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Effects of calcium-phosphate topography on osteoblast mechanobiology determined using a cytodetacher

Shih-Ping Yang [a], Chyun-Yu Yang [b], Tzer-Min Lee [c,*], Truan-Sheng Lui [a]

[a] Department of Materials Science and Engineering, National Cheng Kung University, Tainan 701, Taiwan
[b] Department of Orthopedics, National Cheng Kung University, Tainan 701, Taiwan
[c] Institute of Oral Medicine, National Cheng Kung University, Tainan 701, Taiwan

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ABSTRACT

The Human fetal osteoblast (hFOB) cell morphology, adhesion force, and proliferation on a calcium-phosphate (Ca-P) micropattern surface were investigated and the mechanobiology was investigated by a cytodetachment test. Ca-P-coated groove patterns with 3.0-μm-deep grooves (C3), 4.5-μm-deep grooves (C4), and 5.5-μm-deep grooves (C5) were produced on silicon wafers using photolithography and wet etching techniques. The grooved substrates were coated with a 200-nm-thick layer of titanium (bond coat) and a 200-nm-thick layer of calcium phosphate (top coat) using a sputtering system. Smooth Ca-P-coated Si wafers were used as control surfaces. Analysis of the scanning electron microscopy observations shows that cells on the Ca-P micropattern showed spreading and elongation. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay indicated that C3 and C4 specimens had a significantly higher number of cells than did the control group after 5- and 15-day cultures. The cyto-compatibility of specimens was quantitatively evaluated using a cytodetacher, which directly measures the detachment shear force of an individual cell to the substrate. After 30-min culture, the cell adhesion forces were 38.4 nN for the smooth specimen, 140.8 nN for C3, 124.2 nN for C4, and 67.1 nN for C5. The results indicate that the cell adhesion force is influenced by cell shape and the Ca-P grooved patterns affect the cell shape and cytoskeletal structure, thus influence cell proliferation and cell adhesion force. The cyto-detachment test with nanonewton resolution is a sensitive method for studying cell–biomaterial interaction.

1. Introduction

Shape design and surface properties play important roles in dental implant performance, especially in the early stage of bone healing and long term [1]. Studies indicated that the surface properties of an implant, such as surface chemistry and surface topography, are key factors which mediate bone cell responses in the early stage [2]. When an implant is inserted into tissue, proteins are adsorbed in a few minutes, which mediate and control cell adhesion to the implant. The composition, type, number, and conformation of adsorption proteins dictate secondary phenomena, such as cellular adhesion, and cellular reactions, such as migration, proliferation, and differentiation [3], since cells are capable of sensing mechanical forces and converting them into biological signals via mechanotransduction mechanisms [4]. These kinds of mechanical force, including surface chemistry and topography, can play a critical role in the regulation of cell signaling and function under normal physiological conditions, which is referred to as mechanobiology.

Studies have revealed that cell migration is a multistep cycle on topography substrates that includes the extension of a membrane protrusion, the formation of stable attachments near the leading edge of the protrusion, and the translocation of the cell body forward [5,6]. Surface topography can greatly affect cell physiology. A previous study indicated that surface topography regulates cellular responses from the early stage adhesion and migration to regulate cell physiological responses [7]. The effects of topography on cell shape can also regulate cell growth, gene expression, secretion of proteinases, differentiation, and even life and death [8–10]. An in vivo study found that the fixation of a dental implant might be improved by the incorporation of macro- or microscopic features [11]. Therefore, researchers have attempted to alter the surface properties of artificial implants to maximize biocompatibility.

Microfabrication techniques can be used to precisely control topographies of substrates and masks. Calcium phosphate (Ca-P)-coated titanium alloys are widely used as orthopedic implant materials. They were found to enhance initial bony ingrowth, therefore stimulating osseous opposition to the implant surface, promoting a rapid fixation of the devices to the skeleton [5]. For in vivo study, bioactive ceramics integrate with bone tissue, which is due to the biomineralization of a thin layer of calcium phosphate at the interface between the ceramics and host bony tissue [6]. Therefore, the combination of

* Corresponding author. Tel.: +886 62387321; fax: +886 62387321.
E-mail address: tmlee@mail.ncku.edu.tw (T.-M. Lee).

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topography and Ca-P composition could enhance cell adhesion, especially in the early stage.

Studies have shown that stimuli such as topography exert a mechanical force on a cell and induce cell mechanobiology. In a previous study [7], deoxyribonucleic acid (DNA) and messenger ribonucleic acid (mRNA) levels, cell morphology, and cell adhesion were used to evaluate cell mechanobiology. Anselme [8] indicated that cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, and metastasis as well as tissue integration of biomaterials. Therefore, cell adhesion is an important factor since it is the first step of attachment to materials and results in mechanotransduction.

The cell adhesion force has been used to evaluate cell mechanobiology. Several methods have been proposed for evaluating the cell adhesion force. In our previous study, a cantilever-based technique was used to measure the adhesion force of osteoblasts cultured on various kinds of surface-treated titanium (Ti) alloy disk [9]. This method directly measures the actual strength of attachment between a single cell and its substratum to better assess the cyto-compatibility and mechanotransduction of biomaterials. The objective of the present study is to control cell shape on a Ca-P microgrooved surface. A cytodetacher is used to evaluate the initial adhesion force of osteoblasts and to identify cell mechanobiology. In addition, the cell morphology, area, elongation, and proliferation are also investigated to evaluate the osteoblast behavior on a Ca-P microgrooved surface.

2. Materials and methods

2.1. Microfabrication of CaP-coated grooved pattern

<100> p-type crystal-orientated silicon (Si) wafers were purchased from Wafer Work Corporation, Taiwan. In this study, grooved silicon substrates were fabricated using photolithography and wet etch techniques at National Nano Device Laboratory, Tainan, Taiwan. After a Radio Corporation of America (RCA) cleaning process was conducted to remove native oxide and organic particles from the Si wafer, a silicon dioxide layer was grown on the silicon substrate in a high-temperature oven. The groove pattern was transferred onto the positive-resist-coated silicon wafer using photolithography. The groove patterns were then etched by an anisotropic etchant on the sample with the desired dimensions under various etching conditions. After sonication in isopropanol and acetone, the micropatterns were coated with a 200-nm-thick titanium layer using a sputtering system (Duratek Model 101, Taiwan). The Ti-coated grooved samples were cut into 25 mm^2 pieces and then ultrasonically cleaned in acetone and deionized water for 5 min, respectively. Then, the disks were coated with a 200-nm-thick calcium phosphate layer using a radio frequency (RF) sputtering system. Hydroxyapatite was used as the sputter target. The RF sputtering system chamber was evacuated to a base pressure lower than 10^{-5} Torr, then back-filled with high purity argon gas (99.9995%) until a working pressure of 10^{-2} Torr was obtained. Prior to deposition, the substrate surface was first sputter cleaned for 5 min with argon using a substrate bias. The RF sputter deposition was performed using an RF generator (PFG 1000RF, German) operated at 300 W with a frequency of 13.56 MHz and sample stage heating at 150 °C for 1 h. The distance between the target and substrate was fixed at 5 cm and argon gas flow was fixed at 5.5 sccm. After sputtering, all samples were post-heat-treated at 750 °C for 1 h to obtain the crystalline phases of Ca-P film. The following Ca-P grooved patterns were used in this investigation: (a) the C3 coating pattern had 3.0-μm-deep grooves with a 1.0 μm pitch, (b) the C4 coating pattern had 4.5-μm-deep grooves with a 2.0 μm pitch, and (c) the C5 coating pattern had 5.5-μm-deep grooves with a 5.5 μm pitch. Ca-P-coated smooth Si wafers were used as control surfaces.

The surface and cross-section morphologies and composition of specimens were analyzed using a scanning electron microscope (SEM, Jeol 6390LV, Japan) equipped with an energy dispersive spectrometer (EDS, Oxford 350, England). Thin-film X-ray diffractometry (Rigaku D/Max2500, Japan) was conducted using CuK radiation, operated at 40 kV and 1 mA with an incident angle of 2° and a scan speed of 4° (2 θ)/min to analyze the phase composition.

2.2. Cell culture

A human fetal osteoblast line (hFOB1.19, ATCC number: CRL-11372) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells provide a homogenous, rapidly proliferating model system for studying normal human osteoblast physiology on osteoblast function. The base medium for hFOB1.19 cells was Dulbecco's modified Eagle's medium with F12 (DMEM + F12, GIBICO) supplemented with 10% fetal calf serum (FBS, GIBICO) and 0.3 mg/mL of G418 (GIBICO). The hFOB1.19 cells were maintained in an atmosphere of 5% CO_2/95% air at 34 °C to control the pH value. The medium was changed every 2 days. After cells grew to confluence, they were washed with phosphate-buffered saline (PBS) solution, treated with 0.05% trypsin-EDTA (GIBICO), and centrifuged at 1000 rpm for 10 min to obtain cell suspension. All specimens were packed into autoclaving bags, steam sterilized at 121 °C for 30 min, and dried at 121 °C for 15 min. After sterilization, each specimen was placed in one well of a 24-well plate (Nunclon Nalge Nunc Int., Denmark). For each test, there were 5 specimens in each group (N = 5).

2.3. Cell morphology, area, and elongation

After sterilization, each specimen was placed in one well of the 24-well plate. The cell suspension was cultured on the specimens at a density of 5×10^3 cells/cm^2 for 30 min, 1 h, and 3 h. 500 μL of medium was then added to each well. After incubation, the samples were washed three times with PBS, and fixed with 2.5% glutaraldehyde at 4 °C, 4% OsO_4 at 37 °C, and 1% tannic acid at 4 °C. The samples were dehydrated with a series of graded ethanol solutions and immersed in hexamethyldisilazane (HMDS, SIGMA) for 10 min. The cell immobilization specimens were coated with gold by sputtering, and observed by SEM at an acceleration voltage of 20 kV. The SEM images were analyzed using ImageJ software (Version 1.41, National Institutes of Health) at low magnification. 50–60 cells were used for cell area and perimeter calculations. The length and breadth of cells were defined by the longest chord and longest chord perpendicular to the length, respectively. The cell elongation index was defined as the ratio of the length to the breadth of each cell.

2.4. Cell adhesion force

The adhesion strength between substrates and cells was measured using a cytodetacher combined with a cytodetacher and laser tweezers (Cell Robotics Inc, Albuquerque, NM, USA). A cytodetacher has four major components: (a) a cell selector, (b) a scanning probe microscopy (SPM) cantilever tip (Anosensors NanoWorld AG, Switzerland), (c) a sample holder, and (d) a microscope working station equipped with a motorized stage, a video camera, and a microscope (Nikon, model TE300) (Fig. 1). The whole system was operated in a closed plastic chamber containing 5% CO_2 at 37 °C to maintain the medium pH value. 5×10^3 hFOB cells/cm^2 were seeded onto the specimens. After 30-min culture, the samples were placed into a sample holder with 10 mL of DMEM without phenol red at 37 °C. In the cell adhesion force test, the cytodetacher manipulated the SPM cantilever tip, which detached individual cells. Cytodetachment process images were recorded at twenty frames per second. The maximum deflection of the cantilever tip was calculated using Matlab 7.0 (The MathWorks, Inc., USA) with Hooke's law applied as follows:

$$F_{\text{adhesion}} = K \times \delta_{\max}$$