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In vitro responses to electrosprayed alkaline phosphatase/calcium phosphate composite coatings

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Abstract

Surface modification of titanium implants to improve their fixation in bone tissue is of great interest. We present a novel approach to enhance implant performance by applying important principles of bone mineralization to biomedical coatings. As an attempt to mimic the biphasic biomineralization process, both the enzyme alkaline phosphatase (ALP) and calcium phosphate (CaP) were immobilized onto Ti discs, thereby triggering enzymatically and physicochemically controlled biomineralization pathways. ALP, CaP and ALP–CaP composite coatings with preserved functionality of ALP were successfully deposited using electrospray deposition. In vitro soaking studies in cell culture medium revealed that crystal growth initially proceeded at a faster rate on CaP-coated Ti than on ALP-containing coatings, but mineral deposition onto ALP-coated Ti caught up with the calcification behaviour of CaP coatings upon long-term soaking. Cell culture experiments with osteoblast-like cells, however, demonstrated the opposite effect in mineral deposition on the electrosprayed CaP and ALP coatings. The ALP–CaP composite coatings showed delayed proliferation as well as accelerated mineralization in comparison to cells cultured on the CaP-coated and uncoated Ti. In conclusion, these in vitro results showed that the osteogenic potential of Ti can be stimulated by ALP-containing coatings.

Keywords: Surface modification; Calcium phosphate; Alkaline phosphatase; Composite; Biomineralization

1. Introduction

Titanium and its alloys are the most commonly used metallic materials in the manufacture of orthopaedic and dental implants. However, when applying Ti as an implant material, a non-physiological surface is exposed to a physiological environment. By generating a coating on a Ti surface that mimics organic and/or inorganic components of living bone tissue, a physiological transition between the non-physiological Ti surface and the surrounding bone tissue can be established. In this way, the coated Ti implant stimulates bone formation starting from the implant surface, thereby enhancing early and strong fixation of bone-substituting implants. As such, a continuous transition from tissue to implant surface can be induced. Consequently, modification of the surface properties of Ti to control the interaction between the implant and its biological surroundings has been a major research topic in the past decades [1–7]. Here, we present a novel approach to improve the implant performance by applying important principles of bone mineralization to biomedical coatings.

Mineralization of bone is essentially a biphasic phenomenon [8]. During phase 1, a series of enzymatically directed molecular interactions yield a local enrichment of Ca^{2+} and PO_4^{3-} ions within so-called matrix vesicles, resulting in the formation of the first crystals of apatitic bone. Phase 2 of biomineralization begins with crystal release through the matrix vesicle membrane, exposing the preformed apatite crystals to the extracellular fluid. The constant supply of Ca^{2+} and PO_4^{3-} ions from the extracellular fluid supports

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continuous crystal growth, with the preformed apatite crystals serving as nuclei for the formation of new crystals by a process of homologous nucleation [9]. While phase 1 of biomineralization is under tight enzymatic cellular control, phase 2 is a physicochemical process without cellular control. During phase 1, the essential component in bone mineralization is the metalloenzyme alkaline phosphatase (ALP), which catalyzes the hydrolysis of organic phosphatemonoesters such as β-glycerophosphate (β-GP) [10]. Basically, ALP increases the local inorganic phosphate (P_i) concentration required for physiological mineralization of hard tissues [11]. Previous studies showed that ALP coatings trigger early, enzymatically controlled stages of biomineralization through enzymatic cleavage of organic phosphate precursors, thereby increasing the chemical potential for apatite formation [12]. In order to further mimic the biphasic biomineralization process, the current study proposes the immobilization of both ALP and calcium phosphate (CaP) in a composite coating onto Ti implant surfaces, thereby combining the enzymatically controlled nucleation phase 1 and the physicochemically regulated mineralization phase 2.

The electrostatic spray deposition (ESD) technique has proven to be a successful method for the deposition of (i) CaPs [4,13] and (ii) biomolecules [14–18], offering good control over surface properties. In contrast to the use of ESD for the deposition of either inorganic CaP or organic biomolecule coatings, the current study investigated the feasibility of using the ESD technique to apply biologically active organic–inorganic composite coatings onto Ti implant surfaces. ESD involves atomization of a solution, which contains CaP nanocrystals and/or dissolved ALP, by applying a high voltage to the liquid surface, which then disperses into a spray of micron-sized, charged droplets (<10 μm). These charged droplets are attracted towards a grounded substrate as a result of the applied potential difference. Consequently, when the droplets impinge on the substrate, they lose their charge and dry within milliseconds at 15–25% humidity. Due to this fast dehydration process, a thin film is formed on the substrate surface at room temperature without detrimental effects on the biological activity of the ALP enzyme.

To evaluate the bioactive behaviour of the composite-coated Ti surfaces, both in vitro soaking and in vitro cell culture experiments were performed. The suggested in vitro standard soaking procedure to predict bioactivity of an implant surface involves the incubation of experimental substrates in so-called simulated body fluid (SBF) [19]. The capacity to nucleate CaP formation under in vitro conditions is then interpreted as a first indication of potential bioactivity in vivo. However, since SBF does not contain any organic phosphates for ALP to act upon as present in the human blood plasma, the current study has included soaking experiments in cell culture medium (CCM), supplemented with the organic β-GP and serum proteins [12].

The aim of this study was to deposit ALP/CaP composite coatings onto Ti surfaces using the ESD technique to investigate the feasibility of these electrosprayed organic–inorganic coatings for enhanced mineralization on implant materials. We hypothesized that the combination of both the enzyme-mediated mineralization by ALP and the ongoing crystal growth on top of the already deposited CaP has a stimulatory effect on mineral deposition onto Ti implant surfaces.

2. Materials and methods

2.1. Electrospray deposition of ALP–CaP coatings

Three different ESD coatings were examined: (i) ALP, (ii) CaP and (iii) ALP–CaP composite coatings. Non-coated Ti discs served as a control. Machined, commercially pure Ti discs (Ti–6Al–4V, grade 5; diameter 12 mm, thickness 1.5 mm) were used as substrate material. Substrates were cleaned ultrasonically in acetone (15 min) and ethanol (15 min) prior to the depositions.

2.1.1. Precursor solutions

To preserve the functional properties of the enzyme ALP, deposition of the coatings was performed at low temperatures (<50 °C) and from aqueous solutions [14]. ALP (1 mg ml^{-1}) was dissolved in a 10:90 vol.% ethanol:double-distilled (dd) H_2O solution before electrospraying (Table 1). Ethanol was added to the ALP solution to overcome the high surface tension of ddH_2O, thereby enabling stable spray modes. Previous experiments using circular dichroism spectroscopy showed that 10 vol.% of ethanol did not affect the secondary structure of ALP molecules (unpublished data).

To enable co-deposition of biomolecules at low temperatures, spray suspensions of nano-sized crystalline CaP particles were required. With the CaP already formed prior to spraying, high temperatures during coating deposition and additional heat treatments to crystallize the ceramic were avoided. CaP suspensions were prepared by a precipitation reaction between calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (H_3PO_4) at a Ca/P ratio of 1.67 [20]. A 0.3 M H_3PO_4 solution (Acros Organics, Geel, Belgium) was added dropwise to a 0.5 M $Ca(OH)_2$ solution (Acros Organics) under continuous stirring (350 rpm, Teflon upper stirrer) at room temperature. After complete addition of the reactants, the CaP solution was stirred overnight. After centrifugation of the suspensions (60 min at 5000 rpm in 50 ml tubes), the obtained supernatant was used for electrospraying. Transmission electron micros-

Table 1
Concentration of calcium (Ca) and alkaline phosphatase (ALP) in electrosprayed precursor solutions.

	Ca (mg ml^{-1})	ALP (mg ml^{-1})
ALP	–	1
CaP	0.36	–
Composite	0.18	0.5

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