GROWTH AND DIFFERENTIATION OF HUMAN OSTEOBLAST-LIKE CELLS ON TI-6AL-4V ALLOY MODIFIED WITH ANODIZATION

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Abstract

Titanium and its alloys are widely used as hard tissue implants for their high biocompatibility and suitable physical properties. Along with other surface treatment methods, anodization technique is known to enhance osseointegration. The aim of this study is to evaluate the adhesion, growth and osteogenic differentiation of human osteoblast-like SAOS-2 cells on Ti-6Al-4V samples anodized in electrolytes composed of a stable volume of KOH (336.48 g/l) and variable volumes of liquid glass (124.47 g/l, 84,12 g/l and 37.38 g/l; samples s5, s6 and s7, respectively). Non-anodized Ti-6Al-4V samples, cell culture polystyrene (PS) and microscopic glass coverslips served as control materials. On days 2 and 4 after seeding, the cell number did not differ significantly among the tested samples. However, on day 7, the cell number on s6 samples reached the lowest values, which could be attributed to a non-homogeneous TiO_2 film on s6 samples formed during anodization. Nevertheless, the osteogenic differentiation, estimated by the intensity of fluorescence of collagen I in cells grown in a differentiation medium, was the highest on s6 samples. On s5 samples, coated with homogeneous TiO_2 films, both cell numbers and intensity of fluorescence of collagen I was relatively high. The bone matrix mineralization, evaluated by Alizarin Red staining, was the highest on s5 samples in standard culture medium, and similar on all tested samples in differentiation medium. Thus, the surface modification of s5 samples could be considered the most suitable for application in bone implants.

Keywords: Ti6-Al4-V, SAOS-2 cells, anodization, bone implants, collagen I, mineralization

1. INTRODUCTION

Biomaterials such as titanium (Ti) and its alloys or compounds (e.g., TiO_2) have various medical applications (from implants to drug delivery systems) and are extensively used especially in the fields of orthopedics and dental medicine. For use in hard tissue implants (aside from its favorable mechanical properties and biocompatibility) the materials promoting a better integration with the surrounding bone tissue are desirable, thus the improved adhesion, proliferation and osteogenic differentiation of cells on these surfaces is very important for designing and fabricating the implants. Properties such as surface chemistry, morphology and structure of these materials are important factors to influence the cell behavior. Many techniques are used to modify the titanium implant surfaces (for a review see [1]), e.g. mechanical methods to alter the surface morphology [2-4], and also various biochemical methods using inorganic compounds and biomolecules [5-7].

In this article we tested the response of human osteoblast-like SAOS-2 cells to Ti6-Al4-V alloy samples modified by an anodization technique to form a TiO_2 (i.e., anatase or rutile) film on their surface. Ti oxide layers are known to improve the corrosion resistance of the bulk material. Moreover it has been observed

that an anatase crystalline form of TiO_2 exhibit antimicrobial effects [8] and may play a positive role in the osseointegration process thanks to its effects on calcium phosphate precipitation [9].

2. MATERIALS AND METHODS

2.1. Metallic sample preparation and characterization

Series of Ti-6Al-4V alloy samples were prepared in the form of disks with 14 mm in diameter and 2 mm in thickness. Surfaces of some samples were further modified with anodization (samples s5, s6, s7) in electrolytes of a different ratio composition of KOH and liquid glass (Tab. 1), while samples sC (control Ti-6Al-4V sample group) were left unmodified. Ten samples per each experimental group were used. Anodization was performed using a voltage of 40 V for 60 seconds to form a coating layer of TiO_2.

The chemical composition and morphology of the surfaces of all anodized samples were observed and measured by means of confocal Raman microscopy (XploRA™, HORIBA Jobin Yvon, France) coupled with an optical microscope (Olympus BX41/51, obj. 100x) and 532 nm wave-length laser (20-25 mW, 3B grade).

Table 1 Electrolyte composition for surface treatment

Sample	KOH [g/l]	Liquid glass [g/l]
s5	336.48	124.48
s6	336.48	84.12
s7	336.48	37.38

2.2. Cell culture on metallic samples

The Ti-6Al-4V samples (s5, s6, s7, sC) were cleansed in 70% ethanol, rinsed with distilled and deionized water, sterilized in an autoclave (120 °C, 1 atm., 30 min) and air-dried in a laminar flow box under sterile conditions. The samples were inserted into 24-well polystyrene cell culture plates (TPP, Trasadingen, Switzerland; well diameter 15 mm) and seeded with human osteoblast-like SAOS-2 cells (ATCC-HTB-85, Chemos) in an initial density of 20,000 cells per well (approx. 11,300 cells / cm^2). Polystyrene cell culture wells (PS) and microscopic glass coverslips (sGl) inserted in these wells were used as reference materials. Cultures were supplied with 1.5 ml of McCoy's 5A medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS, GIBCO, Life Technologies) and 40 μg/mL of gentamicin (Gentamicin Lek, Ljubljana, Slovenia), referred as "growth medium". The cells were cultured in a thermostat at 37 ˚C in a humidified atmosphere containing 95 % of air and 5 % of CO_2. On days 2, 4 and 7 after seeding, the cells were rinsed in phosphate-buffered saline (PBS), fixed with 70% cold ethanol and stained by Texas Red C_2-maleimide (20 ng/mL) and Hoechst # 333258 (5 μg/mL) (both Sigma-Aldrich Co.) in PBS for 20 minutes. These samples were evaluated for the cell population density and proliferation by fluorescence microscopy (IX 51 epifluorescence microscope equipped with a DP 70 digital camera, Olympus Corp., Tokyo, Japan). For each experimental group and time interval two samples were used. From each sample 10 images of random areas were acquired and visible cell nuclei were manually counted.

On day 7 after seeding, one half of the samples was supplied with differentiation medium, i.e. the growth medium mentioned above further supplemented with 10 mM β-glycerolphosphate, 2 mM L-glutamine, 50 μg/ml of ascorbic acid, 10^{-6} M dihydroxyvitamin D_3 and 10^{-8} M dexamethasone. The second half of samples was supplied with fresh growth medium. The cells were cultured for additional 9 days.

The production of collagen type I, i.e. early marker of osteogenic cell differentiation, was evaluated using immunofluorescence staining and subsequent image analysis. The cells were rinsed with PBS and fixed with 70% cold ethanol. Non-specific binding sites were then blocked with 1% bovine serum albumin

supplemented with 0.1% triton in PBS for 20 minutes, and then with 1% Tween for additional 20 minutes. The primary antibody, i.e. rabbit anti type I collagen antibody (Cosmo Bio Co., Ltd.), was diluted to the ratio of 1:200 in PBS and applied overnight at 4 °C. After rinsing, the cells were incubated for one hour at room temperature with the secondary antibody, i.e. Alexa Fluor 488®-conjugated F(ab') fragment of goat anti-rabbit IgG (H+L), (Molecular Probes, Cat. No. A11070), diluted 1:400 in PBS with Hoechst # 333258 (5 µg/mL, Sigma-Aldrich Co.) in order to stain the cell nuclei. Images of 10 random areas from each sample were acquired by fluorescence microscope (Olympus IX 51, digital camera DP70, Japan) at the same exposure time using two different filters for each area (one visualizing cell nuclei and the other visualizing collagen). The fluorescence intensity was measured using Fluorescent Image Analysis software (ALICE, version 1.0) [10] and normalized for the number of cells. The fluorescence intensity of the samples stained without primary antibodies was subtracted.

The formation of mineralized matrix, i.e. another indicator of osteogenic cell differentiation, by SAOS-2 cells on tested samples was evaluated by staining with Alizarin Red S (Sigma Aldrich, USA). After staining, the cells with mineral deposits were detached from the sample surface by 10% acetic acid, and the absorbance was measured spectrophotometrically using a Versa Max Microplate Reader (Molecular Devices Corporation, Sunnyvale, California, USA.) at 405 nm wave-length.

3. RESULTS AND DISCUSSION

3.1. Morphology and chemical composition of the sample surface

Surface morphology of the anodized samples is displayed in Fig. 1. It is apparent that after anodization, the samples contain irregularities on their surfaces, while the control non-anodized Ti-6Al-4V alloy were rather smooth (sample not shown).

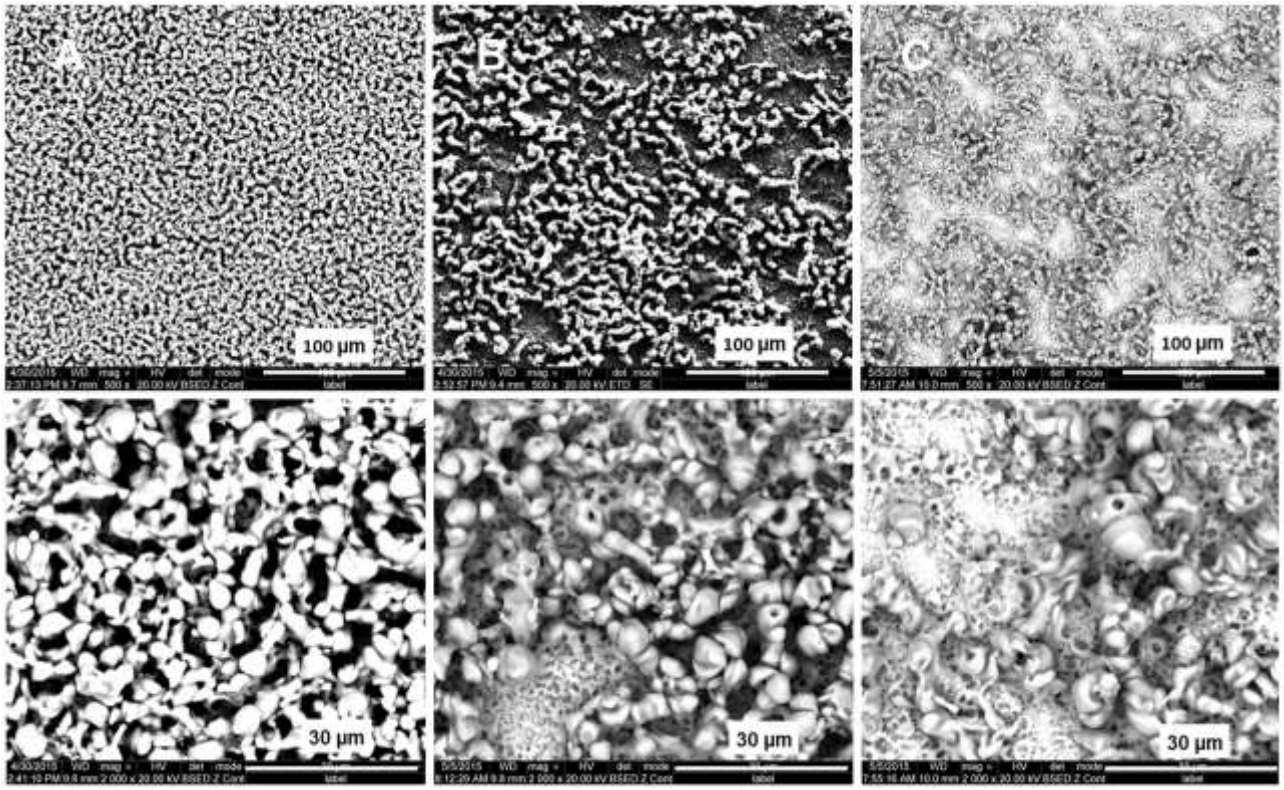

Fig. 1 Surface morphology of s5, s6 and s7 samples (A, B, C respectively). First row: overall view, second row: detailed view.

Raman spectroscopy revealed that the surface layer of anodized samples is composed of TiO_2, predominantly in the anatase crystalline form, although the presence of rutile structures has not been ruled out. Homogenous TiO_2 layer was formed in the electrolyte used for s5 samples. The layers on samples s6 and s7 were increasingly less homogenous. Accordingly, the surface roughness of these samples reached higher values (R_a 0.89 μm, 1.54 μm and 2.28 μm in samples s5, s6 and s7, respectively).

3.2. Adhesion, growth and osteogenic differentiation of SAOS-2 cells

On days 2 and 4 after seeding, the cell number in the growth medium was similar among the tested samples (Fig. 2A). However, on day 7, the cell number on s6 samples reached the lowest values, which could be attributed to a less homogeneous TiO_2 film on these samples. It is known that TiO_2, anatase in particular, support the cell adhesion and growth, especially by its enhanced wettability (for a review, see [11]). The cells on all tested samples on day 7 were well-spread and polygonal, but they formed confluent or at least subconfluent layers only on control unmodified T-6Al-4V (sC) samples (Fig. 3).

The cell number on s6 samples also remained low after additional 9 days of cultivation in the medium promoting osteogenic cell differentiation (Fig. 2B). It is known that if the cells are stimulated to differentiate, they attenuate their proliferation activity (for a review, see [12]), which was apparent on s6 samples and control unmodified (sC) samples. On the other samples, the cell numbers in the growth and differentiation media were similar, and on s5 sample, the cell number in the differentiation medium was even higher.

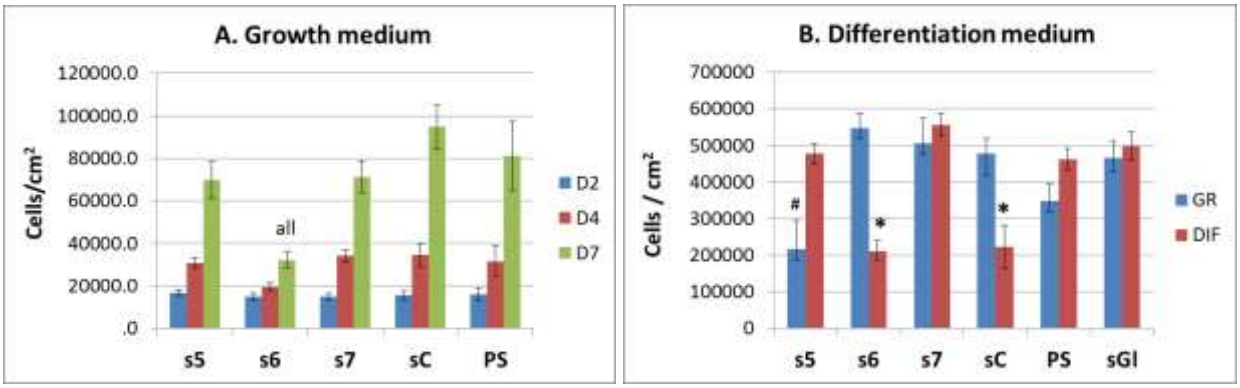

Fig. 2 Number of SAOS-2 cells on s5, s6 and s7 samples, control untreated samples (sC), polystyrene wells (PS) and glass coverslips (sGl) on days 2,4 and 7 after seeding (D2, D4, D7) in the growth medium (A) and after additional 9 days of cultivation in growth (GR) or differentiation (DIF) media (B). Mean S.E.M. from 20 measurements, ANOVA, Student-Newman-Keuls method. Statistical significance ($p \leq 0.05$): ^{all}: compared to all other samples, *: compared to s5, s7, PS and sGl, $^{\#}$: compared to all samples except PS.

The osteogenic differentiation, estimated by the intensity of fluorescence of collagen I in SAOS-2 cells grown in a differentiation medium, was the highest on s6 samples. Also on s5 samples, coated with homogeneous TiO_2 films, the intensity of fluorescence of collagen I in cells grown in the differentiation medium was relatively high, i.e. higher than on s7 samples and control glass coverslips (sGl) (Fig. 4A). In the growth medium, the intensity of fluorescence of collagen I did not differ significantly among the tested samples. Surprisingly, this intensity of fluorescence was on the average higher than in the differentiation medium (Fig. 4A).

The bone matrix mineralization, estimated by Alizarin Red S staining, was generally higher in the differentiation medium than in the standard growth medium (Fig. 4B). However, in the differentiation medium, the mineralization was similar in all tested samples, while in the growth medium, it was the highest on s5

samples. The mineralization was also relatively high on s7 samples and control unmodified Ti-6Al-4V samples in the growth medium (Fig. 4B).

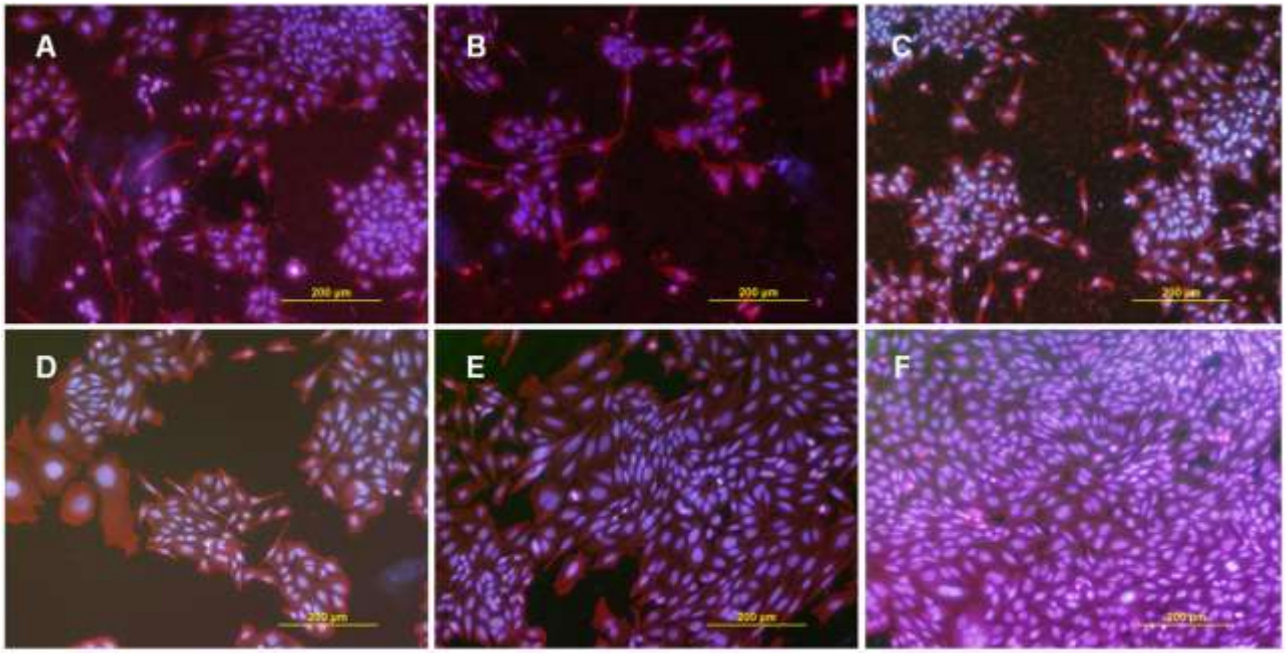

Fig. 3 Morphology of SAOS-2 cells on day 7 after seeding on s5, s6, s7 samples (A, B, C), and on control PS and two control samples sC1, sC2 (D, E, F).

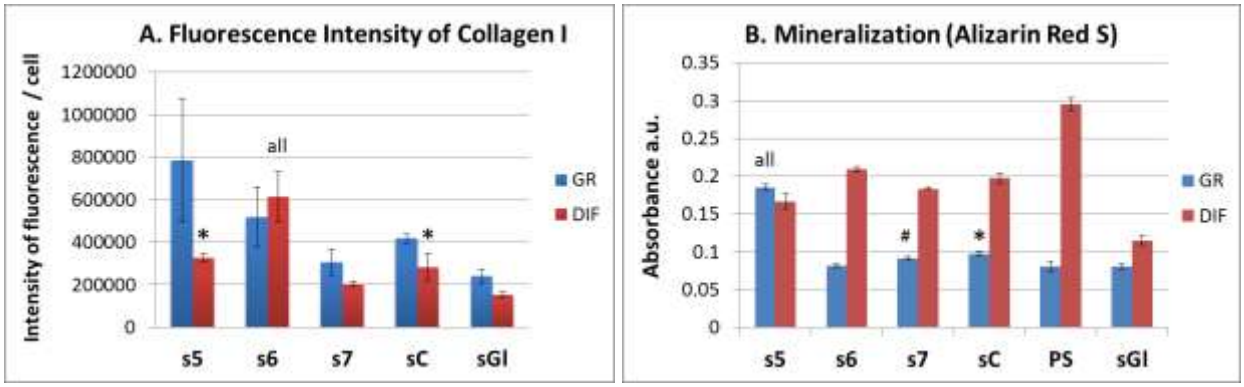

Fig. 4 Osteogenic cell differentiation measured by the intensity of fluorescence of collagen I (A) and mineralization (B) after 9 days of SAOS-2 cell cultivation on s5, s6 and s7 samples, control untreated samples (sC), polystyrene wells (PS) and glass coverslips (sGl) in growth (GR) or differentiation (DIF) media. Mean S.E.M. from 10 measurements, ANOVA, Student-Newman-Keuls method. Statistical significance ($p \leq 0.05$): ^{all}: compared to all other samples, *: compared to s6, s7, PS and sGl, $^{\#}$: compared to s6, sGI and PS.

4. CONCLUSION

It can be concluded that the SAOS-2 cells cultured on s5 samples reached relatively high numbers and showed a relatively high intensity of fluorescence of collagen I, an early marker of osteogenic cell differentiation. At the same time, the bone matrix mineralization, evaluated by Alizarin Red S staining, was

the highest on these samples. Thus, the mode of anodization applied for s5 samples seems to be the most advantageous for the surface modification of bone implants.

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REFERENCES

[1] YANG Z., OH N., LIU Y., CHEN W., OH S., APPLEFORD M., KIM S., KIM K., PARK S., BUMGARDNER J., HAGGARD W. ONG J. Enhancing osseointegration using surface modified titanium implants. Journal of The Minerals, Metals & Materials Society, Vol. 58, No. 7, 2006, pp. 71-76.

[2] DEMNATI I., GROSSIN D., COMBES, CH. REY CH. Plasma-sprayed apatite coatings: Review of physical-chemical characteristics and their biological consequences. Journal of Medical and Biological Engineering. Vol. 34, No. 1, 2013, pp. 1-7.

[3] KIM K., LEE B.A., PIAO X.H., KIM Y.J. Surface characteristics and bioactivity of an anodized titanium surface. Journal od Periodontal & Implant Science, Vol. 43, No. 4, 2013, pp. 198-205.

[4] LI D., FERGUSON S.J., BEUTLER T., COCHRAN D.L., SITTIG C., HIRT H.P., BUSER D. Biomechanical comparison of the sandblasted and acid-etched and the machined and acid-etched titanium surface for dental implants. Journal of Biomedical Materials Research, Vol. 60, No. 2, 2002, pp. 325-332.

[5] ANITUA E., PIÑAS L., MURIAS A., PRADO R., TEJERO R. Effects of calcium ions on titanium surfaces for bone regeneration. Colloids and Surfaces B: Biointerfaces, Vol. 130, No. 1, 2015, pp. 173-181.

[6] DE JONGE L.T., LEEUWENBURGH S.C.G., WOLKE J.G.C., JANSEN J.A. Organic-inorganic surface modifications for titanium implant surfaces. Pharmaceutical Research, Vol. 25, No. 10, 2008, pp. 2357-2369.

[7] SEVILLA P., VINING K.V., DOTOR J., RODRIGUEZ D., GIL F.J., APARICIO C. Surface immobilization and bioactivity of TGF-β1 inhibitor peptides for bone implant applications. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2015. doi: 10.1002/jbm.b.33374.

[8] GIORDANO C., SAINO E., RIMONDINI L., PEDEFERRI M.P., VISAI L., CIGADA A., CHIESA R. Electrochemically induced anatase inhibits bacterial colonization on Titanium Grade 2 and Ti6Al4V6 alloy for dental and orthopedic devices. Colloids and Surfaces B: Biointerfaces, Vol. 88, No. 2, 2011, pp. 648-655.

[9] WANG X.X., HAYAKAWA S., TSURU K., OSAKA A. Improvement of bioactivity of H_2O_2/$TaCl_5$-treated titanium after subsequent heat treatments. Journal of Biomedical Materials Research, Vol. 52, No. 1, 2000, pp. 171-176.

[10] MATEJKA R., ALICE: Fluorescent Image Analyser (ver 1.0); software available at http://alice.fbmi.cvut.cz

[11] VANDROVCOVA M., HANUS J., DRABIK M., KYLIAN O., BIEDERMAN H., LISA V., BACAKOVA L. Effect of different surface nanoroughness of titanium dioxide films on the growth of human osteoblast-like MG63 cells. Journal of Biomedical Materials Research A, Vol. 100, No. 4, 2012, pp. 1016-1032.

[12] BACAKOVA L., FILOVA E., PARIZEK M., RUML T., SVORCIK V. Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. Biotechnology Advances, Vol. 29, No. 6, 2011, pp. 739-767.