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Behaviors of MC3T3-E1 cells on carbonated apatite films, with a characteristic network structure, fabricated on a titanium plate by aqueous spray coating

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ABSTRACT

Four carbonated apatite films having average thicknesses of 1.3–0.11 μm, proportions of network sizes above 10 μm of 41–68%, and average border heights of the characteristic network structure of 0.98–0.29 μm were fabricated on a titanium plate by aqueous spray coating. These carbonated apatite films after heat treatment showed good mineralization ability in Hanks' balanced salt solution. Assessment of initial cell attachment and calcination on these films and on the Ti plate using osteoblastic MC3T3-E1 indicated that the carbonated apatite film heat treated at 600 °C, whose film thickness, proportion of network sizes above 10 μm, and border height were 0.11 μm, 61%, and 0.31 μm, respectively, was most preferred by osteoblastic cells. Field emission scanning electron microscopic observation of the cells attached to the films showed that the wide network and low border height of the network structure on the carbonated apatite film play an important role in the development of the filopodia of the osteoblastic cells.

1. Introduction

Titanium (Ti) and its alloys are used extensively in oral and orthopedic implants for their mechanical properties and biocompatibility with human tissues [1]. Because of the poor osteoinductive properties of Ti, the use of hydroxyapatite (HA; chemical formula, $Ca_{10}(PO_4)_6(OH)_2$) coatings has received considerable attention [2]. From a mechanical perspective, bulk HA is a very brittle bioactive ceramic and cannot be used as an implant in load-bearing applications. The fabrication of HA coatings on metallic substrates, however, combines the advantages of the mechanical properties of metal substrates and the biological performance of HA ceramics [2,3]. Several techniques including plasma spray [4–6], microplasma spray [7,8], sol–gel method [9–11], physical vapor deposition [12,13], electrophoretic deposition [14–16], and electrostatic spray deposition (ESD) [17–21] are effective for the fabrication of dense and uniform HA coatings even on metallic substrates.

Recently, we reported an aqueous spray coating (ASC) method that is simple and inexpensive for fabricating a carbonated apatite (CA; chemical formula, $Ca_{10}(PO_4)_6(CO_3)\cdot 2CO_2\cdot 3H_2O$) film on a Ti plate heated at 70 °C, where CO_2 molecules are inserted into the CA lattice [22]. A stable aqueous solution, in which the Ca/P ratio was adjusted to 1.67 by the addition of phosphoric acid to a calcium hydrogen carbonate solution, was used for spray coating. When the solution (25 mL) was sprayed at the rate of 5 mL min^{-1}, an adhering film with a shear strength of 21 MPa and thickness of approximately 1 μm could be easily deposited on Ti plates. Of note, the shear strength of the sprayed film was improved to 40 MPa, which is sufficient for practical use, by heat treatment at 600 °C under Ar gas flow for a short time (10 min). The resulting CA film, whose chemical component is $Ca_{10}(PO_4)_5(HPO_4)(CO_3)(OH)\cdot CO_2$, has been fabricated. Thus, these CA films can be easily fabricated by ASC. Furthermore, the characteristic network structure on the sprayed film was found to be identical to that observed with the ESD method [23]. The ASC method produced many round and small particles on the CA films.

In this study, the mineralization ability of the four CA films was examined by the immersion test in Hanks' balanced salt solution (HBSS) without organic species, prepared as a simulated body fluid. To evaluate the morphological and heat treatment effects of CA films with different thicknesses, border sizes, and border heights of the characteristic

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network structure spontaneously formed on the films, the initial cell attachment and calcination ability of osteoblastic MC3T3-E1 on the films fabricated on a Ti plate by ASC were investigated. These results were compared with osteoblastic cells cultured on uncoated Ti plates under identical conditions.

2. Materials and methods

2.1. Materials

Calcium hydroxide ($Ca(OH)_2$, 99.9%) (Wako Pure Chemical Industries Ltd., Osaka, Japan) and phosphoric (H_3PO_4, 85%) and citric acids (99.5%) (Taisei Chemical Co., Ltd., Tokyo, Japan) were purchased. Ti plates (99 mass% Ti; Soekawa Chemicals, Tokyo, Japan) of two sizes, with dimensions of $20 \times 20 \times 0.5$ mm^3 and $10 \times 5 \times 0.5$ mm^3, were used for film fabrication. The Ti plates were washed in acetone for 15 min and deionized water for 10 min with sonicated stirring and dried in a drying oven at 70 °C. The surface roughness of the Ti plates was measured using a profilometer (Dektak-3; Veeco/Sloan Technology, Plainview, NY, USA), and the average was approximately 0.3 μm.

2.2. Aqueous spray coating method

2.2.1. Preparation of the ASC solution

An aqueous solution containing Ca^{2+} and PO_4^{3-} was prepared according to our recently reported procedure [22]. The Ca/P ratio was adjusted to 1.67, and the resulting pH value of the ASC solution was 5.57, as reported previously.

2.2.2. CA film fabrication with different amounts of spray solution

The abovementioned aqueous solution was sprayed on the Ti plate of dimension $20 \times 20 \times 0.5$ mm^3 following the procedure reported recently [22]. The Ti plate of dimension $10 \times 5 \times 0.5$ mm^3 was used for film preparation, with the aim of observing the tilt-viewed surface by field emission scanning electron microscopy (FE-SEM). An airbrush, HP-SAR (ANEST IWATA Co., Kanagawa, Japan) was adjusted to the vertical direction of the Ti plate on a stainless steel disk placed on a sheathed heater, with a distance of 200 mm between them. On the Ti plate heated at 40 °C, the solution mist was emitted from the nozzle of the airbrush with a pressure of 0.2 MPa, developed using an air compressor. The heating rate of the sheathed heater was fixed at approximately 7 °C min^{-1}, independent of the applied amount of sprayed solution.

Two types of CA film, **A** and **B**, were prepared by applying 25 and 5 mL of the ASC solution, respectively, with an identical exhalation rate of 5 mL min^{-1}. The surface temperature of a Ti plate placed close to the sprayed substrate was monitored using a thermocouple during the procedure.

2.2.3. Heat treatment of the sprayed films

The other two films, **A′** and **B′**, were prepared by heat treating **A** and **B**, respectively, at 600 °C for 10 min under Ar gas flow of 0.5 L min^{-1}. A tubular furnace, EPKRO-12K (Isuzu, Tokyo, Japan), with a quartz glass tube of 40 mm in diameter and 650 mm in length was used.

2.3. Film thickness and morphology of CA films

To measure the thickness of the CA films, 1/4 of the area of the films was removed from the Ti plate by soaking that part in a citric acid solution of 0.5 mol L^{-1} for 5 s. After rinsing with water and drying at 70 °C, the height difference between the remaining film surface and the appeared Ti surface was measured by continuous scanning every 5 mm on both surfaces using the stylus profilometer.

The surface morphology of the CA films deposited on the Ti plate of dimension $20 \times 20 \times 0.5$ mm^3 was investigated using an FE-SEM instrument (JSM-6701F; Jeol, Tokyo, Japan) at an accelerating voltage of 5 kV. All samples were sputter coated with Au before observation. Data processing of the FE-SEM images obtained using top-view observation was performed using ImageJ [24]. The size distribution of the network structure found in an area of 0.25×0.19 mm^2 in the central part of the films was precisely determined by binarization of the SEM images.

The tilt-view observation of the CA films deposited on the Ti plate of dimension $10 \times 5 \times 0.5$ mm^3 was performed at an inclination angle of 85°. The border heights of the network structure were measured directly from the images, and the average value was determined for each film.

2.4. Mineralization ability of the ASC coating in HBSS

HBSS without organic species was prepared as a simulated body fluid by the method described previously [25]. Each fabricated film, **A**, **B**, **A′**, and **B′**, and the uncoated Ti plate separately immersed in 30 mL of HBSS in a polypropylene bottle at 37 °C. After immersion for 1 day, HBSS on each sample was removed using soft paper, and each sample was immediately dried in a desiccator. The top-view appearances of these samples before and after immersion were pre-coated with Au and observed by FE-SEM.

2.5. Cell culture

Osteoblastic cells (MC3T3-E1, RIKEN BioResource Center, Ibaraki, Japan) were grown in regular culture media consisting of α-modified minimum essential medium (GIBCO/BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (CCB, BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and an antibiotic–antimycotic solution (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B; GIBCO/BRL). The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was changed every 3 days until the cells reached 80–100% confluence.

After 15 days of culture, the cells were detached from the substrate using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25% w/v trypsin/0.025% EDTA, GIBCO/BRL), concentrated, and re-suspended in culture medium.

2.6. Initial cell attachment test

To evaluate cell attachment quantitatively, the above-cultured osteoblastic MC3T3-E1 cells were seeded on the samples at a density of 3550 cells cm^{-2} ($n = 3$). The cells were incubated for 1 and 6 h, and all nonadherent cells were washed out of the samples with a phosphate-buffered saline (PBS) solution prepared by dissolving 9.6 g of Dulbecco's PBS powder (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in water and adjusting to 1 L. The number of adherent cells was determined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) assay (Cell Counting Kit-8; Dojin, Kumamoto, Japan) as follows: the washed samples were transferred to a new culture plate, adherent cells were incubated in a WST-8 solution diluted with regular culture media for 1 h at 37 °C, and 0.1 M HCl (Wako Pure Chemical Industries Ltd.) was added to terminate the reaction. The spectroscopic absorbance of the resulting solution at 450 nm was measured and referenced to a calibration curve of absorbance vs. cell number counted on a hemocytometer by microscopy.

To evaluate cell attachment by FE-SEM, the above-cultured osteoblastic MC3T3-E1 cells were seeded on the other samples at a density of 3550 cells cm^{-2} as described above. After 1 and 6 h of culture, cell layers ($n = 1$) were washed 3 times with PBS solution. The cultured cells were fixed at 0.1 M sodium cacodylate buffer solution (Nacalai Tesque Inc., Kyoto, Japan) containing 2.8% glutaraldehyde