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Nanoscale crystallinity modulates cell proliferation on plasma sprayed surfaces

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ABSTRACT

Calcium phosphate coatings have been applied to the surface of metallic prostheses to mediate hard and soft tissue attachment for more than 40 years. Most coatings are formed of high purity hydroxyapatite, and coating methods are often designed to produce highly crystalline surfaces. It is likely however, that coatings of lower crystallinity can facilitate more rapid tissue attachment since the surface will exhibit a higher specific surface area and will be considerably more reactive than a comparable highly crystalline surface. Here we test this hypothesis by growing a population of MC3T3 osteoblast-like cells on the surface of two types of hip prosthesis with similar composition, but with differing crystallinity. The surfaces with lower crystallinity facilitated more rapid cell attachment and increased proliferation rate, despite having a less heterogeneous surface topography. This work highlights that the influence of the crystallinity of HA at the nano-scale is dominant over macro-scale topography for cell adhesion and growth. Furthermore, crystallinity could be easily adjusted by without compromising coating purity. These findings could facilitate designing novel coated calcium phosphate surfaces that more rapidly bond tissue following implantation.

1. Introduction

Hydroxyapatite (HA) is the main mineral constituent of bone comprising almost 70 wt.% of the mass of our skeleton. Since HA is relatively simple to synthesise using both wet chemical and reactive sintering routes, it has been widely investigated for use as a bone graft replacement [1–3]. The osteoconductive nature of the HA also means that it has found significant use as a coating on metallic implants, where it can facilitate the attachment of both hard and soft tissues and allow firm attachment of the prosthesis in vivo [4].

There are a multitude of methods that have been employed to coat metallic prostheses with HA, which have recently been reviewed in detail [5]. The majority of clinically available prostheses, however, are coated using a high temperature plasma in which the HA is 'melted' before deposition on the implant surface [6]. It is possible to adjust the composition and crystallinity of the deposited coating however, by varying gas phase, atmospheric pressure and anode current. The use of high energy coating conditions can result in a considerable reduction in crystallinity [7], which may be coupled with the deposition of impurity phases within the coating. These impurities may include tetracalcium phosphate ($Ca_4(PO_4)_3O$), calcium oxide (CaO), amorphous calcium phosphate (ACP; $Ca_xH_y(PO_4)_z \cdot nH_2O$), β-tricalcium phosphate (β-TCP; $Ca_3(PO_4)_2$) and oxyapatite ($Ca_{10}(PO_4)_6O$) [8,9]. The formation of these phases within the implant coating has been shown to result in the accelerated localised dissolution of the coating [10]. Numerous workers have striven to eradicate the formation of these impurity phases from the coating [8–10]. As a consequence, the majority of commercially available calcium phosphate coatings are formed from highly crystalline HA. Although easy to characterise using X-ray diffraction and required by some standards, one might expect that the high crystallinity and low solubility of the HA deposited on the majority of prostheses would result in suboptimal early-stage tissue attachment. Other materials which facilitate bone attachment, such as bioglass, are thought to form an intimate adhesion with bone through the dissolution of their surface and their subsequent re-precipitation in surrounding tissues [11]. Whilst undoubtedly the localised dissolution of highly basic phases such as TTCP and CaO could have a deleterious biological reaction and so their formation should be avoided, it would seem surprising that more products are not based around the other more soluble calcium salts (ACP, oxyapatite etc.) that may facilitate this process.

In this study, the attachment of MC3T3 cells to the surface of two compositionally identical commercially available hydroxyapatite coated prostheses of distinct crystallinity has been investigated. The topography of both coatings was characterised using scanning electron microscopy and white light interferometry. Quantification of the crystallinity of the coatings was performed using X-ray diffraction and crystallite size was estimated from the XRD data using the Scherrer equation,

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whilst coating crystallinity was further varied by adjusting the anodic current during the coating process. In addition, elemental composition of the coatings was investigated using X-ray Fluorescence spectroscopy (XRF).

2. Materials and methods

2.1. Preparation of prostheses

Two types of carbonated hydroxyapatite coated hip prosthesis with similar composition but contrasting crystallinities were sourced directly from manufacturers (JRI; Depuy). Both types of prosthesis were sectioned using a diamond saw in order to produce samples of appropriate geometry for seeding in standard 12 well plates. Prior to characterisation, the prostheses were autoclaved for sterilisation. From here on, the prostheses will be referred to as group A (lower crystallinity) and group B (higher crystallinity).

2.2. Cell culture and attachment to prostheses

To evaluate cell attachment to each prosthesis coating, MC3T3 cells (European collection of cell cultures - ECACC) were defrosted and seeded into T150 flasks with Dulbecco's minimum essential medium (D-MEM) containing 10 w/v% foetal bovine serum. After they reached confluency (approximately two days), the cells were removed from the surface of the culture flask using trypsin-EDTA, were redispersed in medium at a cell density of 4.05×10^5 cells per mL and were then passaged 3 times prior to surface seeding. Sterilised (by autoclave and UV radiation) surfaces were seeded with MC3T3 cells at a density of 1×10^5 cells per sample. The number of cells on the surface of the implants were counted on days 1, 9, and 21 and compared with the control (tissue culture plastic). For counting, the live cells were stained using Calcein (a fluorescent green stain which only stains live cells) and were then visualised using a fluorescence microscope. At least five areas of the surface were imaged and then fluorescent cells counted within the field (1 mm^2), and the average cell number per unit area of the surface was subsequently calculated. The data were collected for at least three samples in each sample set, making each result the average of 45 measurements.

2.3. SEM images

The surface morphologies of the coatings were evaluated using scanning electron microscopy. The unembedded prostheses were mounted on aluminium stubs using carbon tape. The surface of the prosthesis was subsequently sputtered with platinum to prevent the sample charging. Following preparation, images were captured using a scanning electron microscope at an accelerating voltage of 10 kV. The embedded samples were examined using a scanning electron microscope forming an image using back scattered electrons, these images were used to determine the thickness of the implant coatings. The samples were unsputtered and charging was prevented by painting the sample surfaces using silver dag adhesive paint.

2.4. Interferometry

In order to enable quantitative characterisation of the topography of the calcium phosphate coatings, interferometric measurements of samples were performed using a MicroXAM interferometer (Scantron, UK), operating using a white light source. Samples were imaged at 31× magnification, acquiring images in a grid array which were subsequently stitched together. The final image had dimensions of 3.64 mm × 2.71 mm. Scanning Probe Image Processor software (Image Metrology, Denmark) was employed for the analysis of acquired images, yielding S_a and S_q values for surface roughness.

2.5. X-ray diffraction analysis

To characterise the crystalline component of the coating, the coating was carefully removed from the surface of the implant using a razor blade and X-ray diffraction measurements were carried out on the resulting powder using a Bruker D8 diffractometer, arranged in transmission mode using Cu Kα1 radiation (= 1.5406 Å) between 5° and 80° 2-θ, with a step size of 0.0197° and a step time of 0.2 s. The collected patterns were compared with JCPDS patterns for HA and any impurity phases likely to be found within the coating. The crystallinity of the coatings was compared by determining the area under the diffraction pattern for a strong peak indicative of the presence of HA. The resulting crystallinity of the material was normalised, so that the raw powder was given a value of 100. It should be noted that this gives a relative and not absolute measurement of crystallinity. To evaluate the influence of process conditions on crystallinity, the coatings were deposited using vacuum plasma spraying and the anodic current was varied between 400–700 A. Crystallite size was estimated from the XRD data using the Scherrer equation:

$$X_s = \frac{0.9\lambda}{FWHM\cos\theta} \quad (1)$$

where λ is the wavelength of the monochromatic X-ray beam (λ = 0.15406 nm for CuKα radiation), FWHM is the full width at half maximum of the diffraction peak under analysis [radians] and θ is the Bragg angle at which the peak is located. Diffraction peaks at $2\theta = 25.784°$ {002} and $2\theta = 32.981°$ {300}, which correspond to the c- and a-axes of the HA crystal lattice, respectively. These peaks were chosen due their isolation from the other diffraction peaks (enabling a more reliable estimation of crystallite size) and as they represent the major axes of HA crystal growth. Fraction of crystalline phase, X_c, was estimated using the following equation:

$$X_c = 1 - \frac{V_{112/300}}{I_{300}} \quad (2)$$

where $V_{112/300}$ is the intensity minimum between the {112} and {300} diffraction peaks of HA, and I_{300} is the intensity of the {300} diffraction peak of HA.

2.6. X-ray fluorescence analysis

To identify the elemental composition of the coatings X-ray fluorescence spectroscopy (XRF) was carried out using a S8 TIGER XRF spectrometer (Bruker Corp., U.S.A.). Samples were prepared as described for the X-ray diffraction measurements.

2.7. Statistical analysis

The data were expressed as the mean ± the standard deviation and analysed using Primer for biostatistics software. For the analysis of the cell attachment data one way analysis of variance (ANOVA) was used with a post hoc Tukey (HSD) test to identify significant differences at a confidence limit of $P < 0.01$.

3. Results

3.1. Cell culture and attachment to prostheses

When seeded onto the surface of the prostheses, cell attachment was noted within one day of seeding on each of the surfaces investigated (Fig. 1). The cells exhibited morphology typical of osteoblasts with an approximate diameter of 20 μm. At day one, the cells attached to the surface of Group A exhibited a polygonal morphology and those attached to the surface of the tissue culture plastic (Control) and Group