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The significance of crystallographic texture of titanium alloy substrates on pre-osteoblast responses

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Abstract

The aim of this study is to investigate the effects of grain orientation in polycrystalline materials on cell-substrate interactions. Samples are prepared from rods and sheets of Ti–6Al–4V substrates with predominately two distinct crystallographic orientations. X-ray diffraction analysis indicates that 36% of the surfaces of rod samples consist of $(1\,0\,\bar{1}\,0)$ plane, while the predominant orientation in the surface of the sheet samples is $(1\,1\,\bar{2}\,0)$ plane (29%). Morphological studies and cell biological experiments including cell attachment, proliferation and differentiation are conducted using MC3T3 pre-osteoblast cells cultured on these two different samples. The number of attached cells on the rod Ti-$(1\,0\,\bar{1}\,0)$ samples (70% after 1 h and 50% after 2 h) is higher than on the sheet Ti-$(1\,1\,\bar{2}\,0)$ samples. Cell proliferation after 3 days is also significantly higher on the Ti-$(1\,0\,\bar{1}\,0)$ samples. Alkaline phosphatase activity, however, shows no significant difference between the two samples. Scanning electron microscopy (SEM) analysis of MC3T3 cells grown on samples with different crystallographic texture demonstrate significant differences in morphology with respect to attachment and growth pattern. This study shows that crystal orientation of the substrate can influence cell responses and, therefore, substrate engineering can be used to improve and control cell-substrate interactions.

Keywords: Titanium alloy; Crystallographic texture; Cell adhesion; Cell proliferation; Alkaline phosphatase; Cell morphology

1. Introduction

The performance of implants depends on how cells and proteins interact with biomaterials. Initial interaction between macromolecules and substrate influences cell responses at the cell-biomaterial interface, which determines long term durability and reduces the possible failure of implants [1,2].

Among various biomaterials, titanium and its alloys are widely used in orthopaedic and oral implantations. Titanium and titanium alloys possess polycrystalline structure with different crystallographic orientation. Physical properties of polycrystalline materials strongly depend on the distribution of crystallographic orientation of grains [3]. Although the cell-substrate interaction between polycrystalline titanium alloys and different cell lines has been extensively investigated [4], there are no reports available in the literature documenting the role of crystal orientation of polycrystalline materials on cell responses.

In studies related to the development and formation of hard tissues such as bone and teeth, it has been shown that in the process of biomineralization, the spacing of the functional group of proteins can match the atomic order of the inorganic cation leading to the nucleation and

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precipitation of minerals [5–7]. Vice versa, the atomic order of the mineral substrate such as hydroxyapatite (HA) can serve as an ordered template to interact with the functional group of proteins leading to polymerization and organized structure. It has been shown that osteocalcin can only interact with calcium ions in specific crystallographic planes in the HA lattice. It is proposed that this specific configuration of the osteocalcin on the HA crystal face enables the binding of receptors responsible for signal transduction functions in osteoblasts and osteoclasts [8]. It has also been reported that cell attachment, spreading, motility and cell–cell aggregation, depend on different crystal faces of the substrate [6,9].

To improve the bone bounding ability of metallic implants, coating material with osteoinductive characteristics such as HA has been widely used [10]. It has been shown that HA precipitated from simulated body fluid creates a specific crystal orientation (1 0 0) [11]. Extreme preferred orientation has been also found in HA thermally deposited coating on metal surfaces [12].

From these findings it can be concluded that any cell-substrate interaction must be controlled by the correlation between macromolecules produced by the cell and atomic order of the substrate confined by specific crystallographic orientations. It is important, therefore, to study the atomic structure of the substrate and its interaction with the cell at the nanoscale level. The crystal orientation of polycrystalline materials used as implants and their interaction with cells and proteins remain largely unknown. In this study, we investigate two polycrystalline Ti–6Al–4V alloys with different crystallographic textures and similar chemical composition described as extruded rod and rolled sheet in order to determine how crystal orientation of metallic substrates can affect the biological responses of cells. This will be assessed through the cell attachment, proliferation, alkaline phosphatase (ALP) activity, total protein content and morphological studies.

2. Materials and methods

2.1. Preparation and surface characterization of Ti alloy substrates

The titanium alloy substrates (rod and sheet of Ti–6Al–4V) were commercially available from McMaster-Carr Company (Los Angeles, CA, USA). Pieces of both rods and sheets were cut in such a way that the surface areas of both types of samples were the same. To eliminate the effect of surface roughness of the substrates, all samples were polished mechanically followed by vibratory polishing to achieve a mirror finish. Subsequently, the samples were cleaned by sonication in ethanol, acetone and isopropanol, respectively, for 20 min and autoclaved before the cell culture. Surface roughness and topography of the samples were analyzed by atomic force microscope (AFM, Veeco Instruments Inc., Woodbury, NY, USA) in tapping mode. Surface wettability of the titanium alloy samples was determined by measuring the contact angles of deionized water (DI) on each sample using a surface analysis system equipped with image analyzer software (VCA Optima 2500, AST Products, Billerica, MA, USA). An auto pipette was used to ensure a uniform volume of DI water droplet (0.5 μl). The experiments were run at room temperature on five samples at three different time points.

2.2. Crystallographic texture analysis

Crystallographic texture is represented by pole figures (PF), orientation distribution functions (ODF) and inverse pole figures (IP), which all show the major orientations present in the material. Pole figure is measured by X-ray diffraction and more than one pole figure is needed in order to calculate ODF [3]. The pole figures of titanium alloy samples were obtained using a Siemens D-500 X-ray diffractometer (XRD, Siemens, Germany). Six pole figures were measured (0 0 2), (1 0 0), (1 0 1), (1 0 2), (1 0 3), and (1 1 0). From these measurements, the ODF was calculated using TexTools software [13]. Normal direction inverse pole figures of both types of samples were also calculated from their ODF's using the same software.

2.3. Culture of pre-osteoblasts

Mouse pre-osteoblast cell line MC3T3-E1 subclone 14 provided by American Type Culture Collection (ATCC, Manassas, VA, USA) was used for this study. The cells were cultured in T25 plastic bottles (Nunc) in alpha minimum essential medium (α-MEM, Invitrogen, Corporation, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air; with the growth media changed every 48 h. Cultured cells were detached by trypsinization, suspended in new culture medium and used for the designed experiments. Controlled experiments were conducted on conventional culture well plates for each set of experiments.

2.4. Cell growth and behaviour on Ti–6Al–4V substrates

2.4.1. Cell attachment

Cell attachment on both types of Ti–6Al–4V samples was evaluated by counting the number of attached cells after 30, 60, 120, and 240 min of incubation. The samples were placed into a 24-well plate and seeded with a density of 7500 cells cm^{-2}. After each time point, the samples were washed with phosphate buffer solution (PBS) to remove unattached cells. Adherent cells were removed from the samples by incubation with 0.25% trypsin in EDTA (Invitrogen Corporation, USA). Trypsin was removed by centrifugation and the cells re-suspended in fresh growth medium. The number of cells in the solution was measured with a hemacytometer.

Fluorescent labeling of nucleic acids was used as a second method of assessing cell attachment [14,15]. Cells were seeded in 24-well plates with a density of 50,000 cell cm^{-2} on each titanium alloy sample and incubated for 30, 60,120, and 240 min. After each time point, the medium was removed and the sample washed with PBS. Subsequently, CyQUANT GR dye in cell lysis buffer (Molecular Probes, Inc., Eugene, OR, USA) was added to each sample and incubated for 2–5 min at room temperature. The emitted fluorescence was then measured directly by FL_X-800 microplate fluorescent reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) with excitation at 485 nm and emission detection at 530 nm. A reference standard curve was created for converting the observed fluorescence values into cell numbers.

2.4.2. Cell proliferation

The proliferation of the pre-osteoblast cells on titanium alloy samples and plastic culture plates (controls) was investigated by measuring the number of cells after 3, 7, and 11 days of culture. Cells were seeded in a 24-well plate onto samples and control plates at a density of 5000 cells cm^{-2}. At each time point, cells grown on the samples and the plates were rinsed twice with PBS, detached from the samples by trypsin and counted using a hemacytometer. The fluorescent labeling of nucleic acids was also performed to measure the cell proliferation rate after 3, 7, and 11 days according to the method described above.

2.5. Alkaline phosphatase activity and total protein content

The differentiation of pre-osteoblast to osteoblast cells was evaluated as a function of alkaline phosphatase (ALP) activity after 5, 12, and 16 days.