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Evaluation of the attachment, proliferation, and differentiation of osteoblast on a calcium carbonate coating on titanium surface

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ABSTRACT

Titanium has been reported to have some limitations in dental and orthopaedic clinical application. This study described a coating process using a simple chemical method to prepare calcium carbonate coatings on smooth titanium (STi) and sandblasted and acid-etched titanium (SATi), and evaluated the biological response of the materials in vitro. The surfaces of STi, SATi, calcium carbonate coated STi (CC-STi) and calcium carbonate coated SATi (CC-SATi) were characterized for surface roughness, contact angles, surface morphology and surface chemistry. The morphology of MG63 cells cultured on the surfaces was observed by SEM and Immuno-fluorescence staining. Cell attachment/proliferation was assessed by MTT assay, and cell differentiation was evaluated by alkaline phosphatase (ALP) activity. MG63 was found to attach favorably to calcium carbonate crystals with longer cytoplasmic extensions on CC-STi and CC-SATi, resulting in lower cell proliferation but higher ALP activity when compared to STi and SATi respectively. Moreover, CC-SATi is more favorable than CC-STi in terms of biological response. In conclusion, the calcium carbonate coatings on titanium were supposed to improve the osteointegration process and stimulate osteoblast differentiation, especially in early stage. And this method could possibly be a feasible alternative option for future clinical application.

1. Introduction

Titanium has been widely used in dental and orthopaedic implants due to its high biocompatibility, excellent mechanical properties and chemical stability [1,2]. The biocompatibility of titanium is attributed to its surface oxide film that spontaneously forms when it is exposed to oxygen [3]. However, this film with a thickness of 3–10 nm is usually very dense and stable, which makes titanium bioinert. The bioinert titanium can only be integrated with bone passively and unable to induce bone apposition [4]. In order to improve the bioactivity of titanium and its alloys, various bioactive coatings have been developed, such as calcium phosphate, bioactive glasses and biologically functional molecules.

Currently, plasma spraying is one of the most popular techniques for the preparation of calcium phosphate coatings [5]. Short-term clinical and experimental studies have shown that plasma-sprayed hydroxyapatite (HA)-coated implants are able to result in stronger fixation and faster bone growth rate compared to noncoated implants. However, there are still some concerns about the long term clinical application of the plasma-sprayed HA-coated implants. Coating delamination and wear debris are reported as major problems associated with these coatings in some long term clinical observations as they cause osteolysis in the surrounding tissue [6,7].

To overcome the drawbacks of plasma-sprayed HA-coated implants, various resorbable coatings, such as biodegradable bone-like carbonate apatite (BCA), octacalcium phosphate (OCP) [8,9], biodegradable Hydroxyapatite [10,11] and resorbable calcium phosphate [11,12] have been developed. Development of the resorbable coatings is based on the thought that an optimal coating should dissolve in a rate similar to bone formation, and should completely disappear after completion of the bone-healing process [9]. The total resorption of the coating may reduce the problems of plasma-sprayed HA-coated implants, which have been mentioned above.

Besides calcium phosphate, calcium carbonate is also an important biomaterial which has been widely used in bone surgery [13,14]. There are three crystalline and one amorphous phase of anhydrous calcium carbonates in nature, which are calcite, aragonite, vaterite and amorphous calcium carbonate. It has been demonstrated that calcium carbonate is a biocompatible and osteoconductive material either in the form of aragonite [13,15–17] or in the form of calcite [18]. Therefore, calcium carbonate might be an applicable candidate to prepare resorbable coating with improved biodegradation rates due to its higher solubility compared to apatite [19]. Recently, Wang et al. [20] successfully fabricated a biologically nacre coating on dental

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titanium implants, where the main component is aragonite. The process of the coating is interesting, but complicated and time-consuming.

The aim of the study was to prepare calcium carbonate coatings on smooth and rough titanium surfaces using a simple chemical treatment, and evaluate the behavior of MG63 osteoblast-like cell on these surfaces. Four groups were investigated: smooth titanium (STi), sandblasted and acid-etched titanium (SATi), calcium carbonate coated STi (CC-STi) and calcium carbonate coated SATi (CC-SATi). After analyzing the properties of the surfaces, osteoblastic cell attachment, morphology, proliferation and differentiation were studied.

2. Materials and methods

2.1. Sample preparation

2.1.1. Preparation of STi and SATi

Commercially pure titanium discs, measuring 15 mm in diameter and 1 mm in thickness, were used as substrates. STi and SATi discs were prepared according to the following steps:

- STi: titanium discs were wet-ground with 120, 600, and 1500 grit silicon carbide abrasive sandpapers, and then were passivated in 50% nitric acid.
- SATi: titanium discs were firstly prepared in the same way as STi, and then were treated as described in our previous article [21]. In brief, the discs were grit-blasted with 0.25 to 0.50 mm corundum grit at 5 bars for 1 min. Subsequently, the discs were acid-etched in hydrochloric acid/sulfuric acid (1: 1) at 65 °C for 30 min.

After the above treatments, the discs were ultrasonically cleaned for 15 min in acetone, ethanol (70%) and deionized water, and finally dried at room temperature.

2.1.2. Preparation of CC-STi and CC-SATi

The experimental setup for preparing $CaCO_3$ films is illustrated in Fig. 1. Two vials, one containing a 20 mM calcium chloride ($CaCl_2$) solution, and the second, containing ammonium carbonate powder, were placed in a desiccator. STi or SATi discs were inverted and placed above the $CaCl_2$ solution. $CaCO_3$ films were then deposited on the STi or SATi substrates via slow diffusion of CO_2, produced by decomposition of ammonium carbonate at room temperature (4 h). The samples were rinsed with deionized water for 1 min after deposition, and then dried in air overnight. According to this process, CC-STi and CC-SATi discs were produced.

2.2. Surface characterization

Surface roughnesses of the four surfaces were measured using a mechanical profilometer (TAYLOR HONSON S4C-3D, England). The contact angles were determined by dynamic contact angle (DCA, Kruss X100, Germany) analysis. Scanning electron microscopy (SEM, Fei QUANTA-200, The Netherlands) was used to investigate the morphology and microstructure of the surfaces. X-ray diffraction (XRD, Bruker AXS D8 ADVANCE, Germany) was employed to determine the crystallinity of the materials. The precipitates of CC-STi and CC-SATi were investigated by Fourier transform infrared spectroscopy (FTIR, Nicolet 170SX, USA).

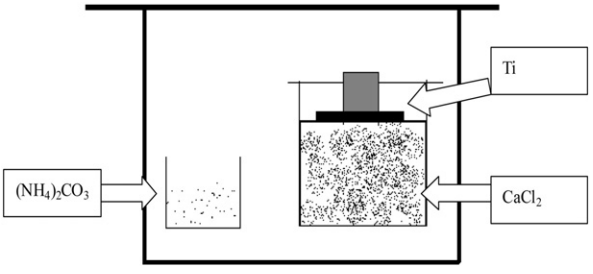

Fig. 1. Illustration of the experimental setup.

2.3. Cell culture

MG63 osteoblast-like cells were used for these experiments because they exhibit several fundamental osteoblast characteristics that are typical of a relatively immature osteoblast [22,23]. As a result, they are a good model for examining the early stages of osteoblast differentiation.

For all experiments, MG63 cells were cultured on disks placed in 24 well plates (Corning, Corning, IL). Cells were plated at 5000 cells cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (In-Vitrogen Corporation) and 1% L-glutamine (In-Vitrogen Corporation) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Media were changed every 48 h until the cells reached confluence. Because of the opacity of the Ti disks, there was no practical way to assess confluency of the cultures. As a result, when cells reached visual confluence on plastic, cultures on all other surfaces were treated exactly as those grown on plastic.

2.4. Immuno-fluorescence staining

After 48 h of incubation, samples were washed twice with pre-warmed PBS, fixed in 4% v/v paraformaldehyde for 15 min. Each of the samples was then permeabilized in PBS, pH 7.4 containing 0.1% Triton X-100 and 100 mM glycine for 30–40 min at room temperature, followed by blocking with 1% w/v bovine serum albumin (BSA) in PBS at room temperature. Actin filaments were stained with rhodamine phalloidin (1:200 in PBS). Nuclei were counterstained with 1 mg/mL 4,6-Diamidina-2-phenylin (DAPI, Sigma) for 10 min. Samples were washed with PBS, mounted with Vectashield (Vector) in glass slides and photographed with an inverted fluorescence microscope Axiovert M100 (Carl Zeiss).

2.5. Cell morphology

Cell morphology was observed by SEM after 2 days of culture for the four surfaces. Tested and control cultures were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 (Sigma) for 30 min. After washing in the same buffer, dehydration was carried out by sequential immersion in serial diluted solutions of 60, 70, 80, 90, and 95% v/v ethanol in water, followed by immersion in 99.8% v/v ethanol. The samples were then air-dried under laminar flow at room temperature overnight, coated with gold and examined by SEM.

2.6. Initial cell adhesion and cell proliferation

Cellular adhesion/proliferation was determined by a MTT assay. For the adhesion/proliferation evaluation, cells were cultured on samples for 4, 24 and 72 h. Then, cells were incubated in a MTT reagent (5 mg/ml in PBS) (Sigma Aldrich) at 37 °C for 4 h. After removing the culture medium, dimethylsulfoxide (DMSO) was added onto the specimens to release the colored product into the solution. Cell numbers can be assessed by measuring the optical density of the solution at 570 nm with an El x 808 Ultra Micro plate Reader (BioTek, USA). The blank reference was taken from wells without cells, also incubated with the MTT solution. Results were expressed as relative MTT activity as compared with control conditions (cells cultured on STi for 4 h).