UDK 620.3:532.78:549.514.6:616-008.852:616.1
Original scientific article/Izvirni znanstveni članek

ISSN 1580-2949
MTAEC9, 53(6)791(2019)

PERFORMANCE OF ANNEALED TiO_2 NANOTUBES IN INTERACTIONS WITH BLOOD PLATELETS

UČINKOVITOST ANATAZNIH TiO_2 NANOCEVK V INTERAKCIJAH S TROMBOCITI

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Prejem rokopisa – received: 2018-11-20; sprejem za objavo – accepted for publication: 2019-05-23

doi:10.17222/mit.2018.249

Titanium dioxide (TiO_2) nanotubes, synthesized by the electrochemical anodization of Ti foil, were annealed to obtain the anatase crystal phase and further characterized as surfaces for vascular stent applications. X-ray diffraction (XRD) was used to analyze the crystal structure of the TiO_2 nanotubes, while the morphology of the nanotubes and biological material was determined by scanning electron microscopy (SEM). The results showed that the surface of the annealed TiO_2 nanotubes acts anti-thrombogenically, since the whole-blood derived platelets do not adhere nor activate readily on such samples, unlike on the surface of amorphous TiO_2 nanotubes and plain Ti foil. Therefore, the anatase crystal phase of TiO_2 nanotubes could be beneficial for stent applications.

Keywords: TiO_2 nanotubes, crystallization, blood platelets, vascular stents

Predstavljena je sinteza TiO_2 nanocevk z metodo elektrokemične anodizacije ter vpliv spremembe kristalne strukture TiO_2 nanocevk na interakcije s trombociti. Analiza odziva trombocitov na kardiovaskularnih vsadkih kot so žilne opornice je ključnega pomena za njihovo uspešno integracijo v biološko okolje; oprijem ter aktivacija trombocitov na površini vsadkov sta nezaželena, saj to lahko vodi v nastanek krvnih strdkov. Z rentgensko difrakcijsko spektroskopijo (XRD) smo analizirali kristalno strukturo TiO_2 nanocevk, morfologijo pa smo analizirali z vrstičnim elektronskim mikroskopom (SEM). Rezultati nakazujejo, da se trombociti na površino TiO_2 nanocevk z anatazno kristalno strukturo ne oprijemajo, medtem ko je površina amorfnih TiO_2 nanocevk ter Ti folije ugodna za oprijem trombocitov.

Ključne besede: TiO_2 nanocevke, kristalizacija, trombociti, žilne opornice

1 INTRODUCTION

Titanium dioxide nanotubes (TiO_2 NTs) manufactured via a simple, yet elaborate, anodization process have gained considerable attention for use in photocatalysis,[1,2] biomedical applications,[3,4] such as an accomplished titania platform, biosensing devices,[5,6] implants[7,8] and controlled delivery systems with targeted drug release.[9,10] In the field of implantable materials TiO_2 is at the forefront of real-life applications due to the favourable spontaneous formation of the oxide layer on its surface, which enables high biocompatibility.[11–14] Nevertheless, despite their non-toxic nature, titania-based implants can still be rejected by the host tissue, the response resulting in, e.g., inflammation or thrombosis.[15] Considering TiO_2 NTs, higher biocompatibility is achieved when the anatase or rutile crystal phase prevails in the nanotube arrays. Indeed, it is well known that the crystal structure of the material affects its performance regarding interactions with biological matter.[16] For example, J. A. Sorkin et al.[17] observed the higher proliferation of neuronal stem cells on TiO_2 NTs with a higher percentage of anatase phase. Similarly, increased adhesion and proliferation of osteoblast cells on anatase TiO_2 NTs in comparison to the amorphous counterpart. Other authors observed the same anatase-related trend in osteoblast behaviour.[18,19,20] Anatase-containing TiO_2 NTs with an anatase crystal structure were also reported to promote adhesion and proliferation of human mesenchymal stem cells.[21]

Despite the favourable effects of TiO_2 NTs' crystallinity on different cells, there are arguments in favour of the hemocompatibility evaluation of blood-contacting devices. Q. Huang et al.[22] showed that rutile-phase TiO_2 NTs reduced platelet adhesion and activation. This particular case is advantageous for vascular stents, for which the inhibition of adhesion and the activation of platelets is preferred, since platelets' aggregation leads to blood-clot formation and in-stent thrombosis. L. Zhang et al.[23] showed that pure anatase in the TiO_2 NTs, lead to better platelet adhesion, which

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could be beneficial for the improved integration of dental implants.

In the present research, the performance of the annealed TiO_2 NTs in interactions with whole blood-derived platelets is presented. These results could provide an additional basis for explaining the discrepancy in the literature concerning platelet adhesion and activation on the annealed TiO_2 NTs.

2 EXPERIMENTAL PART

2.1 Materials

Titanium foil (Advent, 0.1 mm thickness, 99.6 %), ethylene glycol (Fluka, ≥99.5 %), ammonium fluoride – NH_4F (Sigma Aldrich, 28.0-30.0 %), hydrofluoric acid – HF (Sigma Aldrich, ≥40 %) acetone (Honeywell Riedel – de Haen, 99.5 %), ethanol (Sigma Aldrich, 96 %), deionized water (miliQ), phosphate-buffered saline – PBS (tablets, Sigma Aldrich), and glutaraldehyde solution (Sigma Aldrich).

2.2 Synthesis of TiO_2 NTs by electrochemical anodization

The TiO_2 NTs were fabricated by the electrochemical anodization method, as described in the literature[24,25] with the Voltcraft VSP 2653/VSP 2206 laboratory power supply. Experiments were carried out at room temperature (≈ 20 °C) in a two-electrode system (Pt/Ti foils) with a working distance of 15 mm. Before the anodization process, Ti foil was ultrasonically cleaned in acetone. The Ti foil was then dried under a nitrogen stream. The electrolyte used in the first step of the process was composed of ethylene glycol, NH_4F (0.35 *w*/%) and deionized water (1.7 *w*/%). The nanotubular layer grown in this step was then removed with a successive ultrasonication in deionized water. In the second step of the anodization process, the pre-treated Ti foil was used as a substrate to grow TiO_2 NTs with a length of 2.53 μm and a tube diameter of 100 nm. The electrolyte based on ethylene glycol, deionized water (11.0 *w*/%) and HF (1.0 *w*/%) was used. The morphology of the TiO_2 NTs was controlled by applying a voltage of 58 V. The synthesis time was 2.5 h. The as-synthesized TiO_2 NTs were kept in ethanol for 2 hours in order to remove the electrolyte remains and then dried under a nitrogen stream. The as-prepared TiO_2 NTs were further annealed in a conventional furnace at 450 °C for 2 h in an air atmosphere with an annealing/cooling rate = 8 °C/min.

2.3 Characterization

2.3.1 Scanning electron microscope (SEM) analysis

The morphology of the materials was analysed with a scanning electron microscope (JSM 7100F – JEOL) with an acceleration voltage of 15 keV. The elemental analysis was made with an energy-dispersive X-ray spectrometer (EDX) coupled to a scanning electron microscope.

2.3.2 X-ray diffraction spectroscopy (XRD) analysis

The X-ray diffraction (XRD) analysis was performed with a MiniFlex 600 Benchtop X-ray diffractometer (Rigaku) equipped with Cu–K_α radiation (0.1541 nm) over the 2θ range 20–80°, with a step size of 0.017°, divergence slit of 0.218° and counting step time of 25 s in continuous scanning mode. Carbon tape was used to mount the samples on the glass sample holder.

2.4 Interaction of TiO_2 NTs with platelets

The whole blood was obtained from healthy volunteers (the authors of the manuscript) via vein puncture. The blood was drawn into 9-mL tubes coated with trisodium citrate anticoagulant (Sigma Aldrich). The number of platelets of whole blood was counted with a multi-parameter automated hematology analyzer (Cell-DYN 3200, Abbott). The material samples – Ti foil, amorphous and annealed TiO_2 NTs (7 mm × 7 mm in size) were incubated with 250 μL of whole blood for 45 min at room temperature (≈20 °C) in the 24-well cell-culture plates. Afterwards, 250 μL of PBS was added to the incubated samples. The blood with PBS was then removed and the samples were rinsed 3 times with 250 μL of PBS in order to remove weakly adherent platelets. The preparation of the samples for SEM analysis was performed in the following manner: the samples were dipped within the solution of 250 μL of PBS and 250 μL of 0.5 vol/% glutaraldehyde solution for 2 h at room temperature. Then the materials were rinsed with PBS and dehydrated by using a graded ethanol series (50, 70, 80, 90, 100) vol/% and again 100 vol/% of ethanol for 5 min and in the last stage (100 vol/% ethanol) for 10 min. Before observation with SEM the samples were dried with liquid nitrogen and left in a vacuum for 3 h. Before the observation with SEM, the samples were coated with gold/palladium. The test was made in triplicates and the representative images are shown.

3 RESULTS

The results of the XRD analysis are presented in **Figure 1**. TiO_2 NTs are amorphous after electrochemical anodization process. The characteristic peaks observed in the XRD pattern of as-anodized TiO_2 NTs belong to the Ti foil, which was used as a substrate for the NTs' formation. The annealing of TiO_2 NTs in the furnace at 450 °C for 2 h induces the transition of the amorphous phase to the anatase crystal structure, as identified from the characteristic peaks (101), (103), (004), (200), (105) and (211).

SEM analysis revealed altered morphology of the material annealed TiO_2 NTS which, as evident from **Figure 1** and the surface chemistry, as presented in

Table 1. The annealed have thicker walls (**Figure 2**), as already reported elsewhere.[26] X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray (EDX) analysis[27] showed the changes in the surface chemistry (**Table 1**). According to the XPS analysis (depth of analysis of 5 nm) the difference in the concentration of fluorine (F) was observed; annealed TiO_2 NTs do not contain F on the surface, as evident from **Table 1**, while 8.2 x/% of F is present on the surface of the as-anodized TiO_2 NTs. The fluorine comes from the anodization process and these results indicate that it is only weakly bound or absorbed on the surfaces of the TiO_2 NTs, as after annealing F was not detected on the TiO_2 NTs. A slightly lower concentration of carbon was detected on the as-anodized and annealed Ti foil. Increase in the oxygen concentration compared to the Ti foil was observed for as-anodized NTs (about 43.7 *x*/%) and as-anodized annealed TiO_2 NTs (about 49.8 *x*/%). A similar trend was observed for the concentration of Ti, as a slight increase in its concentration was observed on the surface of the annealed TiO_2 NTs. According to results of the EDX (penetration depth 1–2 μm), higher oxygen concentration was detected on the annealed NTs, compared to the as-anodized NTs; about 55 *x*/% and 64.9 at%, respectively. A slight increase in Ti concentration by EDX analysis of the surface of the annealed surface of TiO_2 NTs. F was also detected on the as-anodized NTs, with about 11.9 *x*/%, while after annealing F was not detected. This confirms that F is indeed distributed along the NTs' length as newly TiO_2 NTs are about 2.53 μm in length and this corresponds well with the penetration depth of the EDX analysis. XPS analysis showed a similar concentration of C for the as-anodized and annealed TiO_2 NTs, 30.0 *x*/% and 29.5 *x*/%, while C was not detected by EDX. This result is in agreement with our previous results of depth profile analysis, which showed that carbon is present only in the top surfacelayer (depth of 2 nm).[27]

Interactions between the platelets' and Ti foil, as anodized and annelaed TiO_2 NTs were studied with SEM, **Figure 2**. The morphology of the platelets can be categorized as round, dendritic, spread and fully spread. Platelets adhere to the surface of the Ti foil with lamelopodia and filopodia (**Figure 2**) and their shape can be classified as dendritic. Similar result were observed for the as-anodized NTs. However, platelets could practically not be found on the surface of the annealed NTs. As evident from the SEM image in **Figure 2**, only one round platelet was observed. This result indicates that the platelets do not adhere to the surface of TiO_2 NTs with an anatase crystal structure, nor does such a surface allow them to activate.

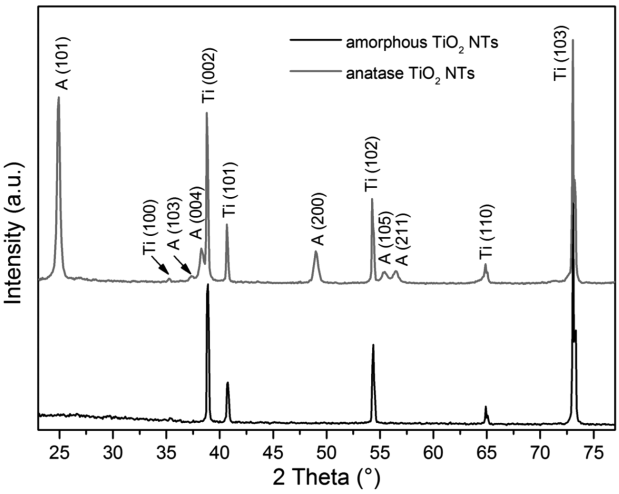

Figure 1: XRD patterns of as anodized TiO_2 NTs amorphous and annealed TiO_2 NTs; Ti=Ti foil, A=anatase

4 DISSCUSSION

A sensible approach of TiO_2 NTs crystal structure manipulation to obtain the anatase/rutile phase is by heat treatment in air. Simply by selecting the appropriate annealing temperature, a tailored crystallization and phase composition of either anatase or rutile can be obtained in the TiO_2 NTs.[29] In the present study, annealing of TiO_2 NTs with 100 nm in diameter was performed at 450 °C for 2 h in a conventional furnace. The results showed that such-annealed TiO_2 NTs crystallize into pure crystal structure without significant alteration of NTs morphology. Contrary to a previous report of improved platelet adhesion on the surface of anatase-TiO_2 NTs,[23] the results of the interactions of platelets and TiO_2 NTs anatase phase obtained in the present study show that such surfaces do not allow for platelet adhesion, unlike the surface of amorphous TiO_2 NTs and plain Ti foil (**Figure 2**). It should be noted the that platelets' adhesion and activation depend on a variety of other factors, such as morphology, surface chemistry, surface topology,[2] surface charge density and

Table 1: XPS and EDX analyses of as-anodized and annealed TiO_2 NTs

XPS analysis (*x*/%)	C	O	Ti	N	F	Si
As-anodized NTs*	30.0	43.7	18.2	0	8.2	0
As-anodized – annealed NTs*	29.5	49.8	20.7	0	0	0
EDX analysis (*x*/%)						
As-anodized NTs	0	55.0	33.1	0	11.9	0.1
As-anodized – annealed NTs	0	64.9	35.1	0	0	0

* Results taken from reference[28]

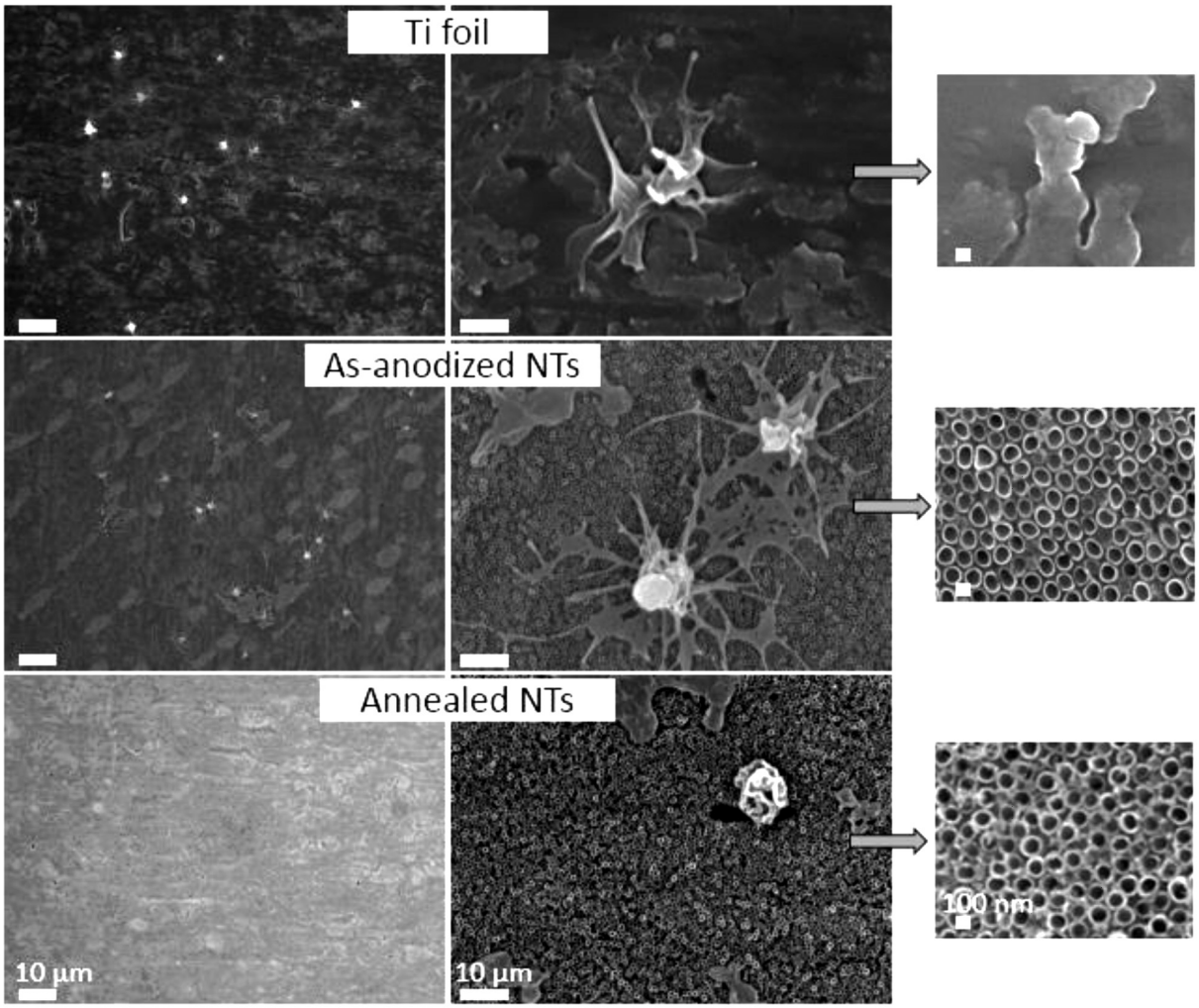

Figure 2: SEM images of Ti foil, as-anodized TiO_2 NTs and annealed-anatase TiO_2 NTs interacting with platelets

potential,[13,14] wettability,[14] of the material and all these parameters might be altered after annealing.[31] For instance, with increasing of the annealing temperature, the concentration of the cell-toxic electrolyte residual – fluorine decreases.[24,27] In our previous studies, we also proved the long-term hydrophilicity of the annealed TiO_2 NTs,[28] in comparison with the less-hydrophilic amorphous TiO_2 NTs and hydrophobic Ti foil.[27] Despite the unaltered structure of the TiO_2 NT after annealing, TiO_2 NTs' surface with anatase crystal structure prepared in the present study do not provide an environment suitable for platelet adhesion and activation, which is from the view point of stent application beneficial, as it could prevent platelet aggregation and blood clot formation, i.e., thrombosis. Additional studies about the performance of relevant cells on the surface of TiO_2 NTs are, however, required. Among others, the surface charge density[13] and surface potential[30] of different types of TiO_2 nanotubes should be determined to provide more detailed information about their interactions with platelets.

5 CONCLUSIONS

TiO_2 nanotubes, prepared by electrochemical anodization of a Ti foil, were annealed in order to obtain the transition of the amorphous to anatase crystal phase. The effect of the crystalline material of the interactions with whole-blood-derived platelets was studied. It has been confirmed that annealed TiO_2 NTs with an anatase crystal structure prevent the adhesion and activation of platelets, unlike the amorphous as-anodized TiO_2 NTs and Ti stent for start. From the view point of stent applications, such results are beneficial due to the ability of the materials to prevent thrombosis reactions.

Acknowledgment

The authors would like to acknowledge the Slovenian Research Agency for financial support, grants No. Z3-4261, J3-9262, J1-9162, J2-8166, J2-8169, J5-7098, P2-0232 and Slovenian Ministry of Education, Science and Sport grant "Public call for encouraging young

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