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Adherence of human mesenchymal stem cells on Ti and TiO_2 nano-columnar surfaces fabricated by glancing angle sputter deposition

Yahya Motemani[a,*,1], Christina Greulich[b,*,1], Chinmay Khare[a,1], Michael Lopian[a], Pio John S. Buenconsejo[a], Thomas A. Schildhauer[b], Alfred Ludwig[a,*], Manfred Köller[b,*]

[a] Institute for Materials, Faculty of Mechanical Engineering, Ruhr-Universität Bochum, 44801 Bochum, Germany
[b] Bergmannsheil University Hospital, Department of Surgery, Surgical Research, Buerkle-de-la-Camp-Platz 1, 44789 Bochum, Germany

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ABSTRACT

The interaction of human mesenchymal stem cells (hMSCs) with Ti and TiO_2 nano-columnar surfaces fabricated using glancing angle sputter deposition was investigated. The adherence and proliferation of hMSCs on different nano-columnar surfaces, including vertical columns, slanted columns and chevrons, were examined with calcein-acetoxymethyl ester fluorescence staining and scanning electron microscopy. For comparison, adherence of hMSCs on compact, dense films was also studied. After 24 h and 7 days, adherent and viable cells were observed on both, Ti nano-columns as well as dense Ti films, which confirms the biocompatibility of these nanostructures. Very small pseudopodia with width of approximately 20–35 nm and length varying from 20 to 200 nm were observed between the nano-columns, independent of the type of the nano-columnar morphology. Large inter-column spacing and effectively increased surface area make these nanostructures promising candidates for bio-functionalization or drug loading on the surface of Ti-based implants.

1. Introduction

Titanium and its alloys have been used as implants such as bone anchoring, hip joint-replacements, bone screws, stents, and fracture fixation devices [1–3]. Ti alloys are of interest as they present biocompatible interface together with relatively low elastic modulus and high strength [1]. The biocompatibility of Ti alloys is attributed to the thin passive oxide layer formed on their surfaces [4]. Particularly, with regard to bone implants various strategies have been followed to improve osseo-integration including surface processing, coating and bio-functionalization [5,6]. However, the surface topography of implants itself has a profound impact on tissue cell adherence and it is also able to improve the quality of implantation [7,8]. Mesenchymal stem cells (MSCs) are closely involved in regeneration of tissue such as bone, cartilage, muscle, ligaments, tendons, adipose tissue, and stroma [9,10]. The capability of MSCs for replication and differentiation into skeletal cell lineages renders them not only as suitable cellular candidates for regenerative medicine and tissue engineering [11] but also as suitable cellular reporters for tissue-biomaterial responses [12,13].

Nanostructured materials have promising applications in orthopedic surgical practices [14]. Various methods have been utilized to fabricate nanostructured Ti surfaces for implant applications [15–19]. Among chemical methods, anodization is one of the promising methods to modify Ti surfaces, where nano-tubular structures are produced on the surface via oxidation process [16,18]. The nanoscale surface morphology has been reported to significantly affect the cellular responses [20–22], making it a critical parameter in optimization of the biocompatibility of implant surfaces. Therefore, in order to activate desirable cellular responses, well-defined topographic features with tunable properties would be required for specially designed implant materials.

The physical vapor deposition technique: '*Glancing angle deposition*' (GLAD) [23,24] has gained attention to synthesize three-dimensional porous, columnar nanostructures in a simple, but well-controllable process. Under GLAD conditions, an obliquely incident particle flux β (usually $\beta \geq 80^\circ$, with respect to the substrate normal) leads to the growth of a highly porous, columnar film as a result of strong influence of self-shadowing mechanism. On stationary substrates, GLAD films tend to become columnar, with column inclination toward the direction of the particle source

* Corresponding authors. Tel.: +49 234 32 27492; fax: +49 234 32 14409.
E-mail addresses: yahya.motemani@rub.de (Y. Motemani), Christina.Greulich@rub.de (C. Greulich), alfred.ludwig@rub.de (A. Ludwig), Manfred.Koeller@rub.de (M. Köller).
[1] These authors contributed equally.

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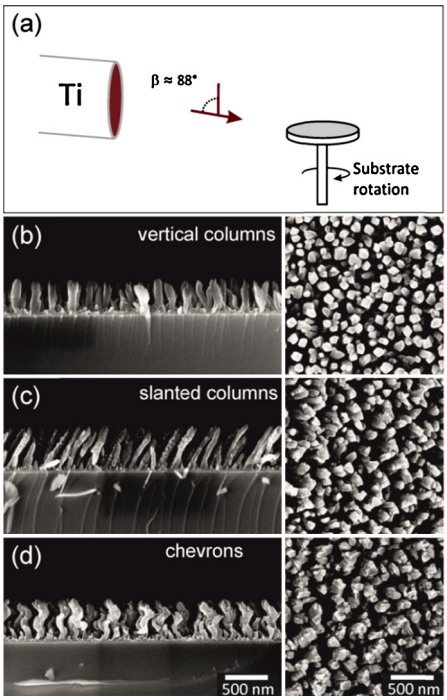

Fig. 1. (a) Schematic (not to scale) of the deposition geometry used to fabricate Ti nanostructures. The Ti target is positioned at an oblique angle ($\beta \approx 88^\circ$) to grow nano-columns, while the substrate can be rotated to obtain the desired shape of nanostructure. (b–d) Cross-sectional and top-view SEM micrographs of vertical, slanted nano-columns and chevron Ti nanostructures, respectively.

[25]. With an appropriate substrate rotation scheme, GLAD offers realization of different nano-structures comprising spirals, screws, vertical columns, and chevrons [25,26]. By utilizing pre-patterned substrates well-ordered and periodically arranged nano-structures can be obtained [27–29]. Such sculptured thin films (STFs) offer variety of applications including polarization filters [30], photonic crystals [31], pressure sensing [32], and photovoltaic [33]. Besides, GLAD nanostructures have also been used in bio-sensing with surface enhanced Raman scattering [34–36]. Recently, Dolatshahi-Pirouz et al. [22] and Pennisi et al. [21] utilized GLAD-grown Pt nanostructures to study adsorption of fibrinogen and proliferation of primary human fibroblast. They observed that cellular responses on Pt nanostructures are dependent upon roughness and nanoscale features. However, detailed study of biological responses on STFs and interaction of MSCs with GLAD-grown Ti and TiO_2 remains to be investigated. The present study aims to examine the adherence and proliferation of human mesenchymal stem cells cultured on Ti and TiO_2 nanostructures fabricated by glancing angle sputter deposition.

2. Experimental methods

2.1. Fabrication of nanostructured thin films

All Ti films were fabricated by magnetron sputter deposition at room temperature from a 4-in. metallic Ti target (purity 99.99%) in a load-locked sputter system (AJA International), at a base pressure $\leq 1.6 \times 10^{-8}$ Torr. For all experiments pieces cleaved from 4-in. oxidized Si (1 0 0) wafers were used as substrates (the 1.5 μm thick thermally oxide serves as diffusion barrier). In order to grow Ti nano-columnar films, the Ti target was positioned at an oblique angle of $\beta \approx 88^\circ$, with respect to the substrate normal (Fig. 1(a)). The target to substrate distance was approximately 19 cm. Subsequently, the Ti target was sputtered at 1 mTorr with 150 W (DC) in 99.9999% Ar. During the deposition of vertical columns the substrates were rotated continuously at ~25 rev/min. In order to grow slanted columns, the substrates were held stationary. Chevrons were fabricated by keeping the substrate motionless in order to grow the first arm (t_{dep} = 50 min) and then rotating the substrate by 180° to grow the second arm. In this way, by altering particle flux direction, four arms of the chevron structures were grown. The reference compact, dense films were fabricated at 1 mTorr with 300 W (DC) in 99.9999% Ar, with the Ti target fixed at confocal inclination angle $\beta \approx 27^\circ$. The substrate was rotated continuously at ~25 rev/min. All films were deposited in metallic form and consecutively, in a second step, oxidized by annealing in air at 500 °C. The temperature ramp-up was adjusted to ~10–12 °C/min. The samples were kept at the desired annealing temperature for 8 h, followed by overnight cooling inside the furnace.

2.2. Cell culture

Human mesenchymal stem cells (hMSC, 3rd to 7th passage, Lonza Walkersville Inc., MD, USA) were cultured in cell culture medium RPMI1640 (Life Technologies, Darmstadt, Germany) containing 10% fetal calf serum (FCS, Life Technologies) and L-glutamine (0.3 g L^{-1}, Life Technologies) using 75 cm^2 flasks (Falcon, Becton Dickinson GmbH, Heidelberg, Germany). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. hMSCs were sub-cultivated every 7–14 days depending on the cell proliferation. Adherent cells were washed with phosphate buffered saline solution (PBS, GIBCO, Life Technologies) and detached from the culture flasks by addition of 0.2 mL cm^{-2} 0.25% trypsin/0.1% ethylendiaminetetraacetic acid (EDTA, Sigma–Aldrich, Taufkirchen, Germany) for 5 min at 37 °C. Subsequently, the hMSCs were collected and washed twice with RPMI/10% FCS. Human MSCs were seeded at 1×10^4 per cm^2 onto the specimens using 24-well cell culture plates (Falcon, Becton Dickinson GmbH, Heidelberg, Germany) and cultured in RPMI/10% FCS at 37 °C under cell culture conditions.

2.3. Characterization methods

In order to determine the effective deposition rate of Ti for GLAD nanostructures and for reference films, a mechanical profilometer (Ambios) was used, which measured the step between substrate and film surface, created by a photoresist lift-off mask. As-deposited Ti films and annealed films were examined by scanning electron microscopy (SEM, Leo 1530VP) at 15 kV acceleration voltage. SEM micrographs were analyzed by using the ImageJ software (ImageJ 1.45s, National Institute of Health, USA) [37]. The TiO_2 (Rutile) phase was detected in the annealed samples using a PANalyticalX'pertdiffractometer with a Cu Kα X-ray tube (Fig. S1, see Supplementary data).

The adherence and morphology of the incubated hMSCs were analyzed using calcein-acetoxymethyl ester (calcein-AM, Calbiochem, Schwalbach, Germany) fluorescence staining. After cultivation of the cells on the thin films for 24 h or 7 days, cells were washed twice with RPMI and incubated with calcein-AM (1 μM) at 37 °C for 30 min under cell culture conditions. Subsequently, the adherent cells were washed again with RPMI and analyzed by fluorescence microscopy (Olympus MVX10, Olympus, Hamburg, Germany). Fluorescence microphotographs were taken (Cell P, Olympus) and digitally processed using Adobe Photoshop® 7.0. In this method, living cells give rise to green fluorescence. After fluorescence microscopy, cells were carefully washed with PBS, fixed with 5% glutaraldehyde (Sigma–Aldrich, Taufkirchen, Germany) overnight at 4 °C and washed again with PBS. Subsequently, the cells were dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 96% and 100%) for 5 min each. After drying, the samples were coated