

Initial responses of human osteoblasts to sol–gel modified titanium with hydroxyapatite and titania composition

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Abstract

Sol–gel thin films of hydroxyapatite (HA) and titania (TiO₂) have received a great deal of attention in the area of bioactive surface modification of titanium (Ti) implants. Sol–gel coatings were developed on Ti substrates of pure HA and TiO₂ and two composite forms, HA + 10% TiO₂ and HA + 20% TiO₂, and the biological properties of the coatings were evaluated. All the coating layers exhibited thin and homogeneous structures and phase-pure compositions (either HA or TiO₂). Primary human osteoblast cells showed good attachment, spreading and proliferation on all the sol–gel coated surfaces, with enhanced cell numbers on all the coated surfaces relative to uncoated Ti control at day 1, as observed by MTT assay and scanning electron microscopy. Cell attachment rates were also enhanced on the pure HA coating relative to control Ti. The pure HA and HA + 10% TiO₂ composite coating furthermore enhanced proliferation of osteoblasts at 4 days. Moreover, the gene expression level of several osteogenic markers including bone sialoprotein and osteopontin, as measured by RT–PCR at 24 h, was shown to vary according to coating composition. These findings suggest that human primary bone cells show marked and rapid early functional changes in response to HA and TiO₂ sol–gel coatings on Ti.

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1. Introduction

The technique of sol–gel processing can be applied to form a uniform and well controlled surface layer on bulk titanium (Ti) implants. This approach aims to enhance the surface characteristics of the Ti implant and improve host tissue bonding strength and corrosive resistance, whilst still maintaining the overall favourable mechanical properties of the bulk Ti substrate, such as high strength and fracture toughness [1]. Hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) and titania (TiO₂) represent two promising coating compositions on Ti surfaces that have been clinically applied.

HA has been widely investigated as a coating material due to its biological and chemical similarity to the inorganic

phases of bones and teeth [2]. HA coatings on Ti result in enhanced bone formation and apposition [3] and improve fixation to adjacent bone in comparison to uncoated Ti [4]. However, the unsatisfactory bonding strength of the HA layer with respect to the Ti substrate, particularly in the case of plasma-sprayed thick coatings, has led to biological concerns regarding HA particulate debris and consequent inflammation reaction [5,6].

The parallel development of TiO₂ coatings on such implants has shown promise. A variety of methods, such as anodization, the sol–gel process and thermal oxidation, have been applied and have shown improved corrosion resistance of Ti and enhanced biological properties [7,8]. Moreover, TiO₂ coatings have been reported to improve the biocompatibility of Ti by allowing the formation of an O–H bond in TiO₂ under moist conditions [9].

The sol–gel technique produces a surface coating of high chemical homogeneity and purity, with the potential to mix

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the molecular percentages of HA and TiO₂ within the coating in order to optimise the Ti implant surface in terms of mechanical and biological performance. Sol–gel process methods additionally have a practical advantage of being applicable at mass-production level and of being a simple, relatively low temperature means of surface modification for complex implant shapes [10]. Moreover, materials prepared by the sol–gel process have been shown to be more bioactive than those of the same composition prepared by other methods, with improved calcium and phosphate precipitation onto the implant surface [11,12].

In previous work, this group has manufactured, characterized and mechanically tested both pure and composite HA and TiO₂ sol–gel coatings on Ti [13]. The HOS osteoblast-like cell line was then used to assess biological response to various coating compositions in terms of proliferation over 5 days and alkaline phosphatase (ALP) production at 7 days. While no significant change was found in proliferation, marked changes in ALP activity were found with HA/TiO₂ coated surfaces [13]. Ramires et al. [14] also found altered expression of other differentiation markers at 7 days using the MG63 osteoblast-like cell line, and confirmed these findings with rat primary osteoblasts [15], although different HA/TiO₂ compositions and manufacture methods were used. An absence of cytotoxic effects for HA/TiO₂ sol–gel coatings, assessed by viability assays in material extracts at 3 days, was also reported [15]. Furthermore, *in vivo* data from an HA/TiO₂ sol–gel composite coated implant in rabbits produced greater torque removal values and bone-to-implant contact than an uncoated Ti equivalent [16].

However, despite such promise for the HA/TiO₂ coatings, and in particular those developed by a sol–gel approach, knowledge of the early biological response of bone to such surface treatments is very limited. Sol–gel derived thin HA films manufactured on Ti showed reduced attachment characteristics for HOS cells [17], while TiO₂ implant coatings, applied by plasma-spray methods, altered attachment characteristics and early morphology of MG63 cells [18]. Variation in the attachment and early growth characteristics of bone cells to different HA/TiO₂ sol–gel coatings could be an important consideration in choosing optimal surface characteristics for future *in vivo* studies. This study aimed to assess, for the first time, the response of primary human bone cells to Ti substrates coated with pure and composite HA/TiO₂ sol–gel coatings. Attention to early cellular responses, including the expression of several bone-associated genes known to play key roles in the growth and function of bone, was also examined.

2. Materials and methods

2.1. Preparation of HA, TiO₂ and their composite coatings

The preparation of the sol–gel coatings was slightly modified from our previous studies [13,19]. As sol–gel precursors for HA, calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O;

Sigma–Aldrich, Dorset, UK) and triethyl phosphite (P(OC₂H₅)₃, Sigma–Aldrich) of 3 M were used. Both were hydrolyzed separately in ethanol and distilled water (DW) for 24 h, and then mixed at a Ca/P ratio of 1.67 and stirred for 2 h. Ammonium hydroxide (NH₄OH; BDH, Poole, UK) was added at 5% to the mixture, in order to improve the gelation and polymerization process [19]. The sol was aged for 7 days to produce a clear HA sol. To produce a TiO₂ sol, titanium propoxide (Ti(OCH₂CH₂CH₃)₄, Sigma–Aldrich) of 1.5 M was hydrolyzed within an ethanol–water solution, containing diethanolamine ((HOCH₂CH₂)₂NH, Sigma–Aldrich), and stirred for 5 days. The ratios of the diethanolamine/Ti and water/Ti were determined to be 1.00 and 2.00, respectively. To obtain HA + TiO₂ composite sols, the HA and TiO₂ pure sols were mixed together at ratios of 10 and 20 mol% with continuous stirring for 24 h.

Commercially pure Ti (cp Ti, grade II) was used as a substrate for the coating after polishing with SiC paper (# 2000 grit) and cleaning in acetone/ethanol. The Ti substrate was dipped into the prepared sols and spin-coated at 3000 rpm for 20 s. After drying, samples were heat-treated at a temperature of 550 °C for 2 h in air at a heating rate of 2 °C/min.

The morphology of the sol–gel coatings was observed with scanning electron microscopy (SEM; JEOL, Peabody, MA, USA). The phase of the coatings was characterized with X-ray diffraction (XRD; PW 3710 Philips, Eindhoven, The Netherlands). Roughness of the coating layer was measured by scanning the surface with a 3 D laser profiler (Proscan 1000; Scantron Industrial Products Ltd., Taunton, UK) [13]. Average roughness was measured on 8–10 different sections for each sample.

2.2. Culture of alveolar bone (AB) cells

Overall, four Ti surface coatings were manufactured for further investigation, with ground commercially pure Ti surface (cp Ti) acting as a non-coated control. The surface coatings consisted of a pure HA coating (HA), a HA composite coating with 10 mol% titania (HA + 10% TiO₂), a HA with 20 mol% titania (HA + 20% TiO₂), and a pure titania coating (TiO₂). Prior to tests the coated surfaces were rinsed with distilled water and sterilised with ultraviolet light (Steristrom 2537 Å, Coast-Air, London, UK) for 1 h.

Fragments of AB were obtained from a male patient aged 26 undergoing routine molar extraction, following a protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital (London, UK). The fragments were immediately placed into alpha-minimum essential medium (α-MEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10% fetal calf serum (FCS), and 25 µg/ml fungizone (all from Gibco Life Technologies, Paisley, UK). After mincing into small pieces (1–2 mm³), the fragments were washed with phosphate-buffered saline (PBS) (Gibco) and placed into 6-well culture plates (Becton Dickinson, Cowley, UK) containing Dulbecco's minimum essential medium (DMEM), supplemented as with α-MEM but without fungizone, then

ID	Title	Pages
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