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Enhanced biocompatibility of TiO_2 surfaces by highly reactive plasma

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Abstract

In the present study the biological response to various nanotopographic features after gaseous plasma treatment were studied. The usefulness of nanostructured surfaces for implantable materials has already been acknowledged, while less is known on the combined effect of nanostructured plasma modified surfaces. In the present work the influence of oxygen plasma treatment on nanostructured titanium oxide (TiO_2) surfaces was studied. Characterization of the TiO_2 surface chemical composition and morphological features was analyzed after plasma modification by x-ray photoelectron spectroscopy and by scanning electron microscopy while surface wettability was studied with measuring the water contact angle. Cell adhesion and morphology was assessed from images taken with scanning electron microscopy, whereas cell viability was measured with a calorimetric assay. The obtained results showed that oxygen plasma treatment of TiO_2 nanotube surfaces significantly influences the adhesion and morphology of osteoblast-like cells in comparison to untreated nanostructured surfaces. Marked changes in surface composition of plasma treated surfaces were observed, as plasma treatment removed hydrocarbon contamination and removed fluorine impurities, which were present due to the electrochemical anodization process. However no differences in wettability of untreated and plasma treated surfaces were noticed. Treatment with oxygen plasma stimulated osteoblast-like cell adhesion and spreading on the nanostructured surface, suggesting the possible use of oxygen plasma surface treatment to enhance osteoblast-like cell response.

Keywords: nanotubes, cells, plasma treatment, implants, surface modification

(Some figures may appear in colour only in the online journal)

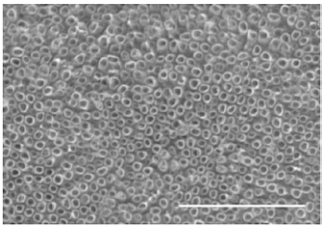
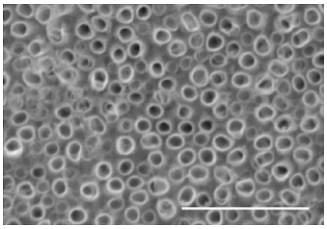
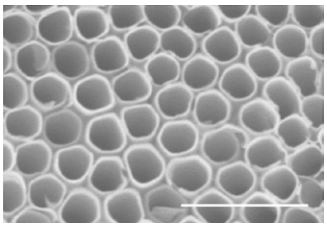
1. Introduction

Titanium (Ti) and its alloys are highly attractive materials for medical applications due to its remarkable tensile strength, flexibility, resistance to corrosion and resistance to body fluids [1]. Thus titanium (Ti) and its alloys are commonly employed in the medical field for orthopaedic implants, dental implants or even cardiovascular implants such as stents [2–4]. The high degree of biocompatibility of Ti is mainly ascribed to the passive oxide film (TiO_2) which is formed on its surface after exposure to air. As most of the Ti implants available on the market exhibit some form of TiO_2 layer on the surface the implant-cell interactions are immediately the interactions between the TiO_2 layer and the host tissue. Therefore

the characteristics of the TiO_2 layer, which has a semi-conducting nature, influence on the biological response [5, 6]. Consequently some attempts have been directed in modification of the oxide layer; mainly by ion bombardment [7, 8] or by treatment with ultraviolet light [9]. Moreover topography and wettability are also important surface characteristics which influence on the biological response. Recently it has been established that nanotopographical features dictate cell-surface interactions, as the cells can sense and interact with random or geometrically ordered surface features from the micro to the nanometer range [10–12]. The main principle behind the nanostructured implantable materials is that such surfaces mimic the natural morphological features of the extracellular matrix with which cells normally interact [12–14]. As nanoscale surface topography stimulates and controls several molecular and cellular events at the tissue/implant interface, specific nanotpographic features have high potential in designing novel medical implants [4, 12, 15, 16]. Lately, as the process of nanotube fabrication enabled rather precise control over their size and shape [3], immense efforts have been done to study the influence of various nanotube morphologies on biological response [2, 12, 17, 18]. Furthermore it has been shown that different cell types react differently to the nanotopographic features [19], which could be used to promote adhesion and proliferation of one cell type over another. Several studies have shown that TiO_2 nanotubes significantly influence on osteoblast differentiation and proliferation [20, 21], thus making them highly prospective for use as orthopaedic or in dental implants. For example in the study conducted by Rubert *et al* [20] it was shown that alginate-coated TiO_2 scaffolds can act as a matrix for delivery of proline-rich peptides, which increase osteoblast differentiation.

Another interesting approach to alter top surface layer of biomaterial without influencing on its bulk attributes is gaseous plasma treatment which is usually employed for vast variety of polymeric materials [22, 23]. However there was not much work done on combine effects of surface morphology (nanotubes, nanorods etc) and plasma modification. By carefully tailoring the features of surface nanotopography and plasma treatment conditions desired biological response may be achieved. Thus the main objective of our work was to employ gaseous plasma treatment technology to alter the oxide layer of TiO_2 nanotubes and to study the influence of such modification on *in vitro* biological response with osteoblast-like cells. For the purpose of our work Ti foil was nanostructured by electrochemical anodization into different diameters of nanotubes. In order to achieve the specific nanotube diameter (15, 50 or 100 nm) appropriate anodizing conditions were applied. These specific diameters were fabricated in order to observe changes in cell-surface interactions, which are mainly governed by the interaction of nanotubes with absorbed protein layer which could enter the hollow interior of nanotubes. For example the narrow 15 nm nanotubes only enable penetration of proteins with less than 15 nm in size (like albumin), while nanotubes with 50 and 100 nm enable penetration of vast number of different proteins, which further dictate cell-surface interactions. In order to study the influence of plasma modification on different nanotubular surfaces gaseous oxygen plasma was employed. The changes in surface properties were assessed using scanning electron microscopy (SEM), x-ray photoelectron spectroscopy (XPS) and water contact angle measurements (WCA). While the *in vitro* biological response of osteoblast-like cells was evaluated by the use of colorimetric assay and by SEM. Our final goal was to determine whether plasma treatment of nanotubes would provide appropriate support for optimal growth and proliferation of osteoblast-like cells, which could be used for orthopaedic or dental implants.

Table 1. Anodization conditions for various diameters of nanotubes and their top morphology by SEM (scale bar: 200 nm).

Diameter of nanotubes	Potential used (V)	SEM of top of the nanotubes
15 nm	10 V	
50 nm	20 V	
100 nm	58 V	

2. Methods

2.1. Fabrication of TiO_2 nanotubes

Ti foils of 0.1 mm thickness (99.6% purity) were obtained from Advent Research Materials, England. Hydrofluoric acid (HF) and the solvents, namely ethylene glycol (EG), ethanol, and acetone, were purchased from Sigma-Aldrich, Germany and used without further purification.

TiO_2 nanotubes were fabricated by electrochemical anodization of titanium foils (Ti foil) as published earlier [4, 24]. Before anodization, the foils were cleaned by successive ultrasonication in acetone, ethanol and deionized (DI) water for 5 min each and then dried in a nitrogen stream. All anodization experiments were carried out at room temperature (~20 °C) in a two-electrode system with titanium foil as the anode and platinum electrode as the cathode, with a working distance of 1.5 cm.

Nanotubes were obtained by a two-step anodization method, where the first step was to obtain a pre-patterned

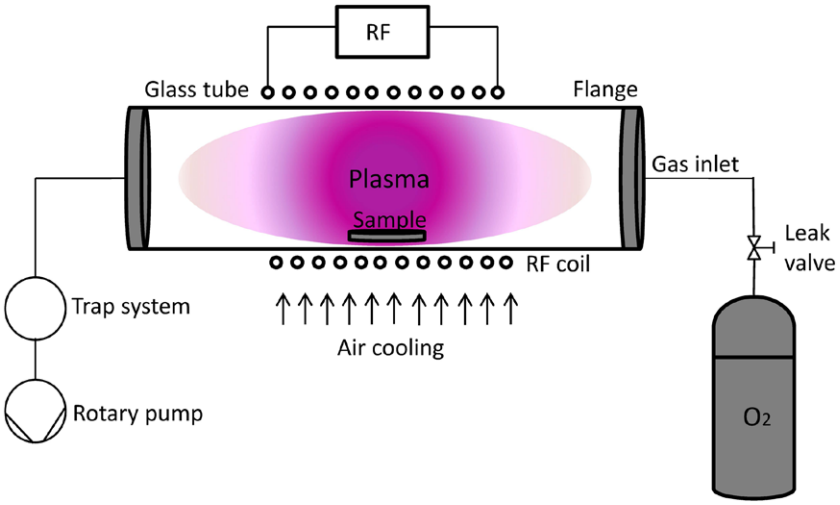


Figure 1. The schematic representation of plasma treatment system used for treatment of pristine Ti foil and nanotubes with 15, 50 and 100 nm in diameter.

surface and the nanotubes were grown in the second step, on the pretreated surface. The first step was carried out by anodization of titanium foil in an ethylene glycol (EG) electrolyte (with 1 M H_2O and 0.1 M NH_4F) at 35 V for 2 h, followed by ultrasonication in water in order to remove the grown nanotubular layer. In this step a pre-patterned surface was obtained which was then cleaned again by successive ultrasonication in acetone and ethanol and used as substrates for the second anodization. The electrolytes used in the second step are based on an EG electrolyte, containing 8 M water and 0.2 M hydrofluoric acid (40% HF solution). All the experiments were carried out for 2.5 h at different potentials in order to obtain desired diameter of nanotubes (see table 1). The as-formed nanostructures were kept in ethanol for 2 h to remove all organic components from the electrolyte, then washed with DI and dried under a gentle stream of nitrogen.

2.2. Gaseous plasma treatment of nanotubes

Treatment of nanostructured materials (nanotubes with 15, 50 and 100 nm in diameter) was performed by oxygen plasma in the plasma reactor. The system was evacuated with a two-stage oil rotary pump with a nominal pumping speed of 4.4×10^{-3} m^3 s^{-1}. The discharge chamber was a Pyrex cylinder with a length of 0.6 m and an inner diameter of 0.036 m. Gaseous plasma was created with an inductively coupled RF (radio-frequency) generator, operating at a frequency of 13.56 MHz and an output power of about 200 W. Commercially available oxygen was leaked into the discharge chamber. The pressure was measured by an absolute vacuum gauge. The pressure was adjusted during continuous pumping by a precise leak valve. The pressure was fixed at 75 Pa which allows a high degree of dissociation of oxygen molecules. At this discharge parameters, plasma with an ion density of about $2 \cdot 10^{15}$ m^{-3}, an electron temperature of 4 eV, and a neutral atoms density of about $4 \cdot 10^{21}$ m^{-3} was obtained. The samples were treated for 60 s in the plasma reactor, as shown schematically in figure 1.

2.3. Water contact angle measurements

The surface wettability was measured 25 h after nanotubes were produced by anodization and immediately after nanotubes were treated by oxygen plasma in order to compare surface wettability on surfaces used for biological experiments (biological experiments were carried on surfaces immediately after plasma treatment). A DI water droplet of 3 μl volume was put on the surface and measured with custom-made apparatus equipped with a CCD camera and a PC computer which enables high resolution pictures of a water drop on the sample surface. For each sample 10 measurements were performed in order to obtain statistically robust data. The relative humidity was kept at 45% and the temperature at 25 °C. The contact angles were determined by our custom-made software which enables fitting of the water drop on the surface in order to allow for a relatively precise determination of the contact angle.

2.4. X-ray photoelectron spectroscopy (XPS)

The x-ray photoelectron spectroscopy (XPS or ESCA) analyses were carried out on the PHI-TFA XPS spectrometer produced by Physical Electronics Inc. Samples were placed on a sample holder and introduced in an ultra-high vacuum spectrometer. The analyzed area was 0.4 mm in diameter and the analyzed depth of about 3–5 nm; this high surface sensitivity is a general characteristic of the XPS method. Sample surfaces were excited by x-ray radiation from monochromatic Al source at photon energy of 1486.6 eV. The high-energy resolution spectra were acquired by the energy analyzer operating at resolution of approximately 0.6 eV and pass energy of 29 eV. During data processing the spectra from the surface were aligned by setting the C 1s peak at 285.0 eV, characteristic for C–C bonds. The accuracy of binding energies was approximately ±0.3 eV. Quantification of surface composition was performed from XPS peak intensities taking into account relative sensitivity factors provided by instrument manufacturer.

Three different XPS measurements were performed on each sample and average composition was calculated.

The XPS spectra were measured for the pristine Ti, plasma treated Ti foil, and the nanotubes (with 15, 50 and 100 nm in diameter) immediately after the anodization procedure and on the nanotubes (with 15, 50 and 100 nm in diameter) immediately after the plasma treatment.

2.5. Human osteoblast-like cells (MG-63)

2.5.1. Cultivation of human osteoblast-like cells (MG-63). The human osteoblast-like cell line (MG-63) were cultured in MG-63 growth medium (DMEM, supplemented with 10% v/v FBS and 4 mM L-glutamine) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cells were routinely sub-cultured 2–3 times per week (when or when cells reached 65–70% confluence) Briefly, cells were washed two times with Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (PBS; Sigma-Aldrich, Steinheim, Germany), harvested with Trypsin-0.25% EDTA (Sigma-Aldrich, Steinheim, Germany) for approximately 1 to 2 min at 37 °C, centrifuged at 200 g for 5 min and distributed in flasks with fresh growth medium.

2.5.2. Exposure of MG-63 to nanotube surfaces. Prior to exposure to cells, the nanorough surfaces were cleaned with ethanol (70%), left to dry, treated by oxygen plasma and immediately packed in sterile containers and used for biological experiments. The reference pristine Ti foil was cleaned with ethanol and sterilized with ultra-violet (UV) light radiation (PHILIPS TUV 36W G13, Philips, Nevada Industrial, USA) at 254 nm and intensity of $1.35 \cdot 10^4$ W cm^{-2} for 30 min prior to incubation. Sterile tweezers (70% EtOH and fire-exposed) were used to transfer plasma treated and untreated nanotube surfaces into four 24-well culture plates in a sterile laminar hood.

Following the same procedure as for the sub-cultivation, the cells were then resuspended in the growth medium and plated at a seeding density of 1.8×10^4 cells per cm^2 of tissue culture 24-well plates (Sigma-Aldrich, TPP®, Steinheim, Germany), containing either the cover glass, pristine Ti foil, or nanorough surfaces (with 15, 50 and 100 nm in diameter), in 1 ml of the growth medium, precisely 2 h after the plasma treatment of the samples. After 48 h growth under controlled conditions (5% CO_2/95% air at 37 °C), the cells were further processed for either fixation for SEM, or the resazurin viability assay (as described in the next subchapter).

2.5.3. The resazurin viability assay. After the end of the incubation period (48 h), samples were removed into clean wells of the 24-well plates. Then, 300 μl of fresh medium supplemented with 20 vol.% resazurin (0.15 mg ml^{-1} dissolved in PBS) was added to each well. After 3 h incubation at 37 °C, 200 μl of the medium in each well was transferred into black 96-well plate and fluorescence intensity was measured using microplate reader (BioTek, Cytation 3, Bad Friedrichshall, Germany; Ex: 560 nm, Em: 590 nm). Background fluorescence values (without cells) were subtracted from the fluorescence values and normalized to the surface area (divided by the estimated surface area of samples (0.2355 cm^2) or cover glass (0.785 cm^2)).

2.7. Preparation of samples for SEM analysis

Concurrently, after 48 h cell growth on the sample surfaces, the cell culture medium was removed, cells were washed 3-times with PBS and fixed for 2 h at room temperature using a modified Karnovsky fixative, composed of 2.5% glutaraldehyde (SPI Supplies, West Chester, PA, USA) and 0.4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in 1 M Na-phosphate buffer (NaH_2PO_4 2·H_2O and Na_2HPO_4 2·H_2O; all the chemicals obtained from Merck KGaA, Darmstadt, Germany). After 3 h, the fixative was removed and 1 M Na-phosphate buffer was added; the samples were left in the fridge over the night. Then the samples were washed in the buffer for 3 × 10 min and the post-fixation of samples was undertaken with 1% osmium tetroxide (OsO_4) (SPI Supplies, West Chester, PA, USA; 1 × 60 min), followed by washing in $d$$H_2O$ 3 × 10 min, incubation in thiocarbohydrazide (TCH; Sigma-Aldrich, Steinheim, Germany) for 20 min, washing in $d$$H_2O$ 3 × 10 min, incubation in OsO_4 1 × 20 min and washing in $d$$H_2O$ 3 × 10 min. Samples were dehydrated with 30% ethanol (EtOH; Merck KGaA, Darmstadt, Germany) (10 min), 50% EtOH (10 min), 70% EtOH (10 min; left overnight at 4 °C), 80% EtOH (10 min), 90% EtOH (10 min) and absolute EtOH (10 min). Further dehydration steps were conducted with a mixture of Hexamethyldisiloxane (HMDS; SPI Supplies, West Chester, PA, USA) and absolute EtOH (1:1; v/v; 10 min), 3:1 (HMDS: absolute EtOH, v/v; 1 min) and absolute HMDS (10 min), which was left to evaporate for 24 h. Prior to SEM analysis the samples were sputtered with gold (5 nm coating) and analysis was done with SEM (Jeol JSM-7600F, USA) at accelerating voltage of 8 keV.

3. Results

The first information about the surface properties of nanotubes and Ti foil was obtained with measuring the water contact angle. It was shown that the freshly fabricated nanotubes possess a hydrophilic character as the water drop entirely spread on the surface; this was observed for all the nanotube diameters samples. The value for the water contact angle was assigned to be less than 5°, as presented in table 2. However, our previous results showed that the wettability of fabricated nanotubes changes over time and that the surface becomes more hydrophobic [24]. Therefore, all the *in vitro* biological experiments were carried out 1 week after nanotube fabrication when the surface was still fully wettable. Such fully wettable surfaces were also observed upon plasma treatment of nanotubes, while the contact angle of about 15° was measured for the plasma treated Ti foil and a contact angle of approximately 101° was measured for the untreated pristine Ti foil.

To study the change in chemical composition between the freshly fabricated nanotubes and plasma treated nanotubes XPS analysis was employed. The results of chemical

Table 2. Surface composition and wettability of pristine Ti foil, freshly prepared nanotubes with 15, 50 and 100 nm in diameter before and after plasma treatment (+P) obtained with XPS and wettability measurements.

Material	Atomic %					Ratio		Water contact angle (°)
	C	O	Ti	N	F	C/O	Ti/O	
Ti foil	38.3	41.2	18	2.5	0	0.93	0.44	101
15 nm	39.2	41.3	15.1	1.3	3.1	0.95	0.36	5 <
50 nm	36.2	42.7	16.1	0.8	4.2	0.85	0.38	5 <
100 nm	37.6	39.9	16.1	1	5.4	0.94	0.4	5 <
Ti foil+P	29.6	53.4	17.0	0	0	0.55	0.32	15
15 nm+P	19.6	57.2	23.2	0	0	0.34	0.40	5 <
50 nm+P	20.1	55.6	23.5	0	0.8	0.36	0.42	5 <
100 nm+P	20.2	55.6	22.7	0	1.5	0.36	0.41	5 <

composition of nanotubes are presented in table 2. For reference, XPS analysis was performed also on the pristine titanium foil, which was used for growth of TiO_2 nanotubes by electrochemical anodization, as well as on plasma treated Ti foil. Low concentration of nitrogen was detected on the surface of pristine Ti foil as well as on the freshly fabricated nanotubes. The pristine Ti foil is composed of about 38% of carbon and 41.2% of oxygen, similar values were measured also for the freshly electrochemically prepared nanotubes. However, nn the surface of the freshly prepared nanotubes, also a low concentration of fluorine was detected, which was shown to increase with the nanotube diameter. Thus 3.1 at% of fluorine was detected on the nanotubes with 15 nm in diameter, while almost two times higher concentration was observed on the nanotubes with 100 nm in diameter (about 5.4%). After plasma treatment a decrease in carbon content and increase in oxygen content was observed, as about 20% of carbon and about 55% of oxygen was detected on the surface. For comparison the survey spectra of untreated and plasma treated nanotubes with 50 nm in diameter is presented in figure 2. Moreover, the fluorine content was reduced on all the plasma treated nanotubes. In the case of nanotubes with 15 nm in diameter, no fluorine was detected on the surface, while about 0.8 and 1.5% of fluorine was detected on the nanotubes with 50 and 100 nm in diameter, respectively. On the plasma treated Ti foil also lower carbon content and higher oxygen content was detected.

Comparisons of the high resolution spectra for Ti 2p, C 1s and O 1s peaks on nanotubes with 50 nm diameter before and after plasma treatment are presented in figure 3. For the case of Ti 2p a doublet peak was observed, containing both Ti $2p_{1/2}$ and Ti $2p_{3/2}$ components which appear at 464.5 eV and 458.8 eV (the difference is 5.7 eV). No significant difference in Ti 2p peaks between untreated and plasma treated sample were observed, presuming that TiO_2 surface layer is similar for both the surfaces. Furthermore, no alterations in oxidation states of titanium between these two surfaces were detected by XPS. However, the differences in C 1s peak for untreated and plasma treated surface were observed. The C 1s for untreated surface exhibits a peak at 284.8 eV corresponding to C–C and C–H bonds and a shoulder peak at 288.9 eV corresponding to C=O or C–F bonds. While in the case of plasma treated surface the C 1s peak at 284.8 eV is broader and two relatively small shoulder peaks appear at 287 eV and 289 eV,

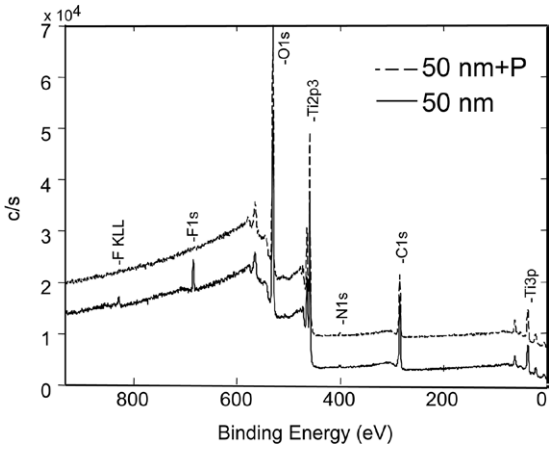


Figure 2. Comparison of XPS survey spectra on TiO_2 nanotubes with 50 nm in diameter before (50 nm) and after plasma treatment (50 nm+P).

corresponding to C–O and C=O or C–F bonds, respectively. This could be ascribed to decreased concentration of F on the surface of oxygen plasma treated nanotubes and oxidation of carbon impurities by oxygen plasma. The O 1s peak appears near 530 eV which is attributed to TiO_2 component.

The cytocompatibility of the TiO_2 nanotubes was investigated by observing growth of osteosarcoma (MG-63) cells on the nanotube surfaces. Human MG-63 osteoblast-like cells were selected as their integrin subunits profile is similar to the one of primary human osteoblasts,therefore this cell type is considered applicable for studying initial cell attachment to surfaces [25]. Viability of MG-63 cells grown on the TiO_2 nanotubes 48 h after cell seeding was tested by the resazurin viability assay (figure 4). The results of the resazurin assay showed no statistically significant differences between cell viability for the different TiO_2 nanotube surfaces, either oxygen plasma treated or untreated, compared to thecontrol surfaces (pristine Ti foil and cover glass).

In order to further assess biological response of osteoblast-like MG-63 cells, possible morphological appearances of the adhered cells (48 h after seeding) were imaged using SEM. Based on the obtained SEM images, substantial differences between the samples were observed. Firstly, as already observed from the low magnification SEM images (figure 5) the largest number of MG-63 cells was present on the pristine

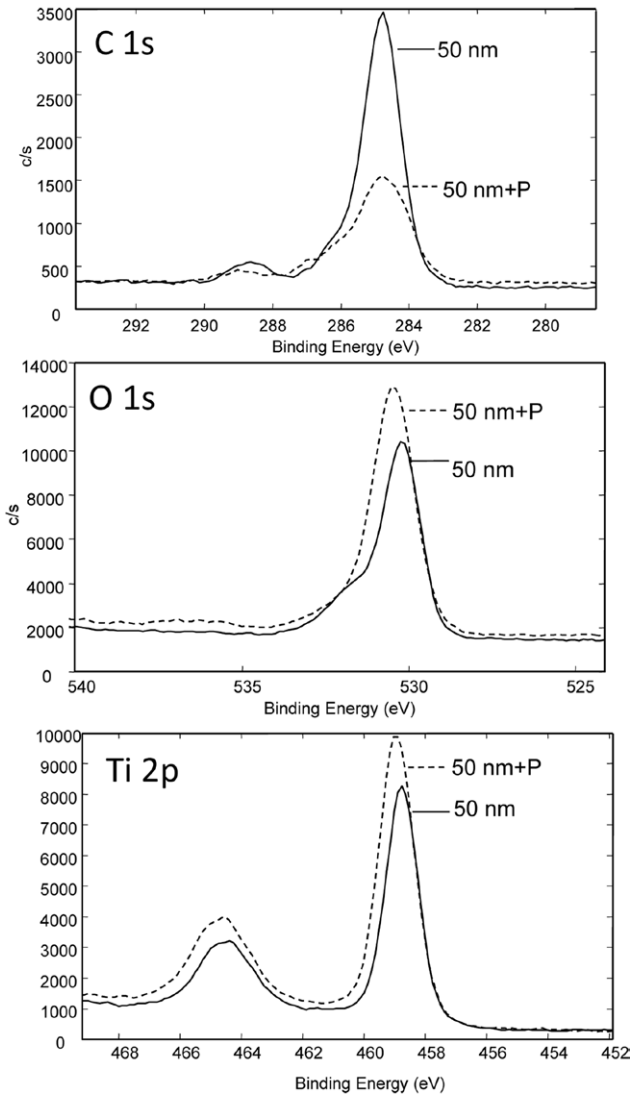


Figure 3. Comparison of XPS high resolution spectra for Ti 2p, C 1s, O 1s on TiO_2 nanotubes with 50 nm in diameter before (50 nm) and after plasma treatment (50 nm+P).

Ti foil and on the plasma treated Ti foil and nanotubes, while the lowest number on the freshly prepared nanotubes. For the case of plasma treated nanotubes no significant alterations in cell adhesion and morphology were observed between different nanotube diameters. However for the case of freshly prepared nanotubes the least spread morphology of osteoblast cells was observed on nanotubes with 100 nm in diameter. Furthermore, no significant differences in cell growth were observed between the untreated and plasma treated Ti foil, as in both the cases similar flattened and spread morphology of cells was observed on the surface.

Higher magnification SEM images provide more information about cell morphology and their interaction with the surface, as seen in figure 6. On the pristine Ti foil, the cells appeared densely grown and spread on the surface, indicating good adhesion and suitable environment for cell adherence and overall cell well-being (which is, as previously mentioned, similar as observed for the plasma treated Ti foils). On the other hand, the cells grown on the freshly prepared

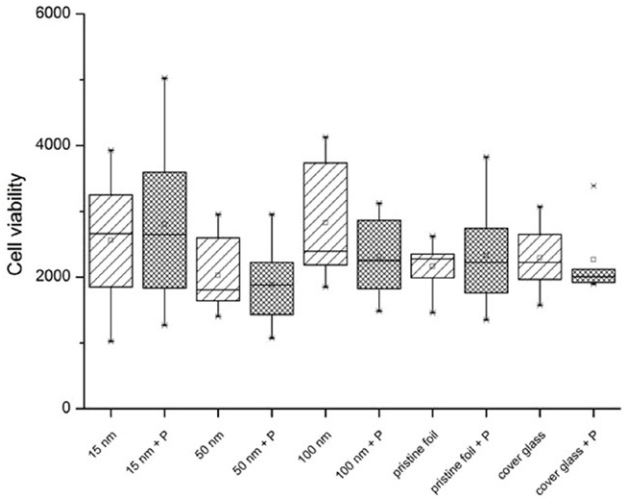


Figure 4. Viability of MG-63 cells after 48 h growth on the samples, estimated by the resazurin viability assay, normalized to the surface area of platelets or cover glass. Abbreviations: pristine foil = pristine Ti foil, P = plasma treated surface, 15 nm = 15 nm nanotubes, 50 nm = 50 nm nanotubes, 100 nm = 100 nm nanotubes. Number of analysed samples were 8 per tested group, except for the 50 nm ($n = 7$) and cover glass +P ($n = 5$).

nanotubes showed abnormal morphology; cells were either both shrunken and rounded, or spread and slightly rounded up, while some of them were even similar to the spread cells, observed on the pristine Ti foil. Regardless the nanotube diameter, the cells appeared to only poorly interact with the nanostructured surfaces and were predominantly in the rounded state, indicating non favorable environment for their adhesion and proliferation. However, this was not the case for plasma treated nanotubes. Namely, the cells were well spread and interacted with the surface and with the surrounding cells, especially observed for the cells grown on the nanotubes with 15 nm in diameter. Similarly to the Ti foil, a wide distribution of different cell morphologies was observed. When comparing cell morphology on the plasma treated nanotube surfaces with the freshly prepared nanotubes, it can be easily noticed that the cells grown on all thee surfaces are spread to a much higher extent and in an active state (viable). Moreover, as mentioned before no distinct differences in their morphology were observed between the cells grown on different nanotube diameters. However on freshly prepared nanotubes slightly more rounded cell morphology was observed for nanotubes with 100 nm in diameter.

4. Discussion

In the present study we discuss surface modification with oxygen plasma on the TiO_2 nanotubes with different nanotube diameters. Surface modification of the external Ti surfaces by plasma discharges appears a promising tool for enhanced cell growth, and for further design of implantable materials with improved and/orcontrolled osteointegration. In our previous study we have just recently showed a significant impact of plasma treated nanostructures on enhanced proliferation and adhesion of human coronary endothelial cells [26]. Surface

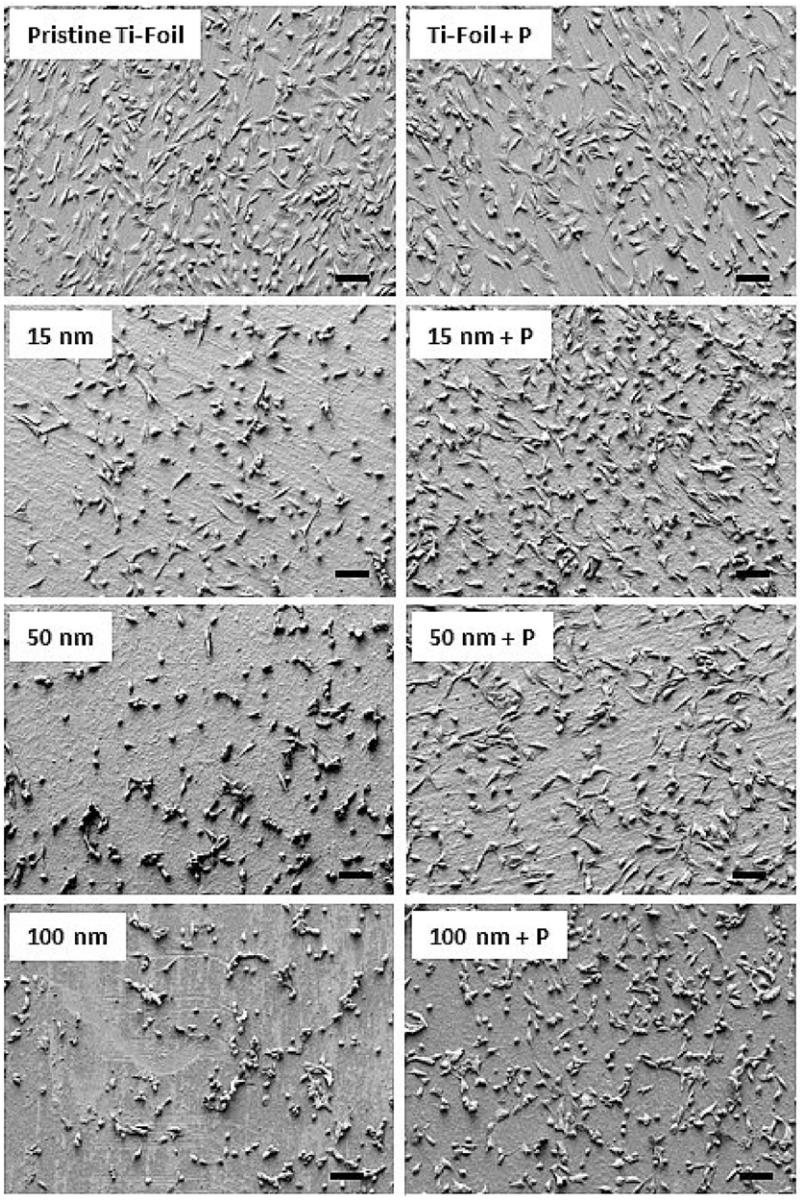


Figure 5. Low magnification SEM images of cells interacting with pristine Ti foil and nanotubes with 15, 50 and 100 nm in diameter before and after (+P) oxygen plasma treatment. (scale bar: 100 μm).

finishing by plasma treatment appears to be a promising way to render surfaces more hydrophilic and to remove surface impurities; as the latter can significantly influence biological response [27–29].

Herein we have shown that wettability of freshly prepared TiO_2 nanotubes and plasma treated nanotubes was comparable, thus all the surfaces exhibited similar hydrophilic character. However, the untreated Ti foil (used as control surface), exhibited higher water contact angle, which decreased after the plasma treatment. Although all the nanotubes showed hydrophilic character, some differences in chemical composition between the freshly prepared nanotubes and the plasma treated nanotubes were observed by XPS. Similarly to the pristine Ti foil, also the freshly prepared nanotubes exhibited higher carbon and lower oxygen content in comparison to the plasma treated ones. We explain this as mainly being a consequence of hydrocarbon impurities present on the surface already before the plasma treatment. Interestingly no significant differences in chemical composition between the pristine Ti foil and freshly prepared TiO_2 nanotubes were observed with XPS. This is mainly governed by the fact that Ti exhibits naturally grown oxide layer, which was confirmed also by XPS [30]. However, a small amount of fluorine (F) was detected on all the TiO_2 nanotube surfaces, and its surface concentration increased with the increasing nanotube diameter. The presence of F on the surface is a consequence of the fabrication procedure; namely, the use of electrolyte during the fabrication. To some extent, the F content on the freshly prepared nanotubes could also be one of the reasons for lower interaction of osteoblast-like cells with these surfaces. It should be emphasized that oxygen plasma treatment resulted in significantly decreased fluorine content. In the case of nanotubes with 15 nm in diameter even no F was detected on the surface which is attributed to the removal of F from

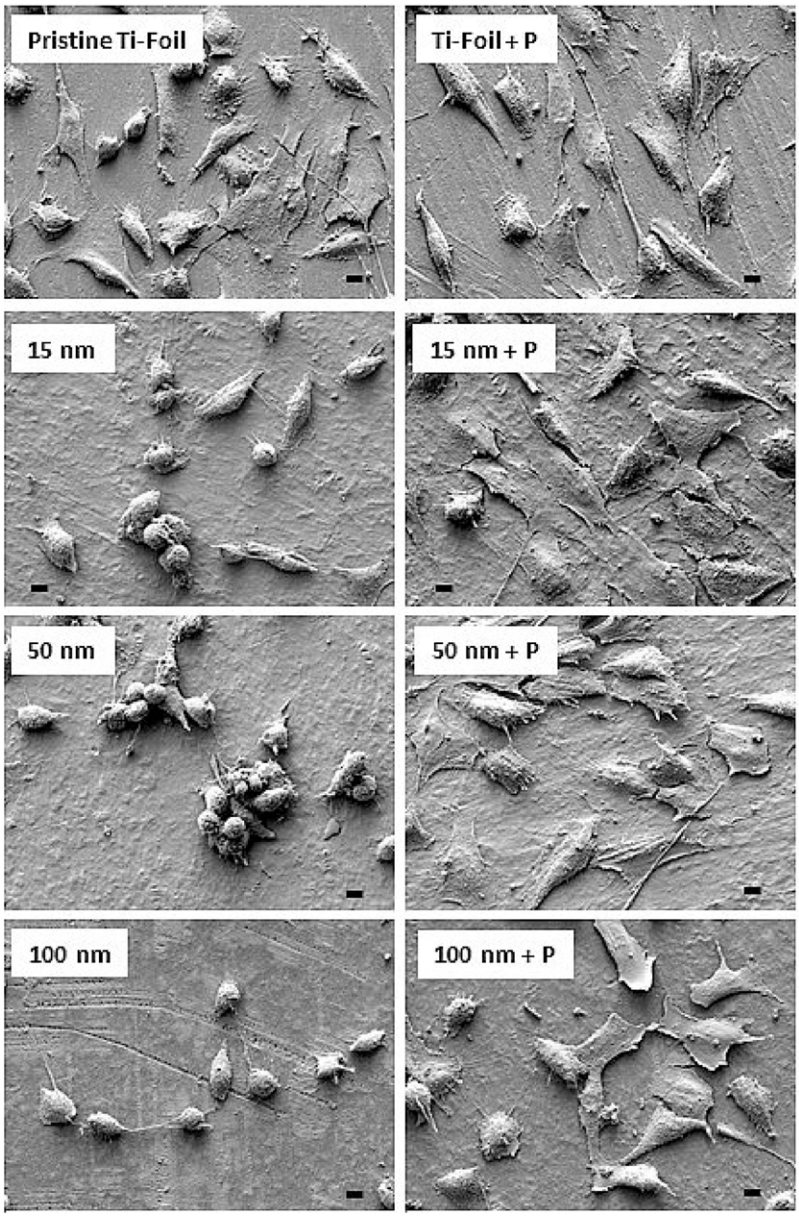


Figure 6. Higher magnification SEM images of cells interacting with pristine Ti foil and nanotubes with 15, 50 and 100 nm in diameter before and after (+P) oxygen plasma treatment (scale bar: 10 μm).

the surface by plasma species. Moreover, on all the plasma treated nanotube surfaces a significantly lower concentration of carbon was detected and a higher concentration of oxygen and no nitrogen. It should be noted that reduced hydrocarbon content is desired for any of the implantable materials, as it is known that lower hydrocarbon contamination increases cellular attachment and promotes osteoblast differentiation during implementation [31, 32]. Thus, our SEM analysis outcomes could be correlated with the observed changes in the plasma treatment-induced chemical composition of the surface, especially regarding the reduced fluorine and carbohydrate content.

Moreover, the SEM analysis clearly showed an important impact of the plasma treatment on cell adhesion, proliferation and morphology. Although MG-63 cells did adhere to the freshly prepared TiO_2 nanotube surfaces, their growth was suppressed. On the other hand, oxygen plasma treatment enhanced cell adhesion and cell proliferation indicating that oxygen plasma treated surfaces provide better environment (less fluorine and hydrocarbons and more oxygen on the surface) required for cell adhesion and growth. As revealed with SEM analysis, significant differences in the cell attachment between oxygen plasma treated and untreated TiO_2 nanotube surfaces were observed. Cells grown on the untreated nanotube surfaces showed more spherical morphology compared to the spread and elongated cells grown on the plasma treated surfaces (figure 6). However, the resazurin viability assay results revealed no statistical significant differences between the oxygen plasma treated and the untreated surfaces. Similar observations for cell growth on plasma treated surfaces with otherwise different chemical composition were reported by other groups [33, 34]. Their studies showed that oxygen plasma treatment increased cell adherence

and cell spreading without altering cell viability. Moreover, our results are also in line with some other studies reporting similar cell viability patterns for cells grown on TiO_2 nanotube surfaces and control surfaces However, some other studies revealed enhanced cell viability on TiO_2 nanotube surfaces in comparison to pristine Ti foil [35, 36]. Although various nanotubes morphologies have already been extensively studied there are still contradictory results and reports by different groups [18, 29, 35–37]. The main reason for this can probably be attributed not only to the type of viability assays applied in individual studies, but also to the different seeding densities, times of incubation and possible presence of impurities on the nanotube surfaces [29, 35].

5. Conclusions

Plasma treatment induces alterations in chemical surface composition of the TiO_2 nanotube surfaces, reflecting in an improved cell adhesion on the surface. In this study, surface treatment with oxygen plasma on electrochemical anodized nanotubes with different diameters was used. As revealed from the SEM analysis, modification of TiO_2 nanotube surfaces with oxygen plasma had a beneficial effect on MG-63 cell adhesion and proliferation. Treatment of samples with oxygen-plasma altered their surface properties, which resulted in a removal of surface contaminants and an increase in oxygen content on the surfaces. Surface finishing of orthopedic or dental implants by oxygen plasma could have an important role in medical implications for design of implants. Nevertheless, prior to *in vivo* studies for a novel biocompatible substitute, additional *in vitro* experiments are still necessary in order to elucidate the behavior of human osteoblasts and osteoblast-like cell monolayers. Furthermore the results of our study show potential of such approach in other medical applications, where it is necessary to promote adhesion of one cell type over another. By fine tuning the surface topography and plasma treatment conditions it would be possible to design appropriate surface conditions for improved/reduced growth of different cell types. This could be especially valuable for development of vascular implants, especially stents, where the main drawback is in insufficient endothelization and uncontrolled proliferation of smooth muscle cells.

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