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Osteoblast cell response to oxide films formed on nanograin 316L stainless steel obtained by two-dimensional linear plane-strain machining

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ABSTRACT

The long-term success of orthopedic implants is strongly determined by their surface characteristics and the interaction of this surface with the surrounding biological environment. The biocompatibility of metallic biomaterials is mainly due to their ability to form an adherent oxide layer on their surface that is in direct contact with the biological environment. In this study, oxide layers were grown on ultra-fine grained 316L stainless steel samples, naturally, chemically and thermally. Samples in three different nanoscale average grain sizes were obtained by severe plastic deformation using linear plane-strain machining technique. Refining the grain size along with growing the oxide layer create surfaces with a wide variety of surface topography, roughness and chemistry. After chemical treatment, a chromium-enriched oxide with island-like topography was formed on the surface and substrates with smaller average grain size demonstrated rougher surfaces. After thermal treatment, micro-scaled oxide grains were formed on the surface and the amount of manganese oxide in its composition was increased. MC3T3 cell adhesion was greatly improved on the native oxide formed on the sample with the smallest average grain size. The oxide layer that is naturally formed on the surface demonstrated higher biocompatibility compared to both thermally and chemically formed oxides.

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1. Introduction

Increasing the average life expectancy and demands for orthopedic devices result in an urgent requirement to developing novel orthopedic devices with higher durability and functionality [1]. Metals present a wide range of favorable mechanical properties for load-bearing orthopedic applications compared to ceramic and polymer compositions; however, they are all inert and do not interact with the biological environment to bond with the bone and enhance the biological interface. Therefore, improving the biological function of metallic surface can open the window to design and develop novel durable orthopedic biomaterials [2,3]. Biological cells are attached to biomolecules initially adsorbed on the surface; composition and configuration of these adsorbed biomolecules conduct signals to cells determining their behavior. Thus, engineering the surface in nanometer scale has been inferred as an attractive technique to manipulate surface characteristics in order to control the absorbance of biomolecules in this scale and improving the cell behavior [4,5].

Traditional methods for altering the surface in nanoscale require multi-step processes, they are chemically complicated or result in uncontrolled surface property changes [6]. A new technique, severe plastic deformation (SPD), is an alternative approach that can produce metals with ultra-fine grain sizes through applying large deformation at high strain rates. SPD techniques do not have complications of other nanofabrication methods and also increase the mechanical strength of the bulk material [7]. Linear plane-strain machining (LSM) is a SPD method whereby a sharp tool quickly removes the surface metal in a predetermined depth and angle. In this method, average grain size can be controlled by three parameters of machining tool angle, tool velocity, and cutting depth [8].

In this study, we studied the MC3T3 (an osteoblast precursor cell line) cellular responses on the austenitic 316L stainless steel samples with three different nanoscale average grain sizes

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obtained by linear plane-strain machining SPD technique. In addition, two types of oxide layers were grown on various nanograin surfaces (*i.e.,* chemical and thermal treatment) to investigate the role of the nanocrystal structures both on surface oxide formation and surface biological function, as well as to compare the MCT3T3 cell adhesion.

2. Materials and methods

Commercially available, annealed and cold-finished 316L stainless steel plate (McMASTER-CARR, Cleveland, OH, USA) was processed by linear plane-strain machining at room temperature using cutting depth of 150 μm and tool velocity of 25 mm/s for three different rake angles of +40°, +20° and 0°. Then, the samples were mechanically and chemically finished to a mirror-finish surface. Oxide layer was chemically grown on all three nanograin samples and the control sample (*i.e.,* coarse-grain samples) using the chemical method reported by Evance et al. [9]. Thermal treatment was also examined to grow the oxide layer on nanograin samples and the control sample via oxidizing the samples in air at 1000 °C temperature for 15 min and rapidly cooling down to the room temperature [10]. Table 1 shows the list of samples prepared in this study using LSM technique along with the surface treatment.

Surface topography and microstructure of all samples was characterized by scanning electron microscopy (SEM) (Jeol-JSM 6610 LV, Japan). Surface chemistry of all samples before and after thermal and chemical treatment was analyzed using X-ray photoelectron Spectroscopy (XPS, ESCALAB 250 XI Thermoscientific, USA). MC3T3 cells at passage number 6 were seeded on the specimens at a density of 3×10^5 cells/ml. Fluorescent images were taken to observe the live and attached cells using fluorescent microscopy (Olympus BX43, Japan) using Calcein AM (life technologies™, NY, USA) staining. Besides, the viability and proliferation of cultured cells was determined using MTT (3- [4,5-dimethylthiozol-2-yl]−2,5-diphenyltetrazolium bromide) (Millipore, MA, USA) colorimetric assay in different time periods (1 h, 2 h, 4 h, 1 d and 5 days).

3. Results

3.1. Surface characterization

Fig. 1 shows the results of SEM imaging on all the samples listed in Table 1. Surfaces with native oxide layer (*i.e.,* no grown oxide either chemically or thermally) showed smooth and mirror-finish topography in micrometer range and no noticeable difference was observed in the surface topography of control sample and the samples obtained by different rake angles (Fig. 1A1, B1, C1 and D1). However, island-like structures, grooves and holes are appeared on the surface after 1hr chemical treatment. The average size of these island-like structures was reduced and the surface roughness increased over decreasing the rake angle (Fig. 1A2, B2, C2 and D2). After thermal treatment, new micro-features were appeared on the surface; however, the size and shape of them were substantially smaller than those observed in the chemically treated samples (Fig. 1A3, B3, C3 and D3).

In addition to surface topography, the chemical compositions of samples were analyzed using XPS technique. Fig. 2(A), D and G show the survey spectra of all samples before and after chemically and thermally surface treatment, respectively. Survey spectra did not change over changing the rake angle before and after chemical and thermal surface treatments. The survey spectra of untreated surfaces showed dominating peaks corresponding to elements of carbon, oxygen, chromium and iron. The lower peak corresponded to the nickel. High resolution spectra of Fe2p and Cr2p in untreated surfaces are presented in Fig. 2(B) and (C), respectively. Fe2p spectra represented a double peak corresponding to free iron and iron oxide compositions. The XPS results for Cr2p region also indicated the presence of free chromium and chromium oxide compositions on the surface. In both chemically and thermally treated samples, peaks corresponding to free iron and chromium disappeared (Fig. 2(E), F, H and I). In chemically treated surfaces, similar peaks to the peaks in the untreated surface were observed; however, the peaks corresponding to chromium oxide and iron oxide were significantly intensified. In thermally treated surfaces, in addition to present peaks observed in untreated surfaces, a new peak corresponding to manganese compositions appeared on the surface and the peak corresponding to chromic oxide was strongly intensified.

3.2. Cell culture study

The results of MTT assay for viability of MC3T3 cells on different substrates over different time periods (1 h, 2 h, 4 h, 24 h and 5 days) are shown in Fig. 3. Fig. 4 shows Calcein AM stained live cells cultured on all the samples before and after surface treatments. Over decreasing the rake angle in untreated samples, MTT results show that cell viability increases in all the time periods (Fig. 3(A)) and the fluorescent intensity also became higher (Fig. 4A1, A2, A3 and A4); the highest number of live cells was spread out on the untreated 0° surface (Fig. 4A4). In the chemically treated surfaces, no significant difference is observed between the viability of cells in different time periods (Fig. 3(B)), besides, the fluorescent imaging also shows no significant difference in the number of live cells in chemically treated surfaces (Fig. 4B1, B2, B3 and 4B4). In thermally treated surfaces, both MTT assay and fluorescent imaging results show that the number of live cells is higher in 0° sample compared to the control sample (Fig. 3(C) and Fig. 4C1, C2, C3 and 4C4). The cell growth on thermally and chemically treated surfaces is noticeably smaller compared to the analogous untreated surfaces.

4. Discussion

Linear plane-strain machining is a two-dimensional SPD technique which can impose a high strain rate altering the material microstructure under a precisely controlled process via varying parameters of rake angle (α), depth of cut and tool velocity. The average grain size of 316L stainless steel is about 22 μm while after plane-strain machining, it reduces to nanometer scale (*e.g.,* 42 nm

Table 1
List of samples prepared by linear plane-strain machining (LSM) technique with different rake angles (*i.e.,* 0°, 20° and 40°) and after surface treatment (*i.e.,* chemical treatment, thermal treatment) (Chem: chemical treatment, Therm: thermal treatment and N: none).

Sample #	1	2	3	4	5	6	7	8	9	10	11	12
Rake angle (°)	0	0	0	20	20	20	40	40	40	N	N	N
Surface treatment	N	Chem	Therm	N	Chem	Therm	N	Chem	Therm	N	Chem	Therm