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Biomimetic apatite formation on calcium phosphate-coated titanium in Dulbecco's phosphate-buffered saline solution containing $CaCl_2$ with and without fibronectin

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ABSTRACT

Calcium phosphate (CaP) thin films with different degrees of crystallinity were coated on the surfaces of commercially pure titanium by electron beam evaporation. The details of apatite nucleation and growth on the coating layer were investigated in Dulbecco's phosphate-buffered saline solutions containing calcium chloride (DPBS) or DPBS with fibronectin (DPBSF). The surfaces of the samples were examined by field emission scanning electron microscopy, X-ray diffraction and X-ray photoelectron spectroscopy. The concentrations of fibronectin and calcium ions (Ca^{2+}) were monitored by the bicinchoninic acid method (BCA) and use of a calcium assay kit (DICA-500), respectively. Apatite initially formed at the fastest rate on the CaP-coated samples with the lowest degree of crystallinity and reached the maximum Ca^{2+} concentration after immersion in DPBS solution for 15 min. After 15 min the concentration of Ca^{2+} decreased with the growth of apatite on the coating layers. For all the samples the maximum Ca^{2+} concentration in the DPBS solutions decreased with increasing crystallinity and immersion time to reach the maximum concentration increased. The presence of fibronectin in the DPBS solutions delayed the formation and affected the morphology of the apatite. Fibronectin incorporated into apatite deposited on the surface of titanium did not affect its biological activity in terms of promoting osteoblast adhesion.

1. Introduction

The clinical success of dental and orthopedic implants is related to their early osseointegration [1,2]. Surface composition and topography are crucial to the short- and long-term success of implants [3–7]. Titanium and its alloys are widely used as biomaterials for dental and orthopedic implants, because they are highly biocompatible materials with relatively low elastic moduli, good fatigue strength, formability and corrosion resistance [8–15]. However, compared with bioactive ceramics such as hydroxyapatite and bioglass titanium is relatively bioinert. Hence, the fixation of such implants in bone needs to be improved.

The application of calcium phosphate (CaP) coatings onto titanium and its alloys is now generally accepted to be advantageous for fast fixation and spontaneous binding to neighboring bone without the formation of a surrounding fibrous tissue, because of its bioactive and osteoconductive properties [16–18]. Following implantation, the release of calcium and phosphate ions into the peri-implant region increases the concentration of these ions in the body fluids, which results in deposition of biological apatite on the surface of the implant [19,20]. Consequently, bone healing around the implant is improved by the biological apatite [21]. Recently, thin hydroxyapatite coating layers with excellent bonding strengths were deposited on titanium and its alloys by an ion beam-assisted deposition (IBAD) method [22,23].

The biomimetic coating method was developed to improve the biological performance of biomaterials through the formation of a bone-like apatite, following the introduction of a coating method involving soaking biomaterials in a simulated body fluid (SBF) with ion concentrations similar to human blood plasma by Kokubo [24]. It has many advantages. The coating is composed of small crystal units, which are more easily degraded by osteoclasts [25]. Most importantly, biologically active molecules (such as osteogenic agents) can be co-precipitated with the inorganic components and thereby become incorporated into the crystal lattice, rather than only being deposited on the surface [26–28]. Fibronectin, one of the earliest cell binding proteins produced by odontoblasts and osteoblasts [29–31], is actively involved in cell adhesion, spreading, wound healing, cytoskeletal reorganization and bone tissue formation [32–34]. Furthermore, fibronectin is known to

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contain calcium-sensitive heparin binding sites, which should interfere with the biomineralization of titanium substrates [35].

Although still a matter of some controversy, apatite forming ability of implants in SBF is still the object of much interest as it could give some indication of in vivo behavior [36–42]. In the present study CaP thin films with different degrees of crystallinity were produced on commercially pure titanium by the electron beam evaporation method. A detailed insight into apatite nucleation and growth was obtained by immersing the samples in Dulbecco's phosphate-buffered saline solutions containing calcium chloride (DPBS) or DPBS containing fibronectin (DPBSF). The effect of the incorporated fibronectin on osteoblast adhesion was also studied.

2. Materials and methods

2.1. Preparation of samples

Commercially pure titanium discs (grade IV, 10 mm in diameter and 2 mm in thickness) with machined surfaces were used as substrates. After sonication in acetone and then deionized water the samples were dried in a nitrogen stream prior to the deposition process [43]. Calcium phosphate films with a thickness of ~500 nm were deposited on the substrates by electron beam evaporation according to Lee et al. [22,23]. Briefly, evaporants of calcium phosphate were prepared by sintering mixtures of powdered CaO (Cerac, USA) and hydroxyapatite (Alfa, USA) at 1200 °C for 2 h in air. For deposition an electron beam evaporator (Telemark, USA) and an end-hall type ion gun (Commonwealth Scientific, USA) were employed. Heat treatment after deposition was performed at three different temperatures, 350 °C (sample M350), 430 °C (sample M430) and 500 °C (sample M500), with a heating rate of 5 °C min^{-1}. After reaching the desired temperature this was held for 1 h and then the samples were cooled to room temperature in a furnace. The thickness of the deposited calcium phosphate film was measured using a a surface profiler (Model P-10, Tencor, Santa Clara, CA).

2.2. Immersion in solutions and characterization

Dulbecco's phosphate-buffered saline (calcium/magnesium free, Gibco-BRL Life Technologies, USA) and reagent grade $CaCl_2$ (100 mg l^{-1}) were dissolved in distilled water to prepare the DPBS solution, since magnesium ions can inhibit apatite growth [26]. Fibronectin (20 μg ml^{-1}, Sigma, USA) was added to the DPBS solution to prepare DPBS containing fibronectin (DPBSF). Each sample was immersed into 2.0 ml of the DPBS solution at 37 °C for at most 24 h. Coated samples were then rinsed with distilled water twice and air dried at ambient temperature. All the solutions were sterilized by filtration using a membrane with a pore size of 0.20 μm before use.

After immersion the surface morphologies of samples were observed by field emission scanning electron microscopy (FESEM) (JSM-6500F, JEOL, Japan). X-ray diffraction (XRD) (Rigaku, Tokyo) using Cu K_α radiation was used to analyze the structures formed on the surface.

The calcium ion (Ca^{2+}) concentration in the solutions was measured using a QuantiChrom™ calcium assay kit (DICA-500, BioAssay Systems, USA). For analyses, 200 μl of work reagent was added to aliquots of 5 μl of sample or standard in a 96-well plate. The plate was incubated for 3 min at room temperature, after which the plate was read at 595 nm using a microplate reader (Tecan Sunrise, Switzerland). Serial dilutions of $CaCl_2$ (0–200 μg ml^{-1}) were used to create a standard curve. Loss of calcium was calculated with reference to the Ca^{2+} concentration of solutions incubated without any samples. It was assumed that any decrease in calcium in solution was all deposited onto the titanium substrates.

The M350 samples were placed into 1.0 ml of DPBSF solution at 25 °C (to avoid protein denaturation of fibronectin) for the determined intervals. The total amount of fibronectin deposited onto the titanium discs was determined using the BCA method (Micro BCA™ protein assay, Pierce, USA). Briefly, 800 μl aliquots of the initial solution and that containing non-incorporated fibronectin after immersion were mixed with 800 μl of freshly prepared working solution in a 24-well plate and incubated at 60 °C for 1 h. After cooling to room temperature, the solutions were removed to tubes and read at 562 nm using a UV/visible spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech). Each protein concentration was calibrated using a standard curve. The incorporated fibronectin was determined by subtracting the residual fibronectin from the initial added fibronectin. Measurements were performed in triplicate for each time point. Additionally, the surfaces of samples before and after immersion in DPBSF solution were evaluated by X-ray photoelectron spectroscopy (XPS) (PHI 5700) with Al K_α X-rays, with the photoelectron take-off angle being set at 45°.

2.3. In vitro cell culture

MG-63 human immortalized osteoblast-like cells were purchased from the American Type Culture Collection (ATCC, USA). The discs were placed in the wells of a 48-well plate after being washed twice with distilled water. The MG-63 cells were suspended in MEM (Minimum Essential Medium, Gibco-BRL Life Technologies, USA) without serum at a concentration of 1×10^6 cells ml^{-1}. Aliquots of 250 μl of the cell suspensions were added to the samples in each well and the plate was incubated at 37 °C in a 5% CO_2 atmosphere for 3 h. After incubation all the non-adherent cells were removed by washing the samples with DPBS solution three times. In the quantitative assay cells adhering to the samples were lysed in 250 μl of lysis buffer (14:1 alkaline buffer solution:4-nitrophenyl phosphate disodium salt hexahydrate) at 37 °C for 30 min and then the lysate collected. Total double-stranded DNA (dsDNA) in the lysate was measured using the Quant-iT™ PicoGreen® dsDNA reagent and kits (Molecular Probes, UK). Briefly, 12.5 μl of dsDNA sample was added to the wells of a 96-well plate containing 37.5 μl of TE assay buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and 50 μl of Quant-iT™ PicoGreen® dsDNA reagent and incubated for 2–5 min. The PicoGreen–DNA complex was detected at fluorescein excitation/emission wavelengths of 480/520 nm using a fluorescence microplate reader (Fluostar OPTIMA, Germany). The total amount of dsDNA related to the number of cells was determined using the standard provided in the kit. In the morphology observations adherent cells on the samples were fixed with 10% neutral buffered formalin. Subsequently, the substrates were dehydrated in a graded series of ethanol and washed with distilled water. Finally, the substrates were dried and sputter coated with platinum and examined using FESEM.

2.4. Statistical analysis

All quantitative data are depicted as the mean ± standard deviation. Tests of significance were performed using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Biomimetic apatite formation in DPBS

Fig. 1 shows scanning electron micrographs of sample M350 incubated in DPBS solution at 37 °C for determined intervals. A homogeneous apatite layer appeared on the coated surface even after incubation in DPBS solution for 15 min, shown in Fig. 1a.