Chemical Surface Modifications of Titanium Implants

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Summary: In the present work, various surface modifications have been applied to titanium surface. The aim of the modifications was to improve cell adhesion and to determine their influence on the properties of titanium surface. The unmodified and modified surfaces were observed using SEM. Subtle changes in modified surface layer of titanium samples were examined using the Auger Electron Microanalysis and Photoelectron Spectroscopy. The properties of surfaces were evaluated by contact angle and roughness measurements. The results revealed large differences in morphology of Ti modified with different procedures whereas only minor differences in the chemistry of the surfaces were detected. Preliminary quantitative measurements (cell number, viability and differentiation) of the MG 63 osteoblast-like cells in the direct contact with the surface of the investigated materials show that both the not pre-treated titanium surface and the surfaces modified by the methods used in the this work are all well tolerated by the living cells. Within the experimental scatter all the surfaces provided good substrate for proliferation and growth of the cells.

Keywords: surface modification; titanium implants

Introduction

Titanium is known as a biocompatible metal[1] which exhibits corrosion immunity as well as good mechanical properties, including high fracture toughness. For these reasons, it is mainly used as bone substitutes, e.g. hip joints and dental roots for which high mechanical strength and corrosion resistance is required to assure negligible ion release from the implant into human body. Nowadays, titanium in the form of porous structures and meshes is taken into account as a potential support for living cells.[2]

Although titanium and its alloys are well known for their biocompatibility,[3,4] still some work is going on forward improvement of its osteoconductivity. Strategies used are based mainly on surface modifications, e.g.: protein immobilization,[5] phosphonic acids grafting,[6] ion implantation,[7] polymer,[8] carbyne,[9] polysaccharide[10] ceramic and bioactive glass[11] coating.

In this experiment the influence of selected surface modifications on cell behaviour in direct contact in titanium is investigated. Particularly consequences of 'piranha' (mixture of sulphuric acid and hydrogen peroxide) etching, sodium hydroxide soaking and dextran coating on titanium were examined in contact with human osteogenic cells in culture. In previous examinations it was shown that these modifications introduce significant changes in surface morphology of titanium samples.[12] Cell response was observed in the case of the bulk samples and also on

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titanium mesh in order to make the system closer to the potential clinical application and to watch additionally the role of the sample architecture.

Materials and Experimental Procedure

The material used in this study was pure titanium in the form of: (1) a commercially available titanium mesh (Tiomesh) commonly used in clinical applications, $\neq$ 0.2 mm and (2) pure titanium (Grade 2) rod Ø 6 mm cut into $\neq$ 2 mm cylinders. The samples were mechanically polished and rinsed in distilled water. Then, three types of modifications have been applied:

- soaking in 5.0 M NaOH aq at 60 °C for 24h; the samples were then gently washed in distilled water, at 40 °C for 48h and additionally annealed at 600 °C for 1 h (Ti/NaOH modification);
- etching in ''piranha'' solution (98% H_2SO_4 + 30% H_2O_2 mixture, the volume ratio 1:1). This type of pretreatment was carried out at room temperature for 4h (Ti/PRT modification)or in boiling solution for 10 min (Ti/PBS modification)
- immersing in a 0.05wt.% water dextran solution (Ti/dextran modification).

The topography of unmodified and modified surfaces were observed using SEM. Subtle changes in modified surface layer of titanium samples were examined using the Auger Electron Microanalysis (AES) and Photoelectron Spectroscopy (XPS). The physical properties of surfaces were evaluated by contact angle measurements. Since titanium is mainly dedicated to the applications in bone surgery, human osteogenic cells were used in the experiments. All investigations were performed twice by using MG-63 cell line and repeated once with human bone derived cells.

The human osteoblastic cell line MG-63 was purchased from American Type Culture Collection (ATCC CRL-1427). Cells were cultured in medium containing DMEM, 10% Foetal Bovine Serum, 1% L-glutamine, 1% Antibiotic-Antimycotic (all substrates from Gibco). Experiments were performed using cells between 97 and 99 passages. Human Bone Derived Cells (HBDC) were obtained from bone tissue harvested during surgery, which would be otherwise discarded. The procedure of HDBC isolation was based on the protocols described by Gallagher et al.[13] with modifications.[14] Cells in the first passage were used. They were cultured in medium supplemented with 0,12 mM ascorbic acid.

In order to determine the effect of surface treatment on osteoblast-like cells in vitro, samples of titanium were put into the wells of 96-well culture plate. Cell population was enzymatically detached and suspended in culture medium and then cells were seeded on the surface of titanium samples. The initial seeding density was 2500 cells per well. After 24 hours medium was change into the medium supplemented with 0,12 mM ascorbic acid (Sigma) and 10 nM dexamethasone (Sigma). Cells on solid samples were cultured under standard conditions for 7 days. Culture on titanium mesh was being continued up to 28 day; medium, additionally supplemented with 14 mM β-glycerophosphate disodium salt (Sigma), has been changed every 2 days.

To estimate **cell viability**, tetrazolium salt-based colorimetric assay was performed. In this test 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) is metabolically reduced in viable cells to a water-soluble formazan product.[15] Medium in experimental culture was changed into XTT and phenazine methosulphate (PMS) solution in DMEM. After 4 hours long incubation in 37 °C, solution was collected and its absorbance was read at 450 nm. Cell viability on the surface of solid titanium samples was investigated after 7 days of culture and on the titanium mesh after 28 days. In both cases cells cultured on tissue culture polystyrene (TCPS) served as a control. Results are shown as a percentage of a control.

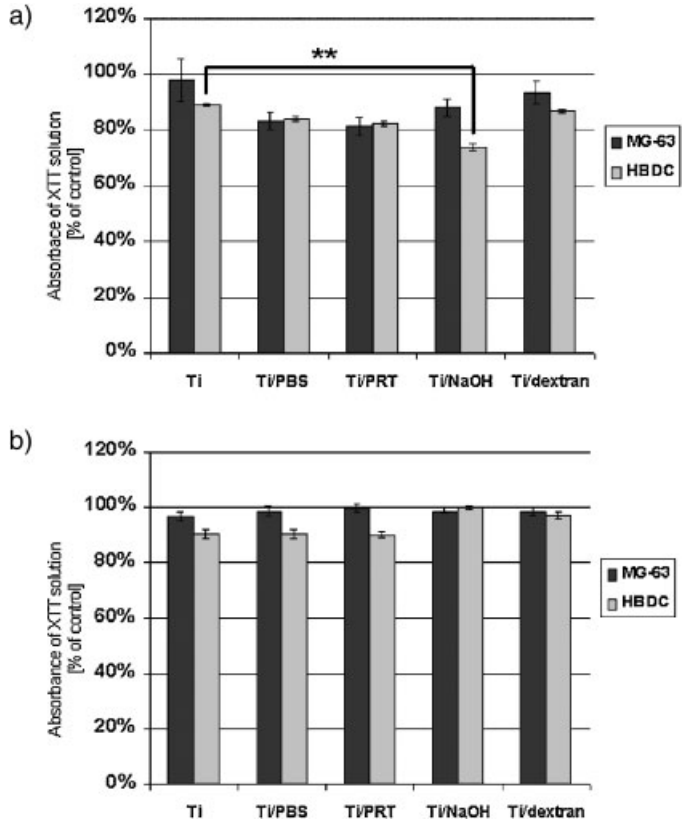

Figure 1.
Viability of MG-63 and HBDC expressed as a percent of control (culture on TCPS): a) cells on solid samples after 7 days of culture, b) cells after 28 days of culture on mesh samples. All data are expressed as means ± standard error. Asterisks show significant difference between the groups at ** $p < 0{,}01$, according to Kruskal-Wallis Test with Dunn's post hoc test.

To estimate the **extracellular matrix production**, collagen was visualised and measured in spectrophotometer. Cells cultured in contact with titanium mesh were rinsed with PBS (Gibco) and incubated with solution of Direct (Sirius) Red (Fluka) for 1 hour. Then cells were rinsed with 10 mM HCl and Direct Red stained collagen was dissolved in 0,1 NaOH. Absorbance of solution was measured at 540 nm.[16] Results are shown as a percentage of a control.

Morphological observations of cells in contact with titanium mesh were performed during culture. After 4 weeks long culture, cells were stained with Hoechst33342 (Sigma) in order to visualise their nuclei. Pictures were taken using inverted microscope Nikon Eclipse 2000-U equipped with Digital Sight DS-U1 camera.

Experiments in which MG-63 cell line was used were performed twice. Results were confirmed in HBDC experiment once. Six samples of any type were used in each particular experiment. Kruskal-Wallis Test (non-parametric ANOVA) with Dunn's Multiple Comparisons post test at a confidence level of 95% was used. Viability of cells cultured on modified titanium samples were compared with viability of cells on non-modified titanium.

Results and Discussion

Viability of MG-63 cells cultured on modified and non-modified titanium samples

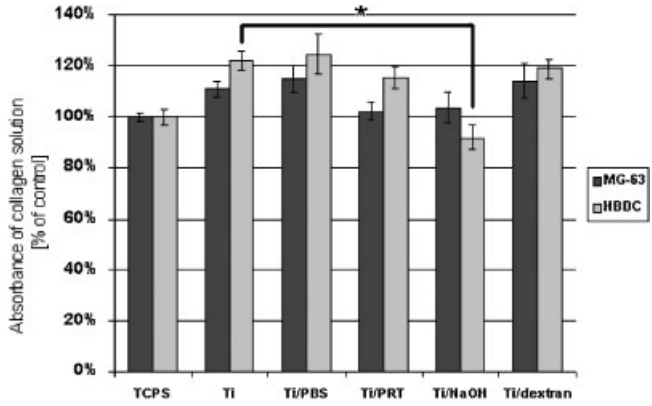

Figure 2.
Synthesis of collagen by MG-63 osteoblast-like cells after 4 weeks long culture. All data are expressed as means ± standard error. Asterisk show significant difference between the groups at * $p < 0{,}05$, according to Kruskal-Wallis Test with Dunn's post hoc test.

does not differ significantly neither after 7-days long culture on the bulk samples (Figure 1a), nor after 28 days on the meshes (Figure 1b). Viability of HBDC cultured on Ti/NaOH is significantly lower than on non-modified titanium after 7 days of culture on the bulk samples.

As can be seen in Figure 2, the amount of collagen on titanium mesh is higher than in the control, on the nonmodified titanium samples as well as on the mesh modified by high temperature piranha and on those coated by dextran, but the differences are not statistically significant. Collagen amount in the culture of HBDCs in contact with Ti/NaOH is significantly lower ($p < 0{,}05$) than on non-modified titanium.

Microscopic observation did not elicit differences between cell cultures on modified and non-modified titanium meshes. Both MG-63 and HBDC cells form a kind of a sheet which is directly adhered to the metal surface. (The example is shown in the Figure 3a). Numerous cells are visible in it as well as on titanium surface (Figure 3b) This view is similar for all investigated titanium samples.

In order to better understand the results of in-vitro study, the surface characteristics has been performed. SEM observations (Figure 4) have shown that Ti/NaOH and Ti/PBS modifications introduce significant changes in surface topography whereas dextran modification leaves titanium sur-

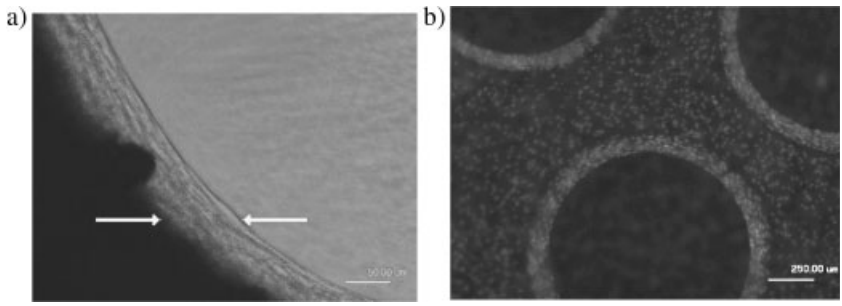

Figure 3.
a) Light microscopy image of MG-63 cells after 4 weeks long culture in contact with titanium mesh soaked in 5 M NaOH. The left narrow indicates the edge of titanium mesh, the right one indicates tissue-like sheet. b) Fluorescent microscopy of HBDC after 4 weeks long culture in contact with Ti/PBS titanium mesh.

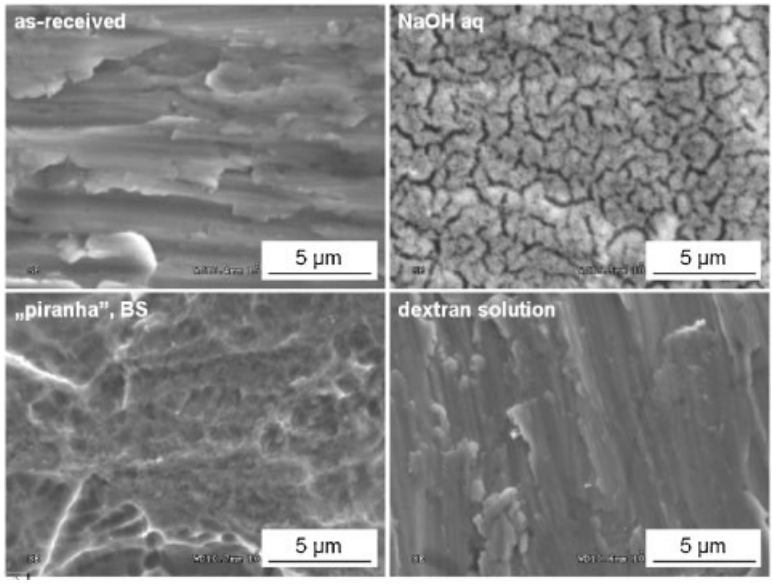

Figure 4.
Surface topography of titanium meshes after modifications: (a) Ti/NaOH, (b) Ti/PRT, (c) Ti/PBS, (d) Ti/dextran

face unchanged in this term (characteristic oriented grooves resulting from grinding can be still seen). The NaOH pretreated surface is the most developed with the small pores (about 0.2 μm in diameter) visible in the ''honeycomb'' islands. In the case of piranha pretreatment sub-microporosity effects can also be seen, e.g. in boiling ''piranha'' solution a high population of shallow depressions about $0.5 \div 1$ m in diameter is formed.

As for the chemical composition, the surfaces modified in both alkaline and acid solution do not differ significantly. The AES survey spectra have shown that Ti is oxidized on all the surfaces (the signals from Ti and O are well distinguishable). Moreover, in the case of Ti/NaOH modification, an incorporation of Ca was also noted (Figure 5). This apparently results from the preparation procedure. Comparison of high resolution Ti LMM spectra

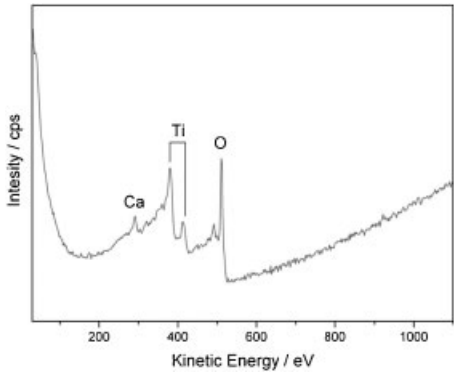

Figure 5.
A typical Auger survey spectra taken at Ti mesh surface after Ti/NaOH modification.

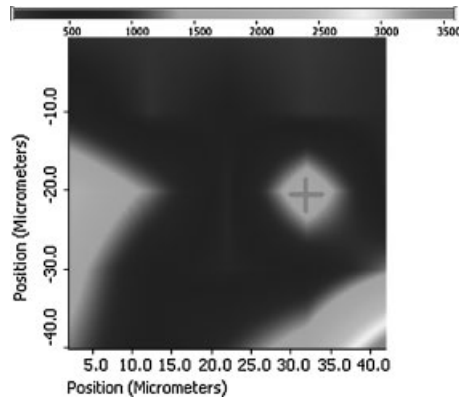

Figure 6.
Intensity of -CH2 Raman signal on the Ti surface.

Table 1.
Values of contact angle measured on unmodified and modified titanium surfaces.

modification	Ti	Ti/NaOH	Ti/PRT	Ti/PBS	Ti/dextran
contact angle	62°	83°	59°	74°	45°

does not reveal any very distinct difference in their shape.[12] This suggests that similar Ti-oxides are formed as a result of all modifications. Ti/dextran modification results in an inhomogeneous coating (Figure 6). However, the advantage of this modification is the presence of hydroxyl groups.

Applied modifications influence also the wettability of surfaces. The value of contact angle is significantly higher for Ti/NaOH and Ti/PBS modifications while it is lower in the case of Ti/dextran modification (see Table 1).

Discussion and Conclusions

Any of the applied modifications resulted in the improvement of the titanium as a support for osteogenic cells in vitro under the specific conditions of this experiment. All modified surfaces were well tolerated by cells, although both cell viability and collagen production were diminished in contact with NaOH-soaked titanium, as compared to non-modified titanium. This was the case only in the culture of HBDCs and not in the culture of MG-63. The worse results for the NaOH-soaked titanium are probably the consequence of the most evident changes in the surface topography after this modification. Any significant differences in cell response to the other investigated modified materials were not demonstrated. Similar cell response to modified and non-modified titanium is probably the result of similar samples' chemistry, which was examined by AES.

The most promising results are those for dextran-coated titanium. It was shown in the preliminary studies that this might promote cell maturation, as can be judged on the basis of collagen production.[17] This result was not confirmed here, but this may be due to the discontinuity in the dextran cover, which was shown in Raman (Figure 6). Good cell viability on dextran covered titanium is anyway a good news in respect to the potential usage of dextran coating as the attractive surface for protein immobilisation.

Dextran was reported to be used as a material for hydrogel and scaffolds for cell culture,[18,19] and also, as a carrier for bioactive molecules, particularly DNA.[20,21] Thus dextran coating of titanium, which was found here to be well accepted by cells, can be taken into account as a carrier for molecules (proteins, DNA) that could promote osteoinductive potential. Results of our experiment encourage to further investigation with using this polysaccharide for coating scaffolds for cell culture and differentiation.

Investigated meshes seem to be a good support for osteoblast-like cells to proliferate and differentiate. It seems that due to its architecture, titanium mesh forms a good support for cells in respect to the production of extracellular proteins, as shown here for collagen. The dense tissue-like sheet found at the edges of titanium mesh may potentially play stimulating role for tissue remodelling after the implantation of the mesh together with cells cultured in vitro.

Acknowledgements: Surface characterizations were performed using a Microlab 350 located at the Physical Chemistry of Materials Center of the Institute of Physical Chemistry, PAS, and of the Faculty of Materials Science and Engineering, WUT. This work was financially supported by the Polish Ministry of Science and Higher Education, the Rector of the Medical University of Warsaw (grant No NZME/W2/06) and by the Foundation for Polish Science through a generous fellowship for M. Pisarek.

[1] X. Liu, P. K. Chen, C. Ding, *Mat. Sci. Eng.* **2004**, *R47*, 49–121.
[2] E. D. Spoerke, N. G. Murray, L. Huanglong, L. C. Brinson, D. C. Dunand, S. I. Stupp, *Acta Biomat.* **2005**, *1*, 523–533.
[3] O. E. Pohler, *Injury* **2004**, *31* (Suppl 4) 7–13.
[4] F. Guillemot, *Expert Rev Med Devices* **2005**, 2, 741–748.

[5] A. Nanci, J. D. Wuest, L. Peru, P. Brunet, V. Sharma, S. Zalzal, M. D. McKee, *J Biomed Mater Res* **1998**, *40*, 324–335.
[6] C. Viornery, H. L. Guenther, B. O. Aronsson, P. Pechy, P. Descouts, M. Gratzel, *J. Biomed. Mater. Res.* **2002**, *62*, 149–155.
[7] D. Krupa, J. Baszkiewicz, J. A. Kozubowski, M. Lewandowska-Szumiel, A. Barcz, J. W. Sobczak, A. Bilinski, A. Rajchel, *Biomed. Mater. Eng.* **2004**, *14*, 525–536.
[8] K. Geckeler, R. Wacker, F. Martini, A. Hack, W. Aicher, *Cell Physiol. Biochem.* **2003**, *13*, 155–156.
[9] G. M. Demyashev, A. L. Taube, E. Siores, *J Nanosci Nanotechnol.* **2002**, *2*, 133–137.
[10] K. Cai, A. Rechtenbach, J. Hao, J. Bossert, K. D. Jandt, *Biomaterials* **2005**, *26*, 5960–5971.
[11] P. A. Ramires, A. Wennerberg, C. B. Johansson, F. Cosentino, S. Tundo, E. Milella, *J Mater Sci. Mater Med.* **2003**, *14*, 539–545.
[12] M. Lewandowska, A. Roguska, M. Pisarek, M. Janik-Czachor, K. J. Kurzydlowski, *Biomolecular Engineering*, in press.
[13] J. A. Gallagher, R. Gundle, J. N. Beresford, Isolation and culture of bone-forming cells (osteoblasts) from human bone. In: *Methods in Molecular Medicine: Human Cell Culture Protocols*, G. E. Jones, Ed., Humana Press Inc., Totowa, NJ **1996**, p. 233–262.
[14] D. Kudelska-Mazur, M. Lewandowska-Szumiel, G. Benke, M. Kowalski, J. Komender, *Eng. Biomater.* **2001**, *15–16*, 51–58.
[15] D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, M. R. Boyd, *Cancer Res.* **1988**, *48*, 4827–4833.
[16] G. Ciapetti, E. Verri, D. Granchi, E. Cenni, S. Gamberini, D. Benetti, M. Mian, A. Pizzoferrato, *Biomaterials* **1996**, *17*, 1703–1707.
[17] M. Lewandowska, H. Garbacz, W. Fabianowski, B. Polak, M. Lewandowska-Szumiel, *Eng. Biomater.* **2004**, *38–42*, 60–62.
[18] S. G. Levesque, M. S. Shoichet, *Biomaterials* **2006**, *27*, 5277–5285.
[19] H. Pan, H. Jiang, W. Chen, *Biomaterials* **2006**, *27*, 3209–3220.
[20] I. K. Park, Y. H. Park, B. A. Shin, E. S. Choi, Y. R. Kim, T. Akaike, C. S. Cho, *J Control Release* **2000**, *69*, 97–108.
[21] H. Hosseinkhani, T. Azzam, H. Kobayashi, Y. Hiraoka, H. Shimokawa, A. J. Domb, Y. Tabata, *Tissue Eng.* **2006**, 4269–7278.

