Biocompatibile TiN-based novel nanocrystalline films

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Abstract. Titanium nitride (TiN) is regarded as a potential biomaterial for blood-contact applications. TiN thin films were fabricated by pulsed laser deposition with the Nd:YAG laser on biologically applied polyurethane. Transmission electron microscopy (TEM) study of 250 nm thick films revealed columnar structure. Such films were observed to be brittle, which led to crack formation and secondary nucleation of microcolumn. TEM studies showed a kinetic mechanism of growth (columnar) in films of 250 nm thickness. It was stated that thinner films were much smoother and uniform than the thicker ones, which could be associated with the surface diffusion mechanism to appear. In order to improve the coatings elasticity, the thickness was reduced to 50 nm, which limited the deposition mechanism operation to the early stage. TEM cross-section observation revealed elastic properties of thin films. A biological test showed that TiN surface film produced on polyurethane is characterized by good biocompatibility and decreased surface affinity for cell adhesion. Films of 0.25 and 0.5 µm thick of TiN were selected for theoretical finite element modelling (FEM) using ADINA program. The micro cracks formation predicted in simulation was verified by phenomena observed in microstructure examinations.

Key words: titanium nitride, thin films, polyurethane, biocompatibility, finite element modelling.

1. Introduction

Blood-contacting materials are routinely used in modern medicine. The bulk and surface properties of biomaterials used for medical implants have been shown to directly influence, and in some cases, control the dynamic interactions that take place at the tissue implant interface [1,2]. These interactions are included in the concept of compatibility, which should be viewed as a bidirectional process between the implanted materials and the host environment that is ongoing through the *in vivo* lifetime of devices. Most biomaterials introduce a non-specific, stereotyped biological reaction. Currently, considerable effort is directed towards the development of engineered surfaces that could elicit rapid and highly precise reactions with cells and proteins, tailored to specific applications.

When blood comes in contact with a foreign material, such as biomaterial, the first clinically manifested process that occurs is the activation of hemostasis. The first step of hemostasis is adsorption of blood proteins, followed by the platelet adhesion and activation. This activation of platelets can be caused by a variety of agents, such as collagen, plasma proteins and products of platelets metabolism [3]. The process is influenced by the fluid mechanical properties of the blood flow. A key issue in this context is the wall shear stress, the force caused by the flow per surface area of the wall.

Materials used in blood contacting devices have often been chosen based on their physical characteristics, such as flexibility or rigidity, mechanical strength, transparency, degradability, etc. Moreover, cost effectiveness, ease of processing and sterilization are important considerations for the choice of the certain material. Thus, it is conceivable, that optimal thrombogenicity is not always achieved. Furthermore, increasing evidence is obtained that the thrombogenic properties of a medical device during clinical use are different from those during the *in vitro* testing under static conditions. The response of the hemostatic system may differ for each application, depending on the flow conditions.

Polyurethane is often used as a material for the current external blood contacting materials. Generally, polyurethanes are linear copolymers of alternating flexible (soft segment) and rigid (hard segment) blocks that are able to separate into amorphous and paracrystalline microdomains [4]. This microphase separation imparts physical and mechanical attributes which make the polyurethane elastomer useful as biomaterials. Polyurethanes used in medicine are proprietary polymeric compositions containing a high proportion of polyurethane elastomer as well as a small proportion of additive materials to enhance both stability and processability. These additives are normally chosen after consideration of their efficiency as processing aids or stabilizers and also their biocompatibility. Polyurethanes can either be thermoprocessed or solution cast into component part. Polyurethane is a bioactive material and due to this fact it can not be used alone in internal device. The other biomaterial which is considered to be used for the internal devices is titanium. Normal tissue concentration of titanium element in humans is 0.2 ppm [5]. No clinical tissular

toxicity has been observed, even at local concentrations higher than 2000 ppm [6]. It was shown that titanium is biologically inert and it includes neither toxic nor inflammatory reactions in connective or epithelial tissues [7]. Titanium is bacteriostatic and does not significantly activate or inhibit different enzyme systems specific for toxic reactions, e.g. β-glucuronidase, lactate, dehydrogenase, glucose-6-phosphatedehydrogenase and acid phosphate. The real biological actions of titanium, if any, have not yet been established. Bioactivity of the polyurethane as well as low wear resistance of titanium and sometimes mentioned inflammatory reactions of titanium were reasons to look for methods which could improve properties of these materials for medical applications.

The biological environment is surprisingly harsh and can lead to rapid or gradual breakdown of many materials. Polymeric components of implantable devices are generally reliable for their intended lifetimes. No polymer is totally resistant to the chemical process and mechanical action of the body. Generally, polymeric biomaterials degrade because body constituents attack biomaterials, sometimes with the intervention of external factors.

Titanium nitride (TiN) is expected as a perspective biomaterial [8–10]. Before being applied it has to be evaluated and meet particular requirements depending on the proposed location in the body. As a blood-contacting material, titanium nitride has to undergo more than the typical biocompatibility tests (cytotoxicity, sensitization, implantation, pyrogenicity, carcinogenicity, mutagenicity, intracuteneous reactivity, systemic toxicity, etc). It has to meet either the hemocompatibility criterion in both: *in vivo* and *in vitro* tests. Several interrelated phenomena contribute to "hemocompatibility" i.e. protein adsorption, platelet retention, leucocyte adhesion, complement activation, haemolytic activity.

The paper focuses on surface modification of polymer implants by fabrication of thin TiN films using pulsed laser deposition (PLD) at room temperature.

2. Materials and methods of examinations

In many cases, the substrate cannot withstand elevated temperature during coating deposition [11]. Thus there is a high demand for developing low-temperature deposition processes, such as pulsed laser deposition (PLD). In the PLD technique a pulsed laser beam is focussed onto a target in order to evaporate in ablation mode its surface layers under vacuum or low pressure gas conditions [12–15]. The vaporized material consisting of atoms, ions and atomic clusters is then deposited onto the substrate. The outstanding advantage of this technique is the possibility to deposit thin films of very high chemical purity and adhesion to various substrate materials at room temperature. Furthermore, a high rate film growth on surface areas situated perpendicular to the target's surface is also possible by using a low pressure process gas. The application of reactive process gases leads to opportunity of varying the film stoichiometry in a wide range.

PU surface modification. The idea behind the surface modification is to create the possibility of the implantation, and thus to reduce the risk of infection. Thin films were deposited on biologically applied polyurethane. Because TiN is more biologically resistant, it was produced to avoid biomaterial degradation. High-purity titanium targets were used in ablation experiments carried out by means of a pulsed Nd: YAG laser system, which provides four beams of 1064 nm wavelength, 0.6 J pulse energy and 10 ns pulse duration at the repetition rate of 50 Hz. Total pressure in the reactive chamber varied from vacuum (10×10^{-5} mbar) to 4 Pa and continuous flow of N_2 of order of 30 sccm (standard cubic centimeter per minute). The thickness of TiN films deposited on polyurethane was related to the number of laser shots.

Methods of examinations. The microstructure of the film material was investigated by a transmission electron microscopy TEM (Philips CM20 Twin) and X-ray diffraction (Philips PW1710). Biological tight examinations of coating were carried out in the range of cell proliferation, death and adhesion (confocal microscopy Olympus FV-500 and laser scanning cytometer – ComuCyte).

Biocompatibility examination focused on the human fibroblast. The fibroblasts were obtained from healthy donors. Cells from the 2–3 passage of the initial culture (the cells which were unstuck by trypsyn with EDTA) were moved to the surface of the investigated materials in the amount of 1.5×10^5 cells/ml in Dulbecco culture medium, consisting 15% of serum (fetal bovie serum, from Gibco company BRL, UK) and antibiotics (penicillin and streptomycin, of the SIGMA company, UK).

When the cell culture was completed, the incubation liquids were gathered and frozen at temp. –70°C. Then, the samples were washed 3 times in buffered physiological salt (of the SIGMA company, UK), the adhered cells on the samples were fixed by 4% paraformaldehyd and 70% methanol. Fixed cells were washed with 0.5% serum (BSA of the ATTC company, USA) and marked by the primary antibody mouse anti-human CD49E or anti-Fn and secondary antibody Alexa Fluor 488 chicken anty-mouse IgG, and coloured by 7 amino-actinomycina (7AAD). The samples with the coloured cells were glued onto the base glass and closed in the medium for the fluorescence investigations. The specimens were analyzed in laser confocal microscopy (Olympus FV-500) and in laser scanning cytometer (ComuCyte).

In the incubation fluids from the cell cultivation the amount of interleukine – 1β (IL-1β) was marked by immunoensimatic method (ELISA). The incubative media or the reference was put onto the microtitracive plates covered by antibody (anty- human) IL-1β, incubated at 4°C. Further they were incubated with the secondary antibody of the goat IL-1β. The appeared complexes were detached by strepavidyna associated with horseradish peroxidase. Coloured reaction was caused by hydrochloride of o-fenylodiamin (OPD). The absorption was checked on the automatical reader of the microplates (Single-channel reader-assay system, type ELX 800; BIO-TEK INSTRUMENTS, INC, USA), with the wave length 492 nm. The obtained results for the specific biomaterial were averaged and the accuracy was calculated. As a reference there were the cells incubated in the cultivationchambers Lab-Tek (Nunc).

Biological examinations were performed on human fibroblast cells in 48 hours culture. Samples of each type of biomaterial were investigated with the same population of cells and analyzed under light and confocal microscope. For light microscopical analysis the cells were harvested from the samples by nonenzymatic chemical Cell Dissociation Solution (Sigma), then analyzed with trypan blue staining and counted using a Burcker's camera. For confocal microscopical investigations the cells adhered to the substrates were fixed in 4% parafomaldehyde followed by ice-cold 70% methanol, then cell nuclei were visualised by incubation with 7-amonioactinomycin D (Merck) and finally were imaged by confocal microscope (Olympus, FV-500 system).

(a)

(b)
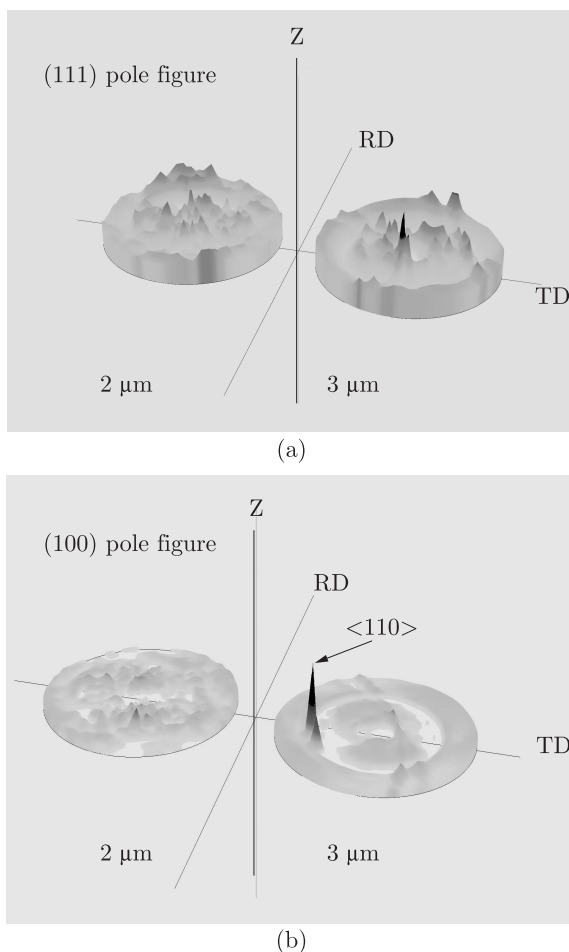
Fig. 1. Texture tomography of (111) and (100) pole figures for the total 3μm thick TiN layer measured on the 2 and 3 μm thickness level

3. Results and discussion

3.1. Crystallographic texture. Texture investigation of thin films is very difficult to perform. It is necessary to apply additional rotation to the material to carry out the examination at the constant level of thickness. This process is called texture tomography (XTT, X-Ray Texture Tomography). XTT is a non-destructive method of texture analysis in the near of the surface area, where the diameter is determined by the penetration depth of the applied radiation. The registration of the constant-depth pole figures can be realized only when a constant incidence angle of the beam on the sample is retained. It can only be achieved with the goniometer in the scanning mode $\theta \leftrightarrow 2\theta$ to the $\omega \leftrightarrow 2\theta$. The results of the texture tomography of the total 3 μm thick TiN films deposited on polyurethane substrate are shown in Fig. 1. The texture was observed to be inhomogeneous, but the dominant components for the 2 and 3 μm level were the same {112}<011>. At the initial stage of the deposition, TiN cluster of the <110> orientation was found at the depth of 3 μm.
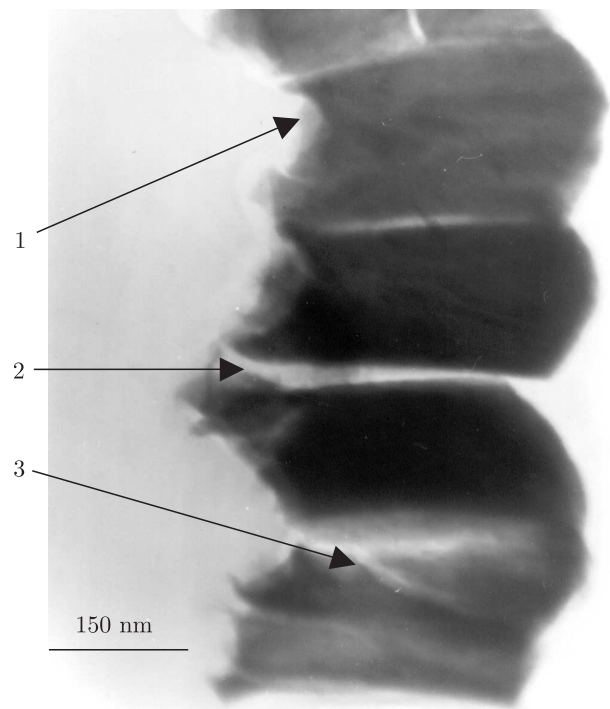

Fig. 2. Micrograph TEM of the cross-section of the 250 nm thick TiN layer deposited on polyurethane substrate; 1 – wrickling, 2 – cracking during growth, 3 – nucleation of new column

3.2. TEM microstructure. 250 nm thick films were deposited on a polyurethane substrate. TEM investigations were performed on the thin foils prepared from the cross-section by means of ultra-microtom method and they revealed the columnar character of the deposited film (Fig. 2). During the deposition, there are two steps of the film growth: initial, typical for the very thin layers, and late model, characteristic for thick ones [11]. The TEM microstructure presents deformation of the substrate, because of the process influence, cracking and additional secondary nucleation after cracking, typical for ceramic coatings (Fig. 2). A precise plasma diagnosis for which the deposition system was equipped and simultaneously performed numerical simulation [16,17] resulted in the preparation of a new layer composition (Polish patent P-371147). The thickness was reduced to 50 nm, with the deposition parameters presented in Table 1. It caused even the change of the layer behaviour (Fig. 3). The selected area electron diffraction pattern revealed nano-crystalline character of the titanium nitride, which proves the uniform layer distribution.
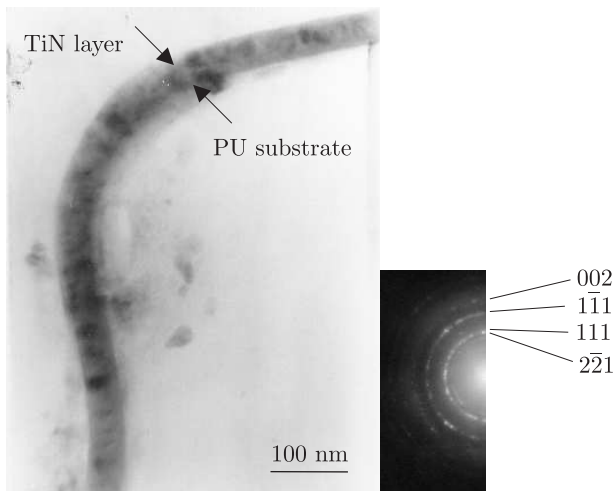
Fig. 3. Micrograph TEM of the cross-section of the 50 nm thick TiN layer deposited on polyurethane substrate
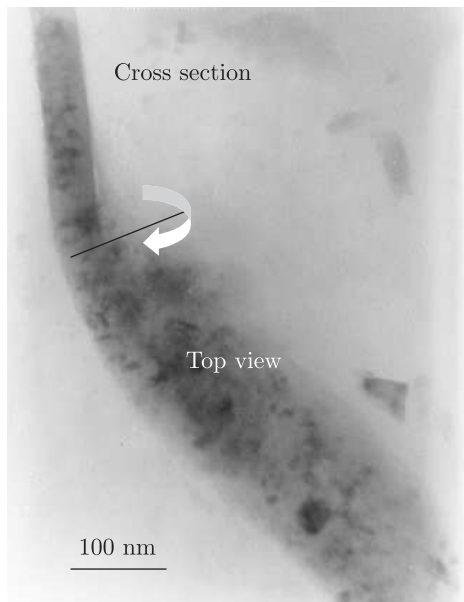

Fig. 4. Micrograph TEM of the cross-section of the 50 nm thick TiN layer showing 90° layer deformation (twisted foil) caused by "microtom" preparation

Table 1
Deposition parameters of the TiN layers coated onto the polyurethane substrate

| Amount of layers	Deposition time	Atmosphere	Total thickness
1	30'	5 sccm Ar, 30 sccm N_2	0.25 µm
1	5'	5 sccm Ar, 15 sccm N_2	0.05 µm

sccm – standard cubic cm per minute

The samples for the TEM investigations were prepared with the ultra-microtom. The microtom knife did not damage

the layer. The TiN layer was observed to be elastic. Even strong deformation caused by the microtom knife did not lead to the delamination of the film in the twisted thin foil (Fig. 4). The flexure parts allowed to obtain the top view of the structure (Fig. 5). The average crystallite size of TiN was estimated (Fig. 5b) on the basis of TEM images. The most probable crystalline size is of 8 nm. It could prove the uniform properties of the film and its constant distribution on the substrate.
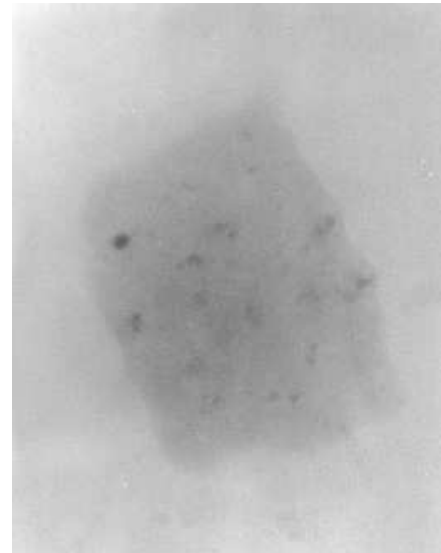
(a)
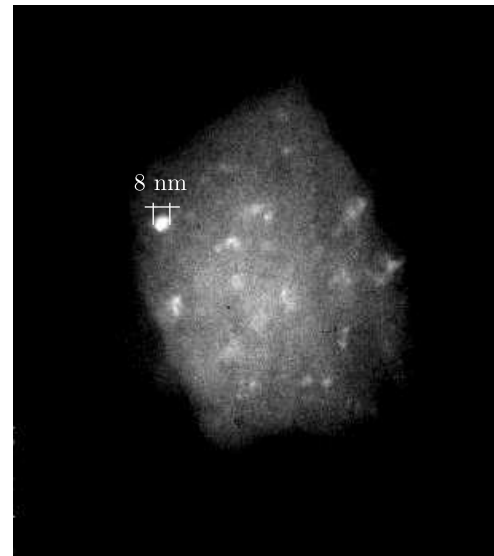
(b)

Fig. 5. Micrograph TEM of top view of the 50 nm thick TiN layer and crystallite size estimation; a – bright field (BF), b – dark field (DF)

3.3. Microanalysis. The Energy Dispersive Spectroscopy (EDS) maps of C,N,O,Ti element distributions in the layer are presented in Fig. 6. Oxygen content was detected in the deposited film which presence in the reactive chamber is due to the residual gas evacuation to 2×10^{-3} Pa. On basis of comparison of the TiN and TiO energy formation, it could be expected that the reaction with oxygen in plasma is very probable even

before the reaction with N atoms. The atmosphere in the reactive chamber is subjected to the interaction with the pulsed laser beam of high energy.
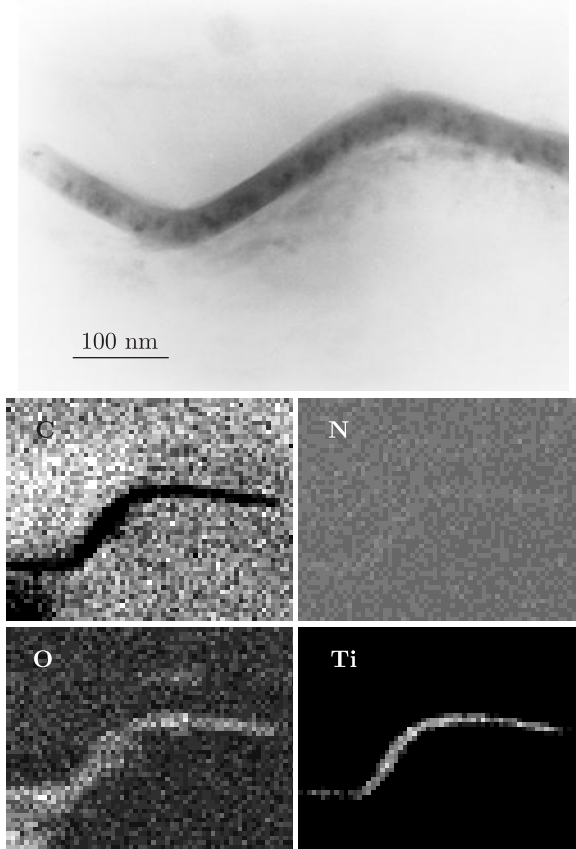

Fig. 6. EDS maps of C, N, O, Ti element distribution in the 50 nm thick TiN layer

3.4. Biocompatibility

Cell adhesion. The data show that the number of fibroblasts (Fig. 7) adhering to polyurethane with a TiN layer was lower than on polyurethane with a Ti layer and the control substrate.

Receptor expression in the cell membrane of a fibroblast for fibronectine (CD49e). Cells are anchored in the extracellular matrix through the receptor present in the cell membrane with the appropriate protein which is in the extra-cellular matrix or in the biofilm. The fibronectine receptor (CD49e) connects with the protein called fibronectin. Receptor expression (CD49e) was investigated in confocal microscopy in adhered fibroblasts on the investigated samples. Similar, slight expression of the receptor was observed in the cultivated cells on polyurethane covered by Ti and TiN, while the expression of the receptor cultivated on a glass substrate was well seen (Fig. 8).

Fibronectine expression. Fibronectine is a glycoprotein which exists outside cells and on the cells surface. It exists also in blood and in other body fluids and on the surface of the cells of connective tissue. This protein associates with the other proteins of the extracellular matrix like fibrinogen, collagen, glicozaminoglicans and with suitable receptors which are in the cell membrane. The expression of the internal and external fibronectine was analyzed in confocal microscopy in

fibroblasts adhering to the investigated samples. It created the net with a different structure on the investigated materials (Fig. 9). Fibronectin on the Ti film deposited on PU substrate created bands with dense fibers laying on the axis of the adhered cells. In the TiN layer, a net with "dots" were formed with dense structure, mainly around the cells. On the control material-glass, the net of the fibronectin created irregular connections which were situated in different directions.

Winculin expression. Winculin is a polypeptyd, which connects the proteins of the cell membrane with the active cytoskeleton of the cytoplasma cortex at the connection point between a cell and the extracellular matrix, i.e. in the place where the so-called adhesive plates are formed (focal contact). Thus, its huge expression provides strong anchoring of the cells in the substrate.

The winculin expression was analyzed in confocal microscopy in the adhering fibroblasts on the investigated samples. Bigger expression of this protein was observed on Ti and PU then on TiN and PU or glass (control material) (Fig. 10).

The level of interleukin – 1β in the incubation medium. Interleukin – 1β (IL-1β) is probably the strongest immunostimulator of the immunological "answer", including the "answer" for the strange material in the biological environment or in the organism. Thus, it is one of the basic factors, which allows to examine immunogenicity of a biomaterial. When the biocompatibility of the biomaterial is low, the cultivated cells synthesise and evolve IL-1β The level of IL-1β was marked in the incubative liquids gained form 48 hours fibroblast proliferation (cultivation) on Ti and TiN deposited on polyurethane. The investigation did not show the presence of IL-1β in the liquid gathered from the surface.
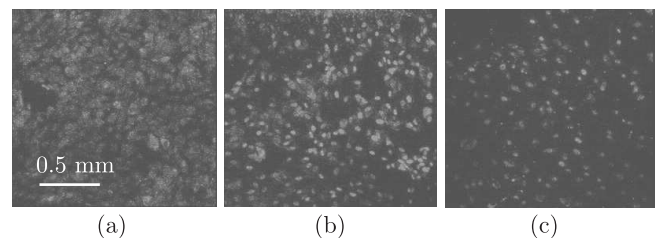

| (a) | (b) | (c) |

Fig. 7. Fibroblast proliferation: (a) – on control culture dish substrate, (b) – on the polyurethane with Ti layer, and (c) – polyurethane with TiN layer
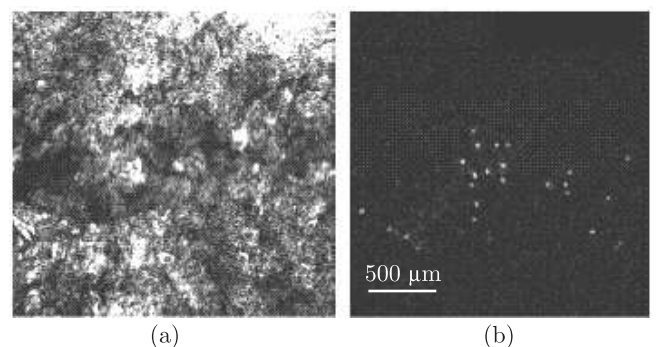

| (a) | (b) |

Fig. 8. Receptor expression in the cell membrane of the fibroblast for fibronectine (CD49e). (a) – glass, (b) – Ti on PU
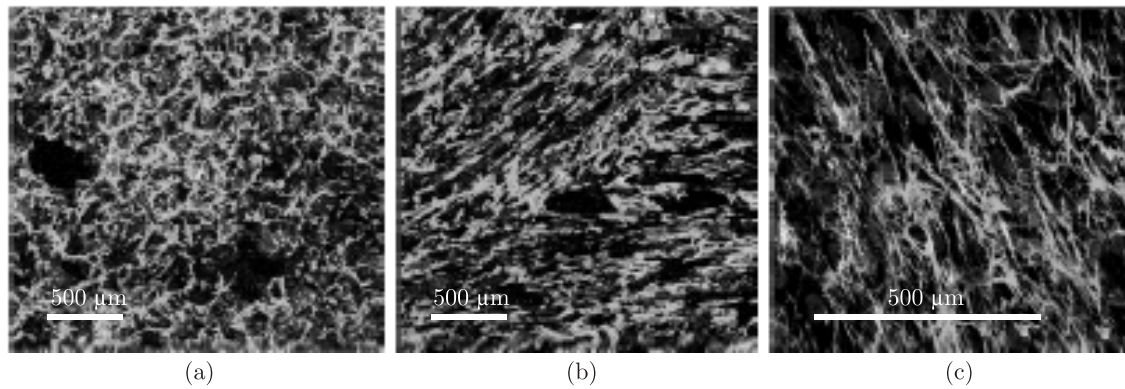
Fig. 9. Fibronectine expression in the fibroblast cultivation (proliferation) on the investigated materials observed in confocal microscopy. (a) – glass, (b) – Ti on PU, (c) – TiN on PU
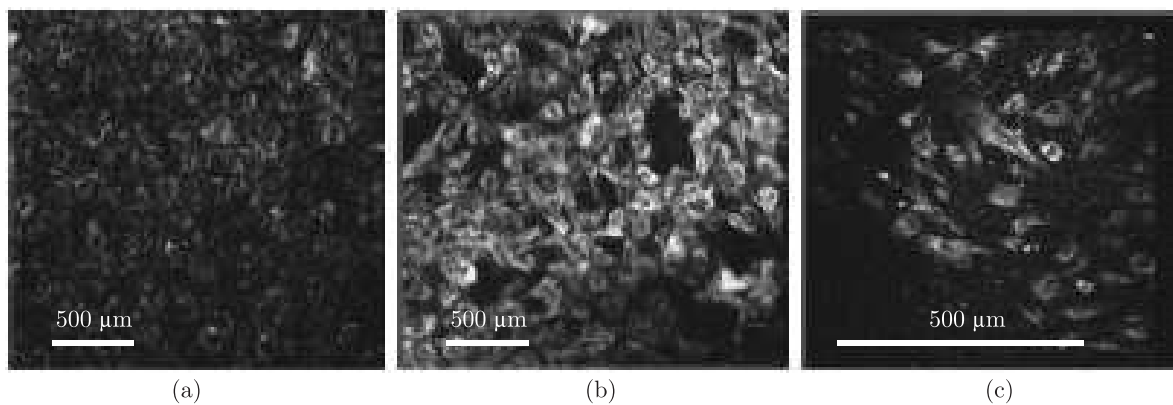

Fig. 10. Winculin expression in fibroblast incubated on the investigated material analysed in confocal microscopy. (a) – glass, (b) – Ti on PU, (c) – TiN on PU

4. Discussion and conclusion

The TEM microstructure of cross section of deposited layer of thickness 50 nm revealed high quality, lack of cracks and elastic behaviour (Figs. 3, 4). On basis of selected area electron diffraction pattern, nanocrystalline structure was identified (Fig. 3). It was possible to find a place with a helical deformation caused by the thin foils preparation by the microtom technique (Fig. 4). No cracks and delamination were observed. Energy dispersive spectroscopy revealed the uniform distribution of element content in the layer (Fig. 6). Oxygen was reminded after evacuation process. Comparing the energy of titanium nitride with an energy of titanium oxide formation it could be expected that there is a mixture of TiN and TiO in the layer composition.

Currently, polyurethane chambers are in clinical use in "the cardiac assist systems" designed and developed in the Foundation of Cardiar Surgery Development in Zabrze. The disadvantage of the solution is that the chambers are situated outside the human body. No polymer is totally impervious to the chemical process and mechanical action of the body. Generally, polymeric biomaterials degrade, because body constituents attack the biomaterials directly or through other device components, sometimes with the intervention of external factors.

The aim of the work was to invent a new biocompatible material. The first examined layer was 250 nm thick, but it turned out to be fragile . On the basis of the gained experience and performed calculation using ADINA program, the thickness was reduced and deposition conditions were improved. The deposited 50 nm thick layers of the nanocrystalline type were observed to be elastic.

The performed investigations of biocompatibility showed, that the probability of the cell adhesion to a Ti layer is higher then to the TiN on PU. It is proved by the number of the adhering cells and the high expression of winculin. Lack of IL-1β in the incubated liquids proves high biocompatibility of Ti and TiN layers. It seems that TiN surface layer produced on polyurethane presents good biocompatibility and decreased surface affinity for cell adhesion, which is expected in blood applications.

Finite element modelling with ADINA system was applied for the transmission electron microscopy results interpretation [16,17]. On the basis of the results of calculations, a new layer composition with the reduced to 50 nm thickness was proposed. Such composition revealed an elastic behaviour of the deposited TiN layer. The early study confirm successful PU modification with depositing of TiN nano-layer which

has nano-crystalline structure, is biocompatible, well adhered to the polymer and flexible.

The technology presented in this work has been applied in the prototype of the new heart assist pump construction designed in the Foundation of Cardiac Surgery Development in Zabrze.

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