JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 411–415

A new way of incorporating silicon in hydroxyapatite (Si-HA) as thin films

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Bioactive silicon-containing hydroxyapatite (Si-HA) thin films that can be used as coatings for bone tissue replacement have been developed. A magnetron co-sputtering technique was used to deposit Si-HA films up to 700 nm thick on titanium substrates, with a silicon level up to 1.2 wt%. X-ray diffraction demonstrated that annealing transformed the as-deposited Si-HA films which were amorphous, into a crystalline HA structure. A human osteoblast-like (HOB) cell model was used to determine the biocompatibility of these films. HOB cells were seen to attach and grow well on the Si-HA films, and the metabolic activity of HOB cells on these films was observed to increase with culture time. Furthermore, mineralisation of the cell layers was observed after 8 weeks of culture. Based on the present findings, Si-HA of different film compositions demonstrate bioactive properties *in-vitro*, and indicate the potential as biocoatings for a wide variety of medical implants including load-bearing applications such as the femoral stem of hip replacement implants.

1. Introduction

The similarity between synthetic hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA] and bone mineral has led to its extensive use in dental, orthopaedic and other medical applications, either in bulk form, as a filler or as a coating [1]. HA-coated metal implants have been widely used in load-bearing applications such as artificial hip replacement implants, due to the mechanical properties of the metal combined with the excellent bioactivity of the HA [2, 3]. Plasma spraying is a commercially popular deposition method to produce thick films and is the most frequently used method for fabricating HA coatings on implant surfaces [4–7]. Despite the clinical success of these plasma-sprayed HA coatings, there remain some problems related to delamination, resorption, chemical or mechanical instability with time if long-term implants are required [8, 9]. As an alternative technique to coat HA on metal implant surfaces, magnetron sputtering has been investigated in order to improve the mechanical and chemical properties of the coatings [10–12]. Magnetron sputtering has the advantages of achieving thin coatings with excellent adhesion, uniform thickness and the ability to coat implants with complex three-dimensional geometries.

Carlisle has demonstrated that silicon (Si) intake is essential for growth and skeletal development in chicks, as a Si-deficient diet resulted in significantly reduced weight gains and profound changes in the cartilage and bone [13]. In addition, Carlisle reported the presence of Si *in-vivo* within mineralising osteoid regions in rats and mice, suggesting that Si plays an important role in the bone mineralisation process [14]. These results were similar to those observed in rats by Schwarz and Milne [15]. In order to take advantage of the benefits of the enhanced biological effects of Si, it is thought that incorporating Si into HA will improve the bioactivity of the ceramic. Gibson *et al.* have demonstrated that the incorporation of Si into HA enhances the osteoblast cell activity and apatite formation in simulated body fluid (SBF) [16] and a study by Patel *et al.* using a rabbit model showed a significant increase in bone apposition at the surface of Si-HA implants [17].

To improve further the osseointegration at the coated metal implant/bone interface, Si has been introduced into the HA coating. In order to produce Si-HA coatings using the plasma spraying technique, Si-HA powder has to be synthesised first. Several authors have demonstrated the possibility of obtaining Si-HA powder via the wet precipitation route, but this proves to be tedious and requires stringent control over the processing conditions and reactants used [18, 19]. Thus, the application of magnetron co-sputtering to deposit Si-HA as thin films on metallic surfaces allows flexibility to control the level of Si in HA whilst retaining

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its phase purity. In this study, the ability of these Si-HA films to support the growth of human osteoblast-like (HOB) cells was assessed.

2. Materials and methods

2.1. Magnetron co-sputtering process

Pure titanium (Ti) plates were used as the substrates. They were roughened using silicon carbide paper of grade 1200, after which they were cleaned ultrasonically for 30 min in acetone before rinsing with deionised water and drying. Pure Si and slip-cast phase pure sintered HA were used as the two sputtering targets.

Full details of the experimental set-up of the magnetron co-sputtering system are described elsewhere [20]. Film composition was controlled by the relative power supplied to each sputtering target: 40–60 W radio frequency (rf) for the HA, and 3 W direct current (dc) for the Si. The total deposition time was 4 h, at an argon (Ar) pressure of 5×10^{-3} Torr, using a constant gas flow. The as-deposited films were then annealed at a temperature of 700 °C for 3 h under a constant flow of moist Ar gas.

2.2. Film characterisation

The film thickness was determined by masking a portion of a Si substrate with a layer of aluminium foil during the deposition and then removing the foil from the substrate, leaving a step in the film. This step was measured using a profilometer. Phase identification was performed using X-ray Diffraction (XRD), with Cu K_α radiation, operating at 40 kV and 40 mA. Data were collected over a 2θ range of 25° to 35°, with a step size of 0.03° and a dwell time of 20 s. Infrared spectra of the films were obtained by Fourier Transform Infrared Spectroscopy (FTIR), utilising the grazing angle technique. The spectra were collected between 700 cm^{-1} and 4000 cm^{-1} at a resolution of 4 cm^{-1}. Energy Dispersive X-ray Spectroscopy (EDS) was used to determine the approximate Si content.

2.3. AlamarBlue™ proliferation assay

The ability of the annealed magnetron co-sputtered Si-HA films to support the growth of human osteoblast-like (HOB) cells (Promocell, UK) was evaluated by the alamarBlue™ assay (Serotec, Oxford, UK). Si-HA coated Ti substrates were sterilised in ethanol for 48 h before treatment with ultra-violet radiation for 30 min. HOB cells (1×10^4 cells/test) were then seeded directly on the surface of Si-HA films and on the surface of roughened Ti substrates (acting as a control) before incubating at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. At days 3, 7 and 14, the metabolic activity of the cells on each sample was determined by the alamarBlue™ medium. The absorbance was measured on a plate reader at a wavelength of 570 nm and a total of 6 replicates were performed for each sample.

2.4. Immunofluorescence of actin and nucleus

After culturing for 4 days, HOB cells were fixed with 4% paraformaldehyde/phosphate buffer solution (PBS) with 1% sucrose for 15 min, washed with PBS and permeabilised at 4 °C for 5 min. Following that, cells were incubated with 1% bovine serum albumin (BSA)/PBS at 37 °C for 5 min to block the non-specific binding. FITC conjugated phalloidin (1:100 in 1% BSA/PBS, Sigma, Poole, UK) was then added at 37 °C for 1 h. After washing three times with 0.5% Tween 20/PBS for 5 min, DAPI was added at 37 °C for 5 min. The cells were then given a final wash (5 min × 3) before mounting on the Vectorshield fluorescent mountant (Vector Laboratories, Peterborough, UK) and viewed under a Digital Leica Fluorescence Microscope (LFM).

2.5. Cell morphology

After 4, 20 and 56 days in culture, the samples were rinsed with PBS after decanting the medium, and finally freeze-dried. They were then coated with a thin layer of carbon before examination with a Field Emission Gun Scanning Electron Microscope (FEG-SEM).

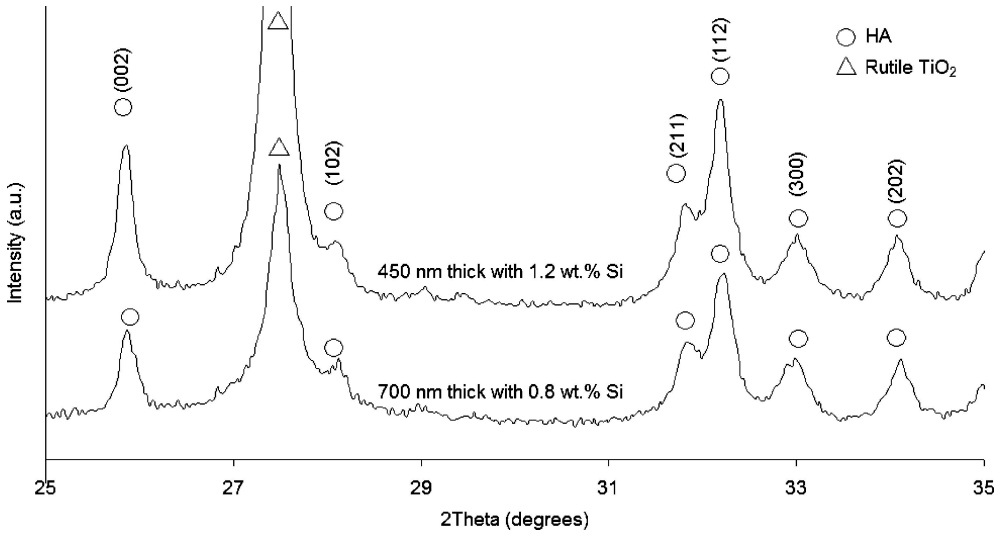

Figure 1 XRD patterns of annealed Si-HA thin films.

2.6. Statistical analysis

A 'pooled' t-test was used in this study to determine whether any significant differences existed between the mean values of the experimental groups. A difference between groups was considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Film characterisation

Si-HA films showed a uniform thickness of 450 nm following deposition with a discharge rf power level of 40 W on the HA target, and 700 nm for a discharge rf power level of 60 W. The Si content was approximately 1.2 and 0.8 wt%, respectively. The films are significantly thinner as compared to plasma-sprayed coatings, which generally range between 50 and 200 μm [21, 22]. Film thickness and Si content may be controlled by the relative power level applied to each target. The thicker film with a lower Si content was obtained at a higher discharge rf power level on the HA target because the HA deposition rate increased. However, this higher discharge rf power reduced the Si content in the film, since the dc power level on the Si target was kept constant throughout the study.

XRD showed that the annealed films were crystalline, with HA diffraction peaks appearing (Fig. 1). In addition, a peak corresponding to rutile titanium oxide (TiO_2) was observed, indicating that the Ti substrate was oxidised during annealing. FTIR spectra revealed that the annealed films showed sharp P─O bonds at 1082, 1037, 962 and 935 cm^{-1}, and an O─H bond at 3571 cm^{-1}; all of which can be attributed to HA (Fig. 2). Thus, by proper adjustment to the processing conditions, Si-HA thin films with a crystalline apatite structure can be achieved by magnetron co-sputtering. It is known that an amorphous coating with the presence of secondary calcium phosphate (CaP) phases tends to decrease the chemical stability and enhance the degradation or resorption of the coating with time [23, 24]. Table I summarises the properties of annealed Si-HA thin films.

3.2. Growth of HOB cells

Fig. 3 shows the cell growth on different samples. The cell activity was found to increase with culture time, indicating that the samples were able to support the growth of HOB cells. When the samples were compared at each time point, the annealed Si-HA films showed

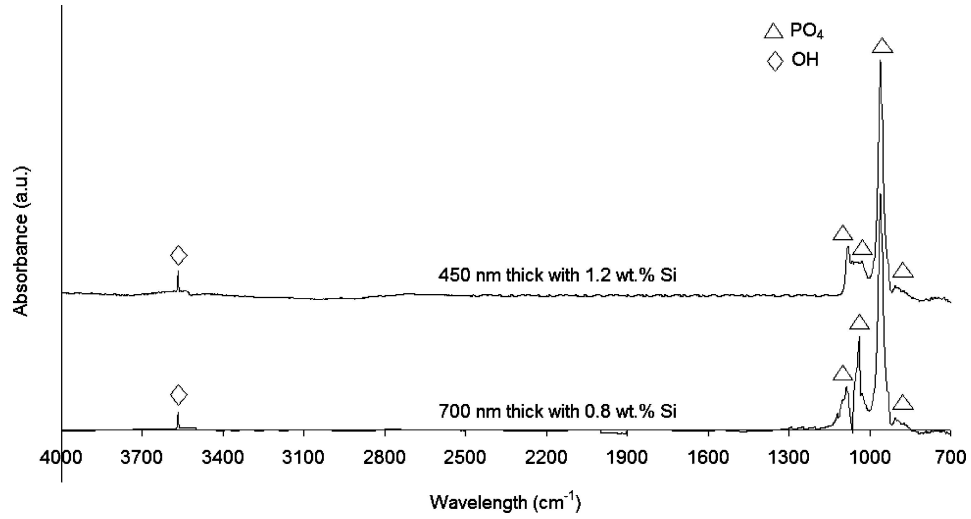

Figure 2 FTIR spectra of annealed Si-HA thin films.

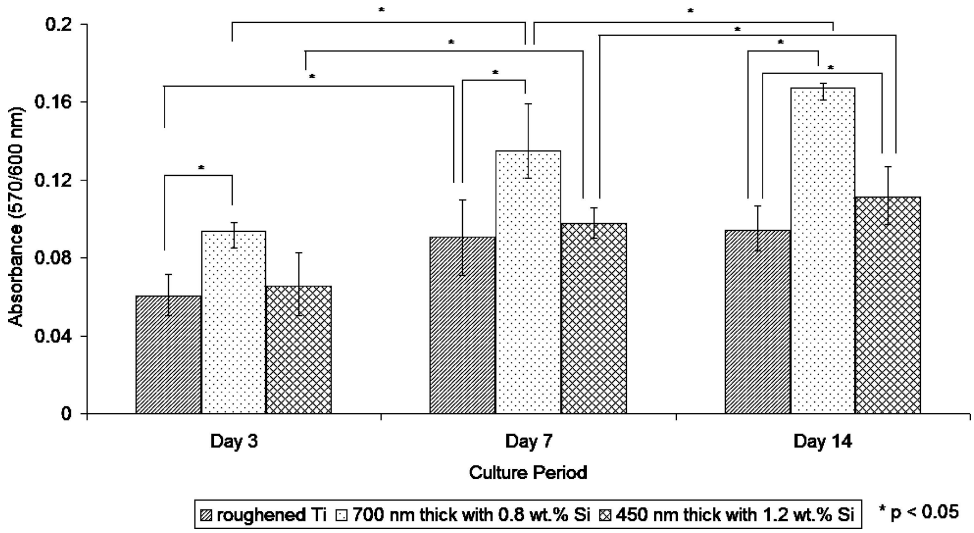

Figure 3 Cell activity on roughened Ti and annealed Si-HA thin films at various culture times.

statistically significant increase at all time points. This effect was most noticeable for 0.8 wt% annealed Si-HA films, and was in agreement with a study which indicated that a substitution of 0.8 wt% Si in HA resulted in the highest level of metabolic activity of HOB cells [16]. In addition, the metabolic activity of HOB cells on 0.8 wt% annealed Si-HA films was higher than that on roughened Ti surface at all time points. However, a significant increase was seen at day 14 when comparing the 1.2 wt% annealed Si-HA films and the roughened Ti surface. This result is supported by a study which found that proliferation and differentiation of rat parietal bone cells were higher on HA (without Si addition) substrates than on Ti [25].

TABLE I Properties of annealed Si-HA thin films

rf (W)	*dc* (W)	*t* (nm)	Si (wt%)	Crystal structure	Molecular structure
40	3	450	1.2	crystalline HA	P—O + O—H bonds
60	3	700	0.8	crystalline HA	P—O + O—H bonds

rf: radio frequency power source at HA target.
dc: direct current power source at Si target.
t: film thickness.

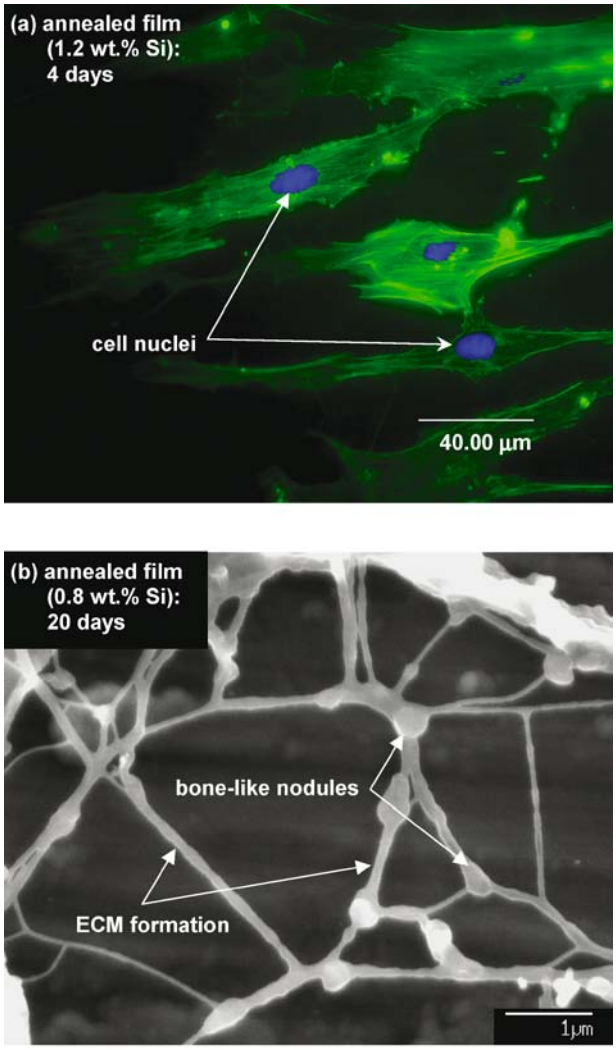

Figure 4 Images showing (a) actin (green) and nuclei (blue) cytoskeleton for HOB cells on annealed Si-HA thin film at culture day 4 (LFM); (b) formation of extracellular matrix (ECM) at culture day 20 (SEM); and (c) mineralisation of cell layers at culture day 56 (SEM).

3.3. Morphology of HOB cells

There was no significant difference seen between the two different film compositions. HOB cells were able to attach readily on the annealed Si-HA films after 4 days of culture, with filopodia anchoring on the film surfaces. Cell nuclei were considered to exhibit normal phenotype morphology. In addition, cells demonstrated well-spread morphology with actin stress fibres seen throughout the cells on the film surfaces (Fig. 4(a)). Confluent and well-spread cells existed on the film surfaces after 20 days of culture. Extracellular matrix (ECM) with the appearance of bone-like nodules, was also produced (Fig. 4(b)). EDS using a spot analysis confirmed the presence of calcium and phosphorus on the ECM, indicating that mineralisation was taking place. The formation of a mineralised layer with the appearance of CaP crystals, was observed on the cell surfaces of the annealed Si-HA films after culturing for 56 days (Fig. 4(c)). All these results indicate that Si-HA coatings enhance the growth of HOB cells, providing preferential sites for cell attachment which in turn, recruit proteins and other growth factors to promote cellular activity [26].

4. Conclusions

Si-HA thin films of less than 1 μm with Si content up to 1.2 wt% were successfully produced via magnetron co-sputtering. XRD revealed that the annealed Si-HA films possessed a crystalline HA structure. These films were able to support the attachment and growth of HOB cells, and subsequently mineralisation of the cell layers during the 8-week *in-vitro* cell culture study. Based on the excellent bioactivity of magnetron co-sputtered Si-HA thin films, it is suggested that this material can be applied as a bioactive coating on metal implants to enhance fixation.

Acknowledgments

Financial support for E. S. Thian from Girton College (University of Cambridge, UK), Cambridge Commonwealth Trust (University of Cambridge, UK), Lee Foundation (Singapore), and Sir Richard Stapley Educational Trust on behalf of GlaxoSmithKline Healthcare

Award (UK) are gratefully acknowledged. Support was provided by The Royal Academy of Engineering (UK), Armourers & Brasiers' Company (UK), and British Alcan Aluminium (UK), to enable E. S. Thian to present this paper orally in the 7th World Biomaterials Congress held in Sydney, Australia. Thanks also to Almath Crucibles Ltd., UK for help in the fabrication of the sintered HA target.

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Received 1 July
and accepted 1 November 2004