Iranian Journal of Materials Science and Engineering, Vol. 20, Number 2, June 2023
RESEARCH PAPER

Acceleration of Bone-Implant Graft by Optimizing the Dimention and Crystalline Structure of Titanium Oxide Nanotubes as the Titanium-Based Implant Coating

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Received: August 2022 **Revised: April 2023** **Accepted: May 2023**
DOI: 10.22068/ijmse.2925

Abstract: *Titanium implants are one of the most durable and conventional orthopedic and dental implants. The aim of this research was to improve the bio-compatibility of these implants by implementing nano-dimension coating of titanium oxide nanotubes (TNT). For this purpose, the effect of dimension and atomic structure of titanium oxide nanotubes on the surface properties and biological performance were examined. TNTs were synthesized by anodizing method on the surface of titanium sheets. Dimension of TNT was controlled by anodizing process parameters. Heat treatment affected the atomic structure of TNTs. Contact angle measurement as one of the important surface properties was conducted on different dimensions and structures of TNTs, to study human blood's physical interaction with the implant surface. In addition, the quality and quantity of bone material sediment on the surface were examined by SBF test and SEM analysis. Finally, cell culture provided informative data on bone cells' response to these nanotubular coatings by analyzing MTT results and SEM photography of cells. As a result, the optimum dimension and atomic structure of TNTs were defined and the required process parameters were introduced to obtain this state. This setup may be used as an optimum state of TNT as a nano- dimension coating on titanium implant with orthopedic functions to enhance the cell adhesion and acquire the highest proliferation rate.*

Keywords: *Titanium oxide nanotubes, Titanium implant, Dental implant, Nanotubes length.*

1. INTRODUCTION

Undoubtedly, medical implants are one of the important alternatives for damaged limbs to continue normal life [1–7] Nowadays, artificial blood vessels, pace-makers, hip joints, dental implants, and artificial organs have become integral parts of modern medicine. On the other hand, the long convalescence period, loosening before complete bonding, infection and pain are the major difficulties of the implantation surgery [8–10]. For instance, 25% of hip replacements are repeated because of implant surgery failure in bonding [8]. The improved bio-performance of the implants can enhance cell attachment, faster bonding and reduce the convalescence time and pain, and increase the chance of success in the implantation surgery. Titanium-based implants are widely used as dental implants because of their good mechanical properties and favorable bio-performance [11–13]. However, improving the bio-performance of the titanium and titanium-based alloys and increasing the cell proliferation on their surface are very important factors to enhance their applicability in orthopedic implantation. The first consequence after locating an implant into tissue is the attachment of proteins from blood and body fluid on the implant surface because of a vessel break. Next, cells would sit on a bed of proteins. The better implant surface properties and contact situations, the better cell attachment. The interface of the implant and the tissue is the critical part of the used alloys and surface engineering can affect the bio properties of the implants [14–16].

Diverse coatings containing titanium oxide nanoparticles are widely used to improve the anti-corrosion performance and bio-compatibility of titanium-based implants. These properties have been significantly affected by other parameters such as the size, morphology, crystallinity, and dispersion of the nanoparticles in the used coatings [15, 17–20]. Porous surfaces can mechanically adsorb the proteins and cells by their particular morphology. Studies proved that nanotubes with 100 nm in diameter are optimum

[DOI: 10.22068/ijmse.2925]

for osteoblast cells' attachment [21].
TNTs are highly ordered porous nanostructures with controlled dimensions which could be optimized to achieve a desired porosity [22–24]. For the first time, titanium oxide nanotubes (TNT) were synthesized by adding Fluoric acid to chromic acid electrolyte through an anodizing method [25]. In follow, a numerous studies were performed to investigate the biological effects of this nanostructure on titanium as a biomaterials [26–28]. It has been demonstrated that, the more surface porosity, the better bio-performance and higher ability for the cell adhesion [12, 16, 19, 29]. Likewise, Alkaline phosphate and MTT analyses revealed that the TNT had a higher cell adhesion performance and proliferation up to 50% compared to the spherical titanium oxide nanoparticles [25]. Some researchers investigated the effect of the diameter and length of the nanotubes on their cell adhesion and bio-performance. For cerebral glioma C6 cell activities, the suitable diameter size is reported in the range of 15~100 nm [30], while for osteoblast cell MG-63, 100 nm has been reported as a proper TNT diameter [21].
The effect of heat treatment on the crystalline structure on different properties of the nanotubes has been studied previously [31–33]. According to these researches, as-fabricated nanotubes are in the amorphous form, while after the heat treatment process the crystalline phases including anatase and rutile are formed. This phase transformation to the crystalline structure leads to an increase in the stability of the oxide film as well as the corrosion resistance [34]. In addition, this process can affect the surface properties such as surface wettability. Since both corrosion resistance and high wettability are desirable properties in biomaterials, the effect of the annealing process performed at 550°C for 2 hours (according to previous studies [35, 36]) on the TNT specimen was investigated in the present study.
The present study tried to gather complete data of all TNTs' parameters for the first time and introduce one model of TNT as an optimum surface nano-coating for titanium dental and orthopedic implants, whereas indicated earlier titanium and Ti-6Al-4V are widely used as dental or orthopedic implants. TNTs were created on the titanium sheets' surface by the anodizing method. TNTs' dimensions were obtained to 100 nm which is the optimum for osteoblast cells by changing in anodizing process's parameters. In addition to implementing previous studies mentioned above, for the first time, the effect of the nanotubes' length on the bio-performance of the titanium-based implant was investigated. Moreover, the heat treatment was performed to modify the titanium oxide crystalline structure and investigate the effect of the atomic structure on the contact angle, wettability with human blood, and bio-performance of the nanotubes. Calcium phosphate deposition on diverse states of TNTs was investigated. MTT and SEM photography of cell culture was performed to see the adhesion, and proliferation of osteoblast cells on the surface and to determine the most appropriate state of TNTs.

2. EXPERIMENTAL PROCEDURES

2.1. Surface Preparation

Commercial pure titanium plates were prepared in dimensions of 20×10×0.5 mm. The chemical and mechanical cleaning methods were used to clean the surface of the plates. According to previos studies [37, 38] and primery knowledge for chemical cleaning, 1 vol.% HF was added to 30 vol.% HNO_3 water base acid solution to remove all stains and sediments. Then, an ultrasonic bath of water, ethanol, and acetone respectively was used to achieve a clean surface of the titanium substrate.

2.2. Fabrication of TNT

Anodizing is a simple method to synthesize titanium oxide nanotubes. The key factor for the TNT fabrication is adding an optimum concentration of fluorine ions into the bath [39, 40]. To supply sufficient fluorine ions for the synthesis of the TNTs, 0.5 wt.% NH4F was added to a 1:1 mixture of distilled water and glycerol solution as the electrolyte. To obtain the nanotubes with 100 nm diameter, 20 V electrical potential was applied between the titanium sheet as anode and graphite as cathode with 3-4 cm distance in the electrolyte as was found in previous experiments [8, 30]. The applied electrical potential leads to the formation of a protective passive layer of TNTs on the surface of the titanium plate and the fluorine ions can react with the TNTs and the titanium substrate as described in Eqs. 1, 2, and 3:

$$Ti + 2H_2O \rightarrow TiO_2 + 4H^+ + 4e^- \quad (1)$$

[DOI: 10.22068/ijmse.2925]

$TiO_2 + 6F^- \rightarrow [TiF_6]_2^-$ (2)

$Ti^{4+} + 6F^- \rightarrow [TiF_6]_2^-$ (3)

These reactions can cause some pits on the surface of the titanium oxide layer. The pits get deeper and a wall of titanium oxide is formed above the pits over time. Therefore, the time span of the anodizing process can determine the length of the formed nanotubes [41]. 5-50 min anodizing was done to synthesize different lengths of the TNTs, as given in Table 1.

2.3. Heat Treatment

The heat treatment was performed on some specimens to investigate the effect of the annealing process on the crystalline structure of the titanium oxide nanotubes. The specimens were annealed at 550°C for 2 h.

2.4. Coating Characterizations

2.4.1. Morphology and structural characterizations

The surface morphology and the elemental analysis of the coatings were characterized by scanning electron microscopy (SEM) equipped with an energy dispersive X-ray spectroscopy (EDS) using TESCAN MIRA3 FEG-SEM with the Thermo Scientific EDS system. In addition, X-ray diffraction (XRD) was performed to determine the crystalline structure of the applied coatings before and after the annealing process. The XRD patterns were recorded using a Bruker 1 D8 diffractometer, with Cu Kα radiation.

2.4.2. Contact angle measurements

The Contact angle is one of the important parameters in medical implants. The contact angle measurements are carried out according to the ASTM D7334 using a human blood droplet dropped on the coated titanium-based implants by SEO Phoenix instrument.

2.5. Bone Matrix Potential Investigation

The simulated body fluid (SBF) analysis can reveal the potential of bone formation on the surface of implants. Calcium phosphate deposition in SBF was implemented for the evaluation of the bio-performance of the applied coatings [42]. According to the ISO 23317 test method, the coated specimens in 6 groups (3 specimens for each group) were soaked in the SBF solvent and incubated for 30 days at 25°C.

2.6. Cell Culture

In this study, the human cell-like category of MG-63 (Human Osteosarcoma) osteoblasts was received from the cell bank of Pasteur institute of Iran (NCBI: C555). The tissue origin of this cell was bone marrow, but these osteoblastic-like cells were stopped in the pre-maturation stage of complete osteoblasts. This cellular class in the laboratory was very similar to the main osteoblast cells and is one of the most common cellular models of osteoblastic culture in biocompatibility studies and morphogenesis (proliferation, migration, differentiation, and death). The cells were cultured in a 75 ml cell culture flask (SPL, Korea) in a DMEM (Gibco, USA) nutrient culture medium containing 10% FBS (Gibco, USA) serum and 1% antibiotic-antifungal (penicillin-streptomycin-amphotericin B). They were incubated in 5% CO_2 and 95% moisture at 37°C. On the first day, each specimen was placed in a 24-cell culture (one plate for 24 h after implantation and one plate for 72 h after implantation) and about 25,000 cells (counting with a hemocytometer slide and the color of tripan blue) were planted on 500 micrograms of the culture medium. The plates containing specimens and cells were transferred to cell culture incubators.

2.7. Viability, Proliferation, and Cytotoxicity

The quantitative method of MTT is one of the most common methods for the determination of cell proliferation and cellular toxicity assay.

Table 1. Anodizing specification to Synthesis titanium oxide Nanotube

Anodizing time (min)	Electrolyte chemical composition	Electrical potential (V)
5	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	**20**
8	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	**20**
10	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	**20**
15	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	**20**
20	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	**20**
30	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	**20**
40	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	20
50	50% H_2O + 50% Glycerol + 0.5%wt NH_4F	20

[DOI: 10.22068/ijmse.2925]

The ISO-10993-2009 standard can be used for the study of cellular toxicity of drugs and medical devices in vitro. In MTT analysis, cell survival is measured by quantifying a metabolic product that confirms the mitochondrial activity of cells and is directly related to cell survival and growth. Viability, proliferation, and cytotoxicity were investigated by MTT test for the TNTs with different lengths. The cell counting was carried out after 24 h and 72 h cell culture. The specimens were rinsed with PBS buffer (Gibco, USA) and then, the culture medium (free of serum and antibiotics) containing 10% MTT dye was added to each well (500 µl per well). The plates were then placed in an incubator for 3 h (until the formation of purple crystals formed). The medium containing MTT marker (Sigma-Aldrich, Germany) was then discharged from each well and added to 300 µl DMSO solvent (Sigma-Aldrich, Germany). 100 µl of the resulting color solution (3 replications of each well) was transferred to the wells of a plate with 96 cell culture cells, and finally, the optical absorption of the colored solutions in each well was read by the Elisa reader.

2.8. Cell Adhesion and Morphology

SEM photography was used to investigate the adhesion and morphology of the cell adhesion on the surface. Each specimen was drained from the corresponding well and transferred to the wells of a new plate. The specimens were rinsed once with PBS buffer and then 2.5% solution (fixator) of glutaraldehyde in PBS buffer (500 µl per well) was added to each well (on cells attached to the surface of the specimens). The plate containing the relevant specimens was then refrigerated for 24 h to fix the cells on the surface of the specimens. At the end of the incubation period, the fixative solution was drained and each specimen was rinsed in the following order and dehydrated with different percentages of alcohol: Distilled water for 10 min, alcohol 30% for 10 min, alcohol 50% for 10 min, alcohol 60% for 10 min, alcohol 70% for 10 min, alcohol 80% for 10 min, alcohol 90% for 10 min, and finally absolute alcohol for 30 min. Finally, the alcohol was drained out of the specimens and the specimens were placed under a hood for 48 h to be dried. The fixed cells on the surface of the specimens were investigated by SEM.

3. RESULTS AND DISCUSSION

In this work, a simple and industrial-compatible method for fabricating a Nano-tubular layer on the titanium surface was used. This method is supposed to improve the biocompatibility of titanium as a common material for orthopedic implants.It can be observed in SEM images, as fabricated TNTs without any other operations like heat treatment are synthesized on the surface of the titanium plates with an average diameter of 100 nm (Fig. 1), which is the optimum diameter for osteoblast cell adhesion [26]. The elemental composition of the Titanium oxide was determined by EDS analysis (Fig. 2).

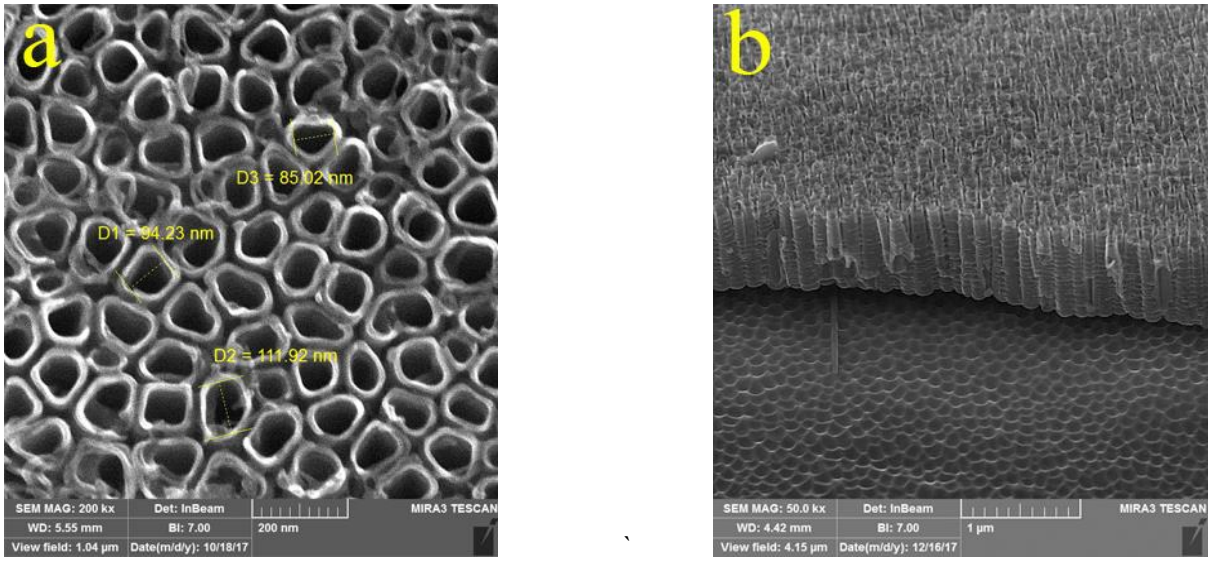
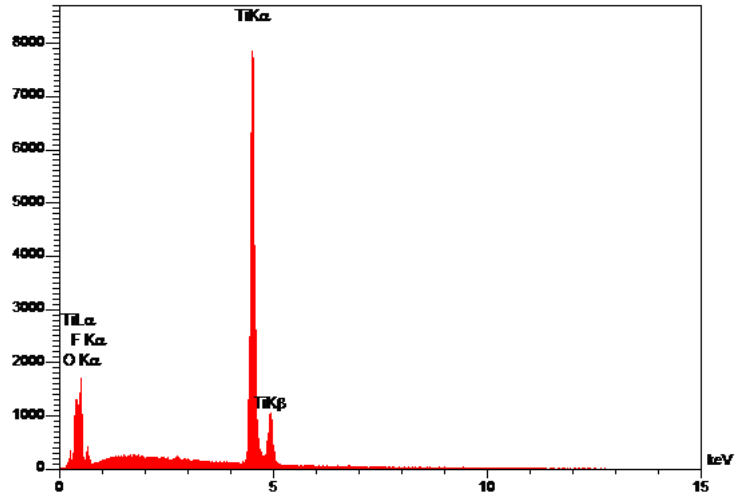


Fig. 1. SEM images of the as fabricated titanium oxide Nanotubes a) surface b) cross section on titanium plate. The average diameter of Nanotubes is 100 nm.

[DOI: 10.22068/ijmse.2925]



Element	Line	W%	A%
O	Ka	34.14	57.37
F	Ka	6.64	9.40
Ti	Ka	59.22	33.23
Total		100.00	100.00

Fig. 2. EDX analysis of synthesized Nanotubes wall on titanium surface

The XRD patterns of the as-fabricated and heat-treated TNTs with 600 nm length are shown in Fig. 3. While in the EDX results synthesis of TiO_2 is observed and SEM photography shows the formation of TNT, there is no sign of crystalline TiO_2 in the XRD pattern. It means that as-fabricated TNTs are created by non-crystalline titanium oxide. Nevertheless, the titanium oxide phase in the form of anatase has appeared clearly beside the pure titanium phase in the crystalline structure of the heat-treated specimen. In fact, the heat treatment process led to crystallization in structure and mostly the formation of the anatase phase.

The cross-section micrographs of the specimens were considered to measure the length of the nanotubes. According to Fig. 4 Fig. 3, each specimen had a different length due to the different anodizing times. Data analysis of more than 50 length measurements at the various anodizing time are given in Fig. 5. The vector shows a direct linear correlation between the time duration of anodizing and TNT's length. The more anodizing time, the longer TNT's length. This diversity in length is investigated in the bio tests of this study.

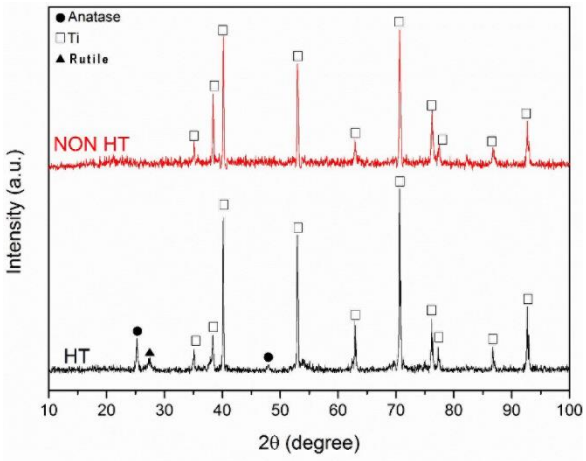


Fig. 3. The XRD patterns of the as-fabricated and heat-treated TNTs with 600 nm length.

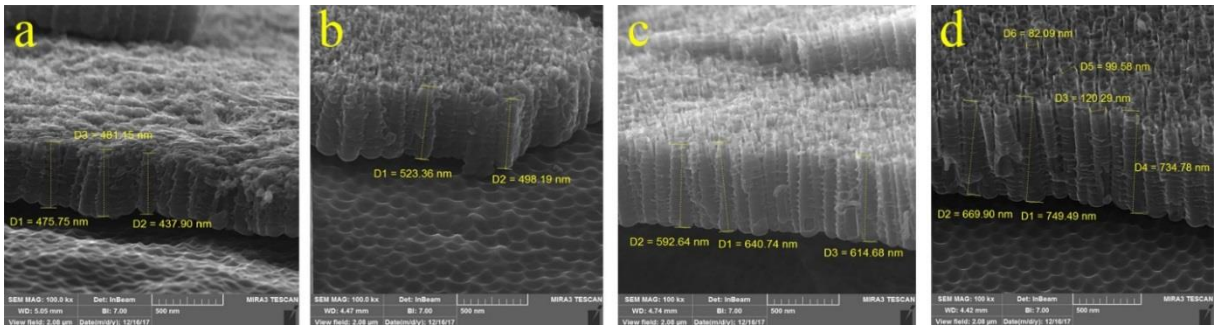


Fig. 4. The TNT height measured by SEM images and software analysis: a) 5 min anodizing, average height of 400 nm, b) 10 min anodizing, average height of 500 nm, c) 20 min anodizing, average height of 600 nm, and d) 50 min anodizing, average height of 950 nm.

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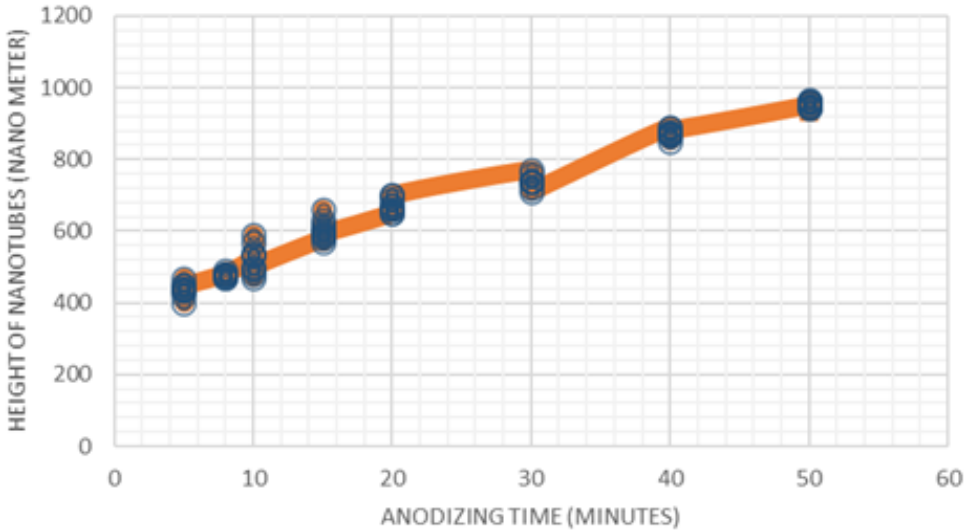

Fig. 5. More than 80 measurements on 8 groups of specimens with various anodizing times. Height of titanium oxide Nanotube showing the direct and liner relation with anodizing process time ($p < 0.05$).

For medical implants, more wettability is one of the important items to establish better bonding in the first contact with bio fluid after surgery and provide a higher cell adhesion. As indicated before, providing a better ground for protein adhesion and cell attachment is a key factor to accelerate implant bonding. Hence, a good wettability of the implant surface can prepare a better situation for bonding with the tissue after the implantation surgery [43–45]. Contact angle measurements in Fig. 6 indicated that the heat treatment of the specimens could significantly increase the wettability of the TNTs. The reason for this phenomenon is related to the TNT's atomic structure. Non-crystalline and rutile structures in TNT have hydrophobic properties, whereas the Anatase phase in TNT has hydrophilic capabilities. A thorough explanation is beyond the scope of this article and has been covered in prior reports [46, 47]. The heat treatment process at 450°C-650°C showed a positive effect on the contact angle results and increased the wettability of the coating surface up to 20° contact angle.

An investigation of the calcium phosphate sediment was carried out through an SBF test using SEM photography after soaking in SBF for a month. Obtained results clarified that the deposition of calcium phosphate on the surface of TNTs was more than that obtained for the titanium oxide compact layer. In addition, it can be seen that the annealed nanotubes provided more suitable conditions for calcium phosphate deposition.

Fig. 7 displays that the deposition was increased by the increment of the length of the TNTs and the most compact layer of calcium phosphate was deposited on the specimen with the length of 600 and 800 nm.

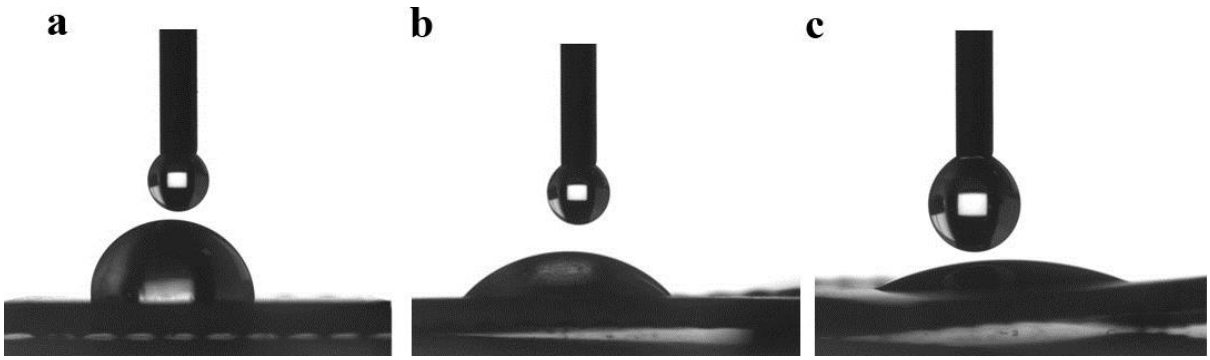

Fig. 6. Fig. 6-Contact angle of a human blood droplet on the surface of a titanium sheet a) 85° average for Coated with titanium oxide b) 44° average on Coated with titanium oxide nanotube c) 26° average angle on Coated with heat-treated titanium oxide nanotube.

[DOI: 10.22068/ijmse.2925]

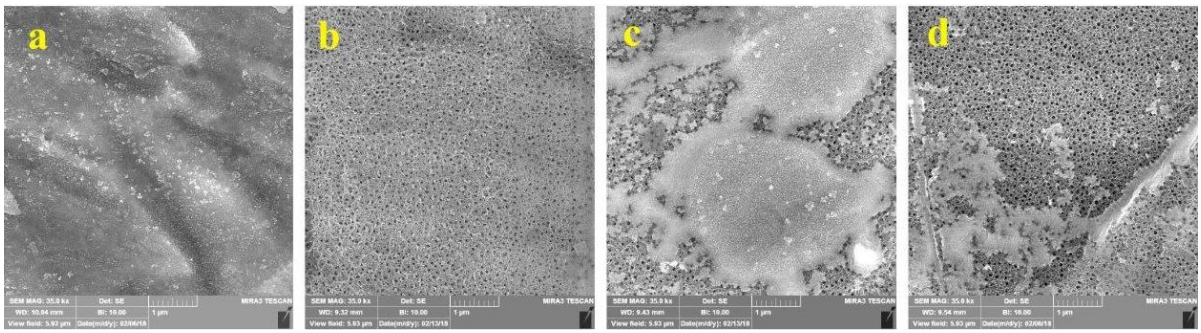

Fig. 7. SEM images of calcium phosphate deposition on a) titanium oxide layer b) titanium oxide Nanotube with 400 nm height c) titanium oxide Nanotube with 600 nm height d) titanium oxide Nanotube with 800 nm height (35k X).

Calcium phosphate deposition as a composition similar to the human bones was investigated in different specimens. In follow, a comparison was performed between heat treated specimens. According to Fig. 8, the heat-treated nanotubes with 800 nm length had a considerable amount of deposited calcium phosphate on the surface more than all non-heat treated specimens. However, the most compact layer of calcium phosphate and the thickest layer is formed on the heat-treated TNTs with 600 nm length, as represented in Fig. 9.
The thickness of the deposited calcium phosphate layer on the surface can be measured using cross-section photographs.

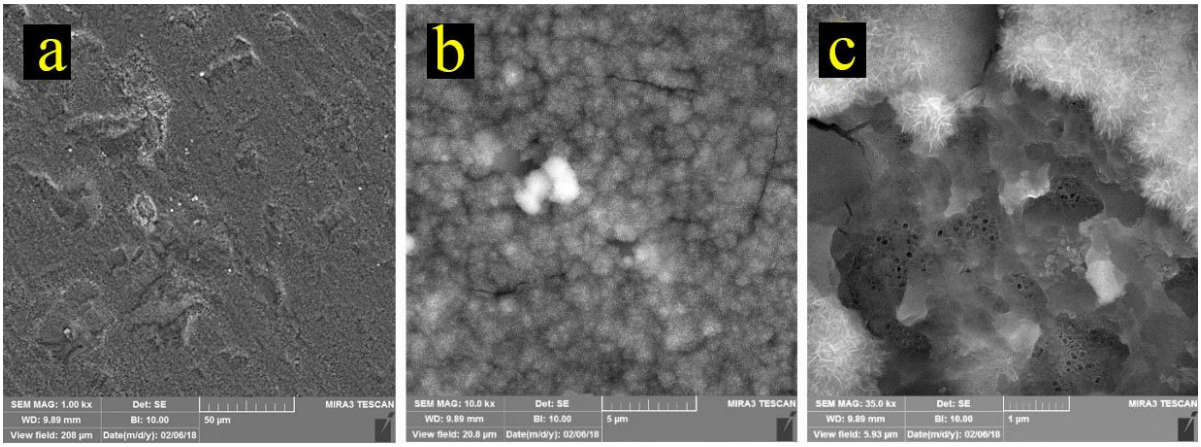

Fig. 8. SEM images of compact calcium phosphate deposited on the heat-treated titanium oxide Nanotube with 800 nm height, a) 1000 X b) 10k X c) 35k X

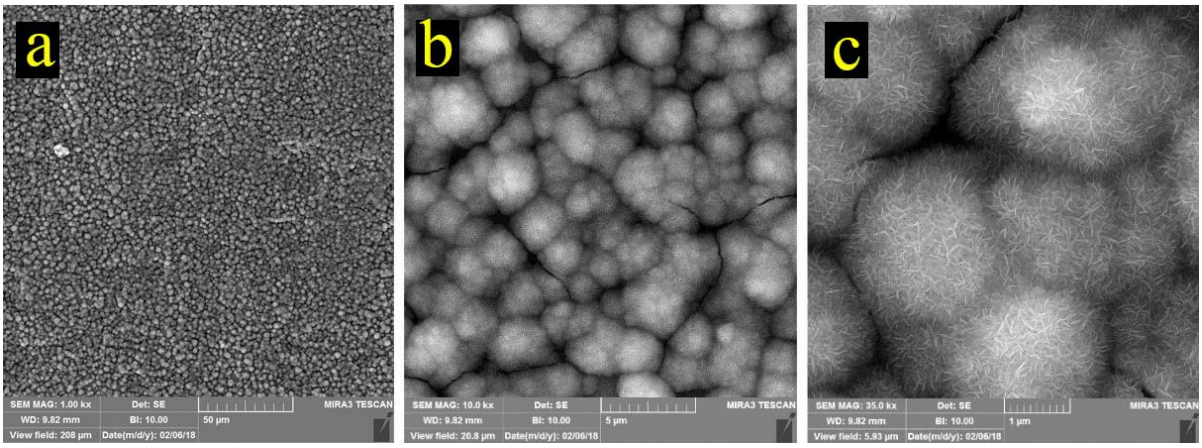

Fig. 9. SEM image of a considerable amount of calcium phosphate deposited on the heat-treated titanium oxide Nanotube with 600 nm height, a) 1000 X b) 10k X c) 35k X

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It can be observed that the heat treatment process can increase the thickness of the calcium phosphate deposition layer in all samples. This is in agreement with the obtained results by Mazare et al. [48]. The thickest deposited layer with about 2 μm thickness was obtained for the heat-treated TNT with 600 nm length, which according to Fig. 10, it was two times more than the thickness obtained for the nanotubes with 800 nm length. The thicker calcium phosphate layer deposited on the heat-treated 600 nm TNT indicates a higher potential for bone formation on this structure in comparison to the compact layer of titanium oxide and other TNT specimens with different lengths.

Some morphological parameters make the surface profile measurable for analysis, including spacing parameters; RSm (Mean width of the unevenness), and depth of unevenness; Rc (mean height of the unevenness). Z. Gong et al. [27] investigated the effect of TNT diameter variation on cell attachment and discovered that TNTs with a 100 nm diameter are the best size for osteoblast cell adhesion. In other words, they investigated RSm of the surface profile. In this study, we supposed fixed 100 nm diameters and investigate the height of the TNTs as another parameter of the surface profile, which addresses Rc. Fig. 7 shows that 600 nm length for TNTs is the best size of surface profile for calcium phosphate deposition. This may be because of the better wettability of the surface profile covered by TNTs 100 nm diameters and 600 nm height shown in Fig. 6. and heat treatment of this nanostructure amplifying this layer deposition (Fig. 9 and Fig 10).

It is been predicted that the higher corrosion resistance of the crystalline structure, as well as the more wettability of the annealed specimen, provide better conditions for maximum cell attach and absorption of the primary cells on the surface. Hence, the crystalline structure of the TNTs affects the bio-performance of the coating [49–51], although higher cell attachment does not necessarily indicate to more number of alive cells and proper proliferation rate.

To investigate the bone cell behavior on the surface of the TNT-based coating, alive cell counting and MTT assay was employed. Two groups of surfaces of 6 different types were selected for these tests. One group of non-nano structure TiO_2 coating in two types of as-fabricated and heat treated TiO_2. Another group was selected from TNT coating containing 3 various lengths (400, 600 and 800 nm) non-heat treated. Since the TNT with 600 nm length showed outstanding results in the initial tests, a heat treated 600 nm TNT was added to TNT group to investigate heat treatment effect on TNTs and comparison with annealed non-nano structure TiO_2. The significance level of the difference was considered as 0.05 ($p < 0.05$) for the cell counting result. The results in Fig. 11 imply no significant difference between the alive cells on the surface of the specimens after the first 24 h, indicating that the time was not enough for the cell division The first 24 h data reveal the proliferation and cytotoxicity. As shown in Fig. 12 the amount of alive cells after the first 24 h was in the same range as all samples.

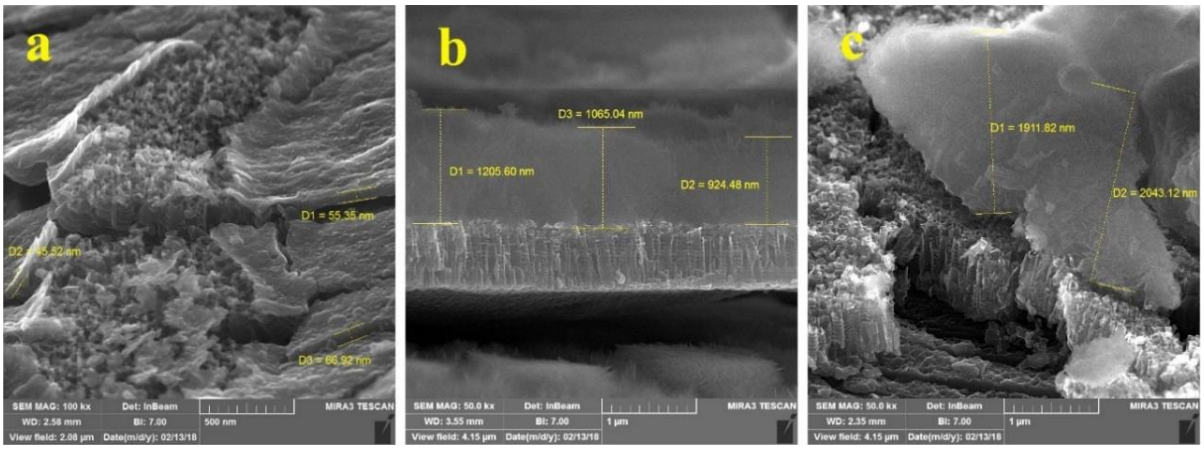

Fig. 10. Cross section SEM images of the calcium phosphate layer deposited in SBF test. Measuring the thickness of the layer a) average 50 nm on titanium oxide Nanotube with 600 nm height b) average 1 micron on heat treated titanium oxide Nanotube with 800 nm height c) average 2 microns on heat treated titanium oxide Nanotube with 600 nm height.

[DOI: 10.22068/ijmse.2925]

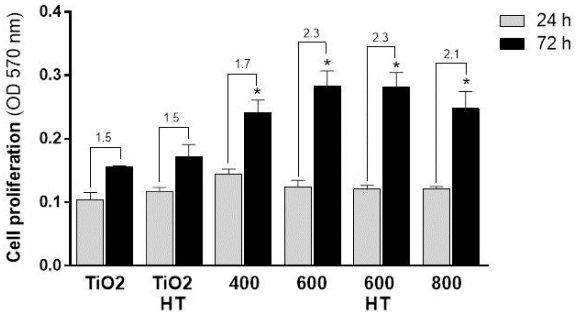

Fig. 11. Proliferation and Viability assay of the cultured MG-63 cells after 24 and 72 hrs. MTT test on titanium plate with titanium oxide compact layer (Tio_2), titanium plate with heat-treated titanium oxide compact layer (Tio_2 HT), titanium plate covered by t

In general, cell culture results of all specimens after the first 24 h, showed no negative point and descendant in reference to the TiO_2 control specimen. Likewise, all groups resulted in proliferation and division after 72 h. A significant and considerable difference was observable in the samples with the nanotube structure. An interconnected cluster of cells can be seen on the TNT structure which is not existing on a continuous titanium oxide surface. This completely matches with a study by Popat et al. [8], the most growth rate in comparison with the reference sample was 230% for the nanotube structure with 600 nm length. Moreover, the most counted alive cell after 72 h cell culture was observed for the TNT with 600 nm length in both as-fabricate and heat-treated forms. The proliferation for the longer and shorter TNTs was less than that was obtained for the optimized sample.

In order to investigate the cell morphology and adhesion on the surface of the specimens, the SEM images of the cells cultured on the surface after 72 h were investigated. Fig. 12a and Fig 12.b show only the spherical shape of the cells on the surface of the as-fabricated and heat-treated titanium oxide specimens.

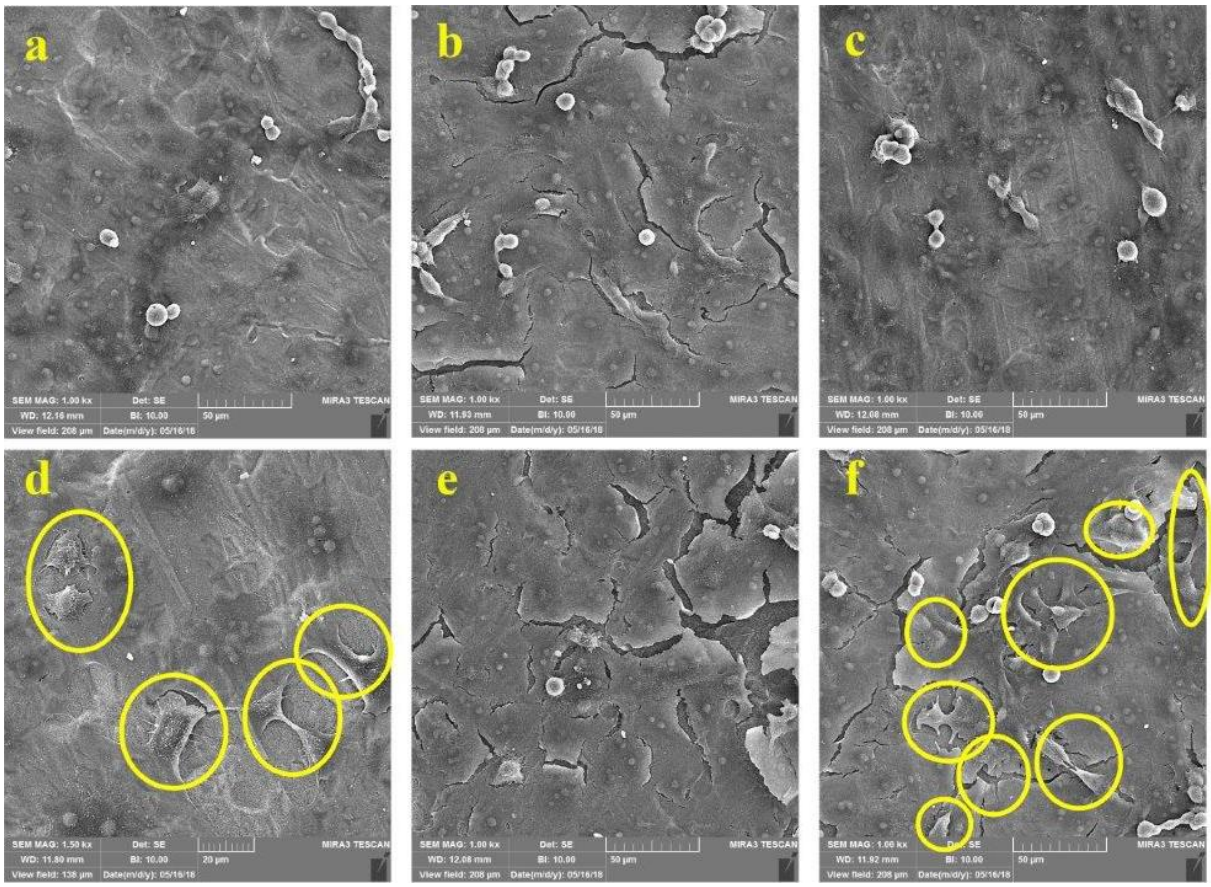

Fig. 12. SEM images of cells cultured on the surface to investigate the adhesion and morphology of cells after 72 hrs on a) As fabricated Titanium oxide compact layer b) heat-treated Titanium oxide c) titanium oxide Nanotubes with 400 nm height d) titanium oxide Nanotubes with 600 nm height e) titanium oxide Nanotube with 800 nm height f) heat-treated titanium oxide with 600 nm height.

[DOI: 10.22068/ijmse.2925]

The spherical shape of the cells without enough contact interface with the substrate is another evidence of the high contact angle of the titanium oxide smooth surface in both as-fabricated and heat-treated specimens and proved contact angel test result in Fig. 6. The spherical shape resulted in the lowest contact area with the flat substrate which is not favorable in biomaterial coatings [52, 53]. The morphology of the MG-63 cells on 400 nm length was almost identical to the titanium surface, which is demonstrated in Fig. 12c. In contrast, the cells that adhered to the TNT surface specimens with 600 nm or higher lengths had different morphologies. The presence of the wide and spindle-shaped cells can be seen in Fig. 12d and Fig. 12f. This indicates the high adhesion and good contact between the cells and the developed surface. It shows that the heat-treated nanotube coating with a length of 600 nm is the most suitable structure as a coating of a titanium substrate for bone cell attachment. The reason is the outstanding results of this structure in all contact angle tests, calcium phosphate deposition, and MTT. Heat-treated 600 nm nanotubes have created a profile on the surface that has the most wettability in the body environment, as well as the heat-treated atomic structure of TNTs and the optimal length of these nanotubes provide the conditions for the maximum precipitation of calcium phosphate, which is the bone composition. The MTT results and the cells' images confirm the above findings. Therefore, the heat-treated structure of the titanium oxide nanotube with a diameter of 100 nm and a length of 600 nm, due to having the most optimal size and surface profile and atomic structure suitable for the deposition of bone composition, is the most suitable coating for the adhesion of bone cells and rapid proliferation in order to create the bone tissue on the titanium implant. The major reasons for this are the TNT's optimum size, surface profile, and atomic structure, which enhance bone composition deposition. In Fig. 13, the suitable adhesion of the cells can be observed which are extended on the implant surface and the high cell proliferation are favorable in biomaterial coatings for orthopedic and dental implants. The high proliferation rate leads to a fast tissue formation on the used implant. This is a key factor especially in orthopedic implants due to the low proliferation rate of the osteoblast cells.

According to Fig. 14, due to the fact that the size of the nanotubes (100 nm) was much smaller than the diameter of the bone cells (Average size of 35 µm), the filament cells can easily penetrate into the nanotube channels. This mechanism assists the cells to acquire a suitable mechanical adhesion on the surface biomaterials.

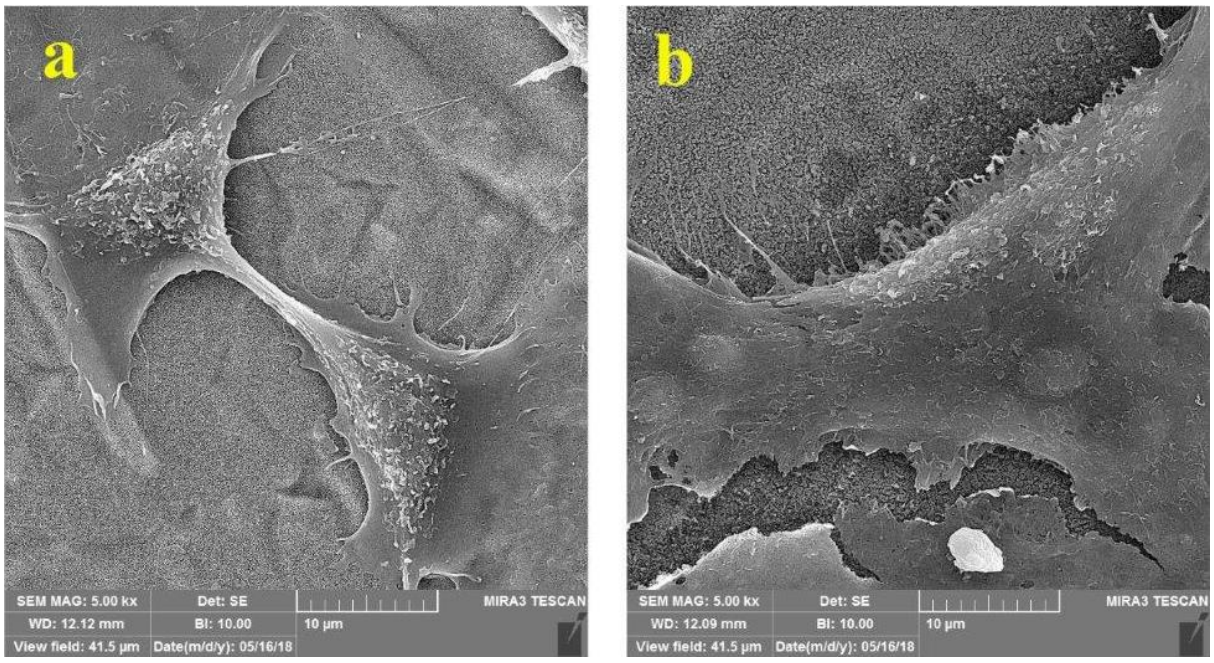

Fig. 13. SEM images with 5000 X from cell proliferation after 72 hrs on a) heat-treated titanium oxide Nanotube with 600 nm height and b) heat-treated titanium oxide Nanotube with 800 nm height shows a elongated shape of cells on the surface.

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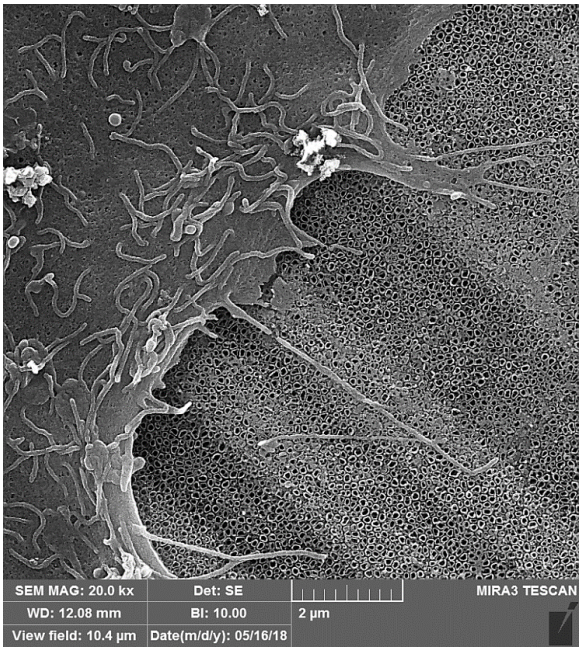

Fig. 14. SEM image with 20 kX, cell filaments penetrated into the nanotubes and a mechanical adhesion was provided for the cells on the biomaterial surface.

3.1. Limitations of the Present Study and Future Research Areas

In the present study, the optimal length of TNTs for bone implants was investigated by in-vitro experiments. However, it seems that the accuracy of the results should be investigated by in-vivo analyses. In addition, the reason for the variations of the biological properties by changing the length of the nanotubes is one of the open questions that understanding of it can help to create more suitable nanostructures. So, more investigations about the reason for the change in biocompatibility and quality of the cell growth with the change of the nanotube length can be continued in future studies.

4. CONCLUSIONS

The Titanium oxide nanotubes were fabricated by anodizing. The longer the anodizing time, the longer was the TNT's length with a direct linear correlation. Heat treatment transformed TNT structure into the anatase crystalline phase. The contact angle measurements showed that the heat-treated TNT had the most wettability and was considered a suitable substrate for calcium phosphate deposition or cell adhesion. The SBF experiments revealed that the heat-treated TNT with 600 nm length had the greatest potential in calcium phosphate deposition as a composition similar to the human bone materials. Cell culture and proliferation analyses indicated similar results about the optimum length of the TNTs. The optimum length for the heat-treated nanotubes was 600 nm. The specimen prepared under the optimum conditions revealed the highest potential for cell attachment and proliferation. All investigations address to 600 nm heat-treated TNTs as the optimum length TNTs that can be used as a coating for titanium implants and may accelerate bone-implant graft and a shorter healing period.

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