dunn test) was used to identify differences between cell viability on the various supports.

2.3. Wettability measurements

For the second set of experiments we have measured wettability with DMEM solution that is a main component of the culture medium. Three samples of each modification were prepared for wettability measurements. Both water and DMEM were used as wettability liquids in our work. Contact angle (CA) measurements were performed before and after sterilization by irradiation. There were no significant differences in the results of CA measurements obtained before and after sterilization. In this paper we present only those results obtained after sterilization, using both DMEM and deionized water as wettability liquids. However, because of the presence of DMEM in cell culture and its interactions between surface and cell, the DMEM wettability results were more interesting for us. Contact angle measurements were performed using goniometric methods. The most important parts of the goniometer are the manual stage where the sample is placed and the optical system with objective and light source. Several sessile liquid drops of DMEM (at least three) were deposited on samples and CAs were measured on both sides of the sessile drop.

2.4. Film thickness measurements

Film thickness was measured using a Tencor Alphastep 200 Profilometer (Institute of Electronic Materials Technology, Warsaw, Poland). A profilometer is a non-destructive, easy to use

and highly sensitive tool for measuring surface properties like topography or thickness. The Alphastep 200 is a computerized step profiler with a manual X–Y stage, and a 9 in video monitor which displays a magnified view of the sample and stylus during the scan, as well as scan profiles and a summary of scan data. A diamond-tipped stylus is in direct contact and is scanned across the mechanically scratched surface. The differences in thickness across the sample are measured.

2.5. Atomic force microscopy (AFM) imaging

The structural features of surfaces were obtained in contact and tapping mode in air with the Nanoscope III from Veeco (Santa Barbara, CA). The mean roughness R of the films was calculated as described elsewhere [18].

2.6. Mechanical testing

All mechanical testing was performed at room temperature using a Hysitron TriboIndenter (Hysitron Inc., Minneapolis, MN). This system is a load-controlled device with an effective force resolution of 100 nN and displacement resolution of 1 nm. Reduced modulus (E_r) values were calculated following the method of Oliver and Pharr [19].

2.7. Raman spectroscopy

The chemical composition of prepared modifications was investigated with Raman spectroscopy (Nicolet Almega XR). Raman spectroscopy enables characterization of the molecular structure of samples. A significant advantage of Raman spectroscopy is that it is carried out in ambient conditions (no high vacuum is required) and the presence of water does not interfere with it, which is important for biological samples. Spectra were recorded on Nicolet Almega XR apparatus (laser 532 nm, exposure time 30 s, number of exposures 4, laser power level 100%, spectrograph aperture 50 μm pinhole).

3. Results and discussion

3.1. Thin film characterization

The thickness of the obtained layers was as follows: for A and AB layers the thickness was less than 50 nm; for the ABA layer the thickness was 50 nm and 80, 120 and 150 nm for ABAB, ABABA and ABABAB, respectively. Si support, Si UV-cleaned supports as well as Si support with an A layer gave optically smooth surfaces. Adding a bentonite layer and the next layers (AB, ABA, ABAB, ABABA and ABABAB films) significantly changed the surface topography. Studied modified surfaces resembled smooth films with rough 'rocks' standing as columns, scattering visible light and therefore yielding milky surfaces. This observation was confirmed with the AFM measurements. Figure 2 shows the AFM image and surface profile of an ABABAB layer. The columnar structures with mean diameters of about 5 μm and heights reaching 2.5 μm are randomly distributed over the surface area. Additionally these observations were confirmed with collected surface Raman spectra. We did not observe valence band absorption originating from PAA around 1700 cm^{-1}. Raman spectroscopy confirmed just the presence of bentonite on each of the top layers with PAA 'hidden' below the bentonite. That is why we can assume that the cell response is a response to bentonite. The bands at 1088 cm^{-1} and about 950 cm^{-1} were assigned to Si–O and Al–OH stretchings, respectively. Surface mapping of all examined layers was performed. Figure 3 presents mapping of the

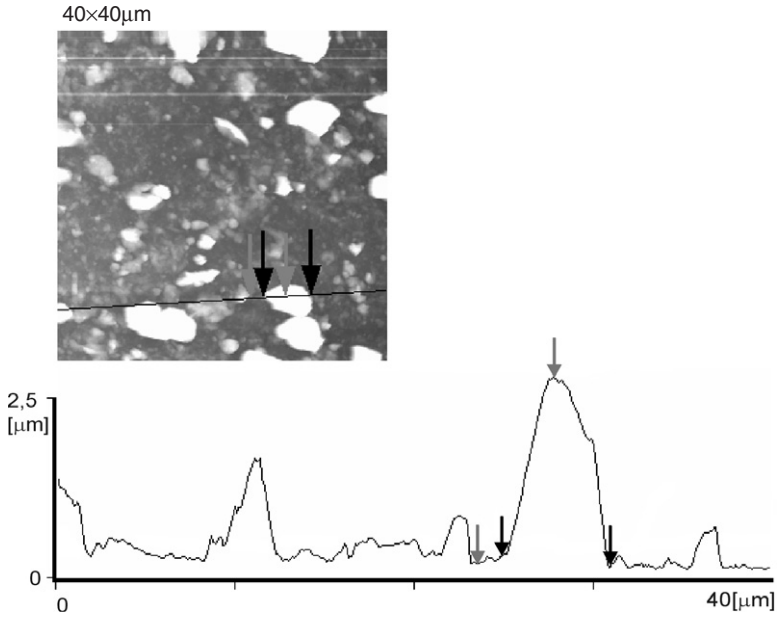

Figure 2. AFM image and surface profile of ABABAB layer after sterilization by irradiation. Grey and black arrows indicate the height and the mean diameter, respectively, of the columnar structure.

ABABAB layer. Figure 4 shows wettability measurements using DMEM and water as liquids. The measurements were performed after sterilization by irradiation (dose 25 kGy). Values of CA depend on both the chemical composition and physical properties of the surface. In our case, after using DMEM as a wettability liquid, the Si control support is hydrophilic. After UV irradiation of the Si wafer the surface became more hydrophilic in comparison with the control one. After Si coating with the first layer A the surface is still hydrophilic, but when we add layer B surface AB becomes more hydrophobic. When we cast the next layer A and the next layer B, surfaces ABA and ABAB are more hydrophobic. This effect can be explained by the presence of bentonite 'rocks' among which PAA is placed during casting from solution. Hydrophilic groups of PAA are hidden below bentonite 'rocks' and they are not 'recognized' by DMEM CA measurements. When 'rocks' were totally covered with PAA the surface again became more hydrophilic (layer ABABA and ABABAB). All obtained surfaces, in the case of DMEM measurements, were slightly hydrophilic with CA values below 60°. The differences between CA results using water and DMEM (with generally higher CA values for DMEM) can be attributed to the surface interactions with DMEM components: inorganic salts, vitamins, amino acids and proteins. During their absorption on the surfaces the hydrophilic character of the supports decreases compared with the water interactions with the same surfaces.

3.2. Cell culture

Human bone derived cells were cultured under standard conditions on studied surfaces. The viability of cells was determined using the XTT test. After UV irradiation of the Si wafer the surface became more hydrophilic than the Si control but at the same time the viability of the cells decreased. On the contrary, in the case of Si modification with a single layer A cell viability was slightly enhanced, although this modification was accompanied by unchanging

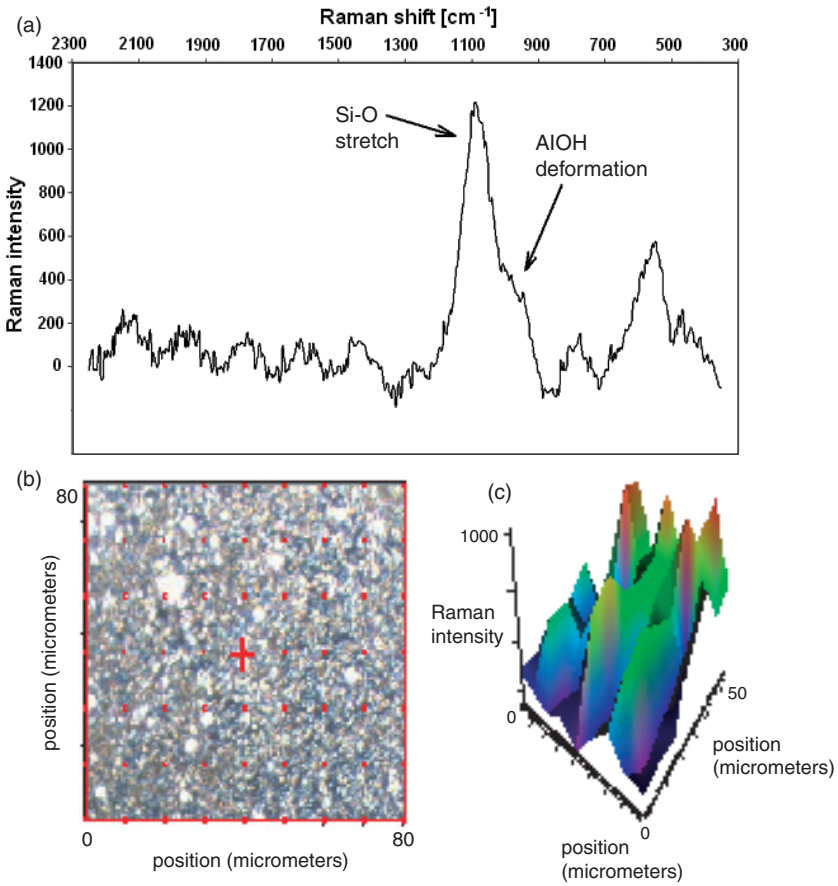

Figure 3. Raman spectroscopy of the ABABA layer and surface mapping (after sterilization by irradiation): (a) Raman spectra of Si–O stretch vibration region at 1100 cm^{-1}. (b) Optical microscope picture (200×) of mapped surface. (c) 3D map of Si–O stretching bond.

(This figure is in colour only in the electronic version)

values of CA. Obtained data for A, AB and ABA layers are scattered over a wide range and result of the XTT test are comparable with the control culture. However, a distinct difference can be seen in the case of ABA, ABAB, ABABA and ABABAB layers. During gradual deposition of layers A and B up to the ABA layerthe viability of cells is at the same level, and for the next layers—ABAB, ABABA and ABABAB—viability decreases. At the same time the CA measurements for ABA and ABAB layers are almost equal. After performing the viability test (XTT) we can state that the best results were achieved for the first five 'sandwich'-like structures—Si, Si/UV, A, AB and ABA layers. For the next layers—ABAB, ABABA and ABABAB—XTT test results decreased significantly (figure 5). The viability of cells, as measured by XTT assay is dependent on the number of cells and their ability to attach and spread on the support. Since HBDC remain alive only when they are able to anchor to a support, the results of the XTT assay confirm the cells' ability to attach and spread on the examined surfaces. On the other hand, the final number of cells is dependent not only on the initial number of cells used for the experiment but also on cell proliferation. It seems that low XTT results on ABAB, ABABA and ABABAB layers are probably achieved not as a

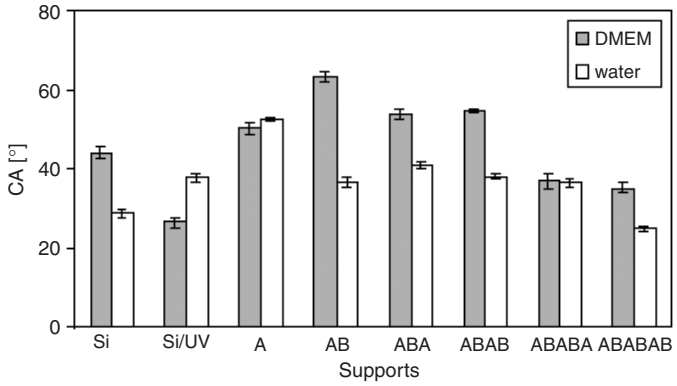

Figure 4. Contact angle measurements of examined alternating layers using DMEM and water as wettability liquids (after sterilization by irradiation).

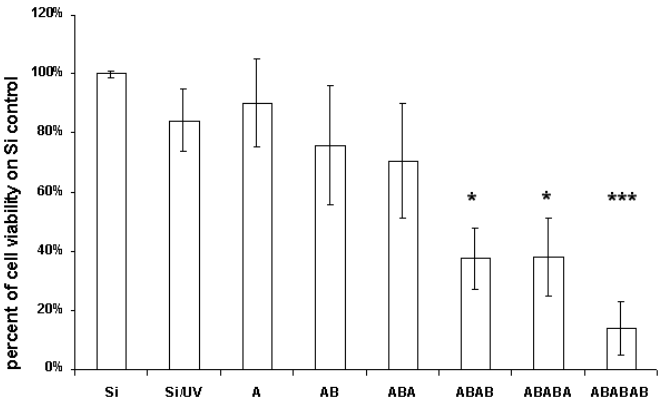

Figure 5. Viability of cells on prepared alternating layers. Results are mean ±SD. Asterisks denote means that are significantly different from control (Si) at $*$, $p < 0.05$ and $***$, $p < 0.01$.

consequence of a cytotoxic effect but because of the diminished ability of HBDCs to attach to these supports and spread on them. This phenomenon seems to be dependent on the surface characteristics and both the elasticity of the support and its topography have an influence.

Measurements of surface CA suggest that it is not wettability that has a crucial effect on cell response in our study. That is why we decided to examine in more detail the surface topography, elasticity and chemical composition.

3.3. AFM examination and mechanical testing

The AFM images in figure 6 demonstrate the surface topography of Si, ABA and ABABAB layers. We have observed that surface roughness increases with gradual deposition of layers. Comparison of the statistical parameters for the surface roughness deduced from AFM is given in table 1. Mean roughness measurements were performed for 1 μm × 1 μm, 5 μm × 5 μm, 10 μm × 10 μm and 20 μm × 20 μm regions. However, the most representative results are from 20 μm × 20 μm regions. Discher *et al* [1, 20] have recently pointed out the importance of surface elasticity for cell behaviour. Therefore we expected that this surface parameter could also play a role in our experiment. That is why mechanical testing of selected layers

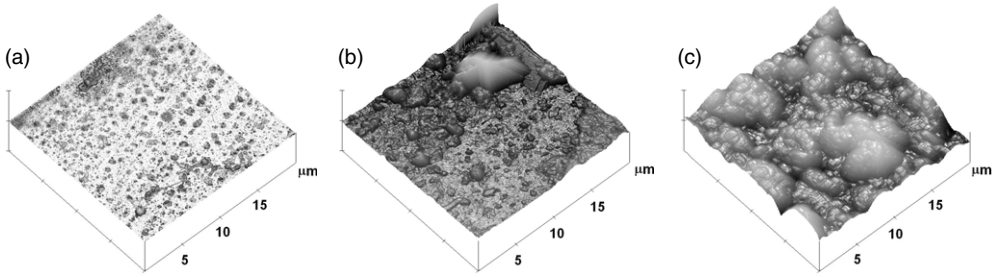

Figure 6. AFM 3D images of: (a) Si support, (b) ABA layer and (c) ABABAB layer.

Table 1. Mean roughness R (nm) deduced from AFM measurements for the following selected areas: 1 μm × 1 μm, 5 μm × 5 μm, 10 μm × 10 μm and 20 μm × 20 μm.

Surfaces	R (nm) 1 μm × 1 μm	R (nm) 5 μm × 5 μm	R (nm) 10 μm × 10 μm	R (nm) 20 μm × 20 μm
Si	37	118	98	411
Si/UV	17	95	183	782
A	37	39	127	188
AB	149	505	783	1400
ABA	79	354	840	2800
ABAB	217	1300	2100	3100
ABABA	336	985	1200	3160
ABABAB	130	1050	1900	3200

Table 2. Results of nanoindentation measurements: E_r, reduced elastic modulus; H, hardness.

Surface	E_r (GPa)	H (GPa)
Si	173.91 ± 0.98	11.73 ± 0.78
Si/ABABAB	118.84 ± 13.93	1.68 ± 0.58

was also performed. Results of reduced modulus (E_r) and hardness (H) measurements for ABABAB and Si layers are collected in table 2. Figure 7 depicts nanoindentation traces on Si (see figure 7(a)) and ABABAB (see figure 7(b)) layers using a Berkovich pyramid tip. All measurements were repeated several times. Increasing the number of layers is accompanied by an increase in elasticity causing a decrease of cell viability. The results obtained in our investigations are in agreement with the previous studies of other authors [1, 20]. Column-like 'rocks' standing in a 'sea' of the PAA structure of hybrid films was also confirmed by the nanoindentation measurements. Hardness results for region 1 on an ABABAB surface (figure 7(b)) were comparable with control silicon ones.

4. Conclusions

We have observed that cell viability varied depending on the surface characteristics. The number of deposited layers has an influence on cell viability. Cell viability was significantly diminished for layers from ABAB to ABABAB. For gradual deposition of A and B layers to obtain A, AB, ABA, ABAB, ABABA and ABABAB surfaces, film stiffness and roughness varied. The mechanical properties and surface roughness of the film have a strong influence

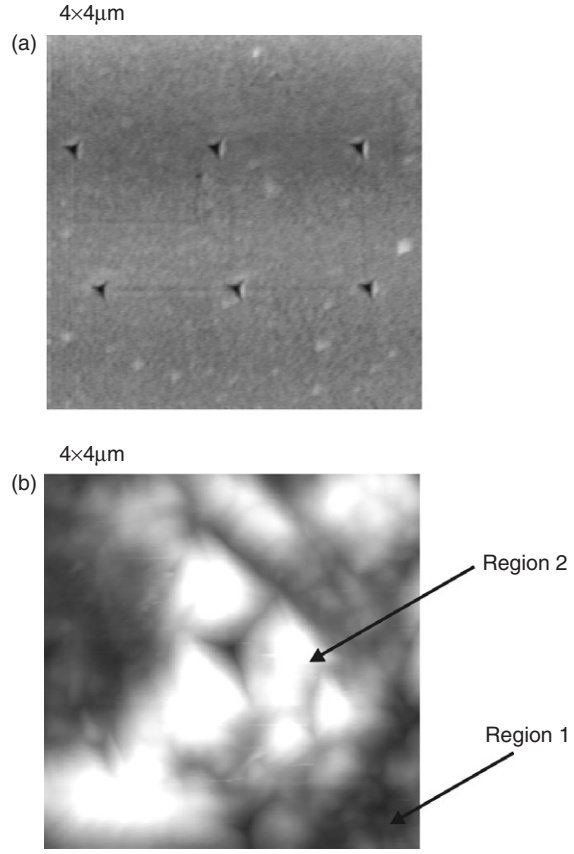

Figure 7. AFM images of surfaces where the mechanical tests were performed. Nanoindentation traces of a Berkovich tip on a Si support (a) and an ABABAB layer (b) are visible.

on osteoblast behaviour, while surface wettability does not play a crucial role for cell viability under the specific condition of this experiment.

Acknowledgments

We would like to thank Mr Lance Kuhn from Hysitron for carrying out the nanoindentation measurements. Research was supported by the State Committee of Scientific Research (grant KBN 4T08E01824).

References

[1] Discher D, Engler A J, Richert L, Wong J Y and Picart C 2004 *Surf. Sci.* **570** 142
[2] Anselme K 2000 *Biomaterials* **21** 667
[3] Dalby M J and Giannaras D 2004 *Biomaterials* **25** 77
[4] LeBaron R G and Athanasiou K A 2000 *Biomaterials* **21** 2575
[5] Brunstedt M R and Ziats N P 1993 *J. Biomed. Res.* **27** 483
[6] Lee J H, Jung H W, Kang I-K and Lee H B 1992 *Biomaterials* **15** 705–11
[7] Daw R, Candan S, Beck A J, Devlin A J, Brook I M, MacNeil S, Dawson R A and Short R D 1998 *Biomaterials* **19** 1717–25

[8] Li B, Ma Y, Wang S and Moran P M 2005 *Biomaterials* **26** 4956–63
[9] Kawase M, Hayashi Y, Kinoshita F, Yamato E, Miyazaki J-I, Yamakawa J, Ishida T, Tamura M and Yagi K 2004 *Biol. Pharm. Bull.* **27** 2049–51
[10] Zhuang H, Zheng J P, Gao H and De Yao K 2007 *J. Mater. Sci. Mater. Med.* (Jan)
[11] Polak B, Fabianowski W and Lewandowska-Szumieł M 2004 *Eng. Biomater.* **38–43** 106–8
[12] Gallagher J A, Gundle R and Beresford J N 1996 *Methods in Molecular Medicine: Human Cell Culture Protocols* ed G E Jones (Totowa, NJ: Humana Press) pp 233–62
[13] Kudelska-Mazur D, Lewandowska-Szumiel M, Benke G, Kowalski M and Komender J 2001 *Eng. Biomater.* **15/16** 51–8
[14] Scudiero D A, Shoemaker R H, Paull K D, Monks A, Tierney S, Nofziger T H, Currens M J, Seniff D and Boyd M R 1988 *Cancer Res.* **48** 4827–33
[15] Breault L G, Schuster G S, Billman M A, Hanson B S III, Kudryk V L, Pashley D H, Runner R R and McPherson J C III 1995 *J. Periodontol.* **66** 545–51
[16] Newell D G (ed) 1996 Core techniques *Cell & Tissue Culture: Laboratory Procedures* (New York: Wiley) pp 4B: 6.1–6.2
[17] Sadler G P, Jones D L, Woodhead J S, Horgan K and Wheeler M H 1996 *World J. Surg.* **20** 822–8 (discussion 828–9)
[18] Schneider A, Francius G and Obeid R 2006 *Langmuir* **22** 1193–200
[19] Oliver W C and Pharr G M 1992 *J. Mater. Res.* **7** 1564–83
[20] Engler A, Sheehan M, Sweeney H L and Discher D E 2003 *Eur. Cells Mater.* **6** 7