

The interaction of osteoblasts with bone-implant materials: 1. The effect of physico-chemical surface properties of implant materials

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ABSTRACT

This comparative study of various surface treatments of commercially available implant materials is intended as guidance for orientation among particular surface treatment methods in term of the cell reaction of normal human osteoblasts and blood coagulation. The influence of physicochemical surface parameters such as roughness, the surface free energy and wettability on the response of human osteoblasts in the immediate vicinity of implants and on the blood coagulation was studied. The osteoblast proliferation was monitored and the expression of tissue mediators (TNF- α , IL-8, MMP-1, bone alkaline phosphatase, VCAM-1, TGF- β) was evaluated after the cell cultivation onto a wide range of commercially available materials (titanium and Ti6Al4V alloy with various surface treatments, CrCoMo alloy, zirconium oxide ceramics, polyethylene and carbon/carbon composite). The formation of a blood clot was investigated on the samples immersed in a freshly drawn whole rabbit blood using scanning electron microscope.

The surfaces with an increased osteoblast proliferation exhibited particularly higher surface roughness (here $R_a > 3.5 \mu\text{m}$) followed by a high polar part of the surface free energy whereas the effect of wettability played a minor role. The surface roughness was also the main factor regulating the blood coagulation. The blood clot formation analysis showed a rapid coagulum formation on the rough titanium-based surfaces. The titanium with an etching treatment was considered as the most suitable candidate for healing into the bone tissue due to high osteoblast proliferation, the highest production of osteogenesis markers and low production of inflammatory cytokines and due to the most intensive blood clot formation.

Keywords: implant, surface properties, proliferation, cell number, osteoblast, cytokine, surface free energy, wettability, blood clot formation

1. INTRODUCTION

Implants replacing a missing or damaged bone or joining bone fragments together must be, contrary to implants for other purposes, not only mechanically resistant, but able to quickly heal into the host organism, i.e. bones as load-bearing structures has to fulfil their role in functioning of living organism as soon as possible (e.g. ribs as breathing apparatus, facial bones serving for swallowing) (Bancroft et al 2001). From the practical point of view, a quick healing of the implant into a recipient's bone is not only a medical problem, but also has a strong socio-economic aspect. The sufficient fast osteointegration does not overload the recipient organism and put the patient back to the normal life without consecutive complications (secondary prevention) and also helps to avoid complications after surgery. The history of the implant application started with smooth polished metal implants. From that time a big part of an effort of scientists has been put on a development of surface treatments leading to acceleration of the implant osteointegration. For example, the implant osteointegration of the polished titanium has been not sufficiently fast for some of the dental clinical applications therefore the implants with very rough surfaces, and thus with a significantly enlarged surface area, leading to faster osteointegration were developed (Franchi et al 2004). There are several surface treatments of implants on the market, especially in a dental implant area. Producers highlight the benefit, their advantages concerning a healing speed, strength of osteointegration as well as a long-term stability in the host bone (Del Fabbro et al 2008, Marchetti et al 2008). Nevertheless, surgeons from clinic don't choose implants according the surface treatment and, in fact, no surface is preferred in a clinical practice.

After the implant insertion into the host body, the foreign material and the living tissue always react with each other (Okumura et al 2001). The pathway of these bilateral reactions depends on the intensity of the mechanic as well as physico-chemical irritation of cells of the affected tissue. This means that the interaction between the implant and the adjacent tissue

depends not only upon bulk material properties, but in particular on the nature of the implant surface. Physico-chemical properties of the surface induce various cell responses what results also in changes in the cellular adhesion onto the implant (Eastlund et al 1995, Jones et al 1999). There is a lot of literature concerning the surface treatments of the particular implant materials, but we find relatively difficult to compare single studies dealing with different materials among each other due to various surface treatment methods used by authors, different sources of used cells or experimental methods.

Also another very important aspect, thought not of such big interest, is the effect of the surface modification on the blood clot formation. The clotting blood is the site of the first connection between the foreign implant material and the living tissue of the recipient Park et al 2001). The quick formation of the blood clot bonds wound together with the implant, isolates the implant bone bed from the external environment and also serves as a barrier against a possible infection. At the same time, fibrin fibres create an early connection between bone bed and the implant surface. The pluripotent cells migrate through these fibres from the bone surface to the implant and differentiate to osteoblasts.

In our previous pilot study, fibroblasts were used as model cells to observe the cell proliferation and the synthetic activity in the course of a initial contact of cells with the surface of commercially available implant materials (Pešáková et al 2007). The comparison of behavior of fibroblasts, representing simple connective tissue cells forming substitutive tissue present at a bone-implant interface where not a tight contact is achieved, and osteoblasts, representing fully differentiated functional bone cells, can show to a possible surface treatment modality for a preferential differentiation of full-value bone cells and reduction of substitutive tissue cells.

In the present study we intend to tabularly demonstrate a main relation between the selected surface characteristics and an early osteoblast response and/or the blood clot formation and thus to facilitate the orientation in a wide range of the commercially available

implant materials already in a practical use. The selected implant materials were investigated with respect to proliferation and the synthetic activity of human osteoblasts. The significant impact of physico-chemical properties of implant material surfaces on the cellular response and blood coagulation have been observed in this follow-up research.

2. MATERIAL AND METHODOLOGY

2.1 Materials, surface treatments and sterilisation.

The materials of a different origin (metal, non-metal) with various surface treatments were selected for this study. As representatives of metal materials were chosen titanium (Ti, polished, machined, sand-blasted, plasma-sprayed, Beznoska, Kladno, Czech Republic) or etched (Fopos, Praha, Czech Republic), Ti6Al4V alloy (Ti6Al4V, machined, Beznoska, Kladno, Czech Republic), CrCoMo alloy (CrCoMo, polished, Beznoska, Kladno, Czech Republic), stainless steel (polished, Beznoska Kladno, Czech Republic). As representatives of non-metal materials were selected ZrO₂ ceramics (ZrO₂, fine-ground, Saint Gobain, Turnov, Czech Republic), polyethylene (PE, Beznoska Kladno, Czech Republic) and carbon/carbon composite (C/C composite, Institute of Rock Structure and Mechanics AV CR, v.v.i., Prague, Czech Republic). All surface treatments of materials (polishing, sand-blasting, etching, plasma-spraying) were provided by the producer. The tissue-culture-grade polystyrene (TCPS, Nunc A/S, Roskilde, Denmark) was used as a positive control in order to compare cultivation properties of the tested materials with this “gold” standard. TCPS represents a material with properties optimised for the cultivation of eukaryotic cells *in vitro*.

For the osteoblast cultivation, small round plates of 30 mm diameter and 1mm thickness were used. Square plates (20 x 10 x 1mm) were used for the measurement of the surface free energy, wettability and surface roughness. Samples were washed in deionised water and in ethanol in an ultrasonic bath and subsequently autoclaved before use for cell cultivation as well as for the characterisation of surface properties.

2.2 *Surface characterization*

The *surface roughness* of samples was measured on the Hommel tester 1000T device (Hommelwerke, Schwenningen, Germany) with a transducer for relative measurements within the scope of 100 μm . The track net length was 4 mm and three samples of each material were analysed. Each sample was measured three times in longitudinal as well as in transversal direction to the direct axis of the sample. The following basic surface characteristics were monitored: R_a – a mean arithmetic average of the profile height; R_z – an average mean profile height in five measured sections; R_{max} – a maximum individual depth of roughness from the five measured sections.

The *surface free energy* γ was estimated by the measurement of the static contact angle (θ) in three different solvents (water, formamide, diiodomethane) using a sessile drop method (video-camera based instrument OCA 20, Dataphysics, Filderstadt, Germany). An average value of θ obtained from the Young-Laplace fitting approach was used for calculation of the surface free energy γ and its polar γ^p and disperse γ^d component according to the Owen-Wendt-Rabel-Kaelble (OWRK) method (Owens 1969).

The *advancing* (θ_A) and *receding* (θ_R) *contact angles* were estimated by the dynamic Wilhelmy plate method (tensiometer Kruss K12, Hamburg, Germany) in water (Andrade et al 1985).

The *surface topography* was monitored using VEGA Plus TS 5135 (Tescan s.r.o., Brno, Czech Republic) scanning electron microscope. The micrographs of tested implant materials are presented in Fig.1.

2.3 *Osteoblast cultivation*

Human osteoblasts (NHOst, Cambrex Bio Science Walkersville, Inc., Walkersville, USA) were cultivated up to the 3rd passage. The tested implant materials were put into a six-

well dish (Nunc A/S, Roskilde, Denmark). Osteoblasts were inoculated on surfaces at density of 15000/cm² and cultured in the OGM BulletKit medium (Cambrex Bio Science Walkersville, Inc., Walkersville, USA) at 37 °C / 5% CO₂ for 4 days. The differentiation of osteoblasts was monitored using the OGM Differentiation SingleQuots set (Cambrex Bio Science Walkersville, Inc., Walkersville, USA). For further estimations, the harvested cells and the culture medium were used.

2.4 *Proliferation and synthetic cell activity*

The proliferation of osteoblasts was established from the mitochondrial oxidising activity of the cellular monolayer cultured on tested materials using the Laughton MTT test (Laughton 1984) after 4 days of cultivation. In parallel, the same MTT test was performed on the concentration series of osteoblasts in order to obtain a calibration curve (Laughton 1984, Mosmann 1983) and the obtained values of absorbance were embedded directly into the software program of the ELISA reader. In this way, absorbance of the tested samples was automatically re-counted for cell number per millilitre of the medium what makes possible more illuminating overview of the cell proliferation in the case of a wide range of the tested samples.

The synthetic cell activity was monitored by evaluation of the selected mediators expressed to the cultivation medium after four days of cultivation. Bone alkaline phosphatase (BAP) isoenzyme was established immunochemically by the ELISA method (Metra Biosystems, CA, USA) and the multi-potent transforming growth factor beta (TGF-β) by an Immunoassay Kit (BioSource International, Camarillo, USA). The levels of mediators of the immune response, i.e tumor necrosic factor alpha (TNF-α) and interleukine 8 (IL-8), were established by the Immulite analyser (DPC, Los Angeles, USA). The evaluation of the expressed adhesive vascular cell adhesion molecule 1 (VCAM-1) was done using the “sandwich” enzyme-immuno-sorbent analysis with a commercially available kit (Bender,

MedSystems, Vienna, Austria); the MMP-1 metalloproteinase levels were established using the enzyme immuno-sorbent analysis kits (NOVITEC, Vienna, Austria).

2.5 *Blood clot coagulation analysis*

The qualitative analysis of the blood clot formation was carried out on all tested samples after 2, 3, 4 and 5 minutes incubation of the surfaces with blood. For a quantitative analysis, the incubation time of materials with blood was set down for 4 minutes and analysis was performed for Ti-polished, Ti-etched, Ti-plasma-sprayed, ZrO₂ ceramics and stainless steel samples.

The samples were put into a 24-well dish (Nunc A/S, Roskilde, Denmark) under sterile conditions. The wells were infused with 3 ml of a freshly drawn whole rabbit blood, covered with a lid and left standing. After the selected incubation time, the samples were taken out, rinsed in a saline solution, fixed in glutaraldehyde, dried and coated with gold. Afterwards, the samples were investigated by scanning electron microscopy (JSM 5500 LV, Tokyo, Japan) (Steinberg et al 1992). The surfaces were analysed using magnification 800 in meander-like patterns because of the sample size. Each side of the sample surface was divided into 16 fields to better describe the clot formation on whole sample surface. The numbers of elements adhered onto the surface, clusters formed by the element aggregation, created fibres and/or developed meshes were statistically evaluated.

2.6 *Statistical evaluation*

The MTT-test for proliferation assay was repeated twice, always with four parallel samples for each surface type. The determination of tissue mediators was done as follows: The cultivation medium from 4 parallel samples of each tested surface was pooled. Then, the ELISA analysis was done using the pooled samples from two separated cultivations, always with two parallel assays using one ELISA kit. Therefore, the statistical analysis of obtained

data was not performed. For the evaluation of the surface free energy (γ), 5 plates of each material were used for particular measurements; every sample was characterised by 6 drops for individual solvents. Five parallel samples of each material were used for all measurements of the dynamic contact angles. Two parallel samples of each material were used for the evaluation of the surface roughness; the measured values were processed using basic statistical tools. The blood clot formation was evaluated using 3 samples of each material and 16 fields on each sample side were viewed and analyzed using Kruskal-Wallis and Mann-Whitney test.

3. RESULTS

3.1 Roughness, surface free energy and wettability of implant materials

The selected parameters of the *surface roughness*, namely R_a , R_z and R_{max} , are presented in Tab.1. Based on the main monitored parameter R_a , the surfaces were divided to surfaces with the polishing, the ablation (i.e. machining, sand-blasting, etching) and the deposition (i.e. plasma-spraying of titanium) surface treatment.

The polished materials (titanium, CrCoMo alloy, stainless steel) and the gently machined ZrO_2 ceramics represent homogeneous surfaces of R_a ranged from 0.07 to 0.225 μm and standard deviation of 10%, what is a value usually observed for surfaces prepared by this surface treatment (Ponsonnet et al 2003). The R_a of the surfaces with the ablation treatment ranges from 0.48 to 3.54 μm with the standard deviation among 10 - 20 %. The sand-blasting led to the increased surface inhomogeneity probably due to a different extent of abrasion in the course of individual treatment cycles. In contrast, the etching provided a relatively homogeneous surface as a result of a constant composition of the etching solution during the treatment. The plasma-deposition of an upper titanium layer onto titanium resulted into an inhomogenous surface with the highest roughness of $R_a = 14,5 \mu m$. The C/C composite

(Pešáková et al 2000) represents also a highly inhomogeneous surface with the standard deviation of R_a overpassing 20 % as a result of the used modification.

The *surface free energy* (γ) and its disperse (γ^d) and polar (γ^p) components were determined for the surfaces with low roughness. The results are presented in Tab.2 where materials are ordered according to the decreasing γ^p . The values of γ ranged among 33.74 and 47.08 mN/m; the highest γ was determined for titanium (47.01 mN/m) and C/C composite (47.08 mN/m). The γ^d was considered very similar for all tested materials (except C/C composite) whereas the significant differences were observed for γ^p , which is considered as an important factor affecting biological interactions. The highest γ^p was determined for the polished titanium, followed by ZrO₂ ceramics, stainless steel and CrCoMo alloy whereas γ^p of non-metal surfaces PE and C/C composite approximated to zero.

The surface *wettability* was measured by a dynamic Wilhelmy plate method which allows calculation of average values of contact angles during the sample immersing into and withdrawing from a liquid across the whole surface. This method allows minimize the impact of the surface roughness on contact angles what often occurs when optical methods are used. All measurements were performed on the samples after autoclaving since it is known that sterilization processes can significantly affect the surface properties. Indeed, autoclaving led to the increase in contact angles approximately for 7 degree in our study (data not presented) what is consistent with the findings of Pegueroles (Pegueroles et al 2008). In the sake of clarity, only the advancing contact angle θ_A (Fig.2), generally considered as an overall parameter of wettability, is discussed in the present work. More detail discussion about both advancing and receding contact angles is presented in our previous work (Pešáková et al 2007).

Based on wettability (Fig.2), the materials were divided into following groups: the surfaces with a moderate hydrophilicity, the hydrophobic metal surfaces and the hydrophobic

non-metal surfaces. Ti-based materials and ZrO₂ ceramics with $R_a < 4 \mu\text{m}$) exhibit the average contact angle θ_A below 70°. This relatively moderated θ_A results from the presence of oxides of titanium or zirconium at the upper layer. The highest observed wettability for the Ti-etched surface is caused by the presence of the upper hydrated TiO₂ layer formed during the surface treatment by the hydrochloric acid/ hydrofluoric acid mixture.

The metal alloys (θ_A upper 80°) and the Ti-plasma-sprayed surface ($\theta_A = 108^\circ$) represent metal hydrophobic surfaces. An observed low wettability of Ti-plasma-sprayed in comparison with other Ti-based surfaces is a result of the high surface roughness (Tab.1). On the other hand, higher θ_A of the machined Ti6Al4V surface ($\theta_A = 83^\circ$) than that of the neat machined Ti ($\theta_A = 73^\circ$) is caused probably by the addition of alloying elements into titanium, and hence a different representation of oxides on the sample surface. Finally, non-metal surfaces are characterized by θ_A over 90°; no significant effect of the surface roughness on wettability was observed in this group.

3.2 *Proliferation of osteoblasts on implant materials*

The number of proliferating cells after four-day cultivation was determined following the cell mitochondrial activity. Fig.3 shows the tested surfaces ordered by a decreasing cell number together with the corresponding surface roughness (R_a).

No distinct differences in proliferation were found among the most of the particular surfaces; however, based on the cell number, tested surfaces were divided into three groups. The osteoblasts proliferated best on the control TCPS and Ti-based materials (the first group of materials). These surfaces also exhibited the highest surface roughness exceeding 3 μm in this study and γ^p higher than 8.88 mN/m (Tab.2). The second group of materials with lower proliferation includes metal materials and ZrO₂ ceramics treated with polishing or machining and the surface roughness below 1 μm . Besides Ti-polished, the values of γ^p ranged from 2.5

to 7.55 mN/m in this group. The least suitable surfaces for osteoblasts cultivation are surfaces included in the third group, namely the CrCoMo alloy of $\gamma^P = 2.46$ mN/m and non-metal materials PE and C/C composite with very low values of the polar component γ^P nearing zero.

3.3 *Metabolic activity of osteoblasts*

The levels of selected tissue mediators expressed by cells were determined from the cultivation medium after four days of the cultivation. Three types of the cellular synthesis were characterised: a) the osteoblast activity (BAP and TGF- β assays), b) the production of metalloproteinase collagenase 1 (MMP-1), and c) inflammatory mediators (TNF- α , IL-8, soluble VCAM-1). The results are presented in the charts (Fig.4 -7), where tested materials are ordered on the x axis according to the decreasing proliferation as shown in Fig.3.

3.3.1 *Synthetic activity of osteoblasts and production of MMP-1*

The production of BAP and TGF- β are shown in Fig.4 and Fig.5. There was an obvious tendency towards an increased expression of both markers with decreasing proliferation. The lowest BAP and TGF- β production was detected on the TCPS control and on Ti-plasma-sprayed. Concerning the group with the highest proliferation (Fig.3), the Ti-etched surface initiated the highest production of osteoblast activity markers. The surfaces with low proliferation, i.e. CrCoMo alloy and PE, exhibited several times higher levels of both markers than TCPS. In the case of the C/C composite, the measured values of BAP and TGF- β did not reach a lower detection limit of the used method.

The expression of the metalloproteinase MMP-1 is depicted on Fig.6. The MMP-1 production on the Ti-based surfaces with the highest proliferation (Ti-plasma-sprayed, Ti sand-blasted) is lower than on the TCPS control whereas osteoblasts on all non-metal materials (i.e. ZrO₂, PE and C/C composite) produced significantly more MMP-1 than on

TCPS. Again, the low proliferation was accompanied by the increased production of metalloproteinase.

3.3.2. Expression of markers of inflammatory response

The expression of *tumor necrosis factor- alpha* (TNF- α) is shown on Fig.7. The TNF- α production on all studied materials significantly exceeded the TCPS control what we found consistent with the fact that TCPS is the material specially developed for the best cell viability. It is also evident that the TNF- α expression on the samples of a high proliferation depends primarily upon the material origin in our study. Surprisingly, the highest TNF- α value were observed on all Ti-based materials, i.e. Ti-polished, Ti-plasma-sprayed, Ti-sand-blasted, Ti-machined and Ti6Al4V-machined. A lower TNF- α production was observed on Ti-etched, where the chemical composition of the surface was altered by etching, and on ZrO₂ ceramics. The low TNF- α value comparable with TCPS were detected for C/C composite, what is in contrast to the TNF- α production on C/C composite by fibroblasts (Pešáková et al 2007).

The next determined markers were the inflammatory chemokine *interleukine 8* (IL-8) (Fritz et al 2002) and the soluble human *vascular cell adhesion molecule 1* (VCAM-1) attracting leukocytes to damaged tissue, both expressed by cells into the cultivation media (Sinha et al 1996). The production of IL-8 (Fig.8) on all materials was a relatively equable and higher than on TCPS. The effect of the surface treatment was not obvious for osteoblasts in the contrast to fibroblasts where a significantly higher IL-8 production on non-metal surfaces was observed (Pešáková et al 2007). The production of VCAM-1 oscillated around the TCPS control (Fig.9). Apparently, more pronounced increase in the VCAM-1 production was mediated also by low surface roughness on Ti-polished, stainless steel and/or CrCoMo alloy. For both markers, the measured IL-8 and VCAM-1 values on PE and C/C composite

were under a lower detection limit of the used analytical method, what could have reflected a low number of proliferating cells detected on these two surfaces.

3.4 The blood clot formation analysis

The qualitative description of the blood clot development on the implant surface with a prolonging incubation (2, 3, 4 and 5 min) with a rabbit blood is presented in Tab.3. The tested surfaces are listed according to the speed of the blood clot formation. The following categories were evaluated: adhered blood elements, clusters formed due to the element aggregation, formed fibrin fibres and/or developed meshes. The category dominating on the particular surface in the selected minute of incubation is noted in Tab.3.

The most rapid blood clot formation was observed on Ti-plasma-sprayed and on non-metal C/C composite and PE samples, where some clusters were already formed in the 2nd minute. The blood agglutinated with a similar rate and with the same dominant category in time intervals on all Ti-based surfaces. The slowest blood clot formation was observed on smooth samples of CrCoMo alloy, stainless steel, ZrO₂ ceramics and TCPS. Blood elements adhered onto TCPS and ZrO₂ ceramics were mostly round-shape with no signs of the platelet activation (Fig.10a).

A more detail quantitative analysis of the developed blood clot was performed for Ti-polished, Ti-etched, Ti-plasma-sprayed, ZrO₂ ceramics and stainless steel. The analysis was carried out after 4 minutes of the sample incubation with the rabbit blood. The same categories of the blood clot formation, as analysed for the qualitative analysis, were determined. Results are presented in Tab.4. Generally, the observed high standard deviation was a result of an inhomogeneous distribution of the investigated parameters on the surfaces.

The highest quantity of all investigated parameters, except clusters, was detected for the Ti-etched surface. This difference was statistically significant at the level $p=0.001$ according to Kruskal-Wallis analysis. Moreover, a dense fibrin mesh with entrapped various blood

elements was typically observed on this surface in many of scanned fields (Fig. 10c). The mesh was developed also on Ti-polished but with a statistically significant lower amount of elements and fibers than was observed on Ti-etched. The Fig.10b illustrates a frequent cluster formation with few fibers created on the Ti-polished surface.

The statistically significant differences among Ti-polished, Ti-plasma-sprayed, ZrO₂ ceramics and stainless steel surfaces were mainly in the formation of fibrin fibres. The Ti-plasma-sprayed and stainless steel surfaces induced formation of higher amount of fibres than ZrO₂ ceramics and Ti-polished ($p = 0.05$ according Mann-Whitney test). No statistically significant differences were observed between the Ti-plasma-sprayed and stainless steel surfaces as well as between the ZrO₂ ceramic and Ti-polished surfaces. However, it was observed using SEM (not presented) that fibrin fibres developed on Ti-plasma-sprayed were almost 10 times longer than fibers on stainless steel.

4. DISCUSSION

In this study we intended to tabularly demonstrate a main relation between surface characteristics and an early osteoblast response and the blood clot formation and thus to facilitate an orientation in a wide range of the implant materials already in a practical use. Besides Ti-based materials, materials not frequently used, such as CrCoMo alloy or the tested C/C composite (Pešáková et al 2005), were also included in to this study for better illustration of an effect of physico-chemical parameters on the cell behaviour. On behalf of better approaching to conditions in a real bone tissue, the human osteoblasts derived from a human connective tissue and from low passages were used for experiments. Two main parameters of an early cell response to an artificial support were studied, i.e. cell proliferation and the synthetic activity, with a respect to three general surface characteristics, i.e. the surface free energy, the surface roughness and wettability.

The tested materials were divided into three groups based on the decreasing osteoblast *proliferation* (Fig.3). Generally, the osteoblast proliferation decreased with a decreasing polar component γ^P of the surface free energy and with a decreasing surface roughness. In the group of the best proliferation (TCPS, Ti-plasma-sprayed, Ti-sand-blasted, Ti-etched), γ , γ^P and γ^D were not directly determined for rough Ti-based surfaces because of the impact of roughness on the contact angles. Yet if we take into account that γ is given by the surface properties based upon the physico-chemical interactions on the atomic level, γ obtained for the smooth Ti-polished surface may be applied with an approximation also to rough titanium surfaces. It concerns, in particular, the effect of high γ^P (15.97 mN/m) on the osteoblast proliferation on the Ti-plasma-sprayed and Ti-sand-blasted surfaces. In the case of the smooth control TCPS surface, we assumed that the supporting effect of the surface roughness on proliferation is compensated by high value of the polar component of the surface free energy ($\gamma^P = 27.06$ mN/m). An importance of γ^P is also clearly demonstrated by the third group of tested materials (CrCoMo alloy, PE and C/C composite) with γ^P near zero (Tab.2), where proliferation after four-day cultivation did not significantly exceed an initial number of cells inoculated on these surfaces.

The observed dependence of osteoblast proliferation onto commercial implant materials on γ^P is in accordance with data published in the literature until now. The surface energy was dominating factor in the cell adhesion strength and proliferation in the presence either absence of proteins (Schakenraad et al 1986). The enhancement of γ^P of the modified zirconia bioceramic played a significant role in the initial cell attachment, thus enhancing the cell growth (Hao et al 2004). Interestingly, higher γ^P of modified calcium phosphate surfaces caused increased osteoblast adhesion but lower protein adsorption (Dos Santos et al 2008). The effects of γ on the cell behaviour were observed also in several long-term studies. MG63 osteoblastic cells cultured on hydroxylated high-surface-energy Ti surfaces exhibited an

increased production of bone alkaline phosphatase and osteocalcin (Zhao et al 2005). The human trabecular osteoblastic cells expressed more collagen on the high-surface-energy hydroxyapatite compared to the low-surface-energy carbonate apatite, though the initial cell proliferation did not differ on both surfaces (Redey et al 2000). The relatively uniform initial proliferation was observed for materials inducing the highest cell number also in our study (see Fig.3, the first group).

The tendency to the enhanced osteoblast proliferation on *rougher* materials (Fig.3) is in the contrast to fibroblasts which preferred smooth surfaces (Pešáková et al 2007). However, this effect is not as dramatic as it was expected and as it is often mentioned in the literature. The deepest irregularities observed on the Ti-plasma-sprayed surface (the highest value of R_z , see Tab.1) surely provided a totally available surface area larger than others, but a significantly higher number of cells was not found on this surface. This is probably because the cells did not utilize an overall available surface and bridge the grooves as it was observed on model smooth and microgrooved polystyrene supports (Walboomers et al 1999). The osteoblasts cultivated on Ti materials with ultra-high roughness (18-74 μm) behaved similarly to those cultivated on the coatings already used in orthopaedics (Borsari et al 2005). The authors concluded that the roughness greater than cell dimensions does not enhance the cell response.

The *surface wettability* is concerned as one of the main factors reflecting an extent of cell adhesion onto the surface (Park et al 2001, Fujisato et al 1996). According to the literature, hydrophilic metal as well as non-metal surfaces are generally favourable for the short-term cell adhesion and proliferation for various cell types such as erythrocytes (Absolom et al 1985), lymphocytes (Groth et al 1994) or human epithelial cells (Allen et al 2006). The maximum adhesion and growth of hepatoma cells, osteoblasts, bovine aortic endothelial cells and fibroblasts on smooth poly(lactide-glycolide) based films appeared at

around water contact angles of 53 to 55° (Khang et al 2002) and these data could serve as an illustration of an extend of the appropriated surface wettability.

However, the contact angles usually observed for Ti-based surfaces vary between 70 to 90° based on the source of the Ti material. Therefore, a big interest is focused on a development of surface treatments leading to more hydrophilic surfaces recently. For example, the treatment of Ti6Al4V or stainless steel by the HPDL laser provided a decrease in θ by 10° and an increase in γ^P and thus the favourable response of human fetal osteoblast cells (Hao et al 2005, Hao et al 2005). The improved cell activity was observed on nanostructured Ti-based materials with wettability of 62° (Faghihi et al 2007) or on sol-gel-modified Ti6Al4V substrates with an extremely high wettability ($\theta = 18^\circ$), $\gamma^P = 30 \pm 3$ mN/m and roughness of $R_a = 0.1 \mu\text{m}$ (Advincula et al 2006). The enhanced mineralization was detected on model hydrophilic flat surfaces relative to hydrophobic ones (Lim et al 2008). Nevertheless, it is important to point out, that all surfaces discussed above are smooth surfaces with the surface roughness not exceeding $0.6 \mu\text{m}$, in comparison with rougher cementless direct bone bonding implants used in the clinic.

Based on the obtained results, wettability seems to have a minor effect on the osteoblast proliferation in our study. The group of materials with the highest proliferation includes the moderately hydrophilic TCPS and Ti-etched surfaces as well as the hydrophobic Ti-sand-blasted and the highly hydrophobic Ti-plasma-sprayed surfaces (Figs.2, 3). This is also in contrast to the fibroblast cultivation where the cells proliferated best on the moderately hydrophilic Ti-polished and ZrO₂ ceramic surfaces with $\theta_A < 70^\circ$ (Pešáková et al 2007). Thus, wettability seems to be an important factor significantly augmenting the osteoblast activity especially on smooth surfaces. However, the surface roughness, though not optimal for proliferation of cells, is highly required from a biomechanical point of view for a long-term successful function of the bone-embedded implants. According to clinical experiences, the

surface roughness leads to better distribution of a loading force onto the bone bed and limits the microcrack formation (Wiskott et al 1999).

The following studied parameter of an early cell response to an artificial support was the *synthetic activity of osteoblasts*. In the course of the synthetic phase of the cellular cycle, cells synthesize not only substances necessary for the cell multiplication, but also substances which affect and determine the future existence of the cells. The *bone alkaline phosphatase* (BAP) is a biochemical marker of the osteoblast phenotype in the stage of early differentiation, and hence also of bone formation and general osteoblast activity. This protein is also involved in the bone mineralisation process (Piattelli et al 1996). The *multi-potent growth factor* TGF- β has regulating effects on migration, proliferation and differentiation of cells, stimulates the extracellular matrix protein expression (e.g. the synthesis of collagen and fibronectin) and also restricts the synthesis of proteases and increases production of their inhibitors (Shaama 2005). TGF- β is also one of the most potent immunoregulatory molecules known. Studies using a simple TGF- β adsorption indicate that delivery of TGF- β to the tissue-implant interface can improve bone formation in the periprosthetic gap and can enhance bone ingrowth into porous coatings (Kloen 1997). The next markers of the osteoblast activity are matrix metalloproteinases (MMP), which catalyze degradation of extracellular matrix proteins and thereby play an important role in many physiological and pathological processes also including tissue remodelling, wound healing or inflammation (Pap et al 1999).

There was an obvious tendency towards an increased expression of BAP, TGF- β and MMP-1 with decreasing proliferation in our study. The increased expression of osteoblastic phenotype markers with the reduced cell proliferation was also found in *in vitro* studies using MG63 osteoblast-like cells (Borsari et al 2005, Boyan et al 2002). It is generally known that, in order to survive, most of the cells need to adhere. Therefore, the contact between individual cells or between the cell and the surface through a specific adsorption is highly required for a

successful cell growth *in vitro*. In the present work, the increased synthetic production observed on CrCoMo alloy and PE is explained as follow: with a low number of the proliferating osteoblasts, the cells try to compensate the deficit in their count by an increased synthetic activity aimed at, at the close, higher expression of extracellular matrix proteins. However, if the surface is not attractive enough for adhesion and proliferation of cells (C/C composite here), it can be assumed that adhered cells are not stimulated to exhibit the synthetic activity. In the group with the highest proliferation in our study, the Ti-etched surface initiated the highest production of osteoblast activity markers.

The expression of the tumour necrosis factor (TNF- α) as a main mediator of the inflammatory response was studied as well. The function of TNF- α should be viewed in three ways: as a stimulus for acute inflammation, as a chronic contributor to autoimmune disorders and/or as a local regulator of bone cell function involved in skeletal catabolic processes in pathophysiology of osteoporosis (Nanes 2003, Rifas 1999). Interestingly, it seems that higher TNF- α expression depends primarily on the material origin, namely Ti-based surfaces, in our study. To our knowledge, this phenomenon has not been mentioned in the literature until now. Among the materials involved in the group with the highest proliferation, the expression of both TNF- α and IL-8 was the lowest on the Ti-etched surface where the chemical composition of the titanium surface has been altered by etching.

The way how the implant surface induces the formation of the blood clot is a very important characteristic of the material, because the clotting blood is the first connection site of the implant and a living host tissue (Park et al 2001). One of the triggers activating coagulation is damage of blood vessels due to surgery. The contact with the implant surface is an additional trigger which starts coagulation cascade and the attractiveness of the implant surface to blood could play a very important role in speed of osteointegration as well as in a sealing of wound. Apparently, the surface roughness seems to be the main factor regulating

the blood coagulation in our study (Tab.3). This dominating effect is demonstrated also by relatively fast formation of the blood clot on C/C composite, which is known, in contrary, to be an extremely tromboresistant material. The used C/C composite material unfortunately exhibited big irregularities on the surface (Fig. 1i) as a result of the final surface treatment. The slowest clot formation was detected on the materials with low surface roughness (TCPS, ZrO₂, CrCoMo, stainless steel, Tab.3) and with no evident effect of wettability or γ^P . On the other hand, the mesh was formed on the smooth Ti-polished surface. Thus we can assume that the particular surface composition/properties, which are not reflected by wettability or γ^P in our study, should be also a trigger of coagulation in the case of smooth surfaces. For example, platelets can also be activated by smooth negatively or positively charged surfaces (Lee et al 1998).

Among the surfaces with higher proliferation (Fig. 3, group 1 and 2, see Part 3.2), the mesh was formed only on all Ti-based surfaces. The highest quantity of all investigated parameters (blood elements, clusters of aggregated elements, fibrin fibres) was detected for the Ti-etched surface (Tab.4). A lower number of formed clusters probably resulted from more homogeneous adhesion of platelets on this surface potentiated by fast platelet activation. This led to very early formation of the fibrin fibres and, consequently, to the development of the mesh. Differences in the fibrin fibre category (Tab.4) observed among Ti-polished, Ti-plasma-sprayed, ZrO₂ ceramics and stainless steel could be explained by combination of the surface and bulk material properties. A lower amount of fibres on the Ti-plasma-sprayed surface could be caused by high surface roughness where formed short fibers were not long enough to bridge the deep irregularities of the surface and thus retarded formation of longer fibrin fibers. Further, we suppose that low amount of fibres developed onto Ti-polished could result from formation of the dense mesh especially around the clusters of aggregated platelets. The stainless steel induced higher formation of the fibrin fibres probably due to material

properties whereas ZrO₂ ceramics is an inert material with adhered but not activated round-shape platelets on the surface (Fig.10a).

From the practical point of view, the study was aimed at looking for the commercially available implant material with the fastest healing. The shortening of healing is an important factor not only from economic aspects (treatment time and costs in the hospital), but primarily from viewpoint of the patient comfort. Concerning e.g. dental applications, the immediate loading concept requires the quickest healing in first few days to stabilize the implant and makes a shield against micromotions. The blood clot can be considered as a natural scaffold augmenting the cell migration and seeding on the bone-implant interface. Therefore, not only a satisfactory high osteoblast proliferation and synthetic activity related to specific surface characteristics should be main factors for selection of an appropriate implant material. However, it is not completely clear from clinic practice, which of these factors is more important for fast healing. Therefore it is desired to search for an optimal intersection point for high osteoblast proliferation, low fibroblast proliferation and fast blood clot formation.

Beyond all convenience of the rough Ti-plasma-sprayed surface, which is characterized especially by the highest proliferation in this study, problems arising during implantation due to bigger amount of bone wear debris evoking immune response cannot be omitted. Also slower blood clot formation makes this surface treatment not optimal. Further, in contrast to our expectation, ZrO₂ ceramics did not belong to the most suitable candidates, neither in term of proliferation and the synthetic activity nor in term of a sufficiently fast blood clot formation. Nevertheless, from the point of view of an appearance of allergy to titanium materials and because of low wear debris, ZrO₂ ceramics is still the promising implant material. The Ti-polished surface represents an advantageous material in term of the blood clot formation but due to its low surface roughness and the moderate wettability is also well colonized by fibroblasts (Pešáková et al 2007) which are undesirable cells on the bone-implant interface. The Ti-etched surface seems to be the most convenient compromise in term

of the osteoblast proliferation and the synthetic activity and the fast blood clot formation due to the TiO₂ structure yielding a high γ^p and the etching treatment leading to the convenient wettability, the surface charge and the surface roughness.

5. CONCLUSIONS

The wide range of commercially available bone implant materials was classified by proliferation and the synthetic activity of osteoblasts and the blood clot formation with respect to the surface properties such as roughness, the surface free energy and/or wettability. Based on proliferation, tested materials were divided into 3 groups. The results indicate that the important factor supporting the osteoblast proliferation activity is the polar part of SFE (γ^p) supported by the surface roughness, here exceeding 3.5 μm (1st group). In the 2nd group of materials (smooth surfaces), the impact of roughness to proliferation is compensated by higher γ^p ($\gamma^p > 8 \text{ mN/m}$). Finally, the lowest osteoblast proliferation (3rd group) was detected on materials with the almost zero γ^p (CrCoMo alloy, PE, C/C composite).

The markers of osteogenesis (BAP, TGF- β) were always higher on tested surfaces than on the TCPS control and were inversely related to the cell number independently on the surface roughness. A dramatically higher expression was detected on surfaces with low proliferation and with γ^p nearing zero, what reflected inconvenient conditions for the cell adhesion. The production of inflammatory mediators (TNF- α , Il-8) is distinctively higher on all surfaces in comparison to TCPS and there is a tendency to an increased TNF- α production especially on the Ti-based surfaces with no respect to the surface roughness.

The analysis of the blood clot formation showed the rapid coagulation on rough titanium surfaces whereas materials with smooth surfaces agglutinated blood more slowly. No evident effect of wettability or the surface free energy on coagulation was observed.

Finally, the titanium surface modified with the etching treatment seems to be the most suitable candidate for healing into the bone tissue among tested materials due to high osteoblast proliferation, the highest production of osteogenesis markers, low production of inflammatory cytokines and due to the most intensive formation of the blood clot.

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- Fig.1** SEM micrographs of tested implant materials: a) Ti-polished, b) Ti-sand blasted, c) Ti- etched, d) Ti-plasma-sprayed, e) stainless steel, f) TCPS, g) ZrO₂, h) PE, i) C/C composite (the objects on the photograph of the C/C carbon surface correspond to the growth cones after the chemical vapor deposition of the pyrolytic carbon), k) Ti-machined, l) TiAl4V-machined, m) CrCoMo alloy
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- Fig.3** Cell number expressed as a number of osteoblasts attached to the surface (☒) after four days of the cultivation compared to the surface roughness (■). Osteoblasts were inoculated on the surfaces at density of 15000/cm². The tested materials are aligned on the x-axis according to the decreasing cell number
- Fig.4** Production of BAP by osteoblasts on tested surfaces after four days of the cultivation. The materials are aligned on x-axis according to the decreasing cell number. NA – not analyzed, under detection limit of the used method. Osteoblasts were inoculated on the surfaces at density of 15000/cm².
- Fig.5** Production of TGF-β by osteoblasts on tested surfaces after four days of the cultivation. The materials are aligned on x-axis according to the decreasing cell number. NA – not analyzed, under detection limit of the used method. Osteoblasts were inoculated on the surfaces at density of 15000/cm².
- Fig.6** Production of MMP-1 by osteoblasts on tested surfaces after four days of the cultivation. The materials are aligned on x-axis according to the decreasing cell number. Osteoblasts were inoculated on the surfaces at density of 15000/cm².

Fig.7 Production of TNF- α by osteoblasts on tested surfaces after four days of the cultivation. The materials are aligned on x-axis according to the decreasing cell number. Osteoblasts were inoculated on the surfaces at density of 15000/cm².

Fig.8 Production of IL-8 by osteoblasts on tested surfaces after four days of the cultivation. The materials are aligned on x-axis according to the decreasing cell number. NA – not analyzed, under detection limit of the used method. Osteoblasts were inoculated on the surfaces at density of 15000/cm².

Fig.9 Production of VCAM-1 by osteoblasts on tested surfaces after four days of the cultivation. The materials are aligned on x-axis according to the decreasing cell number. NA – not analyzed, under detection limit of the used method. Osteoblasts were inoculated on the surfaces at density of 15000/cm².

Fig.10 SEM micrographs of samples treated with rabbit blood for 4 minutes: a) ZrO₂ ceramics – round-shape elements are present, b) Ti-polished - elements and individual fibrin fibres are present, c) Ti-etched - clusters and fibrin mesh are present

Fig.1

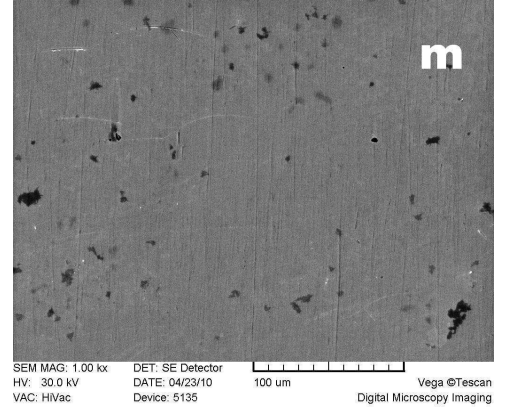
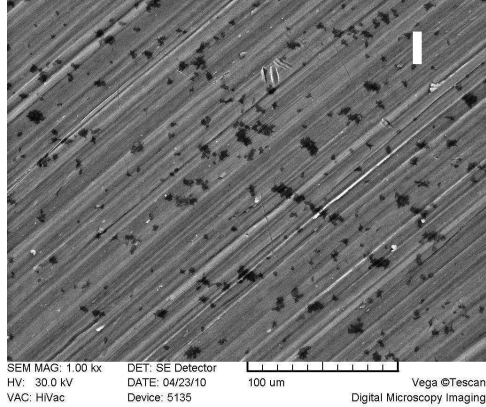
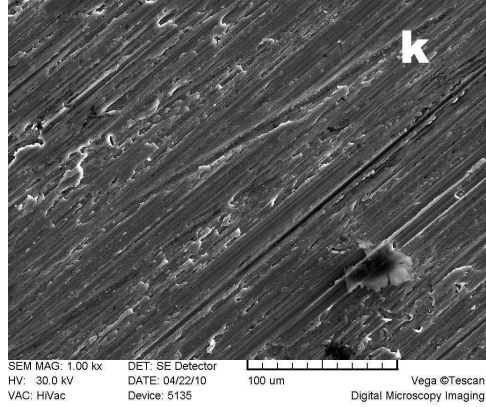
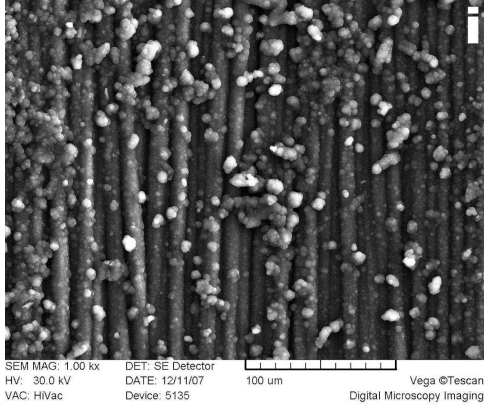
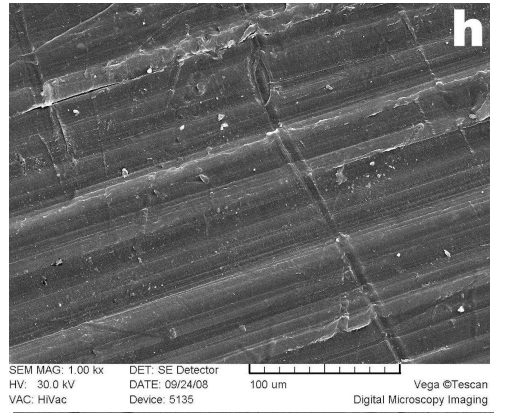
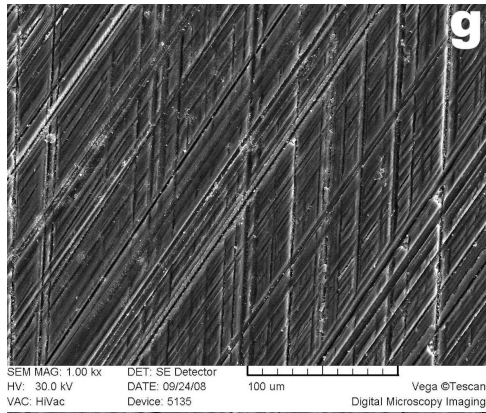
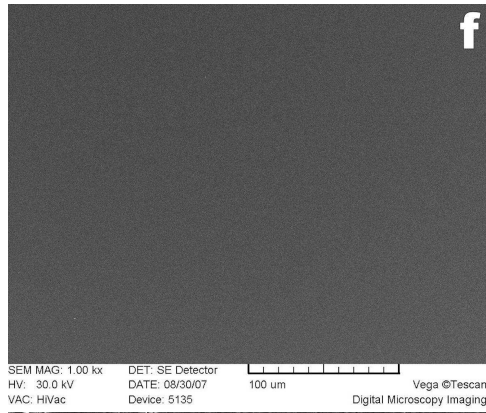
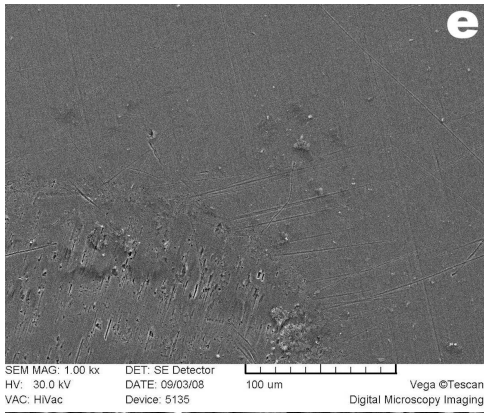
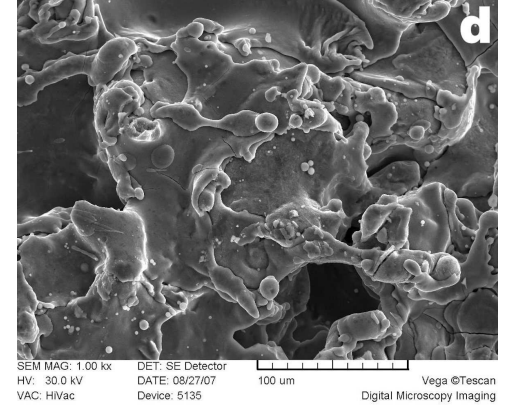
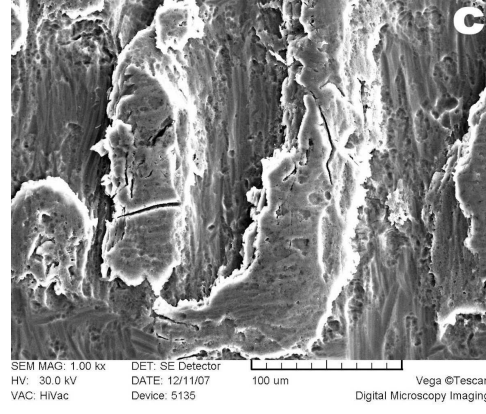
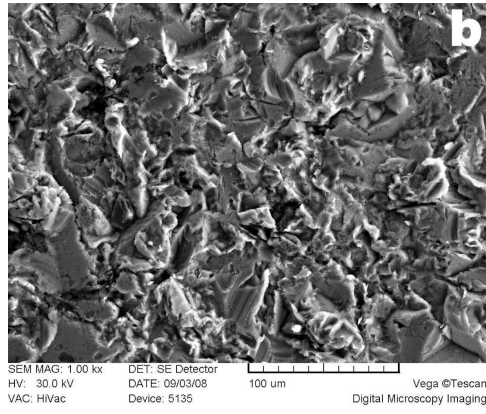
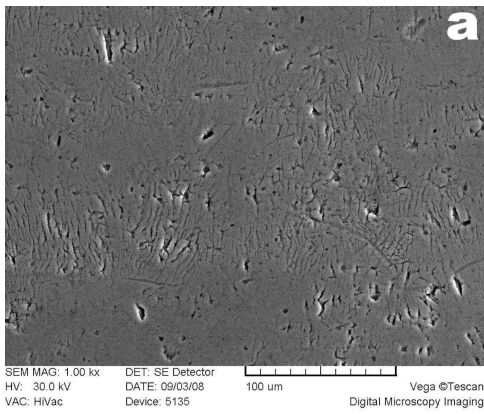


Fig.2

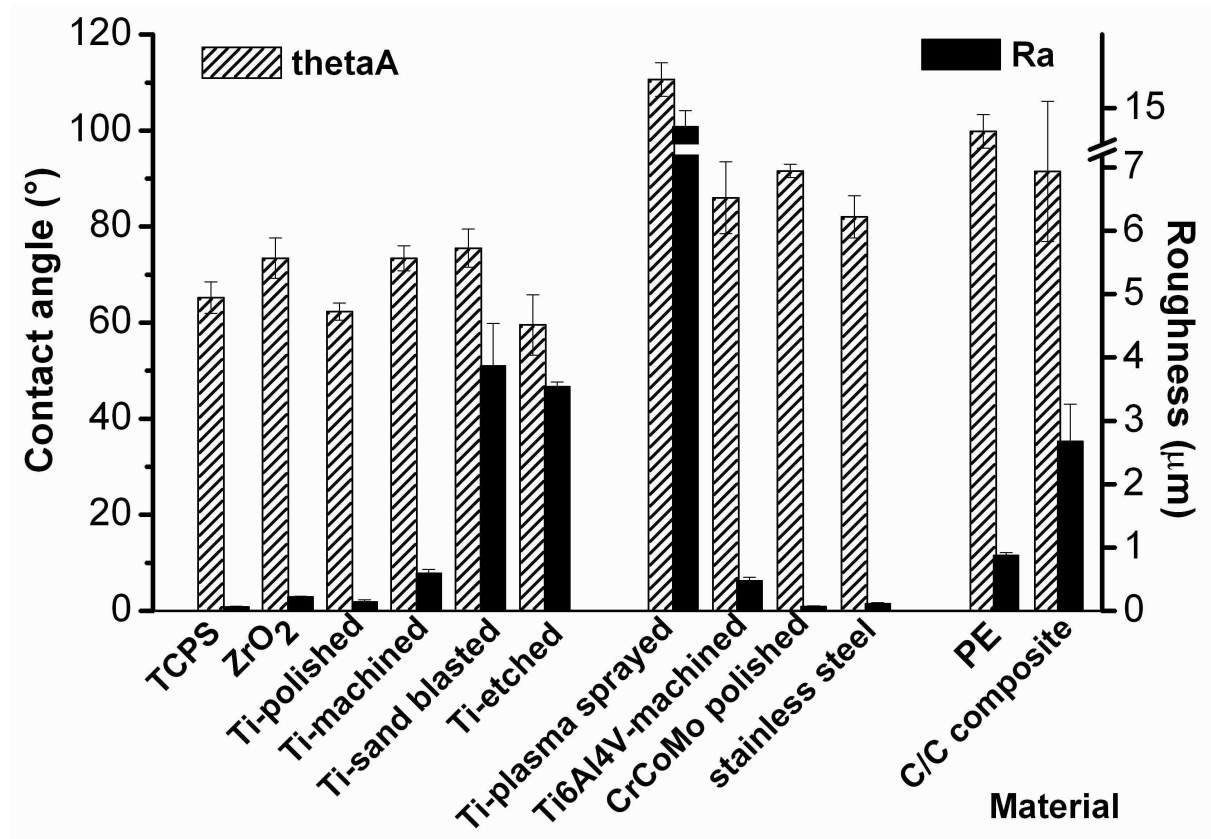


Fig.3

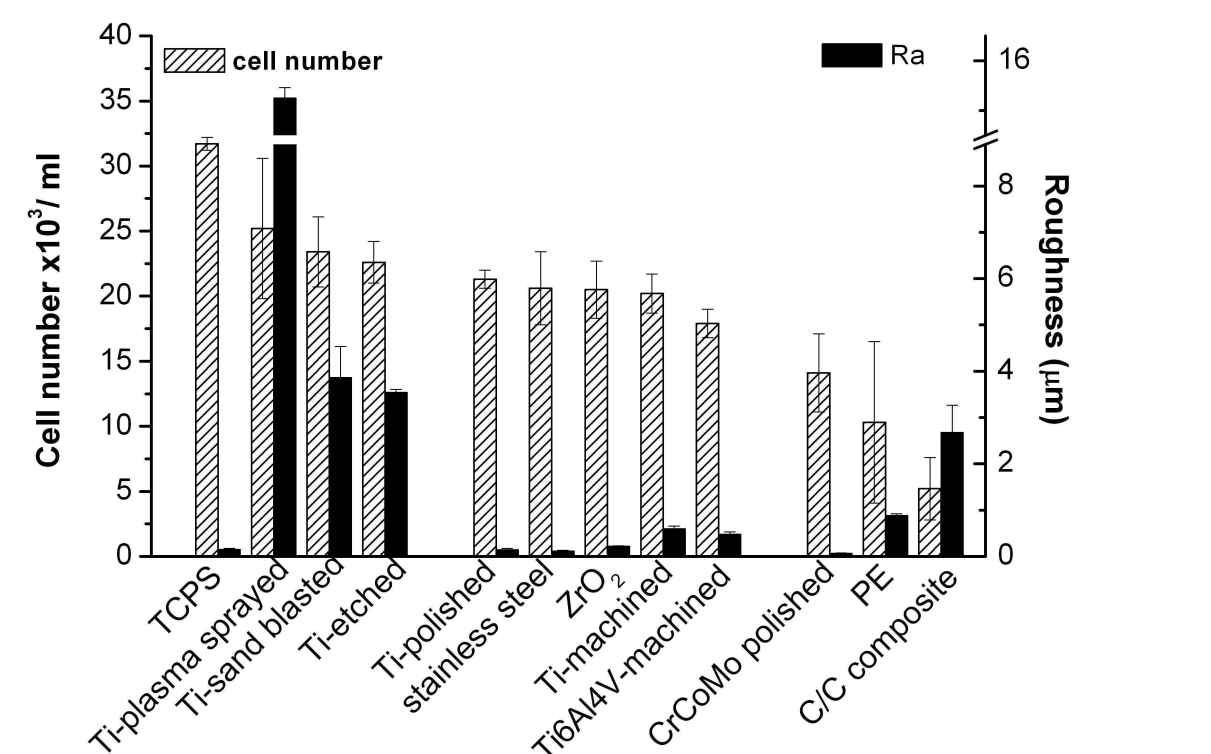


Fig.4

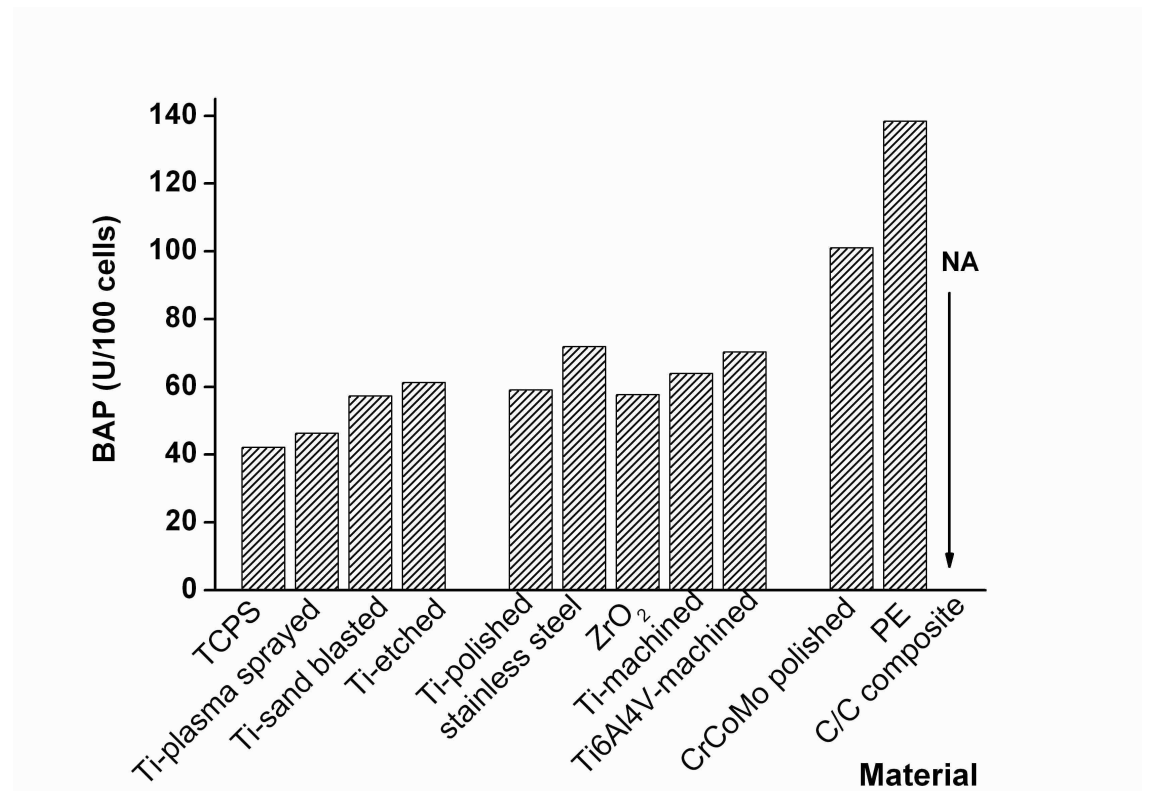


Fig.5

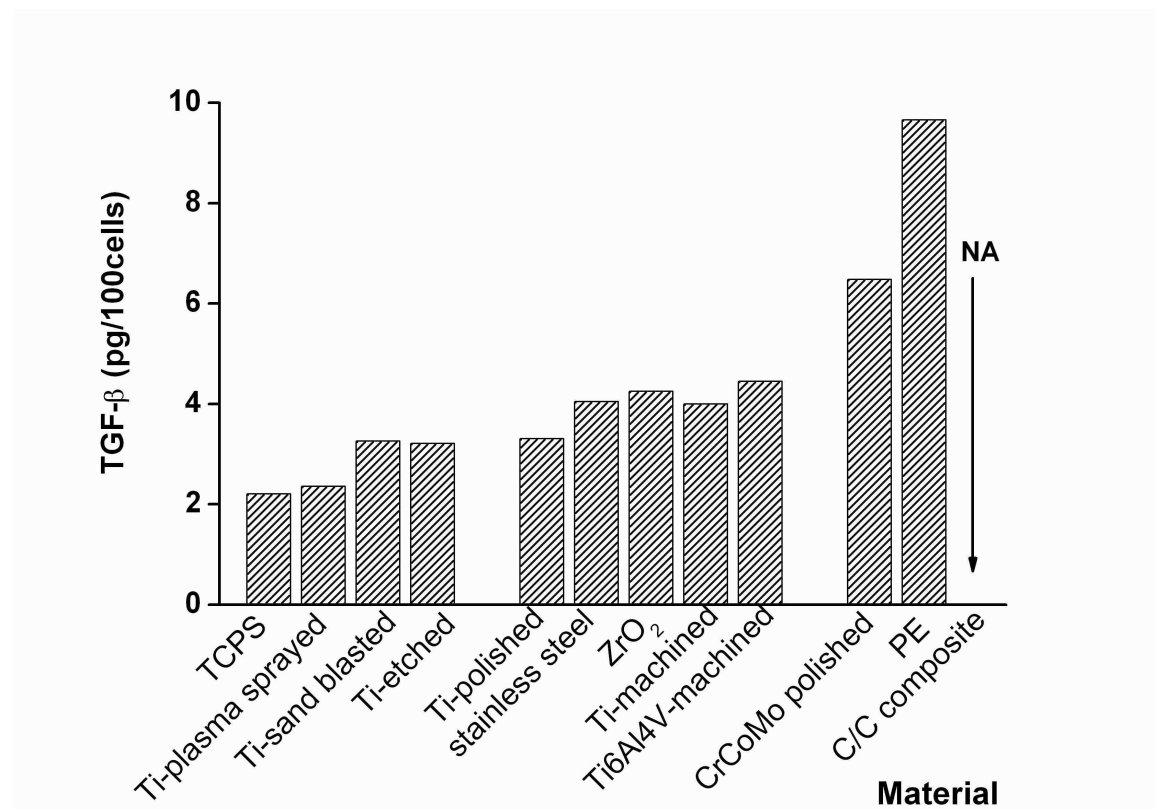


Fig.6

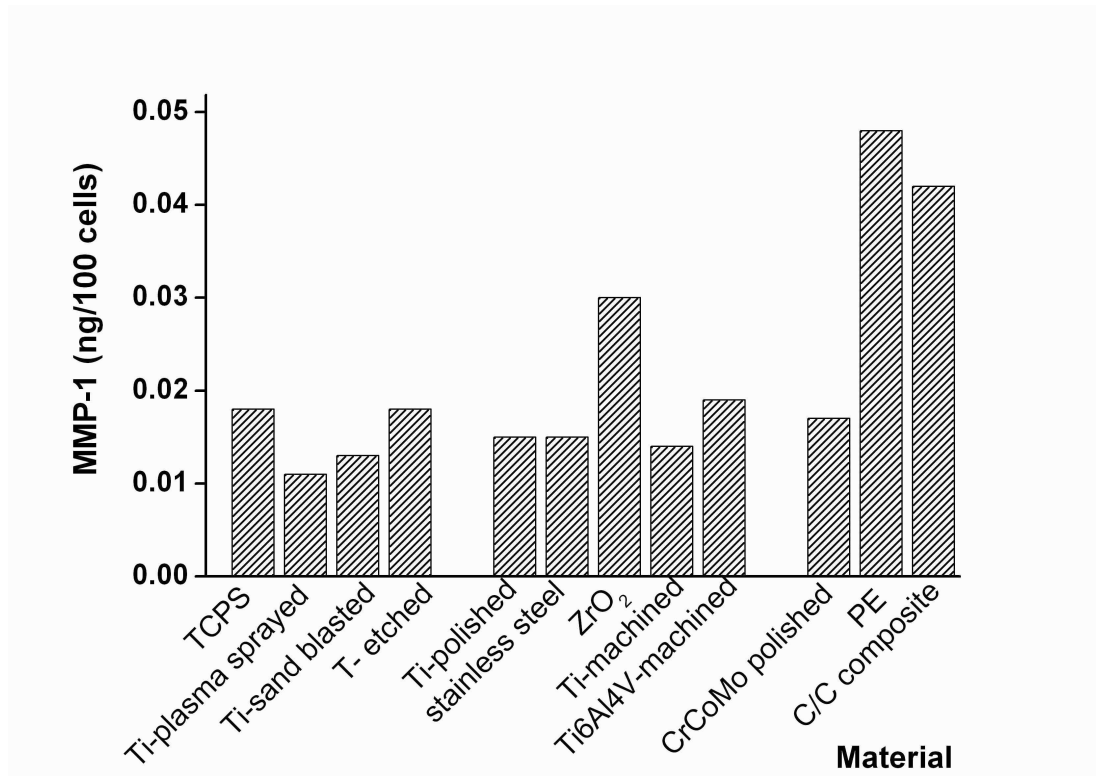


Fig.7

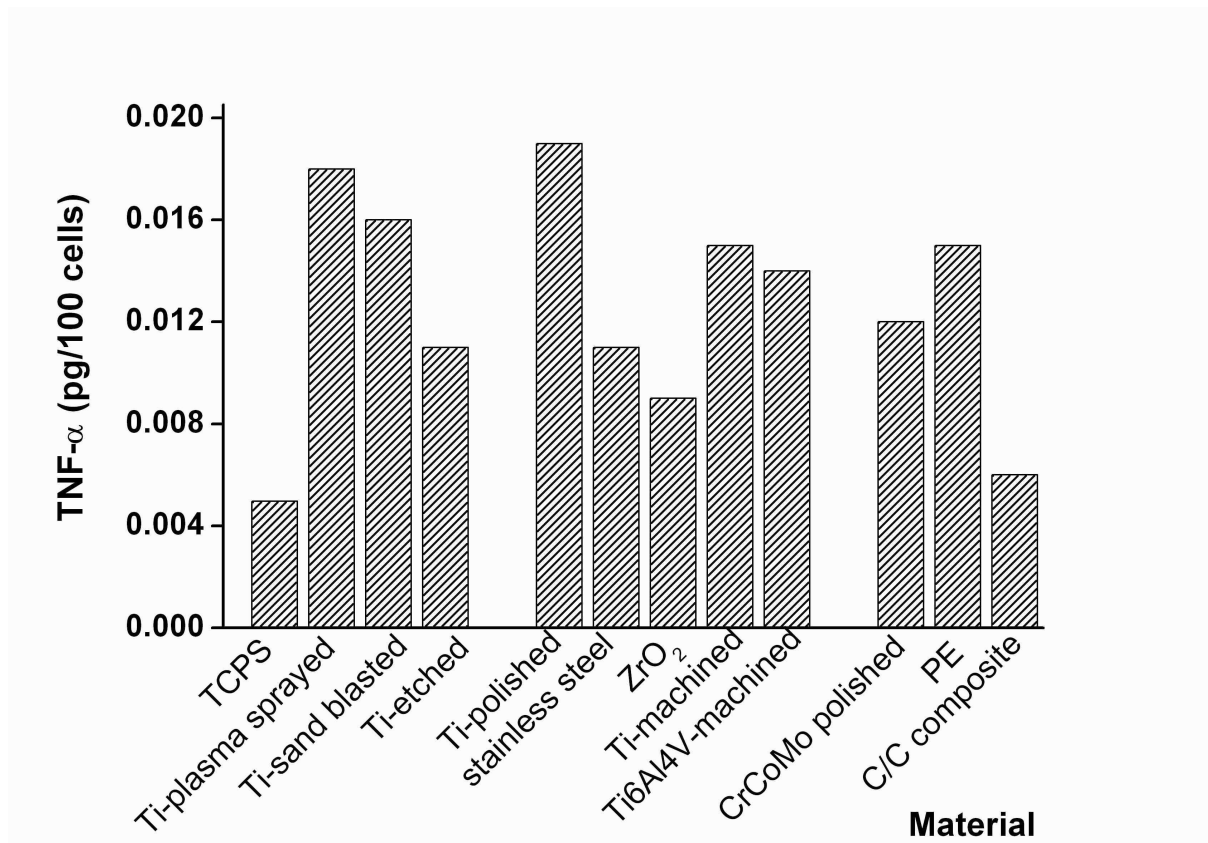


Fig.8

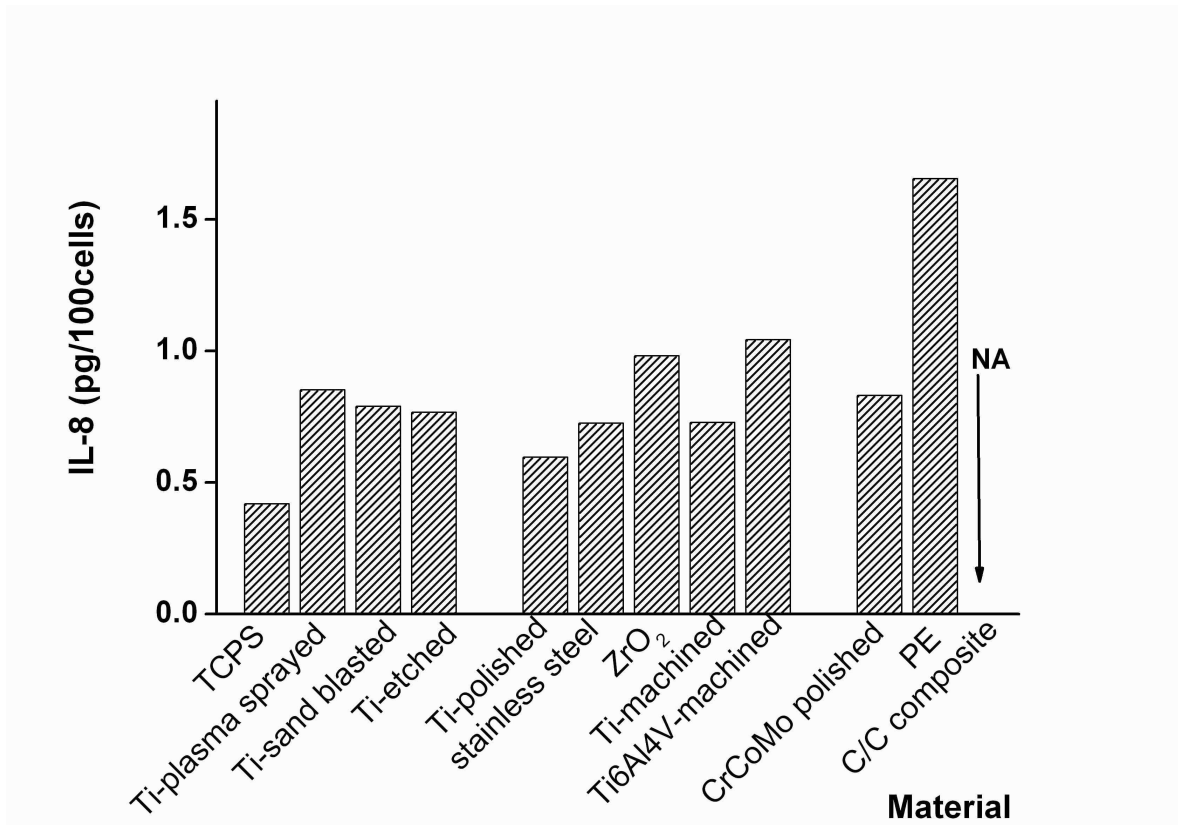


Fig.9

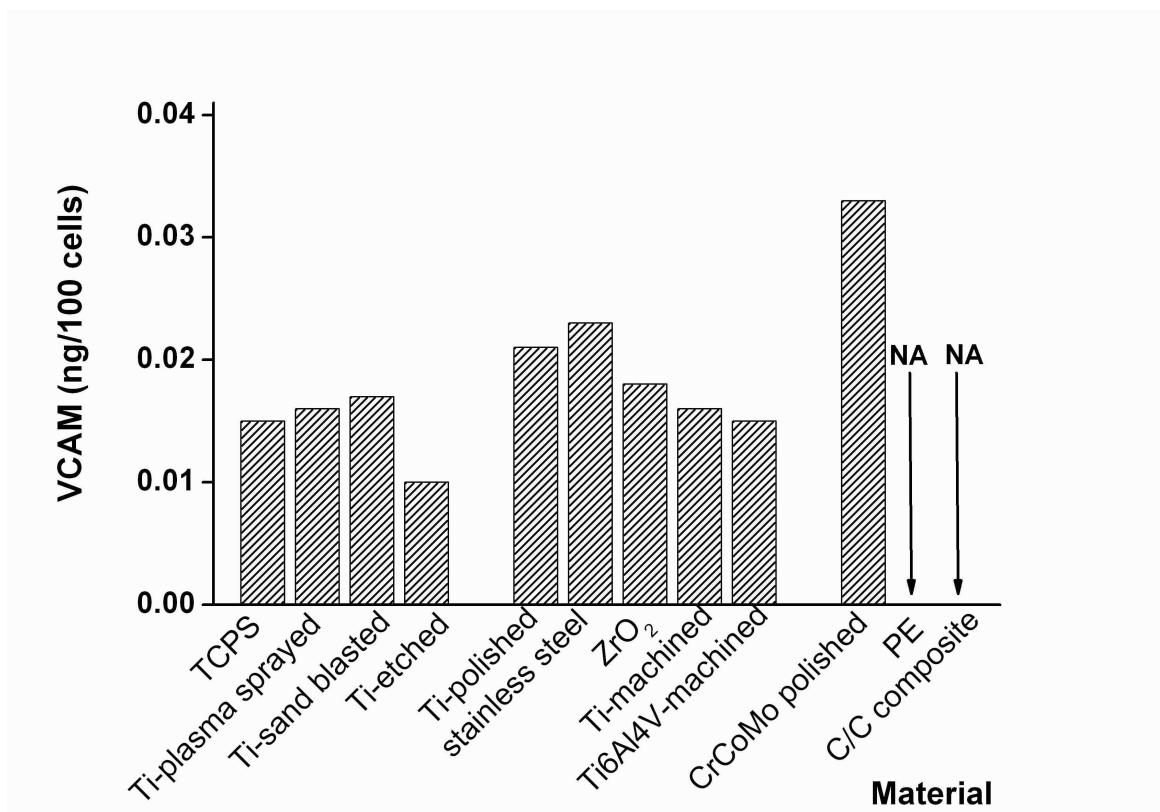
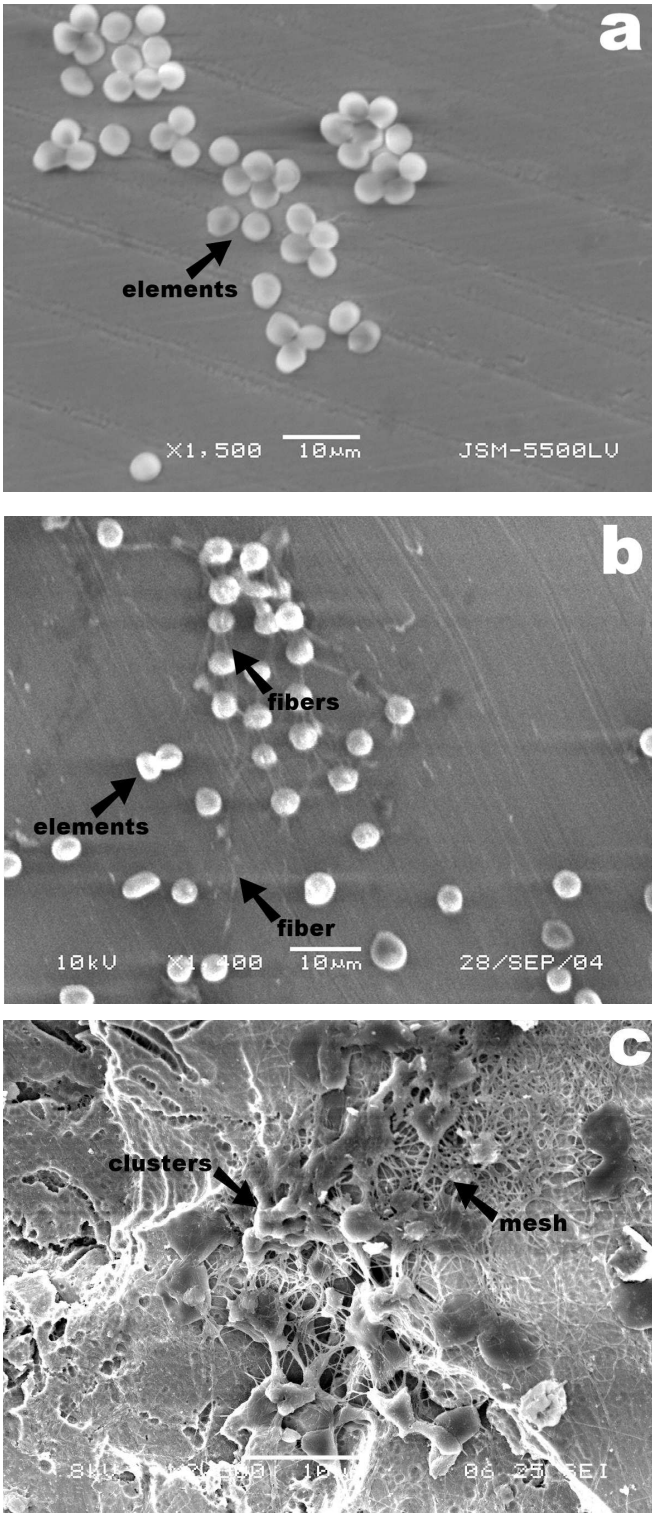


Fig. 10



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Tab.1 Selected surface roughness parameters of tested materials

Tab.2 The surface free energy of selected tested samples

Tab.3 The development of the blood clot on the implant surface with an extending incubation in a rabbit blood – quality description

Tab.4 The character of the blood clot after 4 minute of the incubation in a rabbit blood*

Tab.1 Selected surface roughness parameters of tested materials*

Material	Surface treatment	R_a (μm)	R_z (μm)	R_{max} (μm)
Titanium	polished	0.15 ± 0.02	1.37 ± 0.28	1.98 ± 0.49
	machined	0.60 ± 0.14	3.67 ± 0.43	4.74 ± 0.59
	sand-blasted	3.46 ± 0.66	19.96 ± 4.87	25.15 ± 6.39
	etched	3.54 ± 0.06	15.90 ± 0.40	20.26 ± 0.62
	plasma-sprayed	14.51 ± 2.74	75.18 ± 1.26	96.34 ± 3.12
Ti6Al4V	machined	0.48 ± 0.05	2.48 ± 0.29	3.13 ± 0.50
CrCoMo	polished	0.07 ± 0.01	0.36 ± 0.04	0.49 ± 0.04
Stainless steel	polished	0.12 ± 0.01	0.68 ± 0.03	1.10 ± 0.04
PE	pressed/machined	0.88 ± 0.10	5.54 ± 0.28	7.29 ± 0.72
ZrO ₂	fine-ground	0.23 ± 0.02	1.65 ± 0.04	1.97 ± 0.18
C/C composite	pyrolytic carbon	2.68 ± 0.58	17.84 ± 4.03	25.05 ± 4.14

* data presented as mean \pm SD

Tab. 2 The surface free energy of selected tested samples*

Surface	γ^p (mN/m)	γ^d (mN/m)	γ (mN/m)
TCPS	27.06 ± 1.11	43.15 ± 2.53	70.21 ± 1.92
Ti-polished	15.97 ± 0.54	31.05 ± 0.98	47.01 ± 1.54
Ti-etched	8.88 ± 2.51	32.97 ± 2.11	41.85 ± 4.41
Ti6Al4V	8.59 ± 5.31	30.14 ± 11	38.73 ± 6.01
ZrO ₂	7.55 ± 1.08	34.46 ± 1.53	42.01 ± 1.91
Stainless steel	4.47 ± 0.12	29.61 ± 0.83	34.08 ± 0.84
CrCoMo	2.46 ± 0.51	31.18 ± 0.95	33.74 ± 1.05
PE	0.39 ± 0.02	35.31 ± 1.08	35.7 ± 1.52
C/C composite	0.12 ± 0.03	46.96 ± 1.57	47.08 ± 1.93

* data presented as mean ± SD

Tab.3 The development of the blood clot on the implant surface with an extending incubation in a rabbit blood – quality description

Material		$R_a \pm SD$ (μm)	Incubation time with a rabbit blood (min)			
			2	3	4	5
metal	CrCoMo alloy	0.07 ± 0.01	-	-	elements	clusters and short fibers ^a
	Stainless steel	0.12 ± 0.01	-	-	elements/ clusters	clusters and short fibers
	TiAl4V-machined	0.48 ± 0.05	elements	elements / clusters	clusters	fibers
	Ti-polished	0.15 ± 0.02	elements	elements / clusters	individual fibers ^b	mesh
	Ti-sand-blasted	3.46 ± 0.66	elements	clusters / fibers ^c	fibers	mesh
	Ti-etched	3.54 ± 0.06	elements	clusters / short fibers	fibers/mesh	mesh
	Ti-plasma- sprayed	14.51 ± 2.74	elements / clusters	clusters/ short fibers	fibers	mesh
Non-metal	TCPS	-	-	-	elements	clusters
	ZrO ₂	0.23 ± 0.02	-	-	elements	clusters
	PE	0.88 ± 0.10	elements / clusters	individual fibers	individual fibers	mesh
	C/C composite	2.68 ± 0.59	elements/ clusters	clusters/ mesh	mesh	clots

^a ...individual trombocytes with growing fibers of length app. 2 μm ;

^b ...individual fibers do not overlap but could interconnect trombocytes;

^c ...fibers 4-5 times bigger than trombocytes which overlap and usually connect trombocytes

Tab. 4 The character of the blood clot after 4 minute of the incubation in a rabbit blood*

Surface	Elements (number/mm ²)	Clusters (number/mm ²)	Fibers (number/mm ²)	Mesh (surface coverage %)
Ti-polished	2731 ± 1617	456 ± 181	335 ± 148	20 ± 12
Ti-etched	4187 ± 2116	127 ± 56	2448 ± 1531	22 ± 14
Ti-plasma-sprayed	3169 ± 1303	136 ± 59	1183 ± 638	2 ± 1
ZrO ₂	3111 ± 1023	288 ± 193	81 ± 29	0
Stainless steel	2906 ± 1447	307 ± 164	1418 ± 909	0

*Statistic comments on results are mentioned in the text in paragraph 3.4, data are presented as mean ± SD