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Topographical and chemical effects of electrochemically assisted deposited hydroxyapatite coatings on osteoblast-like cells.

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ABSTRACT: A recently commercialised hydroxyapatite electrochemically-assisted chemical deposition technique (BoneMaster) has been shown to induce increased bone apposition; whether this response is caused by the surface topography or chemistry is unknown. An *in-vitro* examination using human osteoblast-like cells was performed on a series of BoneMaster coated surfaces. The chemistry was separated from the topography using a thin gold coating; Thermanox slides were used as a control. BoneMaster surfaces showed significantly greater alkaline phosphatase activity and osteocalcin production compared with controls; however, no difference was found between the gold-coated and uncoated BoneMaster samples, indicating topography is the main contributing factor.

KEY WORDS: fixation, hydroxyapatite, bone, topography, chemistry, surface

INTRODUCTION

The first hydroxyapatite coating was applied to a primary hip by Furlong and was implanted in 1985 [1]. The purpose of the coating was to mimic the chemistry of bone and thus increase bony fixation at the implant interface; the success of these coatings has resulted in them being widely used throughout the orthopaedic industry. However, aseptic loosening is still a major cause of failure of orthopaedic components [2] which needs to be addressed.

One of the possible issues with any coating is delamination, and there have been reports of hydroxyapatite coatings separating from the underlying metal [3, 4]. Hydroxyapatite coatings are normally plasma sprayed and have a thickness between 70 μm and 170 μm [3]; excessive coating thickness can cause residual stresses to build up within the coating and this can result in delamination [5, 6]. For this reason, plasma-sprayed hydroxyapatite coatings are regularly tested by orthopaedic manufacturers in accordance with ASTM C-633 [7] to minimise the risk of delamination.

Plasma sprayed coatings have a chemical composition which is similar to that of bone, although not exactly the same. The high temperatures used in the coating process can cause extra phases of calcium phosphate to form; these include amorphous calcium phosphate, α - and β - tricalcium phosphate, tetracalcium phosphate and calcium oxide

[8]. These other phases are not present in the mineral phase of natural bone; natural bone consists of hydroxyapatite substituted with small amounts of carbonates, silicon and magnesium.

A novel biomimetic hydroxyapatite (HA) coating, commercially called BoneMaster, has been developed which uses a deposition technique based on an electrochemically assisted precipitation process [9]. The aim of the coating is to closely mimic both the chemistry, and the topography, of natural bone. To create the coating, a current is passed through the implant while it is in a super-saturated solution of ionic calcium phosphate; the current causes a pH increase at the surface of the implant which induces the calcium and phosphate ions to precipitate out onto the surface of the component. The initial phase produced is amorphous calcium phosphate, but with time this crystallizes out into hydroxyapatite needles; mimicking the *in vivo* mineralization process. The coating process is carried out under near-physiological conditions; 37°C and pH 6.4. The resultant coating is only 5 µm thick, therefore reducing the potential for large residual stresses within the coating and the likelihood of delamination.

The biological performance of the electrochemically deposited hydroxyapatite coatings has been investigated previously through *in vivo* studies by Schliephake *et al.* [10]; the study found increased bone-implant contact during the early ingrowth period onto the BoneMaster coated samples. However, the predominant method by which the coating promotes fixation is currently unknown.

The purpose of the current study is to examine whether the osteoinductive properties of the coating can be attributed mainly to the topography or chemistry of the coating surface or whether the combination is crucial. Therefore, three hypotheses will be tested; (1) that the BoneMaster topography causes an osteoinductive response, (2) that the BoneMaster chemistry causes an osteoinductive response, and (3) that the combination of BoneMaster topography and chemistry causes an osteoinductive response in osteoblasts.

MATERIALS AND METHODS

Sample Preparation

Four different surfaces were analysed in this study; BoneMaster-coated titanium alloy (BM), Thermanox coverslips (Thx), gold sputter-coated BoneMaster (BM-Au) and Thermanox (Thx-Au) samples.

The BoneMaster coated samples were prepared by Biomet Deutschland GmbH in the same manner as that performed by Roessler *et al.* [9]. The substrates used were discs of titanium alloy (Ti6Al4V, ASTM 136) of 10 mm diameter and height 2 mm; these were grit blasted prior to BoneMaster coating. The Thermanox coverslips (NUNC) were 13 mm diameter and 0.2 mm thick. Sputter coating of gold onto the BoneMaster and Thermanox samples was performed using a SC7640 Polaron sputter coater at a voltage of 2.2 kV and a plasma current of 17mA in an argon atmosphere at 0.04-0.02 mbar pressure. The coating was applied for 60 seconds with a working distance of 50 mm.

Surface Characterisation

The surfaces were characterised prior to analysis to determine the chemical and topographical nature and to confirm the uniformity of the gold coating. Elemental analysis was performed using X-ray Photoelectron Spectroscopy (XPS). A VG Scientific ESCALAB Mark 1 spectrometer was used with polychromatic AL K α X-rays at an anode potential of 10 kV and a filament emission current of 20 mA. The roughness of the BoneMaster coated samples was quantified using surface profilometry. A PhotoMap 3-D profilometer from Fogale NanoTech was used; four measurements were taken from each sample. Surface and cells were imaged using a Philips XL30 ESM-FEG scanning electron microscope (SEM) in wet mode using an accelerating voltage of 15 kV, a spot size of 3.0 μ m and between 2.3-2.4 Torr operating pressure.

Cell Responses

Human osteosarcoma TE-85 cells from the European Collection of Cell Cultures were cultured to passage 47 in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % v/v foetal calf serum (FCS), 2 % v/v HEPES (1 M), 1 % v/v glutamine (200 mM), 1 % v/v non-essential amino acids (10 mM), 2 % v/v antibiotic/antimycotic and 15 % w/v ascorbic acid. All items were purchased from Invitrogen Ltd. Cells were cultured at 37°C and 5% CO₂. Media was replaced every two days. For the assays, cells were seeded onto the samples at a density of 40,000 cells per cm².

Cell proliferation was measured using an Alamar blue assay, this was performed on the samples after 1, 4, 7 and 14 days of culture (n=3). One millilitre of 10% v/v solution of Alamar Blue (ABD Serotec) concentrate in Hank's Buffered Salt Solution (HBSS) was added to the samples after washing 3 times in PBS. Samples were then incubated for 80 minutes and then shaken at 300 rpm on a Heidolph Titramax 100 plate shaker for 10 minutes. The fluorescence was measured of 100 μ m aliquots of each well using a FLX 800 Microplate Reader; 530 nm excitation was used and emission measured at 590 nm.

In order to examine cell differentiation, alkaline phosphatase and osteocalcin assays were performed and normalised by DNA content. Analysis was performed at predetermined time points. For alkaline phosphatase activity assessment, after 1, 4, 7 and 14 days of culture (n=3), samples were removed from the Alamar Blue solution and washed 3 times in PBS, and then 1 ml of sterile water was added. Samples were then frozen (-20 °C) and thawed (37°C) more than three times. Then 50 μ l of cell lysate and 50 μ l of alkaline phosphatase reagent (Randox Laboratories UK, Cat.No AP307) were added to a well plate. Plates were shaken for 4 minutes at 300 rpm on a Heidolph Titramax 100 plate shaker in a dark environment. The absorbance was then measured using a Bio-Tek ELx 800 colorimetric microplate reader at a wavelength of 405 nm and a 630 nm reference filter.

To quantify the DNA content of the cell layer, 100 µl of cell suspension was mixed with 100 µl of Hoechst 33258 stain (2 mg/ml in TNE buffer). Calf Thymus DNA (Sigma, UK) was diluted in TNE buffer to various concentrations (0.125 µg/ml – 10 µg/ml) to create a calibration curve. The fluorescence emitted at 460 nm after excitation at 360 nm was measured using FLx 800 fluorescence microplate reader, and using the calibration data, the DNA content per ml of cell suspension was determined.

An osteocalcin assay was carried out using an osteocalcin ELISA Kit (Invitrogen KQ1381) according to manufacturer's instructions. Data was normalised by DNA content in the same manner as carried out on the alkaline phosphatase assay. Analysis was performed after 15 and 21 days of culture (n=3).

For SEM examination, cells were fixed with glutaraldehyde (3 % v/v) in sodium cacodylate buffer (0.1M) for 30 minutes, then left in 7% sucrose solution in cacodylate buffer (0.1 M) for 5 minutes at 4 °C and then washed. Cells were then post-fixed with 1 % w/v osmium tetroxide solution for 45 minutes, then dehydrated with; 20, 40, 60, 70, 80 and 90 % v/v ethanol for 5 min, then twice in 100 % ethanol for 5 min, before drying via hexadimethyldisilazane (HMDS). Samples were gold coated, as before, prior to viewing using an FEI XL30 SEM.

Statistical Analysis

Statistical analysis was carried out using Prism 3.0 (Graphpad, USA). Significant differences in cell responses to experimental substrate groups were determined by one-way analysis of variance with a Bonferroni post test. An independent samples Student t-test was used to assess the effect of gold coating on substrate roughness. Differences were considered significant if $p < 0.05$.

RESULTS

Surface Characterisation

Prior to cell culture, the surfaces were characterised using XPS, SEM and optical profilometry in order to examine the elemental composition of the surfaces, the surface topography and nano- and micro-roughness. The XPS spectra (Figure 1) for the uncoated BoneMaster samples identified oxygen, calcium, carbon and phosphate on the surface of the samples. The gold-coated BoneMaster sample spectra only showed gold and carbon.

SEM images were taken of the gold-coated BoneMaster samples to examine the morphology of the surface after gold-coating (typical image shown in Figure 2). The surfaces showed a needle-like morphology, of average approximate length 300 nm.

Optical profilometry assessment of the uncoated BoneMaster and gold-coated BoneMaster samples indicated a very similar appearance (Figure 3) and the R_a values which were calculated from them showed no significant difference between the two sample sets ($p=0.341$, Table 1).

Cell Responses

The HOS cells were imaged using SEM after culture on the different sample surfaces for 1, 4, 7 and 14 days. The cells after 1 day appeared well adhered, lamellopodia could be observed on all surfaces, extensive filopodia were apparent on Thermanox surfaces (Figure 4). By 7 days, a confluent cell layer was present on all samples (Figure 4).

Cell proliferation was assessed using the Alamar Blue assay; the results of which are shown in Figure 5. The cell number on all samples increased with time. After 14 days of culture the BoneMaster samples (BM and BM-Au) showed 27.6% higher cell numbers compared with the Thermanox slides (Thx and Thx-Au); this was a significant difference ($p=0.007$). No difference was found between the BM and BM-Au samples when compared with each other ($p=0.276$); the same was true of the Thx and Thx-Au samples ($p=0.106$).

Alkaline Phosphatase activity (Figure 6), varied between Thermanox substrates and BM substrates over the course of the experiment. Alkaline phosphatase activity on the Thermanox samples peaked at day 4 and was consistently lower than the activity measured for the BoneMaster; at 7 and 14 days the alkaline phosphatase activity on the BoneMaster samples was significantly greater (day 7: $p=0.0012$, day 14: $p=0.0034$, respectively) compared with the Thermanox samples. No difference in alkaline phosphatase activity was observed between the gold-coated and uncoated versions of either the BoneMaster (day 7: $p=0.15$, day 14: $p=0.56$) or Thermanox surface (day 7: $p=0.13$, day 14: $p=0.14$).

The osteocalcin concentration, normalised by DNA, is shown in Figure 7. At both the 15 day and 21 day time points, the BM and BM-Au samples showed significantly greater osteocalcin concentration compared with the Thx and Thx-Au samples (day 15: $p=0.0002$, day 21: $p=0.001$). At 15 days the BM-Au sample showed greater osteocalcin concentration compared to the BM ($p=0.052$); however, by 21 days there was no significant difference between the sample sets ($p=0.29$). The Thx and Thx-Au samples showed no significant difference at either time point (day 15: $p=0.41$, day 21: $p=0.072$).

DISCUSSION

The *in-vitro* experimental approach taken in this study allowed for the assessment of the relative contribution of selected surface properties to osteoblast-like cell responses with some confidence. The XPS spectra of the BM sample showed evidence of calcium, phosphate and oxygen, confirming that hydroxyapatite was on the surface. After gold-coating of the samples, calcium, phosphate and oxygen could no longer be detected, with gold and

carbon being the only detectable elements; confirming that the hydroxyapatite had become completely covered in a continuous gold layer, thus completely altering the chemistry of the surface. Carbon was seen in both spectra; however, it is thought that this was adventitious carbon contamination of the surface and was not related to the coating.

An SEM image of the gold-coated BoneMaster sample illustrated a needle-like morphology, similar to that reported by Roessler *et al.* [9] for electrochemically deposited hydroxyapatite coatings. This indicated that the morphology of the underlying hydroxyapatite coating was retained after gold-coating. The equivalent roughness observed between the BoneMaster samples and the gold-coated BoneMaster samples supported this finding, providing further evidence that the surface morphology was unaffected by the gold coating.

Significant differences in cell proliferation and in markers of osteoblastic phenotype were apparent between BoneMaster and the Thermanox samples after 14 days of culture; both with or without gold coating. This data would indicate that the gold coating of BM surfaces did not significantly alter cell responses to this substrate, which in turn indicates that the osteoblastic phenotype is promoted, not by the chemistry of the BM surface, but by its topography.

These observations are consistent with other studies examining the influence of biomaterial surface texture on osteoblastic phenotype. Such studies have demonstrated the effectiveness of moderate surface roughness (1-2 μm) or micron scale topography in the promotion of osteoblastic attachment, proliferation, differentiation and matrix production [11, 12]. Further studies have recognised the contribution of such surface features in the promotion of bone formation in the surrounding tissue via promotion of specific cytokine and growth factor production by cells in contact with implant surfaces [13, 14]. In addition to the degree of roughness, specific textures or organisation of topographic features have also been demonstrated to be important in the responses described above, both at the micro and nanoscale [15, 16]. The BoneMaster coating is made up of crystallites of length approximately 300 nm and a diameter of 60 nm [9]. Use of laser interference lithography techniques has enabled the responses of osteoblasts to controlled surface morphologies to be assessed; it is known that osteoblasts can detect and respond to surface features as small as 50 nm [17] and that osteoblasts respond particularly strongly to features between 150 nm and 300 nm [17].

There are several biomedical coatings designed to promote osseointegration, which rely on the osteoblast response to the surface topography; these are predominantly used in the dental industry and include; NanoTite (Biomet 3i), TiOblast (Astra Tech), OsseoSpeed (Astra Tech), Lifecore RBM (Lifecore Biomedical), TiUnite (Nobel Biocare), and SLA (Straumann) [18]. There is evidence that these nano-structured titanium surfaces promote osseointegration [19], and the surface texture can upregulate the early expression of bone sialoprotein (BSP) and osteopontin (OPN) [20].

The above consideration should not be taken to exclude any influence of surface chemistry. The results from this study did not see any significant effect due to chemistry; nevertheless, across the range of assays used the results for the BM samples were higher compared with the BM-Au samples. However, the error was too large for these differences to be considered significant in the individual assays. Whilst cells were not in direct contact with the BoneMaster coating in the BM-Au group release of ions from the BoneMaster coating cannot be ruled out. It has been reported in numerous studies that elevated calcium ion levels at implant surfaces promotes osteoblastic phenotype [21]. Calcium ion levels in the culture medium were not measured.

The results shown in this study provide interesting insight into how osteoblast-like cells are affected by the topography of the surface on which they adhere. The needle-like morphology of the BoneMaster could be responsible for the increased osteoblastic activity observed compared with the Thermanox control; or it may have been features on a different scale. However, the similarity of the needle-like morphology of the BoneMaster surface and that of the HA crystals in natural bone may indicate a relation [9]. This may go some way to try and explain the increased bone density seen clinically by Bøe *et al.* [18] during the early stages of bone remodeling as compared to a conventional plasma sprayed HA coating.

CONCLUSION

This study appears to show that direct contact with HA is not necessary for the *in vitro* HOS cell response observed on BoneMaster samples. This does not rule out the influence of locally increased calcium and phosphate ion concentrations on the observed responses both *in vitro* and *in vivo*, but does suggest that topography or texture is a major contributing factor to the *in vivo* response to the BoneMaster coating.

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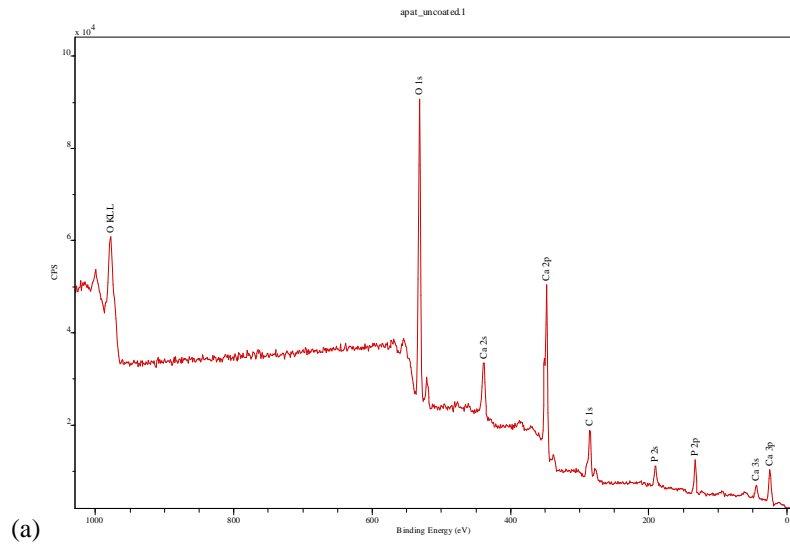
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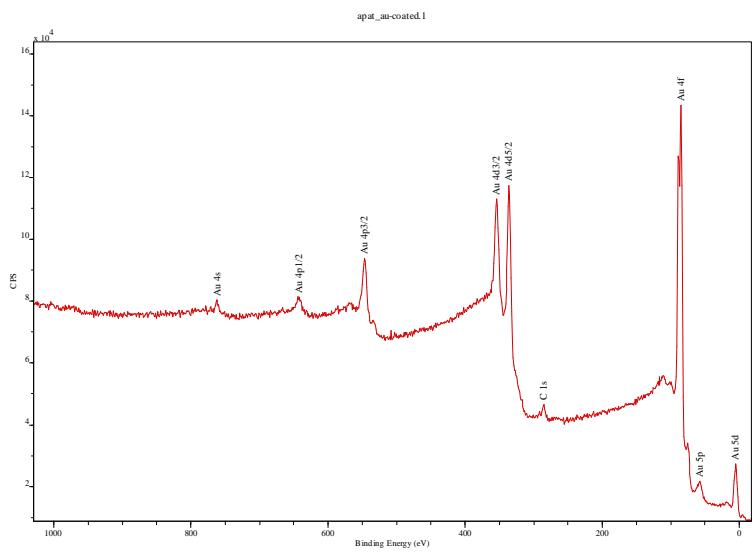
Figures and Tables

| Samples | Surface Roughness, R_a / μm |
|---------|--|
| BM | 2.2 ± 0.13 |
| BM-Au | 2.5 ± 0.30 |

Table 1. Mean micro-scale R_a roughness values measured from optical profilometry scans of the BoneMaster and gold-coated BoneMaster surfaces. The error shown is the standard error in the mean.



(a)



b)

Figure 1. XPS spectra of the uncoated BoneMaster surface (a) and the gold-coated BoneMaster surface (b). Spectra taken with polychromatic Al K α X-rays with a 10 kV potential and a current of 20 mA; elemental peak identification is shown on the images.

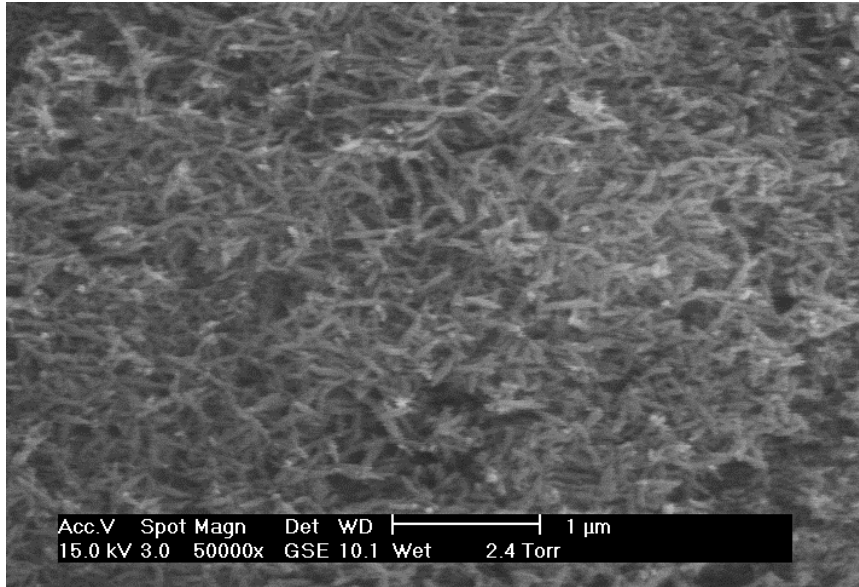


Figure 2. SEM micrograph of the gold-coated BoneMaster surface illustrating needle-like morphology. Image taken with an accelerating voltage of 15 kV and a spot size of 3.0 μm . Scale bar shown on the image.

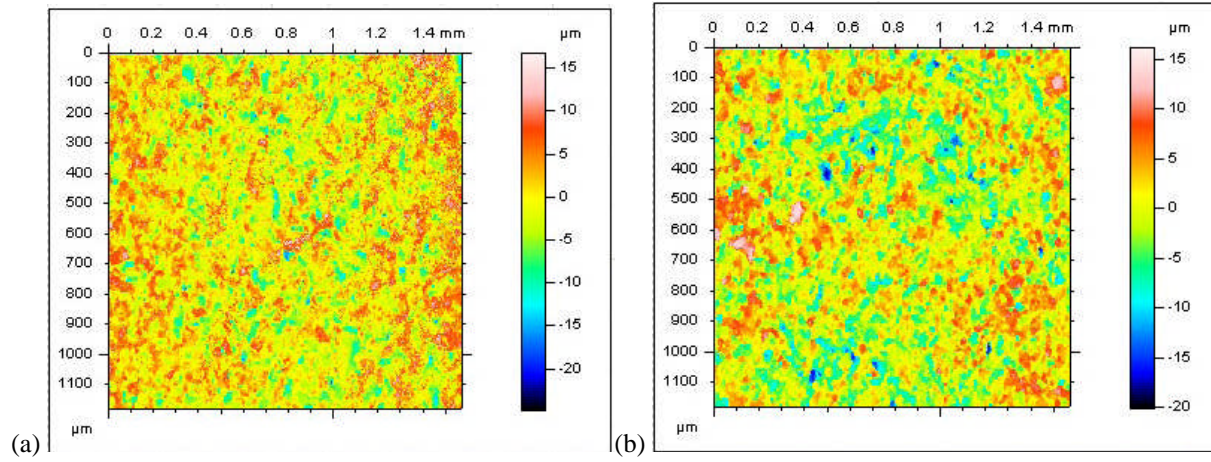


Figure 3. Optical profilometry images of the uncoated BoneMaster samples (a) and the gold-coated BoneMaster samples (b). Scale bars are shown on the images.

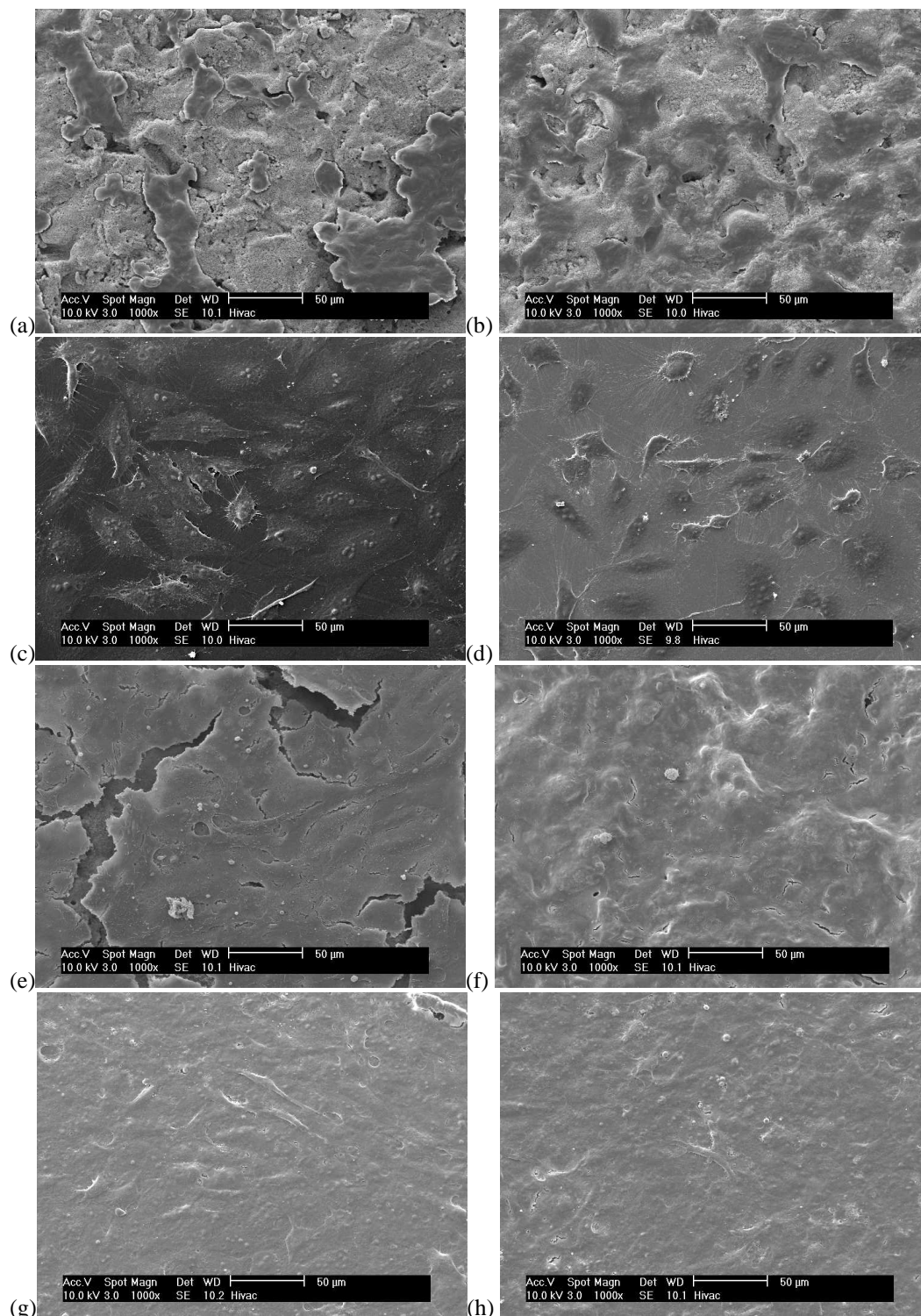


Figure 4. SEM micrographs of the HOS cells after culture on BoneMaster surfaces (a, e), gold-coated BoneMaster surfaces (b, f), Thermanox slides (c, g) and gold-coated Thermanox (d, h) slides for one and seven days respectively. Images taken with an accelerating voltage of 10 kV and a spot size of 3.0 µm. Scale bars shown on the micrographs.

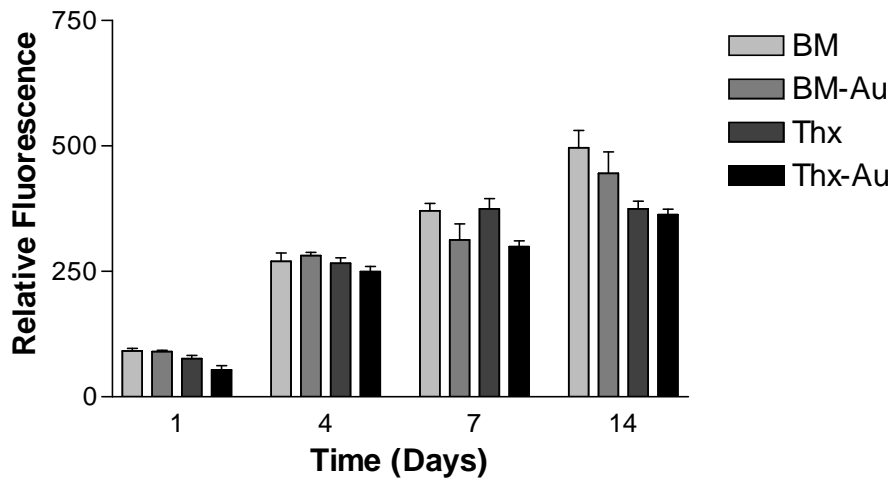


Figure 5. Cell proliferation of HOS cells cultured on BoneMaster, gold-coated BoneMaster, Thermanox and gold-coated Thermanox samples. Values are means and standard error in the mean.

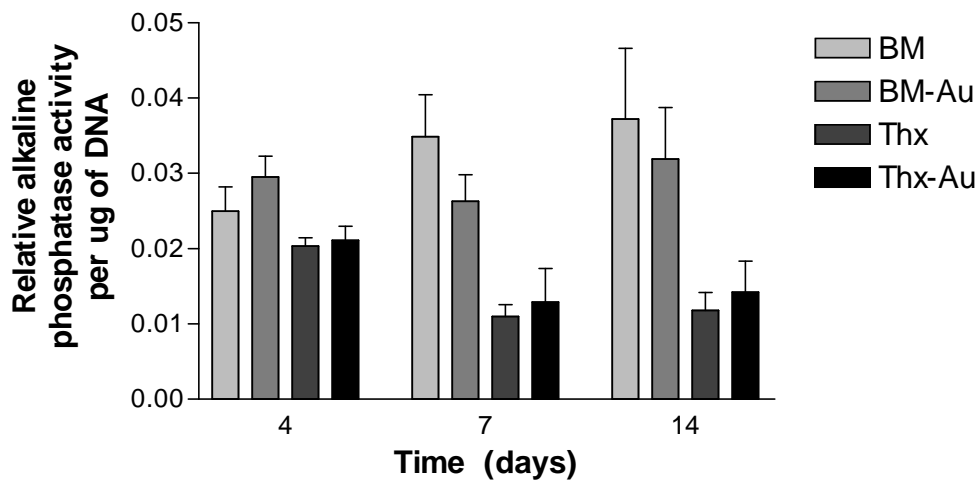


Figure 6. Relative alkaline phosphatase activity of HOS cells (normalised to DNA concentration) cultured for 1, 4, 7 and 14 days on BoneMaster, gold-coated BoneMaster, Thermanox and gold-coated Thermanox samples. Values are means and standard error in the mean.

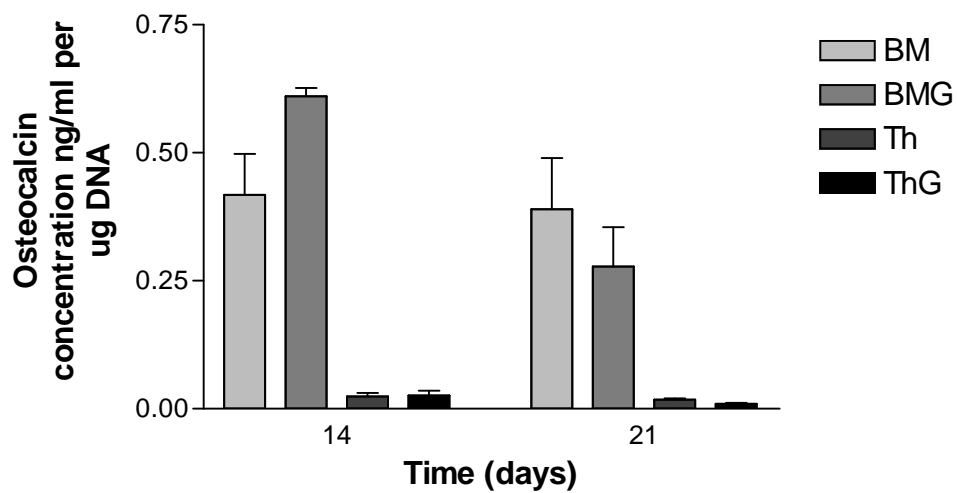


Figure 7. Osteocalcin concentration of HOS cells (normalised to DNA concentration) cultured for 1, 4, 7 and 14 days on BoneMaster, gold-coated BoneMaster, Thermanox and gold-coated Thermanox samples. Values are means and standard error in the mean.