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

Sabina Grigorescu a, Vasile Pruna b, Irina Titorencu b, Victor V. Jinga b, Anca Mazare c, Patrik Schmuki c,d, Ioana Demetrescu a,*

a University Politehnica Bucharest, Faculty of Applied Chemistry and Materials Science, Department of General Chemistry, University Politehnica Bucharest, Faculty of Applied Chemistry and Materials Science, Department of General Chemistry, 1-7 Polizu, Bucharest 011061, Romania
b Institute of Cellular Biology and Pathology “Nicolae Simionescu” of the Romanian Academy, Romania
b University of Erlangen-Nürnberg, Department of Materials Science, LKO, Martensstraße 7, 91058 Erlangen, Germany
c King Abdulaziz University, Chemistry Department, Jeddah, Saudi Arabia

A R T I C L E   I N F O

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

Keywords:
Anodization
TiO2 nanotubes
TiZr alloy
Cell response

A B S T R A C T

Various TiO2 nanotubes on Ti50Zr alloy have been fabricated via a two step anodization method in glycol with 15 vol.% H2O and 0.2 M NH4F 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 nanoarchitectures, 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.

© 2014 Elsevier B.V. All rights reserved.

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 prevent detrimental 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 nanoarchitecture 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 nanoarchitectures 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.% H2O and 0.2 M NH4F. 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
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 TiO2 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 ± 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/cm2. Each contact angle value is the average of minimum 10 measurements. The tests were carried out with an accuracy of ± 1° at a temperature of 25 °C.

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 sulfoxide 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 x 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 genes of interest primers, 1 μg of complementary deoxyribonucleic acids (cDNAs), triphosphate deoxynucleosides, MgCl2 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>
<thead>
<tr>
<th>Primer</th>
<th>Osteocalcin</th>
<th>Osteonectin</th>
<th>Beta actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-3′ sequence of forward primer</td>
<td>AGGCCAGCAGGTAGTGAAG</td>
<td>TGCCTGTCTTAACCTCTC</td>
<td>AGCATTGCGTGAGAGCA</td>
</tr>
<tr>
<td>5′-3′ sequence of reverse primer</td>
<td>AGGCCAGCAGGTAGTGAAG</td>
<td>CCTCCTCTGCTGTTCCTTG</td>
<td>GACCTGACTGACTACTCTC</td>
</tr>
<tr>
<td>Size of PCR product (bp)</td>
<td>252 bp</td>
<td>298 bp</td>
<td>574 bp</td>
</tr>
</tbody>
</table>
observed in the case of Ti40Zr by Kim et al. [9,11] and subsequent nanotube growth on α and β phases.

In Fig. 2 the SEM images of nanotube oxides on TiZr are presented as a function of the fabrication voltages, 15, 30 and 45 V, respectively. As has been explained in literature [26,31] on TiZr with 50% Zr there are 2 types of nanotubes, TiO2 and ZrO2, and in all cases both oxides are present. According to the knowledge about nanotube fabrication via anodization, the general mechanism consists of two stages, the first one being oxide layer formation on the surface of titanium (or titanium alloys) and the second being partially dissolution of the oxide layer in the presence of F− and pit formation. These concurrent processes, anodic oxidation and dissolution lead to the nanotube formation via changes from nanopores to nanotubes [32].

Indifferent of the elaboration potential (15 V, 30 V or 45 V), it is evident that the nanotube elaboration took place according to the above-mentioned mechanism and the diameters of nanotubes are increasing with increasing voltage. The tube diameter and the length of the nanotube oxide layer are summarized in Table 2, including the diameters on both phases of the alloy and the computed porosities.

Porosity was evaluated using the following formula [33]:

\[
P = 1 - \frac{2\pi w (w + D)}{\sqrt{3}(D + 2w)^2}
\]  

where D is the diameter, and w is the wall nanotube thickness. According to literature, the formula is suitable for the case when the tubes are close packed [33], as is the case with the present structures.

It is well known that there are many generations of nanotubes and their morphology strongly depends on anodizing conditions, the nanotubes being very regular, long and with smooth walls when fabricated in organic viscous electrolytes, as was presented in our previous paper [26]. When the two step anodic oxidation is carried out, meaning first

---

**Fig. 1.** SEM images showing the surface morphology of polished Ti50Zr alloy compared to polished Ti.

**Fig. 2.** SEM images showing uniformly distributed nanotubes and their corresponding cross-section obtained by anodization in 2 steps at a) 15 V (TiZr15V), b) 30 V (TiZr30V) and c) 45 V (TiZr45V).
anodization, removal of the layer and second anodization, it is observed that the TiO2 nanotubes are covered with a few nanometer thin nano-porous layer (initiation layer). In the case of two step anodization with a more viscous electrolyte, in the second step a nanoporous structure with no uniform pore diameter and pore density has been observed [34].

The nanostructures presented in the SEM micrographs, put in evidence highly ordered structures for all studied potentials and with the increase of voltage the diameters of the ordered structures increase but are however more evidently covered with a thin initiation layer. It is the same observation as Cho et al. [34], reporting at the top surface of Ti via nano-imprint and successive anodization similar porous structures. The imprints obvious in the thin initiation layer present on all samples have mean diameters of 51.3 nm for 15 V, 93.5 nm for 30 V and 130.6 nm for 45 V. One other aspect to point out is that, due to the presence of the initiation layer, the tube wall thickness of the nanotubular structure is much higher than for nanotubes which were not obtained via a two step anodization method.

The porous initiation structure was attributed to the suppression of F-rich layer dissolution between the cell boundaries in viscous electrolyte. Once the voltage increased at 45 V the reactions are accelerated and for the architecture growth at higher voltage a wider distribution of the structure generates a bigger amount of irregularities, reflected in the same trend of roughness as can be seen in Table 3, where roughness of samples and their contact angles are presented as a function of diameters.

As can be seen from the above table, all the samples have hydrophilic character and this character is much stronger for smaller diameter nanotubular layers fabricated at smaller voltage values.

The novelty of the samples presented in this paper compared to other two step nanotubes is related to the diameters and level of nanotube order, depending on sonication time and voltage-controlled anodization conditions.

In Fig. 3 AFM 3D and 2D images are presented (as well as error images). For the statistical measurements of nanotubular layer obtained at 15, 30 and 45 V, the minimum and maximum heights of the topography were used to determine a mean mathematical value and a median statistic value. The surface irregularities, as can be seen in Fig. 3, are visible as well in the average roughness which varies significantly from 15 V to 45 V as the nanoarchitecture diameters and wall thickness show changes. A statistical analysis of the AFM data is presented in Table 4. A small shift to the right and a higher roughness are visible for 30 V sample and for 45 V sample. This shift is probably due to the stronger forces that the cantilever tip encounters in the larger diameter nanostructures.

### Table 2
Nanotube diameter and porosity as a function of voltage of fabrication process.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Diameter phase 1</th>
<th>Porosity phase 1</th>
<th>Diameter phase 2</th>
<th>Porosity phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range, nm</td>
<td>Mean, nm</td>
<td>Range, nm</td>
<td>Mean, nm</td>
</tr>
<tr>
<td>TiZr15V</td>
<td>14–23</td>
<td>17.6</td>
<td>25–35</td>
<td>27.8</td>
</tr>
<tr>
<td>TiZr30V</td>
<td>27–40</td>
<td>35.3</td>
<td>52–65</td>
<td>58.2</td>
</tr>
<tr>
<td>TiZr45V</td>
<td>45–58</td>
<td>52.2</td>
<td>65–80</td>
<td>76.3</td>
</tr>
</tbody>
</table>

### Table 3
Roughness and contact angles for samples fabricated at nanotubular samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Roughness (Ra), nm</th>
<th>Contact angles, deg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiZr15V</td>
<td>31.4</td>
<td>26.13</td>
</tr>
<tr>
<td>TiZr30V</td>
<td>38.6</td>
<td>64.36</td>
</tr>
<tr>
<td>TiZr45V</td>
<td>46.6</td>
<td>68.87</td>
</tr>
</tbody>
</table>

### 3.2. Cell morphology, colonization level and viability

In order to investigate cell morphology, we cultured the MG63 osteoblast-like cells on TiZr samples for 3 days — as shown in Fig. 4. In the TiZr15V samples, cells showed a developed cytoskeleton, tending to grow in multilayer. The cells presented well spread fibroblast cell morphology and an uninterrupted proliferation after 3 days of
incubation. Cells cultured on all tested samples presented an actin network containing numerous filaments, very thin filopodia and lamelopodia.

Cell proliferation on all anodized samples was higher compared to control sample (TiZr). The best proliferation was observed on samples deposited at 15 V followed by the sample deposited at 30 V and respectively 45 V (Fig. 5).

The higher proliferation was due probably to a larger accessible surface for cell growth caused by nanotube adding, and to a more hydrophilic surface expressed in a lower contact angle. Besides, a hypothetical model for nanotube size dependent interactions with cells suggests that 15 nm nanotubes are a perfect fit to the formed focal adhesion complex of about 10 nm in diameter, while higher diameters like 100 nm do not support cell adhesion and might lead to apoptosis [24,25]. In our case, diameters for TiZr15V samples are the smallest compared to other samples (in the range of 15–30 nm with approx. 17.6 nm on phase 1 and of 27.8 nm on phase 2) and as such have higher proliferation. All proliferation data are in good correlation with literature data for different diameter TiO₂ nanotubes [15,24,25].

Regarding the viability results (see Fig. 6), in all cases the values are higher than control and the best viability was for the nanotubular morphology elaborated at 15 V for both 3 days and 5 days.

Taking into account that both roughness and contact angles are increasing with the elaboration voltage of nanostructure, it can be that voltage is controlling cell adherence, morphology and viability as the following: the best cell proliferation and viability are for samples with smaller roughness and stronger hydrophilic character (elaborated at 15 V).

3.3. Gene expression analysis

At the molecular level, osteoblast characteristics were analyzed by measuring the level of mRNA expression of two osteoblastic specific genes that are considered osteoblast markers: osteonectin and osteocalcin. It is known that osteonectin or SPARC (secreted protein, acidic and rich in cysteine) is a secreted glycoprotein that has the ability to bind Ca²⁺, collagen, and hydroxyapatite, acting as a nucleator for hydroxyapatite deposition [35] and mediating the interactions between cells and extracellular matrix [36]. Osteocalcin is a specific marker to mature osteoblast [37,38]. This protein undergoes γ-carboxylation, via
a K-vitamin dependent mechanism [39] and has calcium binding properties conferred by the two carboxyl groups. Osteocalcin is synthesized by osteoblasts at the onset of mineral deposition [40,41].

After 7 days of cell culture on nanotubes elaborated at 45 V, both osteocalcin and osteonectin genes were expressed as mRNA. 15 V and 30 V samples suppress, at mRNA level, the expression of both osteocalcin and osteonectin. Compared with the control, the 45 V sample maintains a higher expression level of osteocalcin and osteonectin (Fig. 7).

These results showed that cells cultured on 15 V and 30 V nanotube layers acquire a high proliferation potential. In the same time the osteocalcin and osteonectin mRNA levels are under the limit of detection. On the other hand, the cells cultured on 45 V samples, having a lower proliferative rate, present detectable osteogenic properties, in terms of osteocalcin and osteonectin mRNA levels, better than control samples.

4. Conclusion

The experimental data have indicated that voltage controlled the nanostructures elaborated on TiZr as scaffolds reveal a reliable biocompatibility with population of human osteoprogenitor cells utilized in this study. All nanotubular samples sustained a better viability than the control. The higher porosity and hydrophilic character of the samples elaborated at 15 V and 30 V were correlated with the higher rates of proliferation and viability, but not a detectable expression of mRNA for osteocalcin and osteonectin. The less proliferative cultures or probably in stationary state, on 45 V nanotube layers manifest osteogenic differentiation. With few exceptions, a cell population during active proliferation reveals a lower degree of differentiation than in a stationary or less proliferative condition. It is possible that the numerous cell populations grown on 15 V and 30 V nanotube layers, entering the stationary state, became complete osteogenic differentiation and consecutive high utility in bone repair.

Acknowledgments


The authors wish to thank the PCCE 248/2010 program and the Romanian Academy as well.

References


Fig. 6. Viability of MG63 cells cultured 3 days and 5 days on the tested samples: control (TiZr) and nanotubular structures TiZr15V, TiZr30V and TiZr45V.

Fig. 7. MG63 osteoblast bone-specific mRNA expression. Total RNA was isolated from cells at 7 days in culture on: nanotubes elaborated at 45 V (TiZr45V) and on TiZr control samples. Expression levels for osteonectin and osteocalcin are also presented.