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Osteoblast-like cell response to calcium phosphate coating chemistry and morphology on etched silicon surfaces

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Abstract Being able to control the behaviour of osteoblast-like cells on a surface may provide a genuine insight into the material surface characteristics and help in creating a successful coating/cell interface. The possibility of creating a micro-environment that can induce proliferation, differentiation and mineralisation of bone cells in vitro, by successfully combining both chemistry and topography of a micro-fabricated substrate is an area that requires a multidisciplinary approach. Utilising sputter deposition, a process that lends itself to high processability, in conjunction with photolithography allowing for the creation of highly repeatable etched surfaces, we aim to provide a successful combination of chemistry and topography. Correlating the substrate conditions with resultant osteoblast biological function and activity can ultimately be used with a view to modulating the behavior of osteoblast-like cells in vitro.

1 Introduction

Successful cementless fixation of orthopaedic and dental prosthetic devices in clinical practice has been shown to be dependent on the formation of a strong and durable bond between the implant and surrounding tissues. This biologically driven fixation process involves direct and tight apposition of bone tissue to the surface of the implant. The response of the tissue to an implanted device is dependent upon a number of factors including the site of implantation, the mechanical loading upon it and the amount of micro-motion at the interface. Furthermore, certain characteristics of the implant material, particularly its surface chemistry and topography, also affect the extent to which tissue will bond to the implant.

The surface roughness of an implant has been reported to affect the response of tissues such as bone. Surface roughness may be defined as a random variation in the topography consisting of uniformly distributed asperities of more or less similar height and depth, but significantly smaller than the bulk geometry of the solid. It would be useful to correlate and thereby establish the relative impact of surface chemistry and surface roughness upon adhesion of bone cells to surfaces and specifically to those comprised of Calcium Phosphate (CaP) bioceramics which have inherent bioactivity.

Determining the mechanism for the initial adhesion of cells to CaP ceramic surfaces is most readily determined in a cell culture model under well-controlled conditions. Initial events, such as the spreading and growth of anchorage dependent cells, are dependent upon initial adhesion reactions, and have significant connotations for the success or otherwise of subsequent cellular processes, such as migration, proliferation and deposition of extracellular matrix (ECM). Therefore, understanding the behaviour of cultured bone derived cells on a known bioceramic surface may provide a genuine insight into the relationship between the surface characteristics of an implant and the early stage events in creating a successful cell/surface interface. The possibility of creating a micro-/nano-environment that can induce proliferation, differentiation and
mineralisation of bone cells in vitro, by successfully combining both chemistry and topography of a specifically fabricated substrate is the main goal of the research reported here. To this end, the effects that topography and chemistry of CaP bioceramic surfaces can have in modulating the behaviour of cells in vitro have been extensively considered.

A wide range of factors, most notably chemical composition, residual surface energy and the surface topography, can influence the biological response of a biomaterial in vivo. Each factor plays a critical role, with the ability to act both independently and in tandem to regulate key biological reactions. Therefore, in order to estimate the individual contribution that each makes in determining tissue response, it is essential to determine the relevant chemical and physical characteristics of the systems.

There are a number of different methods that can be employed to produce CaP coatings in order to influence the osteoblast response to such materials, including, plasma spraying [1], high velocity oxy-fuel spraying (HVOF) [2], electrolytic deposition [3], sol–gel routes [4], co-blasting [5], and radio frequency (RF) magnetron sputtering [6]. Of these methods, RF magnetron sputtering has been shown to be a particularly useful technique for the deposition of bioceramic thin films (based on Ca–P systems), due to the ability of the technique to provide greater control of the coating’s properties and improved adhesion between the substrate and the coating [6–9].

To this end, a detailed characterisation of the sputter deposited CaP thin film coatings of interest has been undertaken, as a move towards evaluating the performance of these biocermics, when placed in contact with biological processes.

Most cell types used in culture that adhere to substrata reside in an environment with flat topography. Specifically, primary bone cells and osteosarcoma cells are isolated and plated on traditional flat, 2-D dishes to be used for culture study. In these 2-D culture models, cells are deliberately confined to associate laterally on the flat surface of the dish beneath a layer of medium, resulting in the formation of a monolayer of cells. Unfortunately, this distribution does not resemble the natural organization of bone tissue. The onus here is therefore on the development of a suitable tissue culture model incorporating the use of a thin film bioceramic layer and manipulated substrate topography.

A comprehensive range of chemical and physical analytical techniques have been employed to characterise both the substrates and the CaP coating types used herein, in an attempt to correlate these key aspects with cellular response in vitro. This biological activity has been quantified and assessed by a well accepted range of assays, the details of which are described in detail here.

2 Materials and methods

2.1 Production of substrate

In order to produce a substrate with features having well-defined height, depth and degree of regularity, photolithographic procedures were utilised to create an etched surface. Silicon substrates present a relatively uniform surface. The substrate employed for the anisotropic etching procedure was n-type silicon (Okimetic, Sweden—4° N (100) 1–20 Ω/cm, 475–575 μ, single side polished, test grade). The etching procedure employed 30% concentration solution of KOH in water. This was subsequently heated to a temperature of 80°C. At this temperature the etch-rate of (100) silicon is approximately 1 μm/min. An oxide hard mask was used to protect the remaining silicon during the etching procedure, to produce a 1 μm high, repeating pillar and pore effect of known dimensions, i.e. hole length (30 μm), bar (25 μm) and pitch (55 μm). This evolution in substrate condition was continued by the deposition of a CaP coating, to produce CaP features of known chemistry and, crucially, controllable dimensions. CaP coatings were deposited onto the substrates by the process of RF magnetron sputtering from dry-pressed 76 mm Hydroxyapatite (HA) powder targets (Merck KgGA, Germany). To investigate the impact of modifying substrate morphology to induce a positive biological response, a thin film of roughly 1 μm* was used. Sputter deposition was performed using a Kurt J Lesker Co. (East Sussex, UK) custom designed high vacuum deposition system and occurred over 10 h at a power of 150 W, and an argon gas pressures of 15 mTorr, a gas flow rate of 5 Sccm and a target to substrate distance of 60 mm. In order to induce a more crystalline state in the sputter deposited CaP coatings, some samples were heated in air, using post-deposition thermal annealing in a Lenton Muffle furnace, where coatings were annealed to 500°C for 2 h at a ramp rate of 2°C/min. All samples were dry autoclaved at 121°C for 2 h to ensure sterility prior to cell seeding. * Thin film thickness was determined by AFM and profilometry, cross sectional analysis was performed for both the as deposited and annealed samples. As repeatable coating thicknesses of 1013.67 nm ± 21.24 could be obtained, it was decided to use these parameters for further investigation.

Sample key

| Si   | n type silicon wafer          |
| Si Et | n type silicon wafer etched using photolithographic techniques |
| Si AD | n type silicon wafer etched and CaP as deposited |
| Si AN | n type silicon wafer etched and CaP post deposition thermally annealed |
2.2 Analytical techniques

The CaP coatings were evaluated by a variety of analytical techniques before and after thermal treatment, to investigate the change induced chemically and physically by post-deposition thermal annealing. FTIR spectroscopy, XPS and XRD were used to investigate the chemical state of the coatings. Profilometry, atomic force microscopy and scanning electron microscopy were used to illustrate both the coating topography and to determine surface roughness values.

A Bio-Rad Excalibur (FTS300MX Series) FTIR spectrometer equipped with a PIKE Easidiff DRIFTS (diffuse reflectance) accessory was used to investigate the various chemical vibrational modes in the 4,000–400 cm⁻¹ region for the deposited and annealed CaP coatings.

XPS analysis of the RF magnetron sputter deposited CaP coatings was carried out using either a Kratos XSAM-800 instrument or a Sciena 300 XPS system. XPS spectra were obtained from the Kratos Analytical XSAM-800 system (SSL, UU), with excitation via the Mg Kα line (hv = 1253.6 eV). The base pressure in the UHV analysing chamber was below 2 × 10⁻⁹ mbar throughout. Sample charging effects on the measured binding energy (BE) positions were corrected by calibrating the C1 s spectral envelope to 285.0 eV, the value generally accepted for adventitious carbon contamination. Samples were analysed in triplicate. To further the analysis, the calcium to phosphate ratios were calculated by quantification of calcium and phosphate regions, from each components high resolution scan.

A D8 Advance X-Ray diffractometer (Bruker–AXS, UK) was employed to characterise the structure of the thin film sputtered CaP coatings. A grazing incidence measurement was made on each sample with theta (tube) held at 1° and a 2θ scan range of 25–35°, at 0.04° per step, 5 s per step.

A Zyco NewView 5000 3-D Surface Profiler (Zyco Co., USA) was used to characterise and quantify surface texture, step heights and critical dimensions of the various substrate surfaces and coatings thereon (CaP). The optical interferometer is capable of measuring surface roughness and displaying a 3-D image of the surface, with a vertical resolution of 0.2 nm for smooth surfaces and 20 nm for rougher surfaces. Also it has extremely high resolution in the lateral plane of 0.56 μm.

A Burleigh Personal Scanning Probe microscope (PSPM) system operating in contact mode, and fitted with a silicon nitride (Si3N4) tip mounted on a triangular cantilever spring was used in these analyses. Line profiles were taken from 50 × 50 μm AFM images.

SEM images were obtained using a Hitachi S-3200 N variable pressure instrument. The images were generally obtained in backscatter mode, using an acceleration voltage of 20 kV, with a working distance of 15–20 mm at the focus used. For the purposes of examining the morphology of fixed cells on the respective substrates after appropriate time in culture, the substrates were washed three times with ice-cold PBS, and fixed initially with 3% glutaraldehyde in 0.1 M cacodylate buffer for 30 min. The samples were successively dried with increasing concentrations of ethanol (50, 70, 90 and 100%) and finally with hexamethylene disiloxane (HMDS). Dried specimens were coated with a thin conducting layer of gold-platinum of approximately 180 Å/0.018 μm thickness using the Polaron Series II ‘Cool’ Sputtering System Type E5100.

3 Chemicals and reagents

Fetal bovine serum (FBS), minimum essential medium (MEM), penicillin G, and streptomycin were purchased from PAA Laboratories GmbH (Austria). 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical (Poole, UK).

3.1 Cell culture

The human osteoblast-like cell line SaOS-2 derived from human Caucasian osteosarcoma was obtained from American Type Culture Collection (ATCC; Rockville, MD). Immediately upon arrival, cells were thawed and suspended in a T75 culture dish with Minimum Essential Medium (PAA, Austria) supplemented with 10% heat inactivated FBS and antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin). The dish was placed in a humidified atmosphere of 5% CO₂ at 37°C. Cells were subcultured several times when they reached confluence using 0.25% trypsin in Mg²⁺ and Ca²⁺ free PBS before experimental use. This hOS line has been well characterised and shown to exhibit production of mineralised matrix, the major phenotypic characteristic of osteoblastic cells [10]. Similar to bone marrow cells, SaOS-2 cells form a calcifying matrix. Biological mineralisation, as shown by extremely elevated levels of alkaline phosphatase (ALP), is a phenotypic property expressed by SaOs-2 cells, indicating advanced osteoblastic maturation. Similar to osteoblasts observed in situ and in vitro, SaOs-2 cells possess 1, 25(OH)₂D₃ and PTH (Parathyroid Hormone-sensitive Adenylate Cyclase) receptors. The former hormone enhances the production of osteocalcin; the latter stimulates adenylate cyclase which is a parathyroid hormone stimulant. Another bone abundant protein, osteonectin, is also present in SaOS-2 cells. The SaOS-2 cells thus possess sufficient osteoblastic features to make them useful as a
permanent line of human osteoblast-like cells, and as a model for studying biomaterial-cell interactions [11–17].

3.2 Cellular assays

SaOS-2 cells were seeded directly onto substrates at a density of $2 \times 10^5$ cells/ml. The cells were then cultured under standard conditions of 37°C in 5% CO$_2$ for 2, 5 and 10 days. Cell media was changed every 2 days. A number of biological assays were carried out over a 10 day period, including cell proliferation (MTT), cell differentiation (ALP assay) and SEM of fixed cultures to determine cell morphology.

3.3 MTT cell viability assay (MTT)

Cell proliferation and viability is a fundamental measurement of cell response to external factors and can be assayed using the reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). The yellow tetrazolium substrate is reduced metabolically by mitochondrial dehydrogenases of living cells to yield an intracellular purple formazan product which can be solubilised and measured spectrophotometrically. The amount of formazan produced is directly proportional to the number of viable cells. At the respective timepoints, MTT test solution (5 mg/ml in serum-free MEM) was added to the wells in an amount equivalent to 10% of the total cell culture medium. The substrates were then incubated for 4 h at 37°C, 5% CO$_2$, after which the media was aspirated and the intracellular formazan product was solubilised with acidified isopropanol. After dissolution of the formazan crystals, the absorbance was measured on an ELISA plate reader at a test wavelength of 570 nm and a reference wavelength of 650 nm (TECAN Sunrise, TECAN Austria). All MTT assays were performed in triplicate and repeated to confirm results.

3.4 Alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) activity from cell lysates was measured enzymatically with a commercial kit from Sigma Diagnostics (Sigma 221). The production of ALP by the osteoblastic cells after experimental treatments and that on controls was measured at 48 h, 5 and 10 days. Cells were seeded onto substrates and cultured to the required timepoints. The cells were then washed three times with PBS, and total cellular protein concentration was determined by incubation in BCA (bicinchoninic acid) protein assay reagent containing 0.1% Triton X-100, 2 mM MgSO$_4$, and 6 mM PNPP (4-nitrophenyl phosphate). The reaction was stopped by adding 1 M NaOH, and absorbance measured at 405 nm. The percentage change in ALP activity was normalized to the control according to the formula: $M = \frac{\text{value of absorbance at } 405\text{ nm}}{\text{value of absorbance at } 560\text{ nm}} \times 100$ [18].

3.5 Assaying the levels of BMP-4, osteocalcin and type 1 collagen

ELISA kits were used to detect collagen I, osteocalcin and BMP-4 levels respectively. Cells were cultured on substrates for the indicated times. The cell lysates were collected and measured for osteocalcin, collagen and BMP-4 levels. Samples were inoculated in 96 well plates coated with the monoclonal detective antibodies and incubated at room temperature for 2 h. Unbound material was removed by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20). Horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. HRP catalysed the conversion of the chromogenic substrate (tetramethylbenzidine) to a coloured solution where the colour intensity was directly proportional to the amount of the protein present in the sample. The absorbance of each sample was measured on an ELISA reader at 450 nm. Results are presented as the percentage change in activity compared to the untreated control [19].

3.6 Statistical analysis

Data were expressed as means ± S.D. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($P \leq 0.05$) between the means of control and test groups were analyzed by Dunnett’s test.

4 Results

4.1 Physical and chemical characterisation

4.1.1 FTIR spectroscopic analysis of SiAD and SiAN

A typical FTIR spectrum for the as-deposited Ca–P coating is shown in Fig. 1a and clearly illustrates spectral modes associated with P–O stretching vibrations between 1,200 and 900 cm$^{-1}$ and bands associated with O–P–O bending vibrations between 650 and 400 cm$^{-1}$. O–H stretching and librational peaks typically observed at 3,572 and 630 cm$^{-1}$, respectively were not seen here, as would be
expected for coatings produced under the sputtering conditions employed here. After annealing the same coatings to 500°C, spectra that were more indicative of HA were observed, as shown in Fig. 1b. Well resolved P–O stretching vibrations were observed at 1,120, 1,078, 1,020 and 960 cm\(^{-1}\). O–P–O bending vibrations are also present at 578 and 609 cm\(^{-1}\), respectively. Furthermore, the presence of O–H stretching and librational bands is confirmed at 3,571 and 630 cm\(^{-1}\), respectively. It should be noted that the O–H librational band occurs as a weak shoulder rather than a well defined peak [20–22].

Post-deposition thermal annealing at 500°C has brought with it improved resolution in the FTIR signature as evidenced in Fig. 1b. Once more, all the major phosphate peaks are evident as would have been expected for such a coating, with vastly improved resolution resulting from the annealing process. Similarly so OH– peaks at both 3,571 cm\(^{-1}\) and also at 631 cm\(^{-1}\) are greatly enhanced on the silicon substrate for the SiAN sample.

4.1.2 XRD analysis of CaP on n-type silicon

The use of silicon as a substrate type has afforded an opportunity to investigate the impact that post-deposition thermal annealing at higher temperatures (500°C) can have on the crystallinity of a sputter deposited CaP thin film. The XRD pattern for the as-deposited Ca–P (not shown here) exhibits an amorphous background hump as would be expected for coatings produced under the conditions employed here. However, upon annealing to 500°C the Si AN sample, as shown in Fig. 2, exhibits a very different diffraction pattern. The primary crystal orientation planes (002), (102), (210), (211), (112), (300) and (202) that are clearly indicative of HA were seen throughout the pattern with \(2\theta\) values that correspond closely to those observed in the ICDD file #09-0432 for HA. Furthermore, the 002 reflection at 25.9° \(2\theta\) dominates the diffraction pattern, which suggests that this coating may have a 002 preferred orientation [23, 24]. No other Ca–P phases were detected in the XRD pattern for the Si AN sample.

4.1.3 XPS analysis of CaP on n-type silicon

The XPS survey scan for the Si AD coating (Fig. 3) indicates similar peaks to those expected for HA. Peak lines corresponding to C1 s (285.0 eV), Ca2 s (439.1 eV), Ca2P3/2 (347.6 eV), Ca2P1/2 (351.1 eV), P2p (133.3 eV), P2 s (190.8 eV), O1 s (531.2 eV), O AugerKLL (978.0 eV) and Ca AugerLMM (1197.0 eV) are clearly present, and correspond closely to those reported for HA in the literature [25, 26]. The presence of carbon (C1 s) in the survey scan at 285.0 is due to (as previously mentioned) the presence of adventitious carbon contamination in the sample [27, 28]. The Ca/P ratio of the Si AD surface produced was 1.68 ± 0.17 and is very close to the value expected for stoichiometric HA (1.67) [23, 24]. After annealing the samples to 500°C, similar peak lines were observed as compared to the as-deposited coating at the same pressure, as shown in Fig. 4. However, the Ca/P ratio was seen to increase significantly to 2.57 ± 0.41, most likely due to the loss of less strongly bound phosphorous (compared to calcium). For both the Si AD and the Si AN samples no other elemental species were detected at least at the detection limits of the instrument (~0.1 atomic percentage concentration).

XPS analysis was used to investigate the stability of RF magnetron sputtered CaP coatings in cell culture media for the duration of the in vitro cellular analysis. Thermally annealed (500°C) CaP coatings on the n-type silicon substrates were immersed in cell-culture media for a period of 5 and 10 days, removed at the specified intervals and washed in double-distilled water before XPS analysis. Analysis of the annealed HA coated surfaces both before and after exposure to the cell-culture media by XPS was

![Fig. 1](image_url)  
**Fig. 1** FTIR spectra for a Si AD and b Si AN

![Fig. 2](image_url)  
**Fig. 2** XRD diffractogram for Si/CaP AN (International Centre for Diffraction Data (ICDD) File # - 09-0432)
used to evaluate the stability of these CaP coatings as a result of exposure to the cell culture media.

After 5 days exposure to the cell culture media peaks indicative of calcium, phosphorus, oxygen and carbon were observed for the Si AN sample. The Ca/P ratio was also seen to increase to $3.14 \pm 0.3$. No peaks indicative of the underlying substrate (namely silicon–Si2p) were observed, indicating that the coating is still largely intact (Fig. 5).

After 10 days in vitro, the Si AN 10 day sputter coated surface is still present with the XPS wide energy (1,100 eV–0 eV) survey scan illustrating peaks for calcium (Ca2p) in addition to this, peaks indicative of the underlying substrate (namely silicon–Si2p) were observed, indicating that the coating is still largely intact (Fig. 5).

4.1.4 Atomic force microscopy (AFM) analysis of CaP coated etched silicon

AFM analysis of the etched silicon samples and analysis of the topography of the CaP coated samples can be compared with profilometry data (shown below) of the same samples. For the native etched silicon surface (Si Et), it is apparent that both the ‘pillar’ and ‘pore’ surfaces, as illustrated in Fig. 7 (a), have relatively smooth areas. The dimensions of the etched silicon features are apparent. The associated line profile for this image gives a definitive quantification of the height of the etched feature (957.34 ± 35.91 nm).
Figure 7b illustrates an AFM image of the Si AD sample. Immediately apparent are CaP aggregations on the surface of the silicon pillars. Unfortunately, the contrast between the top and bottom of the pillars is not sufficient here to determine whether or not the same type of CaP morphology occurs on the bottom surface. However, from SEM analysis (Figs. 9, 10, 11) of the same substrate it is known that these aggregates exist there also. The line profile shown in Fig. 7b reiterates the height of 1 μm for the etched silicon pillar and provides an estimate as to the average height of the CaP deposits. On this evidence the CaP aggregations are in the region of 1 μm thick, as would be expected for a 10-h deposition run at 150 W.

Figure 7c demonstrates an abundance of CaP aggregations on the surface of a CaP coated pillar, produced as a result of post-deposition thermal annealing at 500°C. Whilst the AFM shown here for the Si AN sample, is similar in appearance to the Si AD sample, the corresponding SEM analysis has indicated that these defined areas of CaP coating are more prominent on the top and bottom of the annealed surfaces.

AFM analysis of a CaP coated 20 × 20 μm area, on the surface of a pillar on the Si AD sample revealed an Ra value of 163.85 nm. For the corresponding annealed sample (Si AN), analysis of the same sized area gave an Ra value of 171.14 nm. No statistical difference in the Ra values for either as-deposited or annealed (500°C) samples was found over the range of samples examined.

4.1.5 Optical profilometry analysis of CaP coated etched silicon

Profilometry was employed as a tool for measuring the roughness of a larger surface area compared to that observable with AFM analysis. In this instance, both the Ra and RMS values have been recorded over an area of 0.6 × 0.6 mm, yielding the results indicated in Table 1.

Figure 8a illustrates the profilometry data recorded for native etched n-type silicon substrate surface. The images support the data shown in both the AFM. The degree of regularity conferred by the etching process is demonstrated by pseudocolouring. The line profile data for both the top
and bottom of the sample region illustrates the flat nature of the underlying substrate, and the regularity of the etched features. The overall Ra roughness measurement recorded for the 0.6 × 0.6 mm area was 0.115 μm.

Figure 8b shows the optical profilometry data for the Si AD sample. Here the upper surface of the ‘pillars’ appear to have lost their regular flattened appearance, compared to the image in Fig. 7a, for the uncoated etched substrate (Si Et). The Ra value recorded for this sample was 0.182 μm over a 0.6 × 0.6 mm area.

Figure 7c shows the profilometry data recorded for the Si AN sample. As evidenced in the SEM images (Fig. 11b), the annealing process has increased the size of the aggregates as compared to the post annealing samples (Fig. 10b). Significantly, the Ra value for this sample has decreased to a value of 0.099 μm for the area of 0.6 × 0.6 mm as compared to the non statistical difference shown by AFM analysis.

4.1.6 SEM analysis of CaP coated etched silicon

Etched silicon was cut into pieces of 20 × 20 mm after the coating deposition had occurred to provide an overall sample area of 400 mm². The SEM images shown in Fig. 9 illustrate the structure of a native etched silicon substrate (Si Et). Figure 9a, b were obtained at ×300 and ×1.2 k magnification respectively, and highlight the regularity of the etched pillars. In addition, they allow for the measurement of the actual dimensions obtained by the etch process. The distance from one side of the pillar to the other is 30 μm. The pitch length is 55 μm, and the bar length is 25 μm.

Figure 9c, d show SEM images of the Si Et sample obtained at a tilt of 55°, and as such illustrate the relative depth that the etching process has created in the substrate. Again the degree of regularity of the features conferred on the substrate is apparent. As the CaP coating is to be
deposited both on the pillars and at the base of the pillars, the relative topography of this part of the substrate will play a key role in determining initial cell attachment and resultant activity. SEM analysis indicates that the etching process has left the base of the silicon relatively unscathed and that it retains the smooth appearance of the native silicon substrate.

The primary concern when depositing a thin film CaP coating onto 1 μm deep silicon etched surfaces was that the topography of the micro-fabricated surfaces might be lost should a sufficient quantity of the coating be deposited. The use of RF magnetron sputtering, however, with a controlled deposition rate meant that this was not an issue here. Since the CaP coating was exposed to the entire surface, i.e., pillars and pores, then all areas were subjected to the same coating thickness, thereby preserving the etch height. The relatively dense, homogenous coating produced by sputtering of CaP is apparent in Fig. 10a, b, at ×300 and ×700 magnification respectively. With no tilt applied to the SEM imaging system there is the appearance of small circular CaP deposits in the flat regions surrounding the pillars. With the tilt applied these deposits are not apparent as illustrated in Fig. 10c, d.

As expected, post-deposition thermal annealing at 500°C has a significant impact on the substrate topography, not least the change in the CaP coating itself. Comparing Fig. 11a–d, obtained for the Si AN sample, with those discussed previously for the Si AD sample, the differences are striking. Where previously the CaP aggregations were noticeable only on the ‘pores’, in the annealed samples these CaP deposits are visible on the pillar surfaces as well. There also appears to be a certain degree of cracking in the annealed CaP coating, especially around 5–10 μm (larger) sized aggregations on the pillar surfaces but also on the “pore” surface, as shown in Fig. 11b. Figure 11c, d, obtained at a tilt of 55° indicate that the thermally annealed (500°C) coating is a dense coating that conforms very well to the underlying substrate, regardless of any change in height or structure that impedes it. As such, in conjunction with XPS, FTIR and XRD analysis, it is certain that the coating covers the entire surface area for in vitro investigation.
5 Biological characterisation

5.1 MTT viability assay

MTT results from SaOS-2 cells on silicon, etched silicon and 15 mTorr CaP coatings thereon, as shown in Fig. 12, indicate that with increasing time in vitro, cell viability is significantly increased on Si Et samples. For the Si Et samples, cell viability reaches its highest point on the sample after 10 days. Si AN samples show significant difference in cell viability when compared to Si AD samples. Comparing coated and uncoated samples, however, there is no statistically significant difference between Si Et and Si AN samples at 10 days.

5.2 ALP assay

ALP assays for SaOS-2 cells on the etched silicon samples and 15 mTorr CaP coatings thereon are illustrated in Fig. 13. The data shows that ALP activity is significantly elevated on the Si ET and Si AN samples after 10 days of cell culture compared with the Si control samples. Si AD samples exhibited significantly lower levels of ALP than controls at all timepoints.

5.3 ELISA

ELISA demonstrated the presence of osteocalcin, type I collagen and BMP-4 in the SaOS-2 whole cell lysates on the four different substrate types used in this study. As can be seen in Fig. 14c there was a significant increase in osteocalcin immunoreactivity over time with levels at a maximum on Si AN samples at day 10.

BMP-4 data presented in Fig. 15 suggests that at 10 days BMP-4 levels are significantly increased on the Si AN surface compared to control substrate. These data correlate well with the ALP assay data shown in Fig. 13, where ALP production was at a maximum on the Si AN sample at 10 days.

Collagen type I expression demonstrated by ELISA as shown in Figs. 16 did not present any appreciable difference in collagen type I levels between all the substrates studied at 48 h.

After 5 days in vitro however ELISA demonstrated a significantly elevated level of protein in SaOS-2 cells cultured on Si AN surfaces at both Day 5 and Day 10, whilst the remaining three substrate types are at levels commensurate with those observed in the control sample.

Fig. 9 SEM images Si Et a ×300 magnification, b ×1.2 k magnification and at a tilt of 55° at c ×700 magnification, and d ×1.5 k magnification
5.4 SEM of SaOS-2 on CaP coated etched silicon

SEM analysis was utilised to confirm osteoblast-like cell adhesion to silicon and the associated modified substrates. Fig. 17a–d illustrates SaOS-2 cells cultured on the four investigative substrates at 48 h. Individual cells with a cuboidal shape can be seen either in the process of spreading, dividing or forming contacts with surrounding cells. Figure 17b show SaOS-2 cells spreading on the Si Et surface. It is apparent that a monolayer of cells has attached to this substrate after 48 h in vitro. At 48 h (Fig. 17c, d), SaOS-2 cells are randomly distributed across the surface and have attached to different aspects of the surface features, including the top of pillars as well as the surrounding ‘base’ area of the pillars. Indeed, there is a high degree of interplay and communication between cells afforded by their proliferations and extensions and by the extracellular matrix that is distributed across the substrate. It is apparent that those cells at early stages of attachment have aligned to the edges and corners created by the etched silicon pillars.

After 5 days culture a monolayer of cells, evenly spread and distributed across the sample surface results for the Si substrate is evident as shown in Fig. 18a–d. All samples both coated and uncoated, demonstrate similarly consistent cell morphologies after 5 days in vitro.

A dense monolayer of cells consistent in depth and thickness, which has enveloped the underlying pillars and assumed the morphology of the substrate, is presented for each of these substrates.

It would appear that the consistency, appearance and definition of this cell monolayer is conserved even after 10 days in vitro. Figure 19a–d illustrate SaOS-2 cell morphologies consistent with the descriptions given for the samples at 5 days described above.

The most apparent difference in the cell layers observed is that the individual substrates have conferred their morphology on the cells and associated matrix formation. Figure 18a shows a dense and homogenous flattened cell layer characteristic of the underlying n-type Si sample, while Fig. 18b illustrates the typical layer seen at all time periods on the Si Et sample. Figure 19c, d show this SaOS-2 monolayer at 10 days on both the Si AD and Si AN samples, in a dense configuration similar to the situation for the Si Et surface.
6 Discussion

In this research, we considered the chemical, physical and biological characteristics of 15 mTorr CaP coatings on a silicon substrate, both in the as-deposited state and also thermally annealed post-deposition (500°C). The assertion by Curtis and Wilkinson that larger numbers of surface discontinuities lead to greater complexity in cellular response, guided the design and realisation of a 12-sided pillar shape utilised in this study. This substrate was then used in the subsequent cellular assays. The topography and morphology of the four different etched silicon samples, namely Si, Si Et, Si AD and Si AN were considered here.

Prior to annealing at 500°C the Si AD coatings had a distinct lack of crystallinity, as might be expected at such a low deposition power [36]. Both the FTIR and XRD data...

![Fig. 11 SEM images Si AN a ×300 magnification, and b ×1.2 k magnification and at a tilt of 55° at c ×700 magnification, and d ×1.5 k magnification](image)

![Fig. 12 MTT assay for SaOs-2 cells on silicon surfaces, as a function of time in vitro (un-coated un-etched silicon control)](image)
Fig. 13 ALP assay for SaOs-2 cells on silicon surfaces, as a function of time in vitro (un-coated un-etched silicon control)

Fig. 14 Osteocalcin detection using ELISA kit where (*) indicates a significant difference ($P \leq 0.05$) when compared to the control

Fig. 15 BMP-4 detection using ELISA kit where (*) indicates a significant difference ($P \leq 0.05$) when compared to the control

Fig. 16 Collagen I detection using ELISA kit where (*) indicates a significant difference ($P \leq 0.05$) when compared to the control
Fig. 17  Si a 2.5 K magnification, Si Et b 1.5 K magnification, Si AD c 1 K magnification, Si AN d 1.2 K magnification. At 48 h

Fig. 18  Si a 0.35 K magnification, Si Et b 1.0 K magnification, Si AD c 1.0 K magnification, Si AN d 1.0 K magnification. At 5 days
results clearly demonstrate this, showing broad, poorly resolved peaks prior to post-deposition annealing, and an amorphous background hump in the XRD pattern for the unannealed Ca–P coating. The AFM and XPS results for these Si AD coatings do, however, indicate that the Ca–P layers are continuous and coherent. In comparison, the data obtained for the Si AN coatings showed distinct differences from the same coatings prior to annealing at 500°C. The XRD results provide direct evidence for a significant improvement in the crystallinity of the coatings. It is also important to note that this improvement in the crystallinity of the coating coincides with increased hydroxylation of the coating, as observed in the corresponding FTIR spectra. It has been documented that the scale of ordering in HA materials is a consequence of the degree of hydroxylation [36–40]. The high Ca/P ratios obtained for these Ca/P coatings deposited using a stoichiometric HA sputter target is not entirely unexpected and has also been reported previously [36]. It has been postulated that this phenomenon can be a result of the evaporation of volatile phosphorus species from the surfaces of such sputter deposited samples [36–38].

Despite this it was evident from the XRD, FTIR, XPS, SEM and AFM data that the Si AN coatings closely resemble HA, rather than an amorphous Ca–P phase. In particular, the XRD data for the annealed coating shows the presence of only a HA phase, albeit with preferred 002 orientation. The corresponding FTIR data also confirms the material to be HA due to the presence of the well-resolved $\text{PO}_4^{3-}$ bands between 1,200 and 900 cm$^{-1}$ and 650–400 cm$^{-1}$ and OH$^-$ peaks at 3,570 and 630 cm$^{-1}$ [20–24]. The XPS data also indicates that the coatings produced here on silicon substrates are not entirely stable, with the substrate clearly visible after 10 days of exposure to the cell culture media. This may have a significant influence on the ability of cells to adhere to the surface and form confluent layers.

Optical profilometry data was acquired for CaP coatings both as-deposited and annealed (500°C) on a native n-type silicon substrate surface (data shown in Table 1). Thermal annealing did not increase the Ra value of the CaP coating, a non statistically relevant increase was shown by AFM and a relevant decrease was shown by optical profilometry. This may be due to a joining or smoothing of the aggregates and is typical of some CaP coatings; it is generally accepted to be due to restructurative grain growth processes caused during thermal processing. It is postulated therefore that if the underlying silicon substrate can be etched in a manner that allows the overlying CaP coating to conform to the substrate geometry. The CaP coating can then confer a bioactive surface to this controllable underlying Si substrate. This may then result in a bioactive
surface with appropriate morphological and chemical parameters that can be used for biological applications [41]. Biological investigations have demonstrated that surface chemistry and topography may induce maturation and improved differentiation in the human osteoblast cell line SaOS-2, without causing a significant detrimental effect on cell viability. Results indicated that osteoblasts cultured on Si AN surfaces caused a significant increase in alkaline phosphatase activity and osteocalcin synthesis. As the synthesis of ALP activity is an early phenotypic marker for mature osteoblast cells our results suggest that our surface characteristics stimulated an early stage of osteoblast differentiation.

In contrast to ALP, osteocalcin is a phenotypic marker for a later stage of osteoblast differentiation, one that coincides with the process of osteoblast mineralisation. Osteocalcin is one of the major noncollagenous proteins specific to mineralized connective tissue in vertebrates [42, 43]. In summary these results indicate that stimulated maturation and differentiation of SaOS-2 cells can be affected at various levels, from early to intermediate and end stages of the cell differentiation process.

BMPs also play an important role in the process of bone formation and remodeling [44]. While the increased expression of markers such as ALP, type I collagen and osteocalcin are indicative of the stimulation of osteoblast cell differentiation [45], the BMPs are also implicated in fracture healing, prevention of osteoporosis and enhancing the response of bone tissue around alloplastic materials implanted in bone [44, 46]. BMP2 has been shown to have bone inductive properties [47] while BMP4 has been shown to induce the differentiation of osteoblast cells [48, 49]. Our study points to the increased production of BMP4 in SaOS-2 cells cultured on Si AN surfaces. Taken together these observations indicate that surface topography and chemistry stimulates osteoblast differentiation at various stages (from osteoprogenitors to terminally differentiated osteoblasts) in SaOS-2 cells. It would, therefore, suggest that chemistry and topography may be beneficial in stimulating osteoblastic activity to promote bone formation.

Whilst a number of varying topographies have been created to capitalise on the effects that a micro-environment might induce in osteoblast-like cells, including primarily grooves and ridges in a wide range of substrate types. Photolithographic etching of silicon to produce regular features with known dimensions has been employed here. In combination with a sputter deposited 15 mTorr CaP coating, a novel substrate type, combining known chemical and morphological features has been created. The results presented suggest that the sputter deposited CaP coatings possess sufficient characteristics for their use in an in vitro situation. Biological analysis suggests that a semi-crystalline CaP coating on a surface with well-defined topographical features is capable of supporting osteoblast-like cell attachment, proliferation and differentiation, for short-term in vitro testing. Therefore an optimal combination of CaP coating chemistry and topography could be utilised for bone-specific tissue engineering applications.

References

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