Materials Science & Engineering C 92 (2018) 268–279

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

In vitro corrosion behavior and biocompatibility of nanostructured Ti6Al4V

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ARTICLE INFO

Keywords:
Surface nanocrystallization
Sliding friction treatment
Corrosion resistance
Osteoblast response
Hemocompatibility

ABSTRACT

Ti6Al4V (TC4) alloy has long been used as a bone interfacing implant material in dentistry and orthopedics due to its excellent biocompatibility and mechanical properties. The performance of TC4 can be further tailored by altering its grain structures. In this study, by means of sliding friction treatment (SFT), a nano-grained (NG) surface layer with an average grain size of ≤100 nm on the topmost surface was successfully generated on coarse-grained (CG) TC4 alloy sheet. It was shown that the NG surface possessed notably enhanced corrosion resistance in physiological solution compared to the CG surface, due to the formation of thicker and denser passive film facilitated by surface nanocrystallization. Additionally, the NG surface with stronger hydrophilicity favorably altered the absorption of anchoring proteins such as fibronectin (Fn) and vitronectin (Vn) that can mediate subsequent osteoblast functions. The *in vitro* results indicated that the NG surface exhibited remarkable enhancement in osteoblast adherence, spreading and proliferation, and obviously accelerated the osteoblast differentiation as compared to CG surface. Moreover, the NG surface also demonstrated good hemocompatibility. These findings suggest that SFT can endure bio-metals with advanced multifunctional properties for biomedical applications.

1. Introduction

Bio-metals (*e.g.*, Ti and its alloys, stainless steel, as well as Co-Cr based alloys) have long been implanted in the human body in order to restore lost functions caused by disease or trauma [1–4]. The continuous increment in the life expectancy and the technological improvement to fix complex fractures further drive the demands for advanced orthopedic devices with long-term stability as well as proper surface and bulk properties to favorably mediate cell-substrate interactions.

Specifically, there are numerous reports on successful application of metal coatings [5,6] and biomimetic coatings [7–10] to Ti or its alloys. Obvious enhancement in surface hardness, wear and friction resistance, corrosion resistance, surface wettability as well as cytocompatibility is reported after these surface modification treatments [1,3,5–9]. Besides, different combinations of surface treatments such as sandblasting, acid etching, and/or anodization have also been used as particular surface modification techniques to obtain irregularly roughened or hierarchical porous surface [11–14], and numerical data have confirmed enhanced corrosion resistance, cell attachment and subsequent cell functions. The grain structure of biomedical implants has also proven to play a key role in mediating almost all characteristics of polycrystalline materials [15]. Grain size reduction through severe plastic deformation (SPD) processes (*e.g.*, severe shot peening (SSP) [16,17], high pressure torsion (HPT) [18,19], equal channel angular pressing (ECAP) [20,21], and surface mechanical attrition treatment (SMAT) [22–24], *etc.*) has gained increasing interest to simultaneously optimize various disparate properties of materials including mechanical strength, corrosion resistance, as well as wear or scratch resistance. Excitingly, S. Bagherifard recently reported that SSP treated 316L stainless steel surface exhibited a significant enhancement in osteoblast adhesion and proliferation as well as a remarkable decrease in the adhesion and growth of gram-positive bacteria (*S. aureus* and *S. epidermidis*) as compared to untreated samples [16]. R. Huang also observed that SMAT-induced NG Ti surface had the potential to alter absorption of proteins that mediate cell adhesion and control/enhance subsequent cell functions [23,24].

Our group has successfully developed a novel SPD technique (named as sliding friction treatment (SFT)) that can easily and successfully generate nanostructure on various metallic sheets (*e.g.*, Mg, Cu and Ta) [25–29]. Our investigation has demonstrated that the average grain size in the topmost surface of Mg, Cu and Ta sheets can be reduced *via* SFT to be 70 nm, 60 nm and 7 nm, respectively [25,26,28], resulting in noticeably increased microhardness as well as tensile/yield strength in these sheets. Obvious enhancement in the corrosion resistance was also obtained in SFT treated AZ31 Mg alloy and Ta [26,29]. A recently published work of our group [30] revealed that the

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https://doi.org/10.1016/j.msec.2018.06.061
Received 11 September 2017; Received in revised form 2 June 2018; Accepted 28 June 2018
Available online 30 June 2018

improved corrosion resistance in SFTed Ti benefited from the formation of a thicker passive film on NG surface, meanwhile, it preliminarily showed that SFT-induced NG layer favorably promoted the earlier response (*e.g.*, cell attachment and proliferation) of osteoblast to pure Ti for implant application.

Excitingly, we found that SFT could also give rise to nano-grains on the TC4 alloy (one of the best implant choices in dentistry and orthopedic implants due to its good combination of specific strength, corrosion resistance and biocompatibility [1,4]). Hence, it is of our special interest to systematically explore the advantages of SFT treated NG TC4 in the respects of both corrosion resistance and cytocompatibility, especially the osteoblast functions associated with cell differentiation. In the current study, the corrosion behavior in two kinds of physiological solutions, as well as the comprehensive cell-substrate interaction including attachment, proliferation and differentiation on SFT treated NG TC4 was investigated. The untreated coarse-grained (CG) TC4 was set as control.

2. Methods

2.1. Sample preparation

A 4 mm thick TC4 alloy sheet was annealed at 1073 K for 1 h before the SFT processing. The details of the SFT set-up and the sequences of the SFT processing were given in Refs. [25,26]. The processing parameters of the SFT process in this study were as follows [31]: 300 N in load, 0.2 m/s in sliding velocity, 100 μm in offset displacement perpendicular to the sliding direction after each sliding, and 100 in cycle. Square samples of $10 \times 10\ mm^2$ cut from both the SFT treated and untreated TC4 sheets were ultrasonically cleaned in acetone, ethanol, and distilled water for 10 min each and dried in air. For *in vitro* biocompatibility studies, all samples were sterilized with alcohol immersion for 3 h and rinsed twice with sterile phosphate buffer saline (PBS; Sigma, USA) before cell seeding.

2.2. Microstructure characterization

The surface microstructure of the SFT treated TC4 sample was characterized by a JEOL JEM-2100 transmission electron microscope (TEM) operated at 200 kV. The TEM samples were cut from the top surface layer and thinned by ion thinning at low temperature. The surface grain structures of the untreated CG TC4 sample were observed by Olympus PMG 3 optical microscopy (OM).

2.3. Electrochemical tests

The electrochemical experiments were performed in both physiological saline (PS, 0.9 wt% NaCl aqueous solution) and simulated body fluid (SBF, prepared according to Ref. [32]) solutions at 37 ± 0.5 °C on an electrochemical workstation (IM6 Zahner-electrik Gmbh, Zenniom, Germany) with the three-electrode configuration: *e.g.*, a test sample, a platinum wire and a saturated calomel electrode (SCE) act as the working electrode, counter electrode and reference electrode, respectively. Prior to electrochemical impedance spectroscopy (EIS), the samples were immersed in the solution for 1200 s and EIS was then carried out at the open circuit potential (OCP) with a 10 mV alternating signal from 10 kHz to 100 mHz. The potentiodynamic polarization (PDP) curves were carried out at a scanning rate of 0.01 V/s. Nyquist and Bode plots were obtained from EIS experiments to evaluate the characteristic of passive films formed on the surfaces of TC4. The solution was refreshed after each test and three replicates for each condition were performed to ensure reproducibility. The specific parameters for EIS were analyzed by ZSimpWin 3.0 software.

2.4. Surface roughness and wettability

The surface roughness of each specimen was recorded by atomic force microscopy (AFM, Dimension ICOM, America) at the nano-level with a scanning size of $5 \times 5\ \mu m^2$. The microscope mode was tapping (probe: RTESP, BRUKER) with an aspect ratio of 1.0 at a scan rate of 0.999 Hz, the cantilever used possessed a nominal force constant and resonance frequency of 60 N/m and 340 kHz respectively. Five measurements were performed at random areas for each group at room temperature.

To characterize how the NG structure changes the surface properties of TC4, the surface wettability test was performed using a Kino SL200B contact angle system. Before contact angle testing, all TC4 samples were rinsed with acetone, ethanol and deionized water, and subsequently dried in air for 30 min. For each measurement, a 2.5 μL droplet of distilled water was suspended from the tip of a microliter syringe and the syringe tip was moved toward the sample surface. When the water droplet came into contact with the surface, an image was obtained with a camera, and the contact angle between the droplet and the surface was measured from the magnified image. The reported contact angle value of each surface is an average of at least five measurements obtained at random locations on the sample surface.

2.5. Protein adsorption assay

After incubation in α minimum essential medium (α-MEM) (Life Technologies, USA) containing 10% fetal bovine serum (FBS) (Life Technologies, USA) for 1 and 24 h at 37 °C, the proteins adsorbed onto the samples were detached into 1% sodium dodecyl sulfate. The fibronectin (Fn) and vitronectin (Vn) concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA). The total protein concentration was determined using a NanoDrop 2000C device (Thermo Scientific, USA). Each test was repeated for four times ($n = 4$).

2.6. Cell culture conditions

The human fetal osteoblasts (hFOB1.19, provided by the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS, 0.3 mg/mL Geneticine418 (Sigma, USA), 0.5 mM sodium-pyruvate (Sigma, USA) and 1.2 mg/L Na_2CO_3 at 37 °C in a humidified atmosphere of 5% CO_2. The medium was refreshed every 2 days during cell culturing. hFOB1.19 suspension of 1 mL with 8×10^4 cells was seeded on the samples placed centrally in 24-well plates.

2.6.1. Cytotoxicity, cell adhesion, proliferation, and morphology

The lactate dehydrogenase (LDH) activity in the culture medium was used as an index of cytotoxicity. After culturing for 1, 4 and 24 h, the culture medium was collected (among this period the medium was not changed) and centrifuged, and the LDH activity in the supernatant was determined spectrophotometrically according to the manufacturer's instruction (Sigma, USA).

The cells were seeded on CG and NG TC4 samples (four replicates) in 24-well plates at a density of 8×10^4 cells/well and the cell counting kit-8 (CCK-8) assay was used to assess the adhesion and proliferation of osteoblasts after culture of 1 h, 5 h, 24 h, 3 days, 7 days and 14 days. After 1, 3, 7, 14 days of incubation, the samples with hFOB1.19 were washed with PBS, fixed in 3% glutaraldehyde, dehydrated in a graded ethanol series, freeze-dried, and sputter-coated with gold prior to observation by the scanning electron microscope (SEM, JSM-6700F, Japan).

Live/dead staining using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, France) was performed to identify viable and nonviable hFOB1.19 on the samples after 3 days of incubation. The cell-adherent