CELL INTERACTION WITH MODIFIED TITANIUM NANOSTRUCTURES

Hynek MORAVEC[a], Marta VANDROVCOVÁ[b], Kateřina CHOTOVÁ[a],

Jaroslav FOJT[a], Luděk JOSKA[a]

[a]*Institute of Chemical Technology Prague, Technicka 5, 166 28 Prague 6, Czech Republic*
moravech@vscht.cz

[b]*Department of Growth and Differentiation of Cell Populations, Institute of Physiology*

Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

Abstract

Electrochemical oxidation of titanium allows preparing TiO_2 tube layer with unique properties. This layer could be further modified to increase bioactivity. The aim of this work was to study the interaction of cells with various modified nanostructures on Ti6Al4V alloy. Nanotubes were prepared in a mixture of $(NH_4)_2SO_4$ and NH_4F.These surfaces were treated with calcium hydroxide solution, phosphoric acid and heat treated. Effect of each adjustment was controlled by SEM, XPS and Raman spectroscopy. To evaluate biological functionality of surface treatments cell line SaOS-2 was used. Cells activities were determined on day 1, 3 and 7 after seeding.

Cell spreading area was evaluated on day 1 after seeding. However, cells on all tested samples displayed only slight reduce in comparison with polystyrene. The viability of the cells on all surfaces, evaluated by the LIVE/DEAD test, was higher than 90 % and there were no significant differences among the tested groups. The cell number was higher on both chemically treated nanostructures after 7 days of cultivation. Therefore, it can be concluded that chemically modified surfaces seem to be promising for biomaterials applications.

Keywords: titanium alloy, nanostructure, treatment, cell.

INTRODUCTION

Electrochemical modification is one of the most common and flexible way to modify metallic materials. Anodic oxidation has been successfully used to transform smooth titanium surfaces into nanotubular structures with diameters up to 100 nm. By adjusting parameters such as the chemistry of the electrolyte, voltage and pH value, one can easily control dimensions of tubes. Surface modification by nanotube layers may be particularly interesting because it allows improving biocompatibility of the surface and also offers the opportunity to additionally fill nanotubes with biologically effective elements (i.e. Ca, F, Na, P, etc.) or active signalling molecules. Next possibility is to convert the crystalline structure from amorphous to crystalline state [1].

Materials are commonly tested in the simulated body fluids (SBF) to get information about their bioactivity. Precipitation of apatite on material´s surface in SBF solution is observed [2]. Interactions of cell lines (CPC-2, TE-85, MG-63, Saos-2) with surface provide more data to characterize materials [3]. Cell morphology, proliferation, adhesion, differentiation, etc. are commonly studied. Cellular response to nanostructured surface can be affected by tube dimension, chemical composition and crystalline form [4, 5]. Nanostructured surfaces give an opportunity to more closely resemble the surface topography of living tissue. From cell in vitro studies it has become increasingly evident that surface morphology could prove an efficient method for controlling cell fate. A mechanism is driven by promoting the adsorption of selected proteins (such as fibronectin and vitronectin), which are important for mediating signals between cell and surface [6]. Changes in the morphology of cells growing on titanium nanotube substrate are also dependent on both the diameter of the tubes and the spacing between them. Many studies have been done to clarify the effect on cell

behaviour on tubes with different diameter (15 – 100 nm), but, this phenomenon still remained unclear [7-10]. Nanotubular structure can possibly exchange of gas, nutrients, proteins flow between the nanotube walls. Larger diameter nanotube (50–100 nm) interspacing quite possibly increases the ECM (extra cellular matrix) production because of the increased storage capacity and ability for flow. In some surveys chemical treatments of nanostructured surfaces are mentioned. Nanotubes could be modified by elements such as calcium and phosphorus. They take part in formation of hydroxyapatite – inorganic component of bone and mineralization of hard tissue. These components are also connected with proper cell function [11-13].

EXPERIMENTAL

Samples of Ti-6Al-4V alloy were used in experiments leaving 2 cm^2 exposed to electrolyte. Nanostructures were prepared in electrolyte consist of sulphate and fluoride ammonium. Calcium were introduced on nanotubes wall by soaking in 0.02 mol/l $Ca(OH)_2$ solution at 60°C. Incorporation of phosphorus into TiO_2 structure was done by adding phosphoric acid (1 mol/l) in sulphate electrolyte. Nanotubes were converted from amorphous to crystalline state by heat treatment. Parameters of process were set as follows: 500 °C, for 3 h (10 °C/min).

Responses of cells to samples of various chemical composition and crystalline structure were followed. Group of 4 different types of samples were prepared: nanotubes with calcium (NT - Ca), nanotubes with phosphorus (NT - P), heat treated nanotubes (NT - HT) and nanotubes without treatment (NT). For the cell culture experiments, the samples were sterilized in 70% ethanol for 3 days. Osteosarcoma cells SaOS – 2 were washed with PBS, trypsinized and inserted in 12 - well polystyrene cell culture plates. Cells were suspended in McCoy medium (Sigma, Cat. No. M4892) with 10% fetal bovine serum (Sigma, Cat. No. F7524), and gentamicin (40 µg/mL, LEK). Cell density was 12 000 cell/cm^2. The cells were cultured for 1, 3 and 7 days at 37 °C in a humidified air atmosphere containing 5% CO_2. Cells were fixed with 70% frozen ethanol at the end points of experiment. Four samples were used for each experimental group and time interval. Cell spreading area, cell counts and their viability were evaluated. As control bottoms of standard polystyrene culture dishes were used.

The surfaces of the samples were documented by scanning electron microscope VEGA 3 SBU (TESCAN Brno, s.r.o.). To evaluate effect of chemical treatments photoelectron spectroscopy spectrometer ESCA Probe P (Omicron) was employed. Crystalline structure after heat treatment was determined by Raman spectroscopy. The number of cells was evaluated for each image using Image J, viability and cell surface using Adobe Photoshop. Statistical analysis SigmaStat (Jandel Corporation, USA) was used. ANOVA and Student-Newman-Keuls methods were applied. The value of $p \leq 0.05$ was considered as significant.

RESULTS AND DISSCUSION

Effect of treatments

The surface composition determined by X-ray photoelectron spectroscopy (XPS) is displayed in Table 1. After the hydrothermal treatment, the original nanotube array architecture are retained. Hydrothermal modification led to enrichment in calcium owing to large active surface area of nanotubes. The results show that the hydrothermal reaction takes place on the entire nanotube surface. The diminishing Ca concentration with depth is due to its insufficient transportation or diffusion into nanotubes. Migration of phosphates to sample (connected as anode) enabled incorporation of phosphorus into growing nanotubes. Heat treatment at 500 °C did not alter the morphologies of the nanotubes. This suggests that the nanotube arrays could bear such a temperature. Pure anatase phase was detected after heat treatment. Content of fluorine has been decreased in nanotube array.

Table 1: X-ray photoelectron spectroscopy analyses of tested samples (concentration in at. %).

Element	NT	NT - Ca	NT - P	NT - HT
Ti	20.5	17.4	21.1	41.3
V	1.5	1.8	0.6	1.2
Al	12.2	12.4	6.8	5.7
O	62.9	56.6	64.9	49.1
F	3.0	2.2	3.5	0.6
P	-	-	3.1	-
Ca	-	9.7	-	-

Cell spreading area

The cell spreading area was measured on day 1 after seeding. Cells were dyed with Texas Red C2-maleimide, which stains proteins of the cell membrane and cytoplasm. The area was evaluated from separated cells. Interconnected or overlapped cells were excluded from measurement. The SaOS -2 cell line on the polystyrene have a more rounded shape, with no cellular extensions or filopodia propagation (Fig. 2), whereas those cells cultured on modified TiO_2 nanotubes became increasingly more elongated and showed a number of filopodia. It is apparent that all tested materials promoted cell spreading, nevertheless cell area was slightly higher on polystyrene standard compared to other samples.

The heat treatment also allows evaporating toxins, such as fluorine, from the electrolyte solution to remain on the surface, which may induce toxicity to the cells. Further studies on the effect of, surface chemistry and crystal structure on cell growth behaviour of different cell types would be valuable for understanding of the nature of cell behaviour on nanotube substrates.

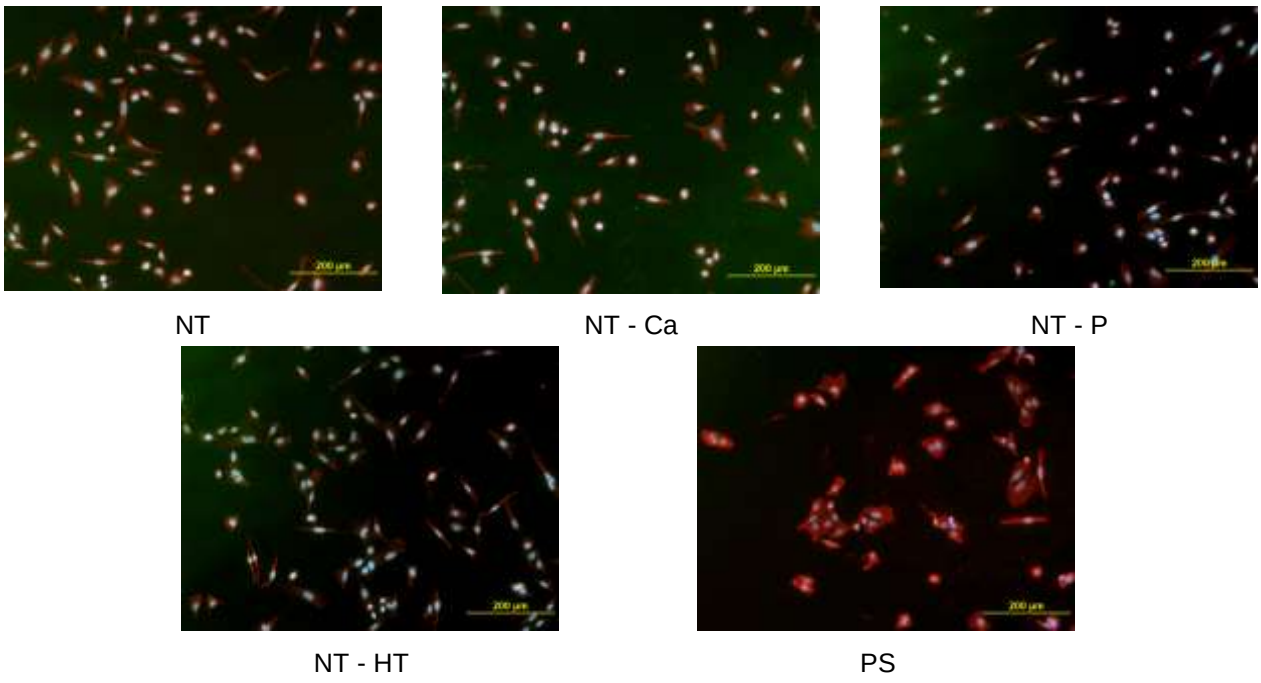

Fig. 1: Human osteosarcoma cell line SaOS-2 after 1-day-old cultures.

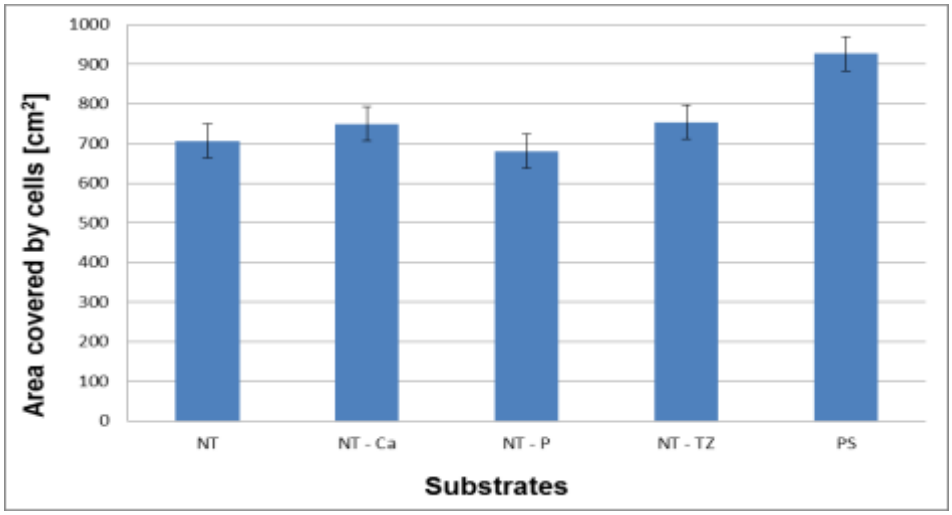

Fig. 2: Size of the spreading area of the SaOS-2 cells on tested materials

Cell number

Cell number was determined after 1, 3 and 7 days of cell cultivation. Dye Hoechst 33258 was applied to stain nuclei of cells. It binds to nuclear that emits blue fluorescence when bound to DNA. The number of nuclei was then manually counted. Initial adhesion on day 1, which is the determining factor for the growth and differentiation of cells on all specimens, was statistically comparable.

All tested materials showed significantly higher value in cell number in comparison with the polystyrene, which is taken as a positive control. Moreover, number of cells increased on the specimens doped with calcium and phosphorus on 7 day after seeding.

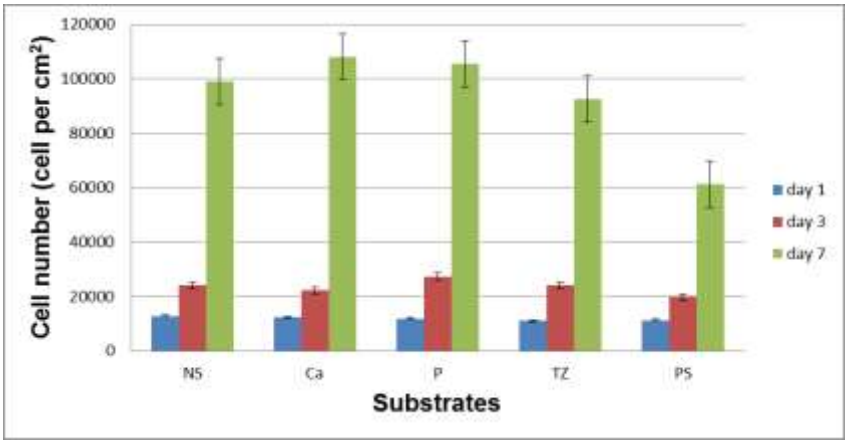

Fig. 3: Number of osteosarcoma SaOS-2 cells on day 1, 3, and 7 after seeding.

Cell viability

Cell viability was assessed using a commercial assay LIVE / DEAD (The LIVE/DEAD® Viability/Cytotoxicity Kit, Invitrogen). The test was conducted immediately after staining to prevent killing of cells by external effects. Dye mixture contained ethidium and calcein AM, where the former bind to the nuclei of dead cells producing red fluorescence and the later one is cleaved only in living cells producing green fluorescence. The viability was determined as the ratio of viable cells to their total number. The results are given as the arithmetic mean.

Modifications did not cause any negative effects on cell activity. The table shows that always 90% cells survived on all tested surfaces. Statistical evaluation shows higher cell viability on polystyrene and nanostructured surface compared to heat treated samples on first day of culturing. Cell viability has gradually grown and reached a maximum on day 7.

Table 2: Percentage of viable SaOS-2 cells cultured on different treated substrates on 1, 3 and 7 day of seeding.

Substrates	Day 1	Day 3	Day 7
NT	98.0±0.3	99.5±0.2	99.8±0.2
NT – Ca	95.6±1.4	93.0±2.6	100.0±0.0
NT - P	96.5±1.3	98.7±0.3	99.8±0.1
NT - HT	91.1±2.6	93.4±1.6	99.9±0.1
PS	98.8±0.6	98.9±0.4	100.0±0.0

CONCLUSION

Modified nanostructured surfaces of the alloy Ti-6Al-4V were tested in this work. Changes in chemical composition of nanostructured surfaces were successfully done. Amorphous character of tubes was turned into crystal anatase phase. Biological tests have been focused on colonization of the surface, cell number and their viability. Cell spreading area measured after 24 hours was lower on all samples compared to polystyrene. Elongated cells were observed on nanostructures samples. The cell count increased uniformly on all modifications, it was on the same or higher level than that of polystyrene Cell viability has moderately changed, but never decreased below 90%. After seven days of culturing, viability was comparable and almost 100%.

ACKNOWLEDGEMENTS

Financial support was provided by specific university research (MSMT No 20/2014) and by TE 01020390 (Technology Agency of the Czech Republic).

REFERENCES

[1] Macak, J.M., K. Sirotna, and P. Schmuki, *Self-organized porous titanium oxide prepared in Na2SO4/NaF electrolytes.* Electrochimica Acta, 2005. **50**(18): p. 3679-3684.

[2] Muller, L. and F.A. Muller, *Preparation of SBF with different HCO3- content and its influence on the composition of biomimetic apatites.* Acta Biomaterialia, 2006. **2**(2): p. 181-9.

[3] Vandrovcova, M. and L. Bacakova, *Adhesion, growth and differentiation of osteoblasts on surface-modified materials developed for bone implants.* Physiol Res, 2011. **60**(3): p. 403-17.

[4] Brammer, K.S., et al., *TiO2 nanotube structures for enhanced cell and biological functionality.* Jom, 2010. **62**(4): p. 50-55.

[5] Chun, Y.W. and T.J. Webster, *The role of nanomedicine in growing tissues.* Ann Biomed Eng, 2009. **37**(10): p. 2034-47.

[6] Sjöström, T., et al., *Initial attachment and spreading of MG63 cells on nanopatterned titanium surfaces via through-mask anodization.* Applied Surface Science, 2011. **257**(10): p. 4552-4558.

[7] Park, J., et al., *TiO2 nanotube surfaces: 15 nm, an optimal length scale of surface topography for cell adhesion and differentiation.* Small, 2009. **5**(6): p. 666-671.

[8] Oh, S., et al., *Stem cell fate dictated solely by altered nanotube dimension.* Proc Natl Acad Sci U S A, 2009. **106**(7): p. 2130-5.

[9] Brammer, K.S., et al., *Improved bone-forming functionality on diameter-controlled TiO(2) nanotube surface.* Acta Biomaterialia, 2009. **5**(8): p. 3215-23.

[10] Brammer, K.S., et al., *Nanotube surface triggers increased chondrocyte extracellular matrix production.* Materials Science and Engineering: C, 2010. **30**(4): p. 518-525.

[11] Xiao, X., et al., *Preparation and bioactivity of TiO2 nanotube arrays containing calcium and phosphorus.* Applied Surface Science, 2012. **261**: p. 312-319.

[12] Xin, Y., et al., *Bioactive SrTiO3 Nanotube Arrays: Strontium Delivery Platform on Ti-Based Osteoporotic Bone Implants.* ACS Nano, 2009. **3**(10): p. 3228-3234.

[13] Oh, S.-H., et al., *Growth of nano-scale hydroxyapatite using chemically treated titanium oxide nanotubes.* Biomaterials, 2005. **26**(24): p. 4938-4943.