Bioelectrochemistry 98 (2014) 39–45

Contents lists available at ScienceDirect

Bioelectrochemistry

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

# The two step nanotube formation on TiZr as scaffolds for cell growth

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ARTICLE INFO

*Article history:*
Received 14 November 2013
Received in revised form 26 February 2014
Accepted 1 March 2014
Available online 10 March 2014

*Keywords:*
Anodization
$TiO_2$ nanotubes
TiZr alloy
Cell response

ABSTRACT

Various $TiO_2$ nanotubes on Ti50Zr alloy have been fabricated via a two step anodization method in glycol with 15 vol.% $H_2O$ and 0.2 M $NH_4F$ under anodization controlled voltages of 15, 30 and 45 V. A new sonication treatment in deionized water with three steps and total sonication time as 1 min was performed after the first anodization step in order to remove the oxide layer grown during 2 h. The second step of anodization was for 1 h and took place at the same conditions. The role of removed layer as a nano-prepatterned surface was evidenced in the formation of highly ordered nanotubular structures and morphological features were analyzed by SEM, AFM and surface wettability. The voltage-controlled anodization leads to various nanoarhitectures, with diameters in between 20 and 80 nm. As biological assay, cell culture tests with MG63 cell line originally derived from a human osteosarcoma were performed. A correlation between nanostructure morphological properties as a result of voltage-controlled anodization and cell response was established.



## 1. Introduction

Due to its performance in various applications titanium is the metal of the century, being as well the implant material of today [1,2], due to its corrosion resistance [3] and long term biocompatibility [4]. As titanium alloys have better mechanical strengths than Ti, a large variety of binary and ternary alloys have been introduced especially for use in biomedical field, the majority of them being with both α and β phases. In this point of view, the alloys with Zr, TiAlZr [5,6] and TiZr [7–10] respectively have been widely investigated, Zr being the best known nontoxic element. TiZr alloys with zirconium contents ranging from 10 to 40 wt.% have only α phase, but with various contents of Zr and different processing, the surface morphology changes [9,11]. In fact, to improve stability and to elicit a desired cell response to preventdetrimental phenomena, surfaces of implants are frequently modified using various types of coatings, thermal procedures or other surface treatments at micro- and nanolevel [12–14]. For Ti and Ti alloys, growing the thickness of the native oxide layer [12] or processing the oxide through electrochemical anodization [15–19] is associated usually with favorable effects at the biointerface, such as improving adhesion [15], gene expression [16,17,19], bioactivity [18], cell adherence and viability [20] and finally, osteogenic potential [21]. Anodization in controlled conditions in order to fabricate nanotubes [22] or nanowires [23] changes the topographical features at interface and subsequently the cell behavior [24,25], depending also on the nanoarhitecture dimensions as a result of different fabrication conditions. The antibacterial effect as a function of various sized nanotubes on Ti50Zr alloy was established as well [26]. Starting from the idea of nanodimension effect on cell response, the present paper presents a novel aspect of a correlation between nanoarhitectures fabricated via a two step anodization on TiZr alloy at several controlled voltages of 15, 30 and 45 V respectively, and cell response. A novel character has been introduced with the sonication protocol which is different from our previous report [26]. This present investigation permits to establish the best voltage value for the best choice in nanostructure dimension and topographical features of the selected scaffold for cell growth.

## 2. Materials and methods

### 2.1. Sample preparation

The Ti alloy used for the experiment contains 50 wt.% Ti and 50 wt.% Zr (ATI Wah Chang Co). Before anodization pieces with 20 × 20 mm as dimensions were ground with abrasive SiC papers up to 1200, and cleaned by sonication in water, acetone and ethanol for 5 min each. Anodization of TiZr samples was performed in a two-electrode setup, with platinum gauze as the counter electrode used as electrolyte ethylene glycol with 15 vol.% $H_2O$ and 0.2 M $NH_4F$. Anodization was carried out in two steps at controlled voltages of 15, 30 and 45 V, the first anodization step duration being 2 h for each sample, and the second step 1 h. Both anodization steps were performed using the same electrolyte recipe. After

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http://dx.doi.org/10.1016/j.bioelechem.2014.03.002
1567-5394/

performing the first anodization step of 2 h at the desired voltage, a sonication treatment in deionized water was performed immediately after the first anodization step, in order to remove the grown oxide layer and expose the underlying titanium surface patterned with dimples from the bottom of the nanotubes obtained in the first step. Subsequently, the obtained pretreated sample was used as a substrate in the second anodization step of 1 h at the desired voltages (15, 30 or 45 V). The sonication time and protocol represent the important difference in sample preparation of nanotubes fabricated in two steps compared to a previous paper when the sonication time was 15 min [26]. The new sonication protocol consists of three cycles, each of them being 20 second ultrasonication plus 10 second break. Total ultrasonication period of time was 1 min and the process was performed with a SONICS VIBRA CELL equipment.

### 2.2. Surface characterization

The morphology of $TiO_2$ nanotubes was observed using a field emission scanning electron microscope (Hitachi FE-SEM S4800) and dimensions of the nanotube diameter have been established from these micrographs. Cross-section images were obtained from mechanically cracked samples.

An electrochemical AFM from APE research was used in contact mode for imaging the anodized and nontreated samples as reference. The roughness of all samples was calculated using a Gwyddion software.

In order to evaluate the contact angle value as an expression of the wettability of the modified surface, contact angle (CA) measurements were carried out with a 100 Optical Contact Angle Meter — CAM 100. Each contact angle value is the average of minimum 10 measurements. The tests were carried out with an accuracy of $\pm 1°$ at a temperature of 25 °C.

### 2.3. Cell culture

For testing in vitro sample biocompatibility, an osteoblast-like cell line MG63 (human osteosarcoma cells) from ATCC (American Tissue and Cell Collection) was used. MG63 cells are human adherent osteosarcoma cells, having a fibroblast-like morphology and are considered to be osteoblast precursors or early undifferentiated osteoblast-like cells [27]. After the samples were sterilized with 70% alcohol for 24 h, they were washed with sterile water and conditioned in MG63 culture medium. This was followed by the sample seeding stage which was performed at a density of 10,000 MG63 cells/$cm^2$. The culture medium used was Dulbecco's Modified Eagle Medium (DMEM) with 1‰ glucose, supplemented with 10% fetal bovine heat-inactivated serum, 100 U/l penicillin, 100 U/l streptomycin and 50 U/l neomycin. Cell cultures were performed in Binder incubators at 37 °C in 5% v/v $CO_2$ in air and a relative humidity minimum of 95%.

### 2.4. Viability of adherent MG63 cells

In order to evaluate the viability of cells the rapid colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay for cellular growth and survival was used [28,29]. This application to proliferation and cytotoxicity is based on the change of the yellow tetrazolium salt MTT via a redox process. The MTT is reduced to the corresponding blue formazan which is solubilized in dimethyl sulphoxide and the process is done both by metabolically active cells, and by the action of dehydrogenases.

In such way the absorbance (OD) measured at a wavelength of 550 nm is proportional to the number of viable, metabolically active cells. The determinations were performed at two different incubation times, namely 3 and 5 days.

### 2.5. Immunolabeling of cytoskeletal components actin and nucleus

Fluorescence microscopy visualization of actin filaments and of the nucleus was used to examine cell morphology and the level of sample colonization with MG63 cells after 3 days in culture. For this, cells were first permeabilized and fixed by keeping them for 15 min in PFA solution 4% v/v in PBS and 0.1% Triton X-100. Staining with FITC-labeled phalloidin for 1 h highlighted the cellular actin filaments and 15 min treatment with Hoechst 33258 dye allowed nuclei observation. After fluorescent staining the cells were examined with a 20× objective at a fluorescence microscope Axio Vert (Carl Zeiss) equipped with a digital camera Axio Cam Mrc5.

The images obtained after phalloidin and Hoechst staining were used for the quantification of the proliferation level. For this purpose we used only the images of cell nuclei, which were loaded into Zen software (Carl Zeiss). All cells were counted in two fields which depicted different areas of the sample with cultured cells.

### 2.6. Gene expression analysis (RT-PCR)

To highlight specific mRNA levels of osteocalcin and osteonectin, cell lysis and isolation of total RNA were performed using Pure Link RNA Minikit Kit from Life Technologies. Subsequently, using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) reverse transcription of 1 μg RNA was performed in order to obtain cDNA necessary for PCR reaction.

PCR reaction was carried out in a mix that contains the gene of interest primers, 1 μg of complementary deoxyribonucleic acids (cDNAs), triphosphate deoxynucleosides, $MgCl_2$ appropriate buffer and Go Taq DNA Polymerase (Promega USA). The thermal cycling conditions were: 1 cycle of 3 min at 94 °C (initial denaturation), 35 cycles with 3 steps per cycle: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s, followed by prolonged elongation at 68 °C for 10 min. In order to normalize the level of PCR products the cDNA of housekeeping beta actin gene was amplified by PCR. The primers used and the size of amplified fragments, are shown in Table 1.

### 2.7. Statistical analysis

Statistical analysis was performed for surface property characteristics (contact angle data, AFM results) and for cell viability.

## 3. Results and discussion

### 3.1. Surface characterization

The surface morphology of Ti50Zr alloy as compared to that of Ti is presented in Fig. 1. It is evident that comparing with the structure of Ti, Ti50Zr alloy presents a needle-like structure, as was also observed by Minagar et al. [30] and it is similar to the needle-like structure

**Table 1**
Primers and the size of amplified fragments used in the experiments.

| Primer | Osteocalcin | Osteonectin | Beta actin |
|---|---|---|---|
| 5′-3′ sequence of forward primer | AGGGCAGCGAGGTAGTGAAG | TGCCTGTCTCTAACCCCTC | AGCATTTGCGGTGGACGA |
| 5′-3′ sequence of reverse primer | AGGGCAGCGAGGTAGTGAAG | CCTCCTCTTCGGTTTCCTCTG | GACCTGACTGACTACCTC |
| Size of PCR product (bp) | 252 bp | 298 bp | 574 bp |