



ORIGINAL RESEARCH ARTICLE

Effect of Anodization Time on the Structure, Corrosion Resistance, and Biocompatibility of TiO₂ Nanotubes Formed at 5 °C

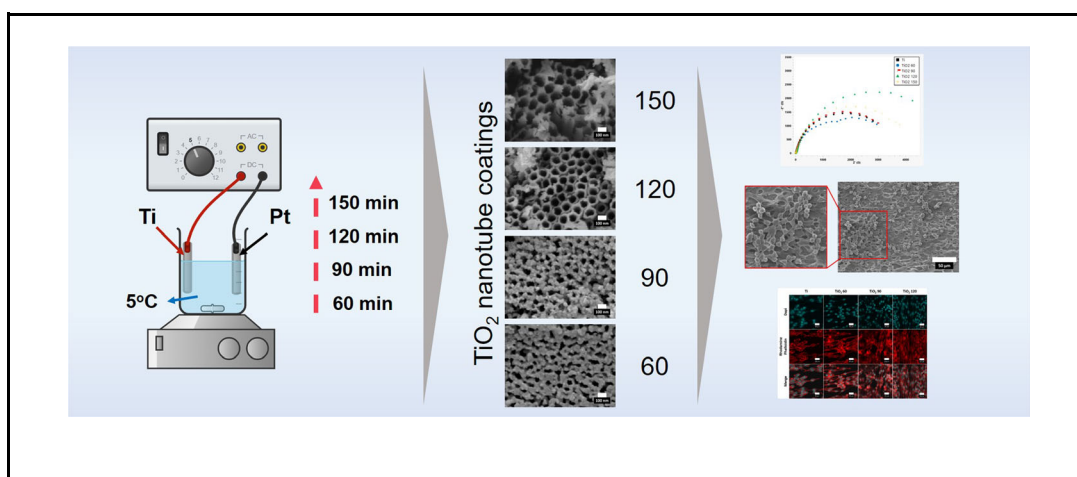
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Despite growing interest in TiO₂ nanotubes coatings on titanium, the influence of anodization time under low-temperature conditions on corrosion resistance and in vitro biocompatibility have not been systematically clarified. In particular, quantitative correlations between nanotube structural evolution and electrochemical performance remain limited. Therefore, this study systematically investigates the effect of anodization time at low temperature (5 °C) on the formation of TiO₂ nanotubes coatings on titanium at four anodization conditions: 60, 90, 120 and 150 minutes. Surface morphology and crystalline structure were characterized using Scanning Electron Microscopy (SEM) and X-ray Diffraction (XRD). Coating hardness was evaluated by the Vickers method, while corrosion resistance was assessed using Potentiodynamic Polarization and Electrochemical Impedance Spectroscopy (EIS) in Simulated Body Fluid (SBF). In vitro biocompatibility was examined through Water Contact Angle measurements and direct cell culture using Baby Hamster Kidney (BHK-21) cells, with cell morphology and attachment further analyzed by SEM and Confocal Laser Scanning Microscopy (CLSM). The results demonstrate that anodization at 120 min under low-temperature conditions (5 °C, 40 V) produces a well-ordered TiO₂ nanotubes layer with optimized performance, exhibiting a low corrosion current density of 0.76 $\mu\text{A}/\text{cm}^2$, a high polarization resistance of $3.42 \times 10^4 \Omega\text{cm}^2$, and a protection efficiency of 90.91%. This condition also shows enhanced surface wettability and significantly improved cell attachment compared to other anodization times. Overall, anodization for 120 min under low-temperature provides an optimal balance between structural stability, corrosion resistance, and biocompatibility, highlighting its potential for biomedical implant applications.

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Graphical Abstract



Keywords biocompatibility, electrochemical anodization, corrosion resistance, low-temperature synthesis, simulated body fluid (SBF), TiO₂ nanotubes arrays, titanium implants

1. Introduction

Titanium and its alloys are extensively used in dental and orthopedic implants due to their high strength-to-weight ratio, excellent mechanical properties, and good biological compatibility. However, long-term implant performance can be compromised by corrosion in physiological environments and insufficient surface bioactivity, which may lead to implant failure. To address these challenges, TiO₂ coatings, naturally formed oxide layers on titanium surfaces, have been widely investigated as protective and bioactive surface modifications. These coatings play a crucial role in enhancing corrosion resistance, promoting osseointegration, and improving overall surface performance in biomedical applications (Ref 1, 2). In the biomedical field, TiO₂ coatings are widely applied in dental and orthopedic implants due to their excellent chemical corrosion resistance, ability to promote osseointegration, and low risk of triggering immune responses (Ref 3, 4). As a result, TiO₂ coatings have received increasing research attention, particularly in relation to their morphology, structural characteristics, and the ability to control these properties (Ref 5). Among various fabrication techniques (Ref 6), anodization has emerged as a promising method for producing TiO₂ in the form of well-aligned nanotubes arrays (Ref 7). The evolution of TiO₂ nanotubes morphology under anodization conditions has been previously investigated by our group (Ref 8), providing a structural basis for understanding nanotubes formation at low temperature. This nanotubular structure significantly increases surface area, supports cell adhesion and proliferation, and serves as a potential platform for drug delivery or enhancing bone integration (Ref 9, 10). Despite this potential, most studies on anodized TiO₂ nanotubes have focused on applications in water treatment, catalysis, and sensing technologies (Ref 11, 12). Nonetheless, their biomedical applications,

particularly in enhancing corrosion resistance and biocompatibility, remain underexplored and require further exploration.

In the context of today's rapidly advancing science and technology, the application of nanotechnology has become essential to meet growing societal demands. Among various approaches (Ref 13, 14), electrochemical technology stands out as a simple, efficient, and highly controllable method that is widely used to fabricate functional coatings on material surfaces (Ref 15). Among these methods of electrochemical technology, anodization stands out as an effective technique for producing highly ordered oxide structures, particularly TiO₂ nanotubes coatings (Ref 16). This technology not only enhances the physicochemical properties of materials but also opens up numerous possibilities in biomedical applications, including implant surfaces, drug delivery systems, and biosensors (Ref 17, 18). Although anodization of titanium offers many benefits, its outcome is greatly influenced by various parameters such as voltage, current density, electrolyte composition, duration and temperature (Ref 19-23). Among these, temperature plays a particularly critical role, influencing the growth, dissolution, and final morphology of the nanotube structures. Furthermore, according to electrochemical theories established in aluminum anodization systems, lowering the temperature of the anodization process (particularly to levels below room temperature) can significantly enhance the uniformity and structural stability of the resulting oxide layer. This is because lower temperatures tend to reduce the reaction rate, allowing for more controlled oxide growth and minimizing the formation of defects or irregularities within the coating (Ref 24-26). However, this factor has not received adequate attention in previous studies, largely due to the difficulty in maintaining stable temperature conditions and systematically evaluating its effects throughout the entire anodization process.

Despite these advances, no systematic study has concurrently evaluated the morphological evolution, corrosion resistance, and biocompatibility of TiO₂ nanotubes fabricated under controlled low-temperature conditions across multiple anodization time points (Ref 27-32).

This study aims to (1) elucidate the influence of anodization time (60, 90, 120, 150 min) at 5 °C on TiO₂ nanotubes morphology and phase composition; (2) quantify corrosion resistance via electrochemical impedance spectroscopy and polarization measurements in SBF; (3) assess in vitro biocom-

patibility through contact angle measurements and BHK-21 cell culture; and (4) identify the optimal anodization time for biomedical titanium implant coatings. Building upon our previous work (Ref 8), which primarily focused on the formation and structural evolution of TiO₂ nanotubes arrays under anodization conditions, the present study systematically extends the investigation to the coupled effects of anodization time and low-temperature processing on electrochemical performance and biological response.

2. Materials and Methods

2.1 Synthesis of TiO₂ Nanotube Coatings Via Anodization

Commercially pure Ti Grade 2 (99.6% purity, ASTM B265 Grade 2) sheets (Kahee Metal, Korea), sized 10 × 10 mm with a thickness of ~ 1 mm, were used as substrates. The samples were sequentially ground with SiC abrasive papers (grit sizes 150, 400, 1000, and 2000), then ultrasonically cleaned in acetone (10 min), followed by deionized water, and air-dried.

Anodization was carried out in 100 mL electrolyte containing 0.5 wt% NH₄F (Sigma, 99.9%), 2 vol% deionized water, and ethylene glycol (Merck, 99.9%) using a DC power supply. The Ti sheet served as the anode and a Pt sheet as the cathode, with ~ 5 cm electrode spacing. The process was performed at 5 °C for 60, 90, 120 and 150 minutes under a constant applied voltage of 40 V using a magnetic stirrer to maintain a constant electrolyte stirring speed of 500 rpm in a temperature-controlled bath. The electrolyte temperature was monitored using a thermometer, and the temperature variation during anodization was maintained within ± 1 °C. After anodization, the samples were rinsed with ethanol and deionized water and then dried. This procedure yielded uniform TiO₂ nanotubes coatings.

2.2 Surface, Corrosion, and in Vitro Biocompatibility Evaluation Methods

TiO₂ nanotubes coatings were fabricated on commercially pure Ti substrates via anodization at different times (60, 90, 120 and 150 minutes) and constant temperatures (5 °C), maintained using a temperature-controlled bath to ensure stable electrolyte conditions. Surface morphology and elemental composition were examined by field-emission scanning electron microscopy (FE-SEM, JEOL JSM-IT800), operated at an accelerating voltage of 3 kV and a working distance of 10 mm. The crystalline structure of the anodized TiO₂ coatings was analyzed by X-ray diffraction (XRD, Bruker D8 Advance) using CuKα₁ radiation ($\lambda = 1.5406 \text{ \AA}$), operated at an accelerating voltage of 40 kV and a tube current of 40 mA. XRD patterns were recorded over a 2θ range of 20°–80°, with a step size of 0.04° and a scan rate of 6°/min.

Surface hardness was measured using the Vickers method (HV5, 5 kgf load, Zwick Roell). Given the nanoscale thickness and porous structure of the TiO₂ nanotubes coatings, the indentation depth under the applied load exceeded the coating thickness. Therefore, the measured hardness values reflect the composite hardness of the coating-substrate system rather than the intrinsic hardness of the TiO₂ nanotubes layer alone. All measurements were performed at multiple locations on each sample to ensure repeatability.

Electrochemical corrosion behavior was evaluated in simulated body fluid (Ref 33) (SBF, pH ~ 7.4) using a

PGSTAT302N potentiostat/galvanostat (Metrohm Autolab). Potentiodynamic polarization measurements were carried out at a solution temperature of approximately 25 °C using a conventional three-electrode configuration, consisting of the sample as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum sheet as the counter electrode. The polarization curves were recorded over a potential range from – 100 mV to + 100 mV with respect to the open-circuit potential (OCP) at a scan rate of 0.1 mVs^{–1} to determine the corrosion potential (E_{corr}), corrosion current density (I_{corr}), and polarization resistance (R_p). Electrochemical impedance spectroscopy (EIS) measurements were performed at the open-circuit potential using a sinusoidal perturbation with an amplitude of 10 mV over a frequency range from 10⁵ to 10^{–1} Hz.

For in vitro biocompatibility evaluation, baby hamster kidney cells (BHK-21, ATCC CCL-10) were used. Prior to cell seeding, all Ti and TiO₂-coated samples were sterilized by immersion in ethanol, followed by rinsing with sterile phosphate-buffered saline (PBS). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and maintained in a humidified incubator at 37 °C with 5% CO₂. The cells were seeded onto the sample surfaces at an appropriate density and incubated for 48 and 72 h. Cell attachment and morphology were evaluated by SEM (SEM, JEOL JSM-IT200) and confocal laser scanning microscopy (CLSM, FV3000RS, Olympus) after fixation, permeabilization, and staining with Phalloidin 555 (actin, excitation at 555 nm and emission at 565–620 nm) and DAPI (nuclei, excitation at 405 nm and emission at 450–480 nm). Quantitative analysis of cell attachment was performed using ImageJ software by counting adherent cells from three independent CLSM images for each sample ($n = 3$). The quantitative data are presented as mean ± standard deviation (SD), and statistical significance among different samples was evaluated using one-way analysis of variance (ANOVA), with $p < 0.05$ considered statistically significant.

3. Results and Discussion

3.1 Surface Morphology of TiO₂ Nanotubes

Fig. 1 presents the FE-SEM images of TiO₂ coatings formed at different anodization times at 5 °C at a magnification of × 100,000. Clear morphological differences are observed among the samples. The coating anodized for 60 min (Fig. 1a) shows irregularly shaped pores with rough edges and sparse distribution, while the 90 min-sample (Fig. 1b) exhibits a slightly more uniform morphology but still contains partially sealed regions. At 120 min (Fig. 1c), a well-defined and highly ordered nanotubular array is observed, consisting of circular pores arranged in a nearly hexagonal pattern with pronounced depth, typical characteristics of a fully developed TiO₂ nanotubes layer. In contrast, the sample anodized at 150 min (Fig. 1d) still retains a tubular form, but many areas display collapsed walls or are covered by aggregated oxide fragments, indicating structural breakdown or re-dissolution processes caused by excessively rapid electrochemical reactions at longer time (Ref 34).

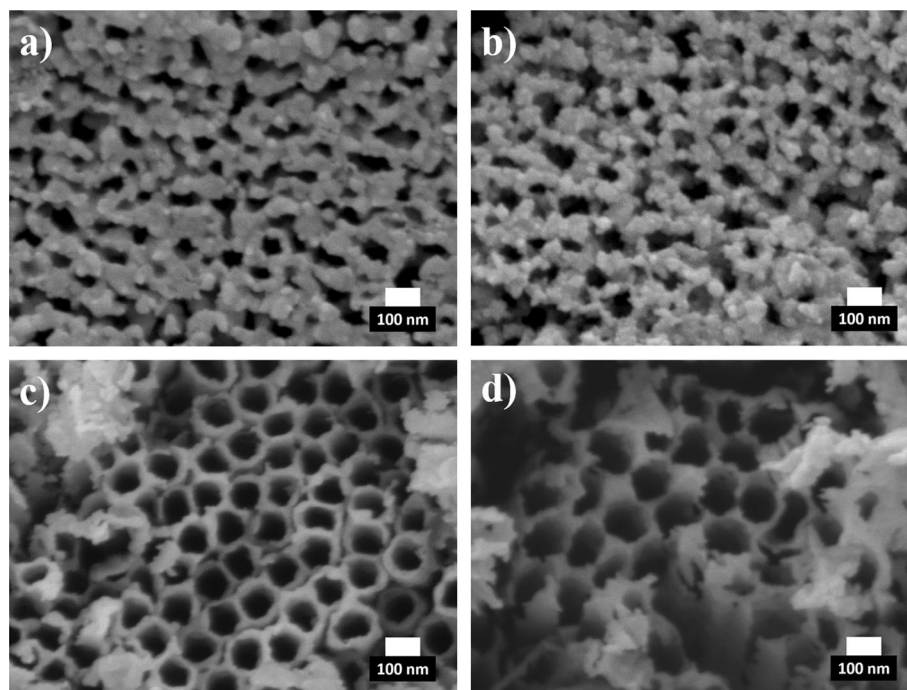


Fig. 1 FE-SEM images (x100000) of TiO_2 nanotubes coatings formed at 5 °C for (a) 60, (b) 90, (c) 120 and (d) 150 min, showing progressive nanotubes development and wall thinning (60 min: ~ 50 nm; 120 min: ~ 20 nm; 150 min: 2–5 nm)

The nanotubes diameter remains approximately 100 nm for all samples; however, the wall thickness decreases notably from about 50 nm at 60 min to 20 nm at 120 min and down to 2–5 nm at 150 min. Prior to FE-SEM observation, all samples were coated with a thin platinum layer to minimize charging effects. The deposited Pt layer was only a few nanometers thick and uniformly distributed; therefore, it does not significantly affect the relative comparison of nanotubes diameter and wall thickness among the samples. The reported values represent average measurements obtained from multiple nanotubes.

Overall, the coating formed at 120 min exhibits the most uniform, stable, and continuous nanotubular structure. In this study, the nanotubes diameter is narrowly centered around 100 nm with a highly ordered and continuous arrangement. In contrast, Prida et al. (Ref 35) reported TiO_2 nanotubes layers with a broader diameter range (approximately 60–100 nm) and a randomly disordered morphology. The narrower diameter distribution and higher degree of structural ordering observed in the present work indicate improved morphological uniformity, this structure enhances both physical durability and biological performance.

3.2 Elemental Composition Analysis of TiO_2 Nanotubes

The EDX results in Fig. 2 show distinct variations in elemental composition and distribution uniformity among TiO_2 coatings anodized at different time at 5 °C. At 60 min (Fig. 2a), Ti dominated (54.5%) while O was 45.5%, indicating limited oxide formation due to slow reaction kinetics. Increasing the time to 90 min raised the oxygen content to 49.3%, suggesting enhanced oxidation (Fig. 2b). The 120 min sample (Fig. 2c) exhibited a balanced composition (53.9% Ti, 46.1% O) and a uniform elemental distribution, implying a stable and homogeneous oxide layer. At 150 min (Fig. 2d), the Ti and O contents were approximately 51.6 wt% and 48.4 wt%, respectively;

however, the elemental mapping revealed reduced uniformity, which may be associated with accelerated electrochemical reactions and partial re-dissolution of the oxide film.

From these data, the calculated O/Ti molar ratios were approximately 2.50, 2.91, 2.56, and 2.70 for the coatings formed at 60, 90, 120 and 150 minutes, respectively. These values represent average ratios obtained from multiple randomly selected EDX measurement areas on each sample surface, with an estimated uncertainty of ± 3 at%, which is typical for EDX analysis, particularly for light elements such as oxygen. These ratios were estimated by converting the EDX-derived wt% values into molar quantities using the atomic weights of Ti (47.87 g mol^{-1}) and O (16.00 g mol^{-1}). It should be noted that EDX analysis is semi-quantitative in nature, particularly for light elements such as oxygen, and the values may be influenced by surface effects and sample preparation. In addition, a thin platinum layer was deposited prior to SEM/EDX analysis to minimize charging effects, which may slightly affect the absolute elemental percentages but does not alter the observed compositional trends. The relatively small variation in oxygen content among the samples therefore reflects general oxidation behavior rather than precise stoichiometric differences. Therefore, the O/Ti ratios exceeding the stoichiometric value of 2.0 should be interpreted within the experimental uncertainty and are likely associated with surface hydroxylation, adsorbed species, and the inherent overestimation of oxygen by EDX rather than true bulk non-stoichiometry.

3.3 Phase Structure Analysis of TiO_2 Nanotubes

The XRD patterns presented in Fig. 3 reveal differences in the crystalline phases of the TiO_2 samples anodized at various times. The untreated titanium substrate shows characteristic peaks corresponding to the Ti phase, representing the typical hexagonal close-packed crystal structure (Ref 36).

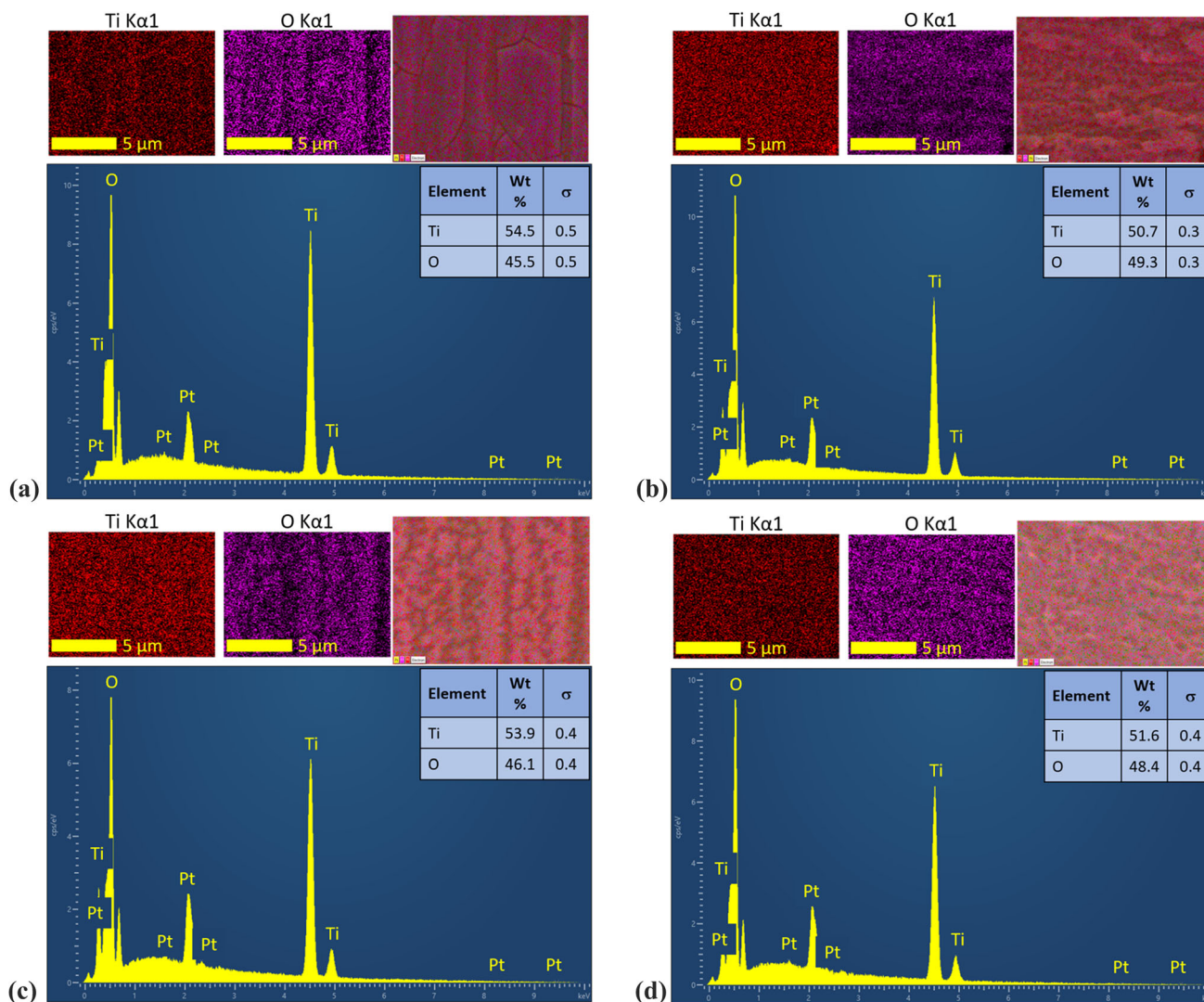


Fig. 2 EDX elemental mapping of TiO_2 nanotubes coatings formed by anodization at 5°C for (a) 60, (b) 90, (c) 120, and (d) 150 min, showing the spatial distribution of Ti and O elements

In the anodized samples, in addition to the residual Ti substrate peaks, which may persist due to the relatively thin oxide layer, TiO_2 oxide phases begin to appear, including both anatase and rutile. The sample anodized for 60 min exhibits predominantly anatase peaks, while rutile is present at low intensity. As the time increases to 90 min, the intensity of rutile peaks slightly increases, suggesting the onset of anatase-to-rutile transformation rather than a complete phase transition.

Notably, the 120 min sample displays the simultaneous development of both anatase and rutile phases with distinct peak intensities, reflecting a stable mixed-phase crystalline structure. Although anatase remains dominant, the presence of a small amount of rutile may contribute to improved mechanical properties and corrosion resistance of the coating (Ref 37, 38).

In the 150 min sample, both anatase and rutile phases are still present, but the increased intensity of rutile peaks compared to the 120 min sample suggests that the phase transformation continues more strongly at longer anodization time (Ref 23). This may negatively impact coating properties if excessive rutile crystallization occurs, as rutile can reduce

surface area and biological activity compared to anatase (Ref 39).

It should be noted that the present phase analysis is qualitative in nature and based on relative peak intensity comparison rather than quantitative phase fraction determination. Overall, the XRD patterns confirm that temperature significantly affects the crystalline structure of the TiO_2 coatings, with the 120 min sample demonstrating the most optimal balance between anatase and rutile, consistent with the favorable chemical and biological characteristics.

3.4 Hardness Results

Table 1 presents the Vickers hardness (HV5) measurement results of the commercially pure Ti substrate and TiO_2 anodized for 60, 90, 120 and 15 min. The uncoated Ti substrate exhibited the lowest average hardness of 132 HV, when TiO_2 anodized at 60 and 90 min, the hardness increased moderately to 140.67 HV and 142.67 HV, respectively. Notably, the sample anodized for 120 min showed the highest hardness, with an average value of 145.67 HV. This enhancement is closely related to the formation of a well-ordered, continuous, and mechanically

stable TiO₂ nanotubes layer with sufficient effective thickness, as observed in the FE-SEM analysis. In addition, the presence of a stable mixed anatase-rutile phase, as indicated by XRD results, may contribute to improved mechanical rigidity compared to predominantly anatase structures.

Although the sample anodized for 150 min also showed improved hardness (140.33 HV) compared to untreated Ti, it was still lower than that of the 120 min sample. This reduction is likely associated with partial collapse, thinning, or re-dissolution of the nanotube walls at prolonged anodization

time, which compromises coating integrity and load-bearing capability despite continued oxide growth.

Overall, the hardness evolution does not depend solely on phase composition but rather on a combined effect of coating thickness, nanotube structural integrity, and anatase-rutile phase balance. These results suggest that 120 min is the optimal anodization time under low temperature, not only for corrosion resistance but also for enhancing surface mechanical properties, which is particularly important for biomedical implant applications.

3.5 Electrochemical Properties of TiO₂ Nanotubes

Consistent with the observed morphological evolution, electrochemical data (Fig. 4) reveal the corrosion behavior of commercially pure Ti and TiO₂ nanotubes coatings fabricated by anodization for 60, 90, 120, and 150 min, as evaluated through potentiodynamic polarization (Tafel) curves in simulated body fluid (SBF).

Among the five samples, the uncoated Ti substrate exhibited the lowest corrosion resistance, followed by the TiO₂ coatings formed at 60 and 90 min. The coatings anodized for 120 and 150 min demonstrated significantly improved corrosion resistance, with the 120 min sample performing the best overall. These findings are consistent with the SEM and EDX analyses discussed earlier. At short anodization time (60 and 90 min), the anodization process proceeds slowly, which likely results in incomplete or poorly developed TiO₂ nanotubes layers on the Ti surface, leading to reduced protective ability compared to coatings formed at long time. However, even these incomplete coatings offer better corrosion resistance than bare Ti.

Although the polarization curve of the sample anodized for 150 min appears to shift toward lower current density, quantitative analysis based on Tafel extrapolation indicates that its corrosion resistance is slightly inferior to that of the 120 min sample. This behavior can be attributed to partial structural degradation of the TiO₂ nanotubes layer at prolonged anodization times. Accelerated electrochemical reactions may promote re-dissolution of the oxide, leading to morphological instability and a less compact protective layer, which ultimately limits the corrosion resistance despite the apparent reduction in current density. Overall, the TiO₂ coating formed at 120 min offers the most effective corrosion resistance among the tested conditions, highlighting it as the optimal anodization time for achieving a stable and protective nanotubular structure under low-temperature conditions for biomedical applications.

According to the data in Table 2, the TiO₂ nanotubes coatings formed at different anodization times clearly show improved corrosion resistance compared to pure Ti. The uncoated Ti sample had the highest corrosion current (I_{corr}) at 8.36 $\mu\text{A}/\text{cm}^2$, indicating the lowest level of protection. Coatings

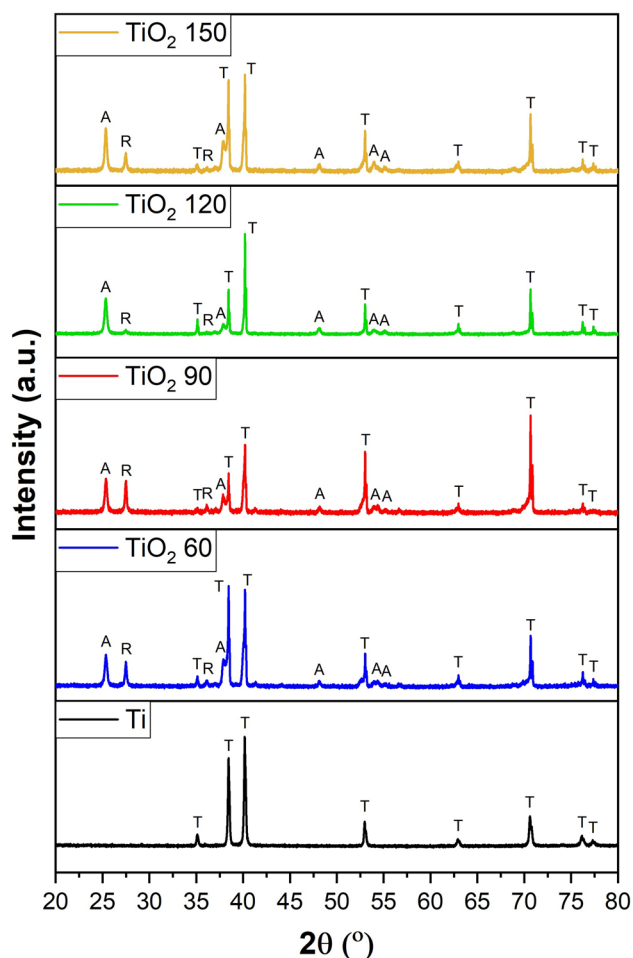


Fig. 3 XRD patterns of Ti and TiO₂ coatings obtained at different anodization times (CuK α_1 radiation, $\lambda = 1.5406 \text{ \AA}$, $2\theta = 20\text{--}80^\circ$), showing diffraction peaks corresponding to the Ti substrate (T, PDF#44-1294), anatase TiO₂ (A, PDF#21-1272), and rutile TiO₂ (R, PDF#21-1276) phases

Table 1 Vickers hardness (HV5) values of pure Ti and TiO₂ nanotubes coatings formed by anodization at 5 °C for different anodization times

Samples	Hardness 1	Hardness 2	Hardness 3	Average
Ti (HV)	132	131	133	132 $\pm$ 1
TiO ₂ 60	141	141	140	140.67 $\pm$ 0.58
TiO ₂ 90	141	143	144	142.67 $\pm$ 1.53
TiO ₂ 120	148	143	146	145.67 $\pm$ 2.52
TiO ₂ 150	137	143	141	140.33 $\pm$ 3.06

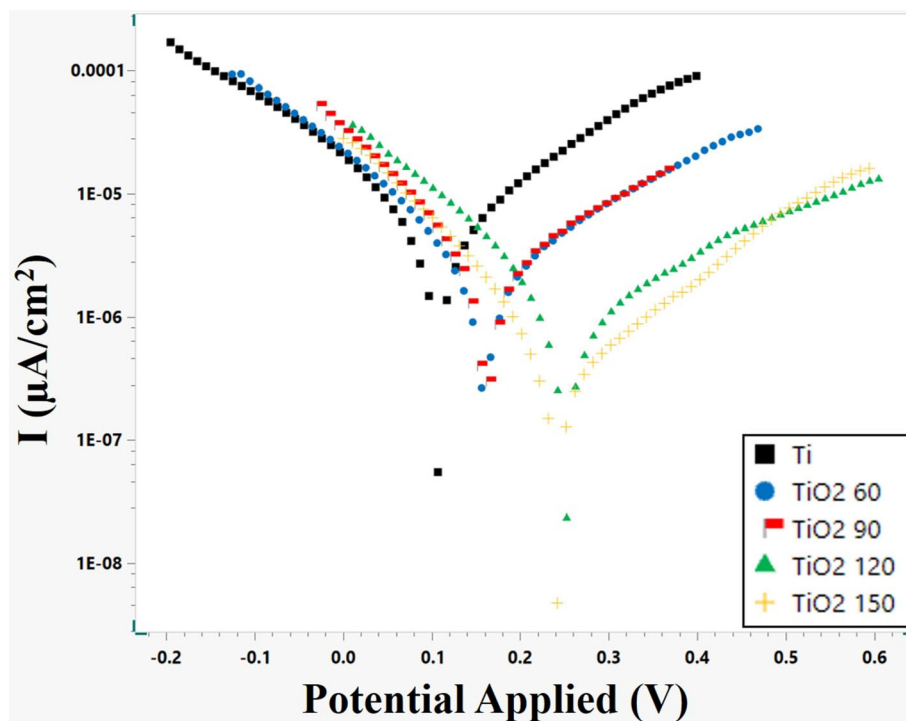


Fig. 4 Potentiodynamic polarization curves (Tafel plots) of Ti and TiO₂ coatings in simulated body fluid (SBF, 37 °C, pH 7.4), showing the corrosion potential (E_{corr}) and corrosion current density (I_{corr})

Table 2 Potentiodynamic polarization parameters of pure Ti and TiO₂ nanotubes coatings anodized at 5 °C for different times, measured in simulated body fluid (SBF, 37 °C, pH 7.4)

Samples	E_{corr} V	I_{corr} $\mu\text{A}/\text{cm}^2$	R_{corr} $\Omega \text{ cm}^2$	Corrosion rate, mm/year	H , %
Ti	0.107	8.36	3.11×10^3	0.097	0
TiO ₂ 60	0.160	3.50	7.43×10^3	0.041	58.13
TiO ₂ 90	0.158	2.87	9.06×10^3	0.033	65.67
TiO ₂ 120	0.251	0.76	3.42×10^4	0.009	90.91
TiO ₂ 150	0.241	2.17	1.20×10^4	0.025	74.04

Each polarization measurement was performed in triplicate ($n = 3$). The reported values represent mean values. Experimental variation was within $\pm 5\%$ for I_{corr} and ± 5 mV for E_{corr} , which is within the typical uncertainty range of potentiodynamic polarization measurements in SBF

formed at 60 and 90 min significantly reduced the I_{corr} values, with corresponding protection efficiencies ($H\%$) of 58.13% and 65.67%, respectively.

Among all the samples, the coating produced at 120 min stood out, showing the lowest I_{corr} ($0.76 \mu\text{A}/\text{cm}^2$), the lowest corrosion rate (0.009 mm/year), and the highest corrosion resistance ($R_{\text{corr}} = 3.42 \times 10^4 \Omega \text{ cm}^2$), resulting in a protection efficiency of 90.91%. This suggests that 120 min is the optimal time under low temperature for anodizing Ti to achieve the best corrosion protection, even outperforming the results reported by Acar et al. (Ref 40). Although the coating at 150 min also performed well ($H\% = 74.04\%$), its effectiveness was slightly lower than that of the 120 min sample. This might be attributed to morphological deformation or disruption of the nanotubes structure caused by re-dissolution at longer anodization time, where accelerated reactions can compromise the integrity of the layer and reduce its protective performance.

The Nyquist plots obtained from the untreated titanium and the anodized samples at various times clearly reveal differences

in their surface electrochemical behavior in SBF solution (Fig. 5). All samples exhibit characteristic semicircular shapes, with the sample anodized for 60 min showing the smallest semicircle diameter. This indicates that the oxide layer formed at this time under low temperature provides poorer corrosion protection than even the native oxide layer on untreated titanium.

The untreated Ti sample presents a slightly larger semicircle, corresponding to a modest charge transfer resistance provided by the spontaneously formed native oxide. As the anodization time increases to 90 min, the semicircle expands slightly, indicating that the oxide layer begins to develop and enhance its protective function, although not significantly. The sample anodized for 120 min displays the largest and most well-defined semicircle, corresponding to the highest charge transfer resistance. This suggests the formation of a dense and uniform anodic oxide layer that serves as an effective barrier against ionic penetration, thereby greatly improving corrosion resistance. At 150 min, although the semicircle remains larger than

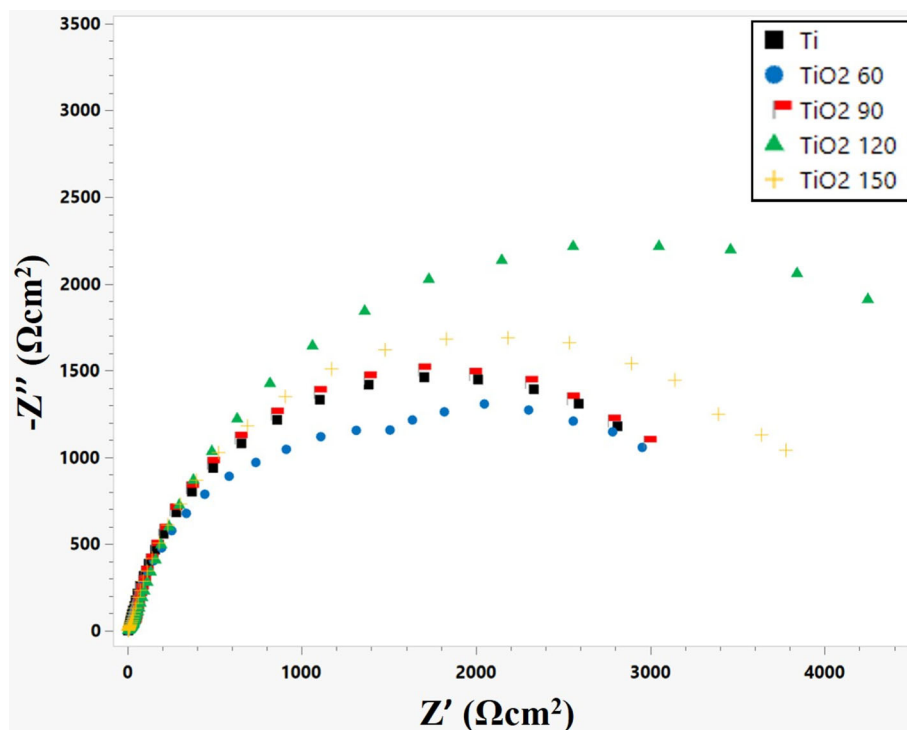


Fig. 5 Nyquist plots of pure Ti and TiO₂ nanotubes coatings anodized at 5 °C for 60, 90, 120, and 150 min, obtained from EIS measurements in simulated body fluid (SBF, 37 °C, pH 7.4)

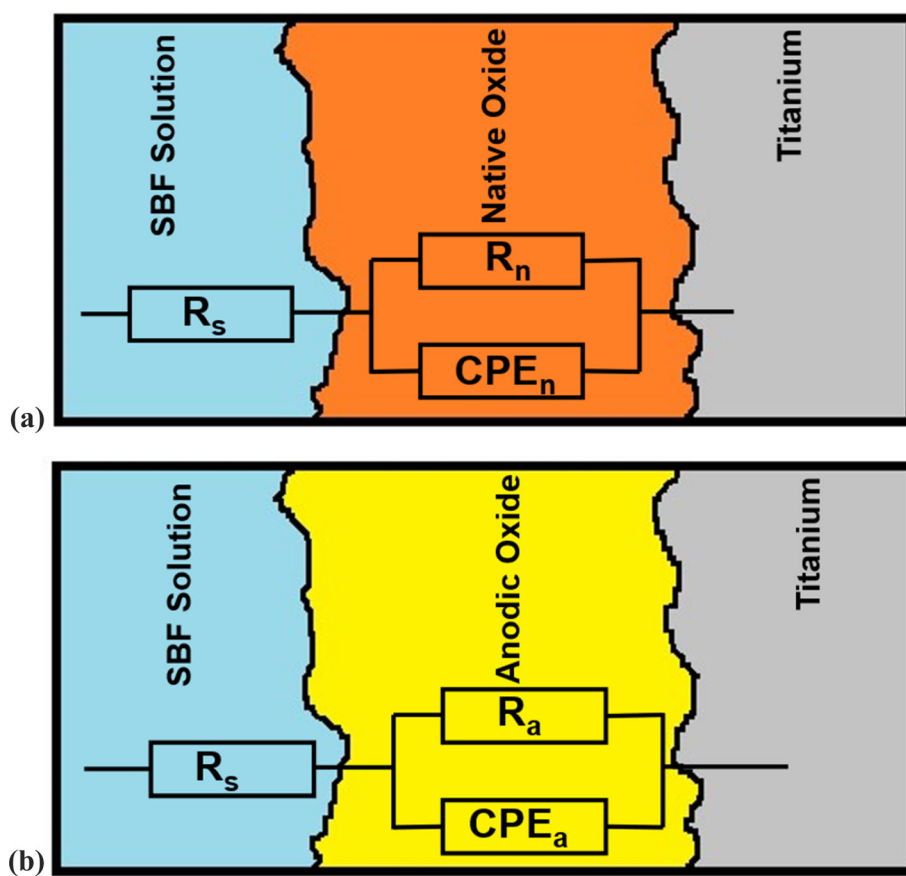


Fig. 6 Equivalent circuit models employed for fitting the EIS spectra of pure Ti and anodized TiO₂ nanotubes coatings fabricated at 5 °C, based on measurements performed in simulated body fluid (SBF, 37 °C, pH 7.4)

that of the untreated sample, it is clearly smaller than that of the 120 min sample. This may be attributed to structural degradation of the oxide layer at longer time, such as increased porosity, microcracks, or defects, which reduce its protective efficiency.

The EIS results presented in Fig. 6, Table 3 and the Nyquist plot clearly demonstrate the influence of anodization time on the electrochemical behavior of the oxide layer formed on titanium. The sample anodized for 60 min exhibits the lowest polarization resistance ($R_p = 3.51 \text{ k}\Omega$), slightly lower than that of the untreated Ti substrate ($3.76 \text{ k}\Omega$), suggesting that the anodic oxide layer is still poorly developed at this stage and contains a high density of structural defects or discontinuities. Such defects can facilitate localized charge transfer at the coating/electrolyte interface, leading to a reduced R_p despite the presence of an anodic oxide layer. It should be noted that this behavior does not contradict the Tafel polarization results. While EIS primarily probes interfacial charge-transfer resistance under small perturbations, potentiodynamic polarization reflects the overall corrosion kinetics over a wider potential range. As a result, even a partially formed TiO_2 layer can reduce the corrosion current density and provide moderate protection, as evidenced by the 58.13% protection efficiency observed for the 60 min sample.

The untreated Ti sample shows a slightly larger semicircle and a higher R_p than the 60 min sample, indicating that even the thin native oxide layer offers better protection than a poorly formed anodic oxide. As the anodization time increases to 90 and 150 min, the R_p values rise to 3.99 and 4.66 $\text{k}\Omega$, respectively, indicating gradual improvements in the protective

properties of the oxide layer. These trends are consistent with the increasing semicircle diameters observed in the Nyquist plots. The sample anodized at 120 min stands out with a significantly higher R_p of 11.8 $\text{k}\Omega$, 2 to 3 times greater than the other samples. Its Nyquist plot shows the largest semicircle, confirming that the anodic oxide formed at this time under low temperature is well-developed, uniform, and exhibits excellent insulating properties. Although the n value decreases to 0.681, suggesting increased non-ideality, this may be attributed to a thicker and more complex oxide structure.

In conclusion, 120 min appears to be the optimal anodization time under low temperature within the studied range, resulting in an anodic oxide layer with superior corrosion resistance. In contrast, too short a time, such as 60 min, significantly reduces R_p , even below that of the untreated titanium, and thus provides inferior protection.

3.6 Water Contact Angle of TiO_2 Nanotubes

The water contact angle measurements presented in Fig. 7 clearly demonstrate the distinct difference in surface wettability before and after surface modification. The untreated Ti sample (Fig. 7a) shows an average contact angle of approximately 64° , indicating a moderately hydrophilic surface. In contrast, the anodized sample with a TiO_2 nanotubes coating (Fig. 7b) exhibits a significant reduction in contact angle, down to around 9.6° , revealing a superhydrophilic surface. This transformation is attributed to the formation of a dense and uniform nanotubular structure, which increases surface energy and enhances water spreading. All contact angle values were recorded within 5 seconds after droplet deposition to minimize time-dependent spreading effects. For the TiO_2 nanotubes surfaces, the contact angle decreased rapidly and reached a stable value within this time interval, indicating fast wetting behavior.

In this study, the sample anodized for 120 min is presented as a representative for all anodized conditions, as the contact angle results were found to be nearly identical across all TiO_2 -coated samples. The significant enhancement in surface wettability after anodization is attributed to the well-organized, continuous nanotubular structure of the TiO_2 coating, which provides a high surface area. This morphology facilitates capillary effects within the nanotubes, allowing better water retention and spreading (Ref 41). Such superhydrophilicity not only improves the material's biocompatibility but also plays a crucial role in promoting cell adhesion and spreading upon

Table 3 Fitted EIS parameters derived from equivalent circuit modeling of pure Ti and TiO_2 nanotubes coatings anodized at 5°C for 60, 90, 120, and 150 min, based on EIS measurements performed in simulated body fluid (SBF, 37°C , pH 7.4)

Samples	R_s (Ω)	R_p ($\text{k}\Omega$)	CPE ($\mu\text{Mho.s}^n$)	χ^2	n
Ti	4.26	3.76	224	0.025	0.861
TiO_2 60	4.90	3.51	141	0.129	0.816
TiO_2 90	4.66	3.99	211	0.072	0.837
TiO_2 120	5.68	11.8	246	0.125	0.681
TiO_2 150	5.15	4.66	128	0.022	0.820

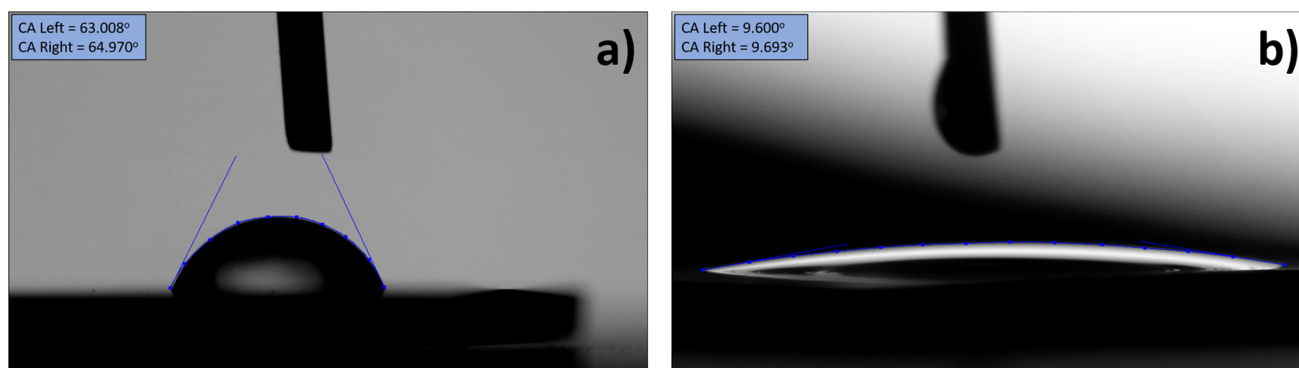


Fig. 7 Water contact angle measurements of pure Ti and anodized TiO_2 nanotubes coatings formed at 5°C for different anodization times, measured using a sessile drop method at room temperature

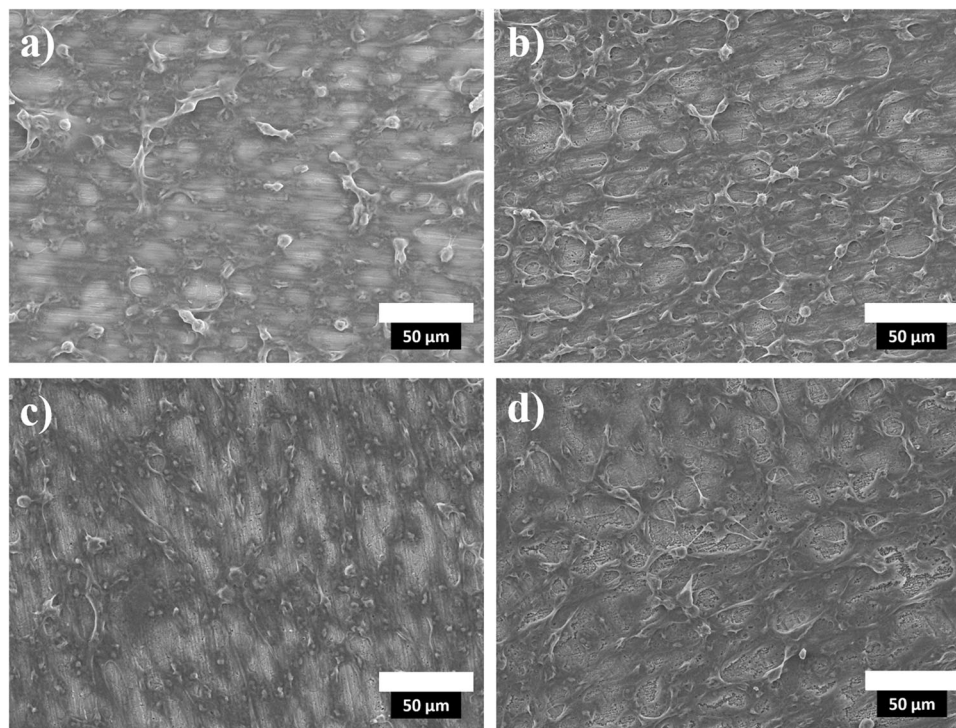


Fig. 8 SEM images of BHK-21 cell adhesion on (a) pure Ti substrate and TiO_2 nanotubes coatings anodized at 5 °C for (b) 60, (c) 90, and (d) 120 min after 48 h of in vitro culture at 37 °C

contact, an essential factor for the success of biomedical implant applications.

3.7 In Vitro Biocompatibility Evaluation of TiO_2 Nanotubes

In this study, baby hamster kidney (BHK-21) cells were used to evaluate the biocompatibility of the samples through direct cell culture on the material surfaces under in vitro conditions. The investigated samples included commercially pure Ti and TiO_2 coatings formed by anodization at times of 60, 90 and 120 min under low temperature (5 °C). The sample anodized for 150 min was excluded from the biological testing due to excessively rapid electrochemical reactions during anodization, which led to re-dissolution phenomena that destroyed the coating structure. This resulted in an unstable oxide layer that was prone to delamination from the substrate. Such insufficient mechanical stability could negatively affect the cell culture process, distort the results, and pose risks to the cell culture environment. Therefore, only samples with stable and well-adhered coatings were selected to assess their ability to support cell attachment and proliferation, ensuring the reliability and relevance of the findings for potential biomedical applications.

Fig. 8 shows SEM images of cell culture results after 48 hours on pure Ti substrates and TiO_2 coating layer samples anodized for 60, 90 and 120 min. In Fig. 8a, cells exhibit low density and poor distribution, with a rounded and retracted morphology, indicating weak adhesion and limited biocompatibility. This suggests that the untreated pure Ti surface does not provide a favorable environment for effective cell attachment and growth. In contrast, Fig. 8b and Fig. 8c, corresponding to TiO_2 layers anodized for 60 and 90 min, show a noticeable increase in cell density and morphological changes, including the appearance of filopodia, which implies better surface

interaction. However, the uneven distribution and cell aggregation suggest that these surfaces still lack optimal conditions for uniform adhesion and proliferation. The sample anodized for 120 min (Fig. 8d) presents the most favorable outcome, with high cell density, even distribution, flattened cell morphology, and an interconnected filopodial network. This reflects strong adhesion and robust cellular development, demonstrating the high biocompatibility of the TiO_2 coating formed for 120 min at 5 °C.

Compared to the 48-hour time point, extending the cell culture period to 72 hours (Fig. 9) revealed a clear increase in both cell density and overall cellular development across all samples, though differences in morphology, structure, and surface characteristics continued to produce varying levels of biocompatibility. In Fig. 9a, the pure Ti substrate shows a slight increase in cell density compared to 48 hours, but cells still appear mostly rounded and retracted, with minimal filopodia formation-indicating limited adhesion and poor cellular growth. In contrast, Fig. 9b, representing the TiO_2 coating anodized for 60 min, exhibits better cellular performance than the pure Ti, with more visible filopodia, suggesting improved cell-surface interaction. However, due to the uneven surface texture, overall cell density remains relatively low, and cell distribution appears irregular, reflecting incomplete adhesion. Fig. 9c, corresponding to the TiO_2 coating at 90 min shows a similar trend to that at 60 min, but cells are more widely spread across the surface, suggesting a slight improvement in biocompatibility. The most striking result appears in Fig. 9d, which represents the TiO_2 sample anodized for 120 min. Here, cells are densely packed, widely spread, and cover nearly the entire surface, forming an intricate network of interlaced filopodia. This indicates strong adhesion and robust interaction between the cells and the material surface. Altogether, these observations clearly demon-

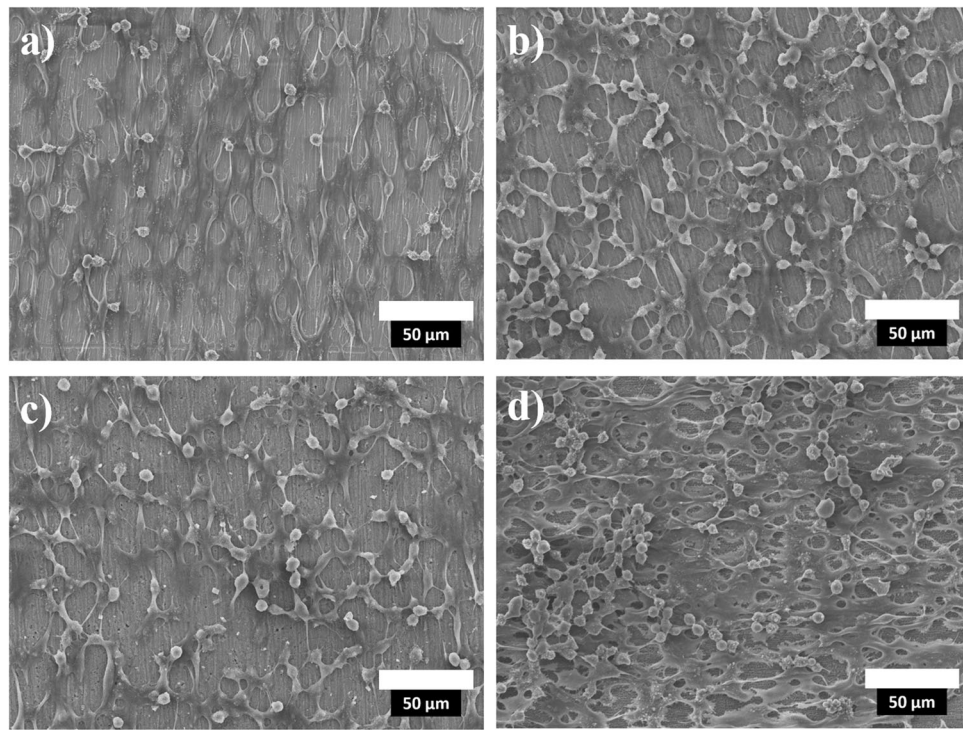


Fig. 9 SEM images of BHK-21 cell adhesion on (a) pure Ti substrate and TiO₂ nanotubes coatings anodized at 5 °C for (b) 60, (c) 90, and (d) 120 min after 72 h of in vitro culture at 37 °C

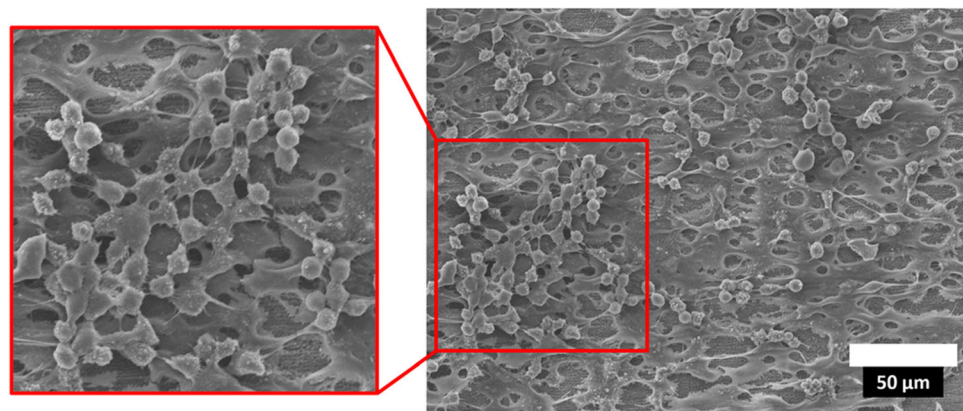


Fig. 10 SEM images highlighting BHK-21 cell adhesion and morphology on TiO₂ nanotube coatings anodized at 5 °C for 120 min after 72 h of in vitro culture at 37 °C

strate that the TiO₂ coating formed at 120 min offers the most favorable surface for cell attachment and growth, confirming its structural, chemical, and biological superiority in supporting long-term cell development.

A deeper analysis of the TiO₂ sample anodized for 120 min, particularly focusing on the red-highlighted region in Fig. 10, reveals robust cellular growth, indicating an exceptionally high level of biocompatibility. The image clearly shows the presence of distinct cell clusters growing on the material's surface. Rather than being scattered individually, the cells tend to aggregate into groups, interconnected through a network of fine bridges or thread-like structures between spherical bodies. These connections are strong indicators of active cell-cell and cell-material interactions (Ref 42, 43). The magnified region further emphasizes this feature, showing that the adherent cells

appear to be surrounded by a mesh-like extracellular matrix (ECM) (Ref 44). This ECM functions as a biological scaffold that stabilizes and supports cellular adhesion, while also confirming that the cells recognize the material surface as a suitable environment for attachment and development (Ref 45, 46). Moreover, the observed cell density across the entire area is relatively high, reinforcing the excellent biocompatibility of the TiO₂ at 120 min at 5 °C and its capability to support cell proliferation and sustained growth throughout the culture period.

The CLSM images after 72 hours of cell culture (Fig. 11) provide a more detailed and intuitive visualization than the SEM results regarding the adhesion, distribution, and spreading of cells on the surfaces of Ti and TiO₂ samples anodized at different temperatures. The top row shows cell nuclei stained

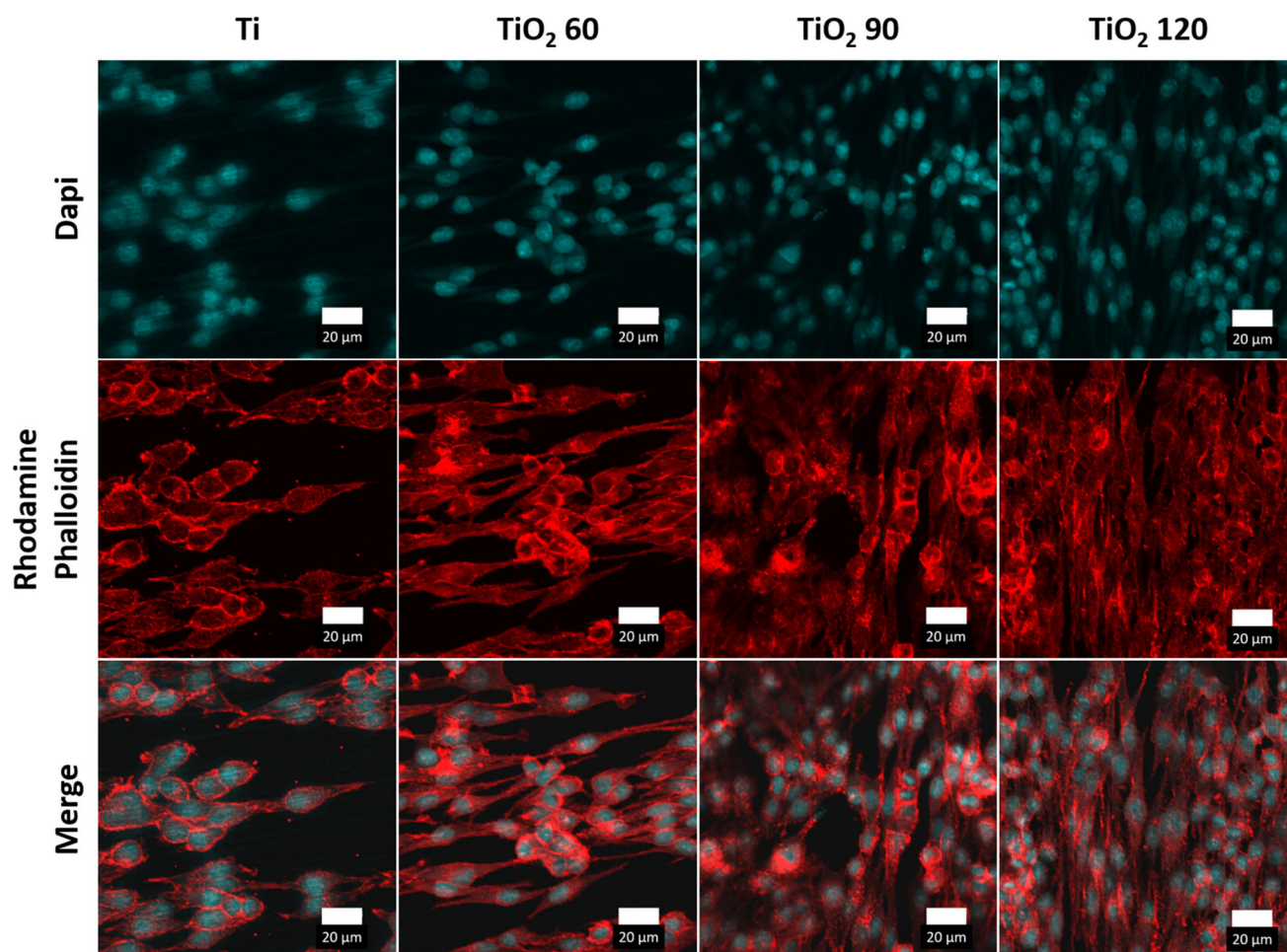


Fig. 11 CLSM images of BHK-21 cells cultured for 72 h on pure Ti substrate and TiO₂ nanotubes coatings anodized at 5 °C for 60, 90, and 120 min under identical in vitro condition (DMEM, 37 °C, 5% CO₂), stained with Phalloidin (actin) and DAPI (nuclei)

with DAPI (blue), the second row displays actin cytoskeleton structures labeled with phalloidin (red), and the third row presents merged images combining both fluorescence channels.

In the pure Ti sample, cells appear sparsely distributed, with a rounded shape and scattered arrangement, showing only a few thin actin filaments, indicating poor adhesion and minimal interaction with the material surface. This clearly reflects the limited biological performance of untreated titanium surfaces, which typically have low surface energy and lack the porous structure needed to provide mechanical support for effective cell attachment.

When moving to the TiO₂ sample anodized for 60 min, the number of adhered cells increases significantly, and the cell morphology begins to elongate with the formation of more actin structures. However, the distribution remains uneven, reflecting an improvement in biocompatibility that is still suboptimal. This may be attributed to the underdeveloped TiO₂ coating, as the low temperature slows the nanotubes formation process, resulting in a discontinuous surface structure. At an anodization time of 90 min, cells exhibit greater spreading with clearly extended actin filaments, increased density, and a more stable adhesion network, indicating more effective interaction between the cells and the coating. This improvement is related to the more uniform TiO₂ nanotubes structure and the larger effective surface area compared to the 60 min sample.

The sample anodized for 120 min demonstrates outstanding results: cells are densely distributed, covering nearly the entire surface, with a flattened morphology and well-developed actin networks organized in a clear structural pattern. This performance is attributed to the highly uniform and continuous arrangement of the TiO₂ nanotubes, which form a capillary-like system capable of retaining water and biological proteins. Such a structure enhances surface recognition, thereby supporting cell adhesion and spreading. The high-density, evenly distributed, and appropriately sized porous nanotubes structure provides a favorable biological environment, functioning as a “nano-scaffold” that increases surface hydrophilicity, retains extracellular proteins, and anchors cellular filopodia (Ref 47).

Quantitative analysis of CLSM images after 72 hours of cell culture, as shown in Fig. 12, reveals statistically significant differences in cellular attachment among the tested samples. Statistical analysis was performed using one-way ANOVA based on cell counts obtained from three independent CLSM images for each sample ($n = 3$), confirming a significant effect of surface modification on cell adhesion ($p < 0.001$). As shown in Fig. 12, the number of adherent cells increases progressively across the samples, with the lowest count observed on the pure titanium surface, followed by the TiO₂ anodized for 60 min.

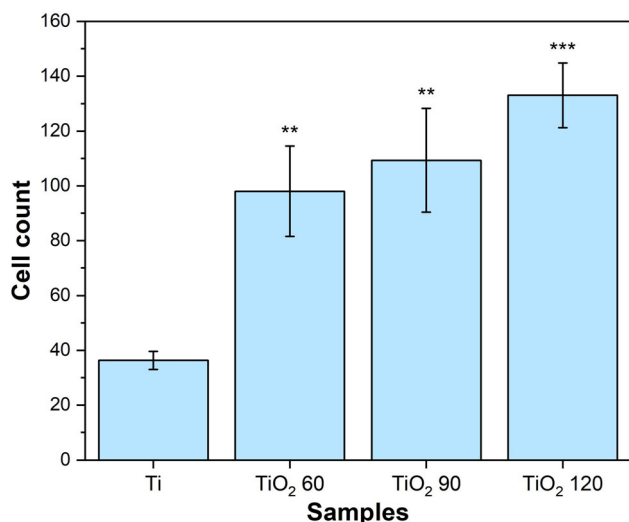


Fig. 12 Quantitative cell counts of BHK-21 cells after 72 h of culture on pure Ti and TiO₂ nanotubes coatings anodized for different durations. Data are presented as mean ± SD ($n = 3$). Statistical analysis was performed using one-way ANOVA. Asterisks indicate statistically significant differences compared with the Ti control (** $p < 0.01$, *** $p < 0.001$)

Compared with the Ti control, the TiO₂ sample anodized for 60 min shows a statistically significant increase in cell attachment ($p < 0.01$), corresponding to an increase of approximately 170%. A further enhancement in cell adhesion is observed for the TiO₂ sample anodized for 90 min, which exhibits a significantly higher cell count than pure Ti ($p < 0.01$), with an increase of about 201%. Notably, the TiO₂ coating anodized for 120 min demonstrates the highest level of cell attachment, showing a highly significant difference compared to the Ti substrate ($p < 0.001$) and an increase of approximately 266%. Although the TiO₂ samples anodized for 90 and 120 min display similarly high cell counts, the 120 min sample consistently exhibits the greatest mean value, indicating superior support for cell adhesion and proliferation among the investigated conditions.

4. Conclusions

This study systematically investigated the influence of anodization time at low temperature (5 °C) on the formation and functional performance of TiO₂ nanotube coatings on titanium, with emphasis on their structural characteristics, electrochemical behavior, mechanical properties, surface wettability, and in vitro biocompatibility. Among the investigated conditions, anodization for 120 min produced a well-ordered and uniform TiO₂ nanotube architecture, as confirmed by FE-SEM observations, together with a favorable mixed anatase-rutile phase composition identified by XRD analysis.

This coating exhibited the most balanced functional performance, characterized by the highest surface hardness (145.67 HV) and superior corrosion protection in simulated body fluid, with a corrosion current density of $0.76 \mu\text{A cm}^{-2}$, a polarization resistance of $3.42 \times 10^4 \Omega \text{ cm}^2$, and a protection efficiency of 90.91%. In contrast, a shorter anodization time (60 min) resulted in underdeveloped nanotube layers with

higher defect density and reduced corrosion protection efficiency (58.13%), while excessive anodization (150 min) led to partial structural collapse and diminished electrochemical stability due to accelerated oxide dissolution. In vitro biocompatibility evaluation using BHK-21 cells further demonstrated a pronounced enhancement in biological performance for the 120 min coating, showing an approximately 266% increase in cell adhesion compared to the pure Ti substrate.

Overall, these findings demonstrate that anodization at 120 min and 5 °C provides an optimal and quantitatively validated balance between structural integrity, electrochemical protection, mechanical enhancement, and biological response, highlighting its strong potential for application in biomedical titanium implant surfaces.

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Data availability

All data that support the findings of this study are included within the article (and any supplementary files).

Conflict of interest

The authors declare that they have no conflict of interest.

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