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Evaluation of Osteoblast-like Cell Responses to Surfaces Patterned with Hydroxyapatite Nanopowders on Ti Surfaces

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ABSTRACT

Hydroxyapatite (HA) is a widely used biomaterial for coatings on prostheses which are used to repair, replace and restore the function of traumatised or diseased bone. In the present study, template-assisted electrohydrodynamic atomisation (TAEA) was used to pattern Ti surfaces (convex, concave and flat) with different HA nano particles in order to evaluate the cellular responses. A comparison of biocompatibility between surfaces coated with locally prepared (SL) and commercially available HA nano powders was carried out. The cytotoxicity and cellular responses to these substrates (ie., cell adhesion, morphology and proliferation) were evaluated using human osteoblast-like cells (HOS) in vitro. The results demonstrate that Ti surfaces coated with HA (SL) and HA (commercial) supported HOS cell attachment and proliferation without eliciting any deleterious substances. Moreover, retention of cell viability and morphology of osteoblast-like cells were observed once cells were in contact with the surfaces and were comaparable to that of the tissue culture control. Interestingly, cells attached to HA (SL) elicited a better response. Proliferation of cells on convex surfaces was relatively higher in comparison to that of the concave and flat surfaces; which could be due to increased surface area of the HA (SL).

Keywords

Hydroxyapatite, Diseased bone, Titanium, Cells.

Introduction

The ability of the human body to regenerate or reconstruct damaged or abnormal bone is limited. Therefore, biomaterials which may be of biological or chemical origin and biocompatible are being developed to repair, replace and restore the function of traumatized or diseased soft and hard tissue. However, over the years, there has been a significant change in emphasis on biocompatibility. A common characteristic of bone is the strong affiliation between the inorganic and the organic phases [1] while diverse bone substitutes affect proliferation and differentiation rates of osteoblasts and also have an effect on their morphology [2]. Due to biocompatibility and bioactivity, calcium phosphate materials have gained significant attention as bone substitutes [3] while hydroxyapatite (HA) has been used in many clinical conditions [4]. However, major drawbacks of porous calcium phosphate ceramics including HA is their mechanical weakness and difficulty in manufacturing specific shapes, limiting their applications as bone analogue material [5].

Therefore, due to mechanical properties being closer to those of bone, titanium (Ti) and its alloys are being used as artificial joints and teeth roots and have been commercially available for over three decades. In addition to its high strength-to-weight ratio, corrosion resistance, biocompatibility and cost effectiveness have been recognized and thus have a strong technical choice by the medical profession.

However, independently of substrate chemistry, substrate topography has been reported to have significant effects on cell behaviour. Studies have revealed that with the appropriate microstructural dimensions, bioactive HA is ideal as it encourages direct bone apposition rather than fibrous tissue encapsulation of

the implant [6]. Interestingly, Anselme and Bigerelle [7] reviewed the role played by the surface topography on cell attachment to biomaterials in which it was concluded that surface roughness enhances bone cell attachment and adhesion. Another study showed that morphology of the roughness was also a highly significant factor [8]. Meyle et al. [9] have demonstrated that even small modifications in roughness prompt cell responses. However, according to the review done by Anselme and Bigerelle, the majority of work has revealed that cell proliferation was increased on smooth surfaces [7]. They also stated that changes of the surface roughness modify the surface chemistry which would influence the results. This fact was supported by another study carried out by Herath et al. [10]. Accordingly, interferences done to change surface roughness, could frequently fabricate surface chemical changes and these residues may interfere with original adsorbed protein conformation [10].

However, their study on osteoblast response to zirconia surfaces with different topography which were prepared with great care eliminating such contaminations, demonstrated that the initial cell attachment, spreading and bone formation were much better on abraded surface whereas the long-term cell proliferation and expression of phenotypic markers were high on polished surfaces.

According to Kuroda & Okido, it would take a comparatively long time to attach the metallic implant to bone when implant surfaces do not undergo surface modification [11]. Therefore, many approaches have been made to improve osteoconductivity while one of those is applying coatings of bioactive compounds. This approach would accelerate bone formation on metallic implants and enhance bone in-growth and hence implant materials with bioactive coatings have been used in clinical set ups. Thus, introduction of surface structures using biocompatible HA supported to the better candidate for enhanced osteoblast response.

In the present study, template-assisted electrohydrodynamic atomisation (TAEA) spray-patterning was used to prepare coatings on Ti surfaces [12]. Further, this method allows the production of interlocked bioactive coatings on orthopaedic metallic substrates allowing better adhesion of the coating. In addition to using commercial HA, HA nanoparticles were prepared using modified calcium sucrate route for the application on Ti surfaces [13]. Finally, cellular responses were determined when in contact with the material surfaces (in order to evaluate the biocompatibility of the materials).

Experimental Details

Materials

Square shape Ti samples with dimensions of 10 mm x 10 mm were cut using commercially available flat Ti substrate (Sigma Aldrich, UK). Thickness of the samples was 0.5 mm. Curved Ti samples were cut using commercially available pure Ti tubing (Advent Research Materials, Oxford, UK). The diameter of both the convex (outer) and concave (inner) was 10 mm and the thickness of the samples was 0.5 mm. The Ti plates were then polished using silicon carbide grinding papers (No.800, No.1200, No.2400 and No.4000). Prior to TAEA deposition, each plate was degreased with acetone and rinsed with the solvent used for deposition, and then air-dried.

Synthesis and characterization of HA nanoparticles

The synthesis of HA was done through calsium sucrate route [13,14]. In this, 100 ml of 0.5M calcium sucrate solution was prepared using 2.8 g of calcined CaO (Sigma, UK) supernatant and impurities were removed by centrifugation. Followed by, 100 ml of 0.3 M ammonium dihydrogen orthophosphate (Sigma, UK) was added drop wise while stirring at 500 rpm and mixture was further stirred for 12 hours at ambient conditions. The pH of the mixture was maintained at a range of 8-12 using conc. NH3 solution whilst the addition of ammonium dihydrogen orthophosphate. Finally, precipitate was collected by centrifugation and washed three times with 50 ml of distilled water. The resulted precipitate was air-dried for 12 h and ground to fine powder.

Preparation of HA suspensions and patterned HA

Ethanol was added as a liquid carrier for both prepared and commercial HA powder to create a suspension with 6wt% concentration. The HA and ethanol mixtures were then stirred using a magnetic stirrer (Thermoscientific, USA) for 60 minutes. A concentration of 6wt % was prepared and kept constant to achieved electrohydrodynamic spray deposition in the stable cone jet mode, as found in the literature [15].

Ten samples from each type (flat, convex and concave) were patterned with commercially available HA (Sigma Aldrich, UK) as well as that produced in Sri Lanka using TAEA technique [16].

Suspension Characterization

The physical properties of the suspensions such as density, viscosity, surface tension and electrical conductivity were measured.

Deposit and cellular Characterisation

The morphology and microstructure of the TAEA deposits were observed and characterised using optical microscopy (OM, Olympus, Japan) and field emission scanning electron microscopy (SEM, Zeiss, German) to determine their geometry.

Cellular Responses to material surfaces

A human osteosarcoma cell line (HOS ATCC® CRL-1543TM) was used to determine the cellular responses. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Dorset, UK), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, 150 µg/ml ascorbic acid, 0.02 mM L-glutamine, 0.01 M HEPES, 100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin. Thermanox™ cover slips (TMX, Life Technologies, Paisley, UK) were used as the tissue culture control. HOS cells were seeded at a density of 2×10^5 cells ml^{-1} on test samples [flat (Type 1), concave (Type 2) & convex (Type 3) surfaces coated with hydroxyapatite, synthesised in Sri Lanka (SL) and by commercial and on controls [Thermanox™, C_2H_5OH] materials. The culture plates containing cell seeded materials were incubated at 37°C in a humidified atmosphere with 5% CO_2

and medium was replaced at intervals chosen to minimize the disturbance of the culture conditions.

MTT assay was performed to determine the cell viability and cytotoxic effects of the material. Extracts for the assay were prepared by eluting test samples in complete DMEM at 37°C for 1, 7, 14, 21, 28 and 35 days. HOS cells were then cultured in the extracts at 37°C in a humidified air at 5% CO_2 for 24 h and further 4 h incubation in the presence of 10% MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, USA). Dimethyl sulfoxide (DMSO, Sigma, USA) was added after removing the spent culture medium and mixed for 10 min to certify complete dissolution of crystals. Absorbance was measured on a micro-plate reader (Thermo, MultiskanEx, China) at a test wavelength of 570 nm and reference wavelength of 630 nm.

Cell proliferation was determined by performing an AlamarBlue™ assay. The test materials were seeded with HOS cells at a density of 2×10^5 cells ml^{-1} and were incubated at 37°C in humidified air with 5% CO_2 for 1, 7, 14, 21, 28 and 35 days. At regular time intervals the spent culture medium was replaced with care to minimize the disturbance of the culture conditions. At each time point the spent culture medium was removed from all the test wells and replaced with one ml of 10% alamarBlue™ solution (Sigma, USA) and was incubated at 37°C in a humidified air at 5% CO_2 for 4 h. The absorbance was read at 570 nm with a reference wavelength of 630 nm in the micro plate reader (Thermo, Multiskan Ex, China) after transferring 8 × 100 µl aliquots from the original well to a 96 well plate. The wells containing test samples and controls were washed with PBS after the incubation period and fresh culture medium was added and was further incubated. This was carried out serially for 1, 7, 14, 21, 28 and 35 days of incubation.

Samples were prepared for scanning electron microscopy by seeding HOS cells at a density of 2×10^5 cells ml^{-1}. They were incubated at 37°C in humidified air with 5% CO_2. Followed by 48h of incubation, the cells were fixed with 1.5% glutaraldehyde in 0.1 M sodium cacodylate, stained in 1% osmium tetroxide in 0.1 M sodium cacodylate and 1% tannic acid buffer and dehydrated using a series of aqueous alcohol solutions starting from 20%–70% in 10% increments. The samples were stained in 0.5% uranyl acetate alternative and further dehydrated in 90%, 96% and then 100% ethyl alcohol containing Na_2CO_3 and with hexamethyl-di-salazane and finally air dried. The sample surfaces were gold coated for examination under the scanning electron microscope (Zeiss, German) in duplicate at each time point for observation of cellular morphology and attachment.

Results and Discussion

Suspension characterization

Results showed that the synthesised hydroxyapatite created a denser, and electrically conductive suspension, whilst with less viscous and a lower surface tension. These characteristics aid electrodynamic spraying, resulting in more homogeneous and dense patterns and coatings.

Coating geometry

Scanning electron micrographs of interlocked coatings (TiO_2 pattern with HA overlay) are shown in the following Figure 1. Results showed that patterns yielded from HA (commercial) had mean diameter 96.5 ± 4µm, whereas the HA (SL) had mean diameter 98.6 ± 5µm, suggesting that the synthesised HA had more accurate patterning via TAEA.

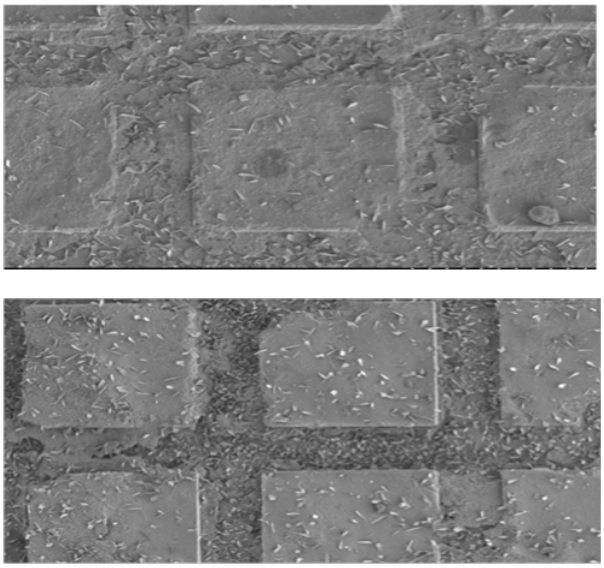

Figure 1: Optical micrographs of square mesh copper template with strut width 50µm and square spacing of 100µm depicting interlocked TAEA coatings of TiO_2 square pattern with HA overlay: 6wt.% HA commercial and HA SL (For both, flow rate, applied voltage and collection time, 20µl min-1, 10kV and 300s respectively) on convex substrate of titanium with diameter 10 mm.

Cellular Responses

Cytotoxicity to cell membrane

MTT assay confirms that HA synthesized in Sri Lanka (SL) and HA (Commercial) was not toxic to cell membranes (Figure 2). This colorimetric assay which is rapid and versatile, is based on the tetrazolium salt MTT which measures living cells quantitatively as the tetrazolium ring is cleaved in active mitochondria [17]. Furthermore, the appropriateness of this assay to measure the cytotoxicity to cells has been established [17-19]. Mosmann [20] shows that MTT is cleaved by all living, metabolically active cells but not by dead cells. According to the results obtained, high levels of metabolic activity were observed in cells attached to surfaces coated with HA of both types of HA.

There was a significant difference between the cytotoxicity of the toxic positive control (ROH) and the test samples, SL & commercial. Furthermore, pattern of the results of the test samples during the 35 day period was comparable to that of the negative non-toxic control, Thermanox™ (TMX). However, results of MTT assay showed a highly significant increase ($p<0.001$) in SL samples when compared to commercial samples. In contrast, when the values for samples of SL and TMX for the period were compared, the difference did not show a significant difference while difference was highly significant between commercial HA

and TMX ($p<0.001$). Accordingly, it is evident that test materials are non-toxic while metabolic activity of cells attached to SL was significantly higher than that of commercial HA.

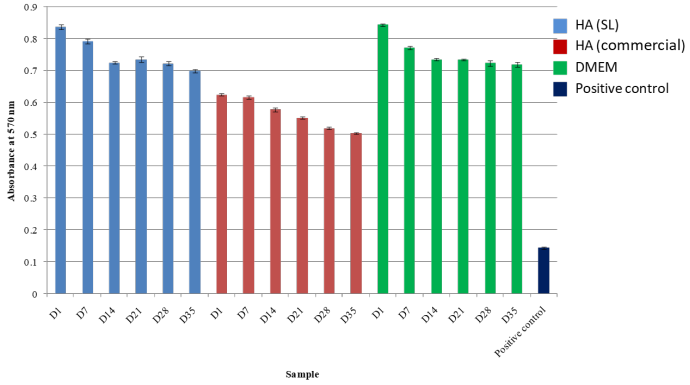

Figure 2: Histogram of MTT assay results for HA (SL), HA (commercial), negative (DMEM) and positive (C_2H_5OH) controls up to 35 days of incubation. Mean data plotted (error bars represent 95 % confidence limit, n = 8).

Cell growth and proliferation

SEM results demonstrated that the surfaces coated with both types of HA (SL and commercial) favoured cell adhesion and were capable to support regular osteoblast growth (Figures 3 and 4) and by 48 hours of incubation, HOS cells have spread on the material surface. Moreover, cells were approaching confluence on both surfaces by 48 hours indicating favourable atmosphere and greater survivability. Numerous cell filopodia were observed extending and spreading across the surfaces indicating a favourable surface for cell attachment.

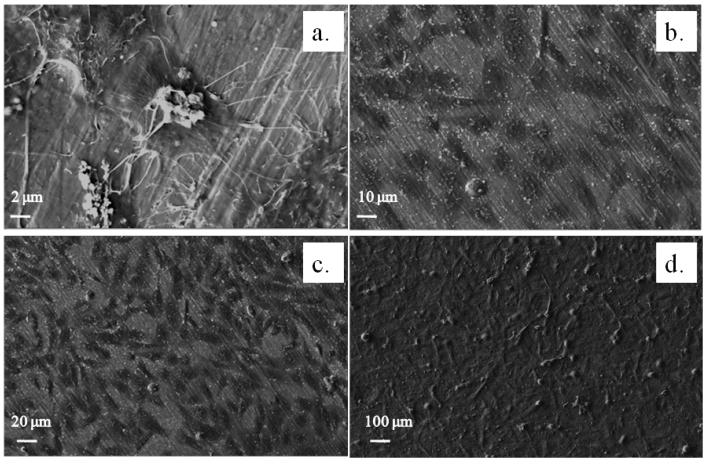

Figure 3: SEM images of HOS cells on surfaces coated with HA (SL) after 48h incubation under different magnifications.

As described by Dalby et al, HOS cells on all surfaces maintained the usual polygonal shape with few morphological variations which may be a response to the surface characteristics [6]. Lamellipodia which were attached to HA surface have made the cells flat and evenly spread on the surfaces. The rounded cells which were on the surfaces may be undergoing cell division initiating cell proliferation.

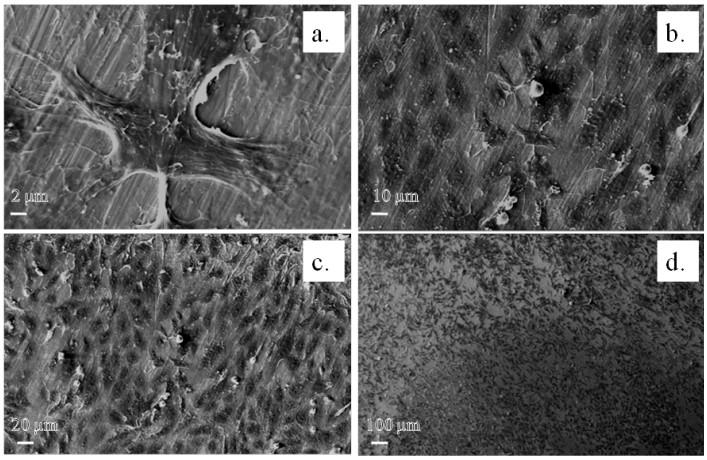

Figure 4: SEM images of HOS cells on surfaces coated with HA (commercial) after 48h incubation under different magnifications.

The pattern and the levels of proliferation of cells attached to different surfaces determined by alamarBlue™ assay [15,21,22,23] were comparable (Figure 5) throughout the study period. General pattern of the AlamarBlue™ results of HA (SL) and HA (commercial) were comparable to that of the TMX. Accordingly, proliferation of cells attached to the test samples and the negative non-toxic control was significantly higher than that on the positive toxic control ($p<0.0001$), while the proliferation reached the maxima between day 21 and day 28 followed by a decrease towards day 35. This indicates that cell proliferation on the test samples have reached their maximum levels well before the negative control (TMX).

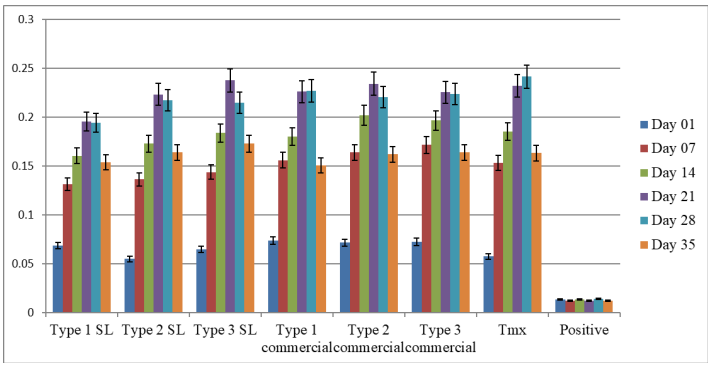

Figure 5: Histogram of alamarBlue™ mean assay results for HOS cells cultured on type 1 SL, type 2 SL, type 3 SL, type 1 Commercial, type 2 Commercial, type 3 Commercial, negative control (TMX) and the positive control (C_2H_5OH). (Error bars represent 95 % confidence limit, n = 8).

However, initial cell proliferation (day 1) was significantly higher on flat surfaces of both SL and commercial HA than that of the TMX ($p<0.001$ & $p< 0.0001$ respectively). However, the difference was not significant towards end of the study period. In contrast, a highly significant difference ($p<0.0001$) was observed when compared with the positive toxic control.

When the flat (Type 1) and concave (Type 2) surfaces of HA (SL) were compared, it was significantly high ($p <0.0001$) on Type 1 on

day 1, 14 and 28. The convex surfaces showed a marked increase ($p<0.01$) on day 7, 14, 28 and 35 while it was highly significant on day 21 ($p<0.001$). Moreover, the values for HA (commercial) convex (Type 3) surfaces were higher than that of the commercial flat (Type 1) surfaces while the difference was significant on day 14, 21 and 35 ($p<0.001$, $p<0.05$, $p<0.01$, respectively). These results indicate that performance of convex surface was better than that of flat and concave.

When the convex (Type 3) surfaces of commercial and SL were compared, cell proliferation on the former was significantly higher than that of the latter by day 14 ($p<0.01$) and day 28 ($p<0.05$) while it reached equal levels on rest of the time points indicating initial and long term proliferation was similar on both the surfaces.

However, when the proliferation between cells attached to SL concave (Type 2) and convex (Type 3) is compared, the latter was significantly high ($p<0.01$) on day 7. When concave and convex surfaces of commercial HA were compared, proliferation was high on concave surfaces ($p<0.01$) by day 14. According to overall performances, the highest proliferation was achieved by SL convex (Type 3) and commercial concave (Type 2) by day 21.

The overall results revealed that cells on convex surfaces showed better proliferation than on concave and flat surfaces. This may be due to the availability of more surface area on convex surfaces, allowing cells to undergo different developmental stages without being affected by the phenomenon, contact inhibition.

Conclusion

The test materials patterned with HA (SL) and HA (commercial) were non-toxic and comparable to the tissue culture control, TMX while metabolic activity of osteoblast-like cells on HA (SL) was higher, compared to that of HA (commercial). The proliferation of cells attached to flat, concave and convex surfaces of HA (SL) and (commercial), showed a gradual increase, reaching the highest between day 21 to 28. However, the results indicate that the curvature of the surface has not exerted a significant effect on the cellular responses although cells adhered to convex surfaces showed better performance. This could be due to the contact inhibition experienced by the cells attached to the concave and flat surfaces which is a common phenomenon encountered by anchorage dependent cell line.

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