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Ectopic bone formation by 3D porous calcium phosphate-Ti6Al4V hybrids produced by perfusion electrodeposition

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ABSTRACT

Successful clinical repair of non-healing skeletal defects requires the use of bone substitutes with robust bone inductivity and excellent biomechanical stability. Thus, three-dimensionally functionalised porous calcium phosphate-Ti6Al4V (CaP–Ti) hybrids were produced by perfusion electrodeposition, and the *in vitro* and *in vivo* biological performances were evaluated using human periosteum derived cells (hPDCs). By applying various current densities at the optimised deposition conditions, CaP coatings with sub-micrometer to nano-scale porous crystalline structures and different ion dissolution kinetics were deposited on the porous Ti6Al4V scaffolds. These distinctive physicochemical properties caused a significant impact on *in vitro* proliferation, osteogenic differentiation, and matrix mineralisation of hPDCs. This includes a potential role of hPDCs in mediating osteoclastogenesis for the resorption of CaP coatings, as indicated by a significant down-regulation of osteoprotegerin (OPG) gene expression and by the histological observation of abundant multi-nucleated giant cells near to the coatings. By subcutaneous implantation, the produced hybrids induced ectopic bone formation, which was highly dependent on the physicochemical properties of the CaP coating (including the Ca^{2+} dissolution kinetics and coating surface topography), in a cell density-dependent manner. This study provided further insight on stem cell-CaP biomaterial interactions, and the feasibility to produced bone reparative units that are predictively osteoinductive *in vivo* by perfusion electrodeposition technology.

1. Introduction

Despite the advances in biomaterials and tissue engineering, clinical repair of critical-size or non-healing skeletal defects remains challenging. This is due to the lack of essential biological and biomechanical entities at the deteriorated defect site, whereby osteoprogenitor cells, extracellular matrix, signalling molecules/growth factors, and mechanical stability of the fracture, are the four vital constituents that are necessary for an effective bone defect healing [1]. Other parameters, including vascularity, comorbidities, and the physiological profile of the patient, are recently proposed to have strong interactions with these constituents, and thus constituting a conceptual framework for enhancement of bone repair [2]. Potentially, this framework can be translated into a clinically-relevant setting, through *in vitro* production of a robust bone

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reparative unit that possesses both the necessary biological and biomechanical characteristics. In this context, engineering a three-dimensionally (3D) functionalised calcium phosphate-titanium (CaP–Ti) hybrid that combines both the superior mechanical integrity and osteoinductivity, with or without the use of stem cells technology, may appear as an ideal synthetic bone substitute that could overcome certain limitations and clinical complications related to the standard bone grafting procedures [3].

Indeed, titanium (Ti) and calcium phosphate (CaP)-based biomaterials have respectively gained promising clinical track records in orthopaedic and dental applications over the years, either due to their well-known biocompatibility, biomechanical strength, osteoconductivity or the increasing scientific evidences on their osteoinductivity [4]. These have provoked strong interests in the research and development of various surface functionalisation strategies to modify Ti-based biomaterials with CaP layers, aiming to promote osseointegration and to induce bone formation within a superior mechanical framework at the defect site [5]. So far, most of the existing functionalisation methods have failed to produce such an osteoinductive hybrid, with the exception of biomimetic deposition of CaP coatings, where bone formation was induced intramuscularly [6]. However, this technique has limited control over the deposition of biomimetic apatite with desirable physicochemical properties, and the process is relatively time consuming. Alternatively, electrodeposition or electrolytic deposition appears to be a promising technique that can promptly functionalise three-dimensional (3D) porous Ti structures with CaP layers and offers higher controllability and reproducibility on the coating properties [7]. Unfortunately, the produced coating often lacks of osteoinductivity *in vivo*, although higher osteogenicity has been reported using *in vitro* culture systems [8]. This is mainly due to the lack of in-depth understanding on the necessary material properties of CaP that would effectively trigger osteogenesis in terms of the *in vitro* stem cell-material and *in vivo* cell-host-material interactions [9], as well as the absence of a comprehensive technological knowledge on electrodepositing CaP layers with excellent bone induction properties.

Previously, we reported on the integration of perfusion and electrodeposition technology to deposit CaP layers onto additive manufactured 3D porous Ti6Al4V scaffolds in a controllable and reproducible manner [10]. In this study, we describe the use of a six-channel perfusion electrodeposition system (^{6}P-ELD) as a laboratory up-scaling functionalisation tool, to produce three-dimensionally (3D) functionalised porous CaP–Ti hybrids with different physicochemical properties. The *in vitro* biological performance of the produced hybrid was assessed by analysis of the *in vitro* proliferation and osteogenic differentiation (i.e. alkaline phosphatase activity and osteogenic gene markers expression) of human periosteum-derived cells (hPDCs). The *in vivo* ectopic osteoinductivity was evaluated by seeding hPDCs onto the hybrids followed by subcutaneous implantation in nude mice. After 8 weeks of implantation, samples were retrieved, characterised by nanofocus X-ray computed tomography (nanoCT) and processed for histological analysis for *de novo* bone formation. To correlate the *in vitro* and *in vivo* biological outcomes, the physicochemical properties of the deposited CaP coatings (including coating distribution, surface morphology, dissolution kinetics and thickness) before and after *in vitro* studies were analysed by scanning electron microscopy (SEM), inductive-couple plasma-atomic emission spectrophotometry (ICP-AES) and microfocus X-ray computed tomography (μCT). These data are essential as feedback for optimisation of the ^{6}P-ELD production parameters, in order to produce a bone reparative unit that is predictively osteoinductive *in vivo*.

2. Materials and methods

2.1. Production of three-dimensionally functionalised porous CaP–Ti hybrids using six-channel perfusion electrodeposition system (^{6}P-ELD)

A six-channel perfusion electrodeposition (^{6}P-ELD) chamber was fabricated for lab-scale production of 3D porous CaP–Ti hybrids, based on the single-channel experimental prototype as reported previously (Fig. 1A) [10]. The six channels were designed in a cylindrical array (Fig. 1B), and each channel (10 cm length) consisted of a cathode (a 3-pin holder for Ti-scaffold) surrounded by platinum ring anode (10 mm in height and diameter). A supersaturated calcium phosphate solution [11] was used as electrolyte and perfused through the 6 channels using a peristaltic pump (Ismatec®). Current densities (I) of 1.54, 5, 10 or 20 mA/cm^2 were applied on each of the six scaffolds in order to deposit CaP coatings with different physicochemical properties onto the 3D porous Ti6Al4V scaffolds [Ti-scaffolds, fabricated by selective laser melting (designed pore size = 1000 μm)] [12], while other parameters were fixed at optimum conditions: deposition time (t) = 6 h, electrolyte flow rate (f) = 10 ml/min, and the process temperature (T) = 50 °C. Scaffolds of 3 mm height × 6 mm diameter (type A) were used for the *in vivo* ectopic bone formation assays, and 10 mm height × 6 mm diameter (type B) for *in vitro* coating characterisation and cell culture experiments (Fig. 1E). The pH change of the bulk electrolyte was recorded using a pH measurement kit (Pico® Technology, U.K.), and the deposited CaP coating mass was calculated from the dry weight of the scaffolds before and after electrodeposition (n = 3) to represent deposition efficiency.

2.2. Physicochemical characterisation of the deposited CaP coatings

2.2.1. Microfocus X-ray computed tomography (μCT) analysis

The distribution, morphology and thickness of the CaP coatings deposited on the Ti6Al4V scaffolds were characterised using high-resolution μCT on a Skyscan 1172 system (Skyscan NV, Kontich, Belgium) at an isotropic voxel size of 4.5 μm^3. The samples were scanned using a source voltage and current of 60 kV and 167 μA, with a filter of 0.5 mm Al and a rotation step of 0.3° over a total of 180°. The obtained radiographic images were reconstructed using NRecon (Skyscan NV, Kontich, Belgium) software and the coating thickness was measured using CTAn (Skyscan NV, Kontich, Belgium) software. Briefly, the coating thickness at 20 different locations on a reconstructed uCT image was measured, and in total 10 representative μCT images were used for each produced hybrid. Then, the obtained values were subtracted by the value measured on the plain Ti-scaffolds (due to partial volume effect (pv)), and the average coating thickness was determine for each produced hybrid.

2.2.2. Dissolution test

The produced CaP–Ti hybrids (n = 3) were incubated in 15 ml phosphate buffered solution (PBS, Biowhittaker®, without Ca^{2+} and Mg^{2+} ions) in free floating condition on an orbital rotator at 10 rpm inside a 37 °C incubator. The solutions were refreshed at 3 and 6 h, and at 4, 7, 14 and 21 days, and the pH of the solutions were recorded before storage at −80 °C. The released [Ca^{2+}] and [PO_4^{3-}] over 21 days were measured by Inductive Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) (Varian 720-ES) at 393.366 nm (for Ca^{2+}) and 253.561 nm (for PO_4^{3-}) respectively. After 21 days of incubation, the dried weights of the hybrids were recorded, and the change in coating thickness were characterised by μCT as described above.

2.2.3. Scanning electron microscopy analysis (SEM)

The morphology of the CaP coatings before and after dissolution was assessed by SEM coupled with an energy dispersive X-ray (EDAX) analysis (FEI XL30 FEG) at 10 kV. The samples were coated with gold to increase conductivity of the deposited CaP coatings for better visualisation.

2.3. Characterisation of in vitro biological behaviours of hPDCs seeded on CaP–Ti hybrids

2.3.1. Isolation and expansion of hPDCs from human donors

Periosteal biopsies (10 mm × 5 mm) were harvested from the medial side of the proximal tibia of male and female of adolescent patients [6 donors, age 14.9 ± 2.1] during distraction osteogenesis. The periosteum was stripped from the tibia with a periosteal lifter and transported in growth medium (GM) [DMEM + GlutaMAX™-1, Invitrogen] containing 10% foetal bovine serum (Invitrogen™), 1% antibiotic-antimycotics solution and 1% sodium pyruvate. The biopsies were finely minced and digested overnight at 37 °C in 0.2% type IV collagenase (Invitrogen) and subsequently centrifuged at 1300 rpm for 10 min to collect the deliberated periosteal cells. All collected cells were pooled together and seeded in T175 flask in GM. Non-adherent cells were removed after 4 days by changing the medium and the adherent cells were subsequently expanded in GM. Upon confluence, the cells were harvested and resuspended in DMEM with 10% FBS and 10% DMSO, and stored in liquid nitrogen. All procedures were approved by the ethical committee for Human Medical Research (KU Leuven), and the patient informed consent forms were obtained as described previously [13].