

Hemocompatible Thin-Film Materials Recreating the Structure of the Cell Niches with High Potential for Endothelialization

Roman Major, Klaudia Trembecka-Wojciga, Juergen Markus Lackner, Boguslaw Major

Abstract—The future and the development of science is therefore seen in interdisciplinary areas such as biomedical engineering. Self-assembled structures, similar to stem cell niches would inhibit fast division process and subsequently capture the stem cells from the blood flow. By means of surface topography and the stiffness as well as microstructure progenitor cells should be differentiated towards the formation of endothelial cells monolayer which effectively will inhibit activation of the coagulation cascade. The idea of the material surface development met the interest of the clinical institutions, which support the development of science in this area and are waiting for scientific solutions that could contribute to the development of heart assist systems. This would improve the efficiency of the treatment of patients with myocardial failure, supported with artificial heart assist systems. Innovative materials would enable the redesign, in the post project activity, construction of ventricular heart assist.

Keywords—Bio-inspired materials, electron microscopy, haemocompatibility, niche-like structures, thin coatings

I. INTRODUCTION

PROGRESS in the field of biomedical engineering solutions can effectively combine materials science with alive cells. The evaluation criteria are: a long lasting time, easy storage, avoid risk of infections transmitting, simple preparation, high rate of acceptance [1]. In tissue engineering many different types of cells are taken under consideration. Currently, most attention is put to the stem cells. Stem cells are of great interest because of their biological properties and clinical application. Stem cells are capable of self-renewal, they have a high potential to differentiate into other cell types. The selected stem cells isolated from the early embryo can develop into an embryo. Pluripotent stem cells, isolated from the body, have the potential to differentiate into all tissue types. In the near future treatment associated with stem cells will allow incubating cells in vitro for transplantation. Stem cells are defined as self-renewable and producing differentiated, specialized cells [2]. Each stem cell is capable

for proliferation, self-renewal and differentiation through divisions, which are asymmetrical and therefore give rise to two- derived cells, which differ from each other. One of them is the same as the original stem cell, so that supports the population; while the other goes into more mature. Each cell, which grows and matures, has its own niche. Each niche is a spatial structure of cells and extracellular material. It forms a micro-environment that keeps the cells and gives signals to the main cell proliferation, maturation, or to self-renewal. Stem cell niche refers to an anatomical and functional structure, including cellular and extracellular components, local and systemic factors that are integrated to regulate stem cell proliferation, differentiation, survival and localization [3]-[5]. In 1978, Schofield proposed the concept of “stem cell niche” in studies of the hematopoietic stem cells (HSCs) [6]. Since then, this hypothesis has been validated by a number of studies. The in vivo evidence of the existence of stem cell niche was first provided in studies using invertebrate models [7] and in the *Drosophila* germline stem cells [8], [9]. In mammals, stem cell niches have been identified in different tissues over the past several years, including bone marrow, brain, hair follicles, intestines, and teeth [10]-[15]. Theoretically, a stem cell niche is composed of the stem cells themselves; stromal support cells; extra cellular matrix proteins; blood vessels and neural inputs. The interaction of the biomaterial with the stem cells is described in the literature [16]-[21]. Scaffolds are manufactured from suitable biocompatible materials which will allow disintegration and absorption in the body. Surface modification of polymers by the use of thin layers allows forming new features of the material while maintaining or slightly changing the physical properties of the polymer [22]-[24]. The main attention of the surface modification of medically used polymers is put on improving the stability of the material in the direct contact with tissue. Adhesion of the layer to the substrate is one of the most important factors which classify material for the biomedical engineering. In order to reconstruct the niche like structures the surface wrinkling are regarded. Reducing the overall strain energy in the layers subjected to compressive stress, so-called surface "Wrinkling" appears. The suggested explanation considers a common deformation of the substrate surface and the layer [24]. "Wrinkling" is described as the forming of sinusoidal elevations on the surface which do not cause the loss of adhesion to the substrate. The main mechanism of wrinkling is the substrate or subsurface areas of the substrate with the applied layer uplifting. The reason of the

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folding of the deposited layer comes from the mechanical instability. This can be compared to the problem of instability of elastic layer on a flexible substrate medium. In this model, both the base layer and the substrate have a biaxial stress. The result of compressive stress relaxation is the formation of the wavy structure of the surface [24]-[26]. Layer structure of the cluster with adjustable arrangement of folds leads to relaxation and stress compensation through the process of deformation. The effective area of the surface is larger, which promotes the growth of a network parameter. The total disappearance of residual stress is only possible in some areas. The formation of the corrugated surface of the material is the result of induced stresses during growth. The loss of the crystal structure in the subsurface areas affects the lower density of atoms, hence weaker binding, which results in easier deformation through greater flexibility and a lower level of stress. Theoretical considerations of the folding effect of the soft layer- substrate showed that folding mainly depends on mechanical properties of the substrate and the interfacial area [24]. Different values of residual stress are formed on substrates having different values of Young's modulus. The most visible consequences of the stress existence of the coatings will be visible for coatings of the lowest values of the elastic modulus [25]. The following aspect is stiffness dependent differentiation. The researchers demonstrated that the specific type of cells of ESCs was enhanced on stiff substrates compared to soft substrates [27]. They illustrated that the mechanical environment can play a role in both early and terminal ESC differentiation. Their results suggest a fundamental role for mechano-sensing in mammalian development and illustrate that the mechanical environment should be taken into consideration when engineering implantable scaffolds.

II. THE VASCULAR STEM CELL NICHE- NICHE ORGANIZATION AND STRUCTURE

Stem cells in adult organs reside in specialized niches that regulate their proliferation and differentiation. The actual word 'niche' means the medium was "live" in vivo or in a lab or in-vitro microenvironment [28]. Stem cell niches are extraordinarily complex which support many aspects of stem cell identity, including multipotency or quiescence and provide necessary signals for their regulation [29]. The structure of the niche varies between stem cell types, its composition ranging from a single cell or cell type to many cells of varying cell types [30]. In addition to specialized cell types, the extracellular matrix (ECM) is a crucial component of stem cell niche. It is believed that the stem cell niche provides a complex array of physical signals, including cell-cell contacts and cell-matrix adhesions, and biochemical signals, such as growth factors, to stem cells in a temporal and spatial manner; the integration of both local and systemic cues in the niche guides these cells to proliferation and fate specification [31], [32]. The structural, chemical and mechanical properties of niche can also direct cell function. Cells are highly responsive to the structural properties of their surroundings such as topographical surface features and 3D structure. Physical stimuli such as mechanical stiffness and

topography are known to significantly impact stem cell behaviors; being translated through adhesions, intracellular tension and mechano-transduction, which can alter gene expression and thus cell fate [33]. The mechano-structural environment is the architecture in two and three dimensions as well as mechanical forces such as stress and strain, all of which act in a non-linear but fairly constant manner.

III. DIAGNOSTIC METHODS OF MICROSTRUCTURE AND MECHANICAL PROPERTIES

The main criteria while designing the blood contacting materials are no degradation of the substrate, bio and haemocompatibility, high adhesion and minimal effect on changing of the mechanical properties of the polymeric substrate. The main challenge is to design and elaborate the protective coating which would protect the polymer from biological degradation. Thin film materials, deposited on polymer substrates should be analyzed using highly specialized research equipment.

A. Methods of Microstructure Analysis

The layer structure and the mechanisms of the coating anchoring to the substrate are analyzed using electron microscopy techniques. Scanning electron microscopy are used to describe the morphology of the coatings. Thin foils for transmission electron microscopy (TEM) analysis are prepared on the QUANTA 200 3D device (using a focused gallium ion beam; technique FIB-Focused Ion Beam) equipped with a micromanipulator OmniProbe, giving the film exactly the places of interest (defect or phase boundary). The cross section study is performed by the transmission electron microscopy equipped with field emission gun. The physical surface structuring is done using plasma based techniques, like laser ablation and ion beam milling. The physical structuring consider the controlled residual stress generation in order of the niche-like structure formation. The value of the residual stress is normally analyzed in grazing incidence mode by the X-ray diffraction technique with the highly collimated incident beam radiation. Atomic Force Microscopy- the technique, which enable the visualization of the surface. Both the analysis of the initial surface after deposition as well as the surface after tensile straining is of an importance. Scanning acoustic microscopy technique is used as a support technique for the stress analysis. It allows to visualize the endanger of the delamination caused by the ion surface modification. The study provides two screening analysis based on the dynamic interaction of blood. These tests simulate physiological phenomena occurring in the blood vessel. The first selection considers coatings deposited on the polymer substrate. For this purpose flow chambers, arterial flow condition simulator could be proposed which is originally designed and elaborated. It is equipment of biomedical engineering laboratory of the Institute of Metallurgy and Materials Engineering. The second screening test considers niche-like structure analysis and the probability of the stem cell capturing from the flowing blood its differentiation and analysis. For this reason bio- robotics are developed. So called large

mechanical model was developed. It simulates large circulation blood flow. The blood-material interaction assay considers the gas exchange as well as the influence of the blood pressure change into the quality of the tested element. The material duration is regarded as the function of time and pressure. The final considered aspect is focused on the adhesion, proliferation and differentiation of stem cells, captured from the whole blood. In this study the ability to differentiate mesenchyme stem cells (MSC) into the endothelial cells are evaluated. The antibodies which are taken to analyze the stem cells mainly consider anti CD133+, anti CD45+, anti CD133+ VEGFR2+. The cells and the cell-material interaction are treated by the application of the fluorescent techniques, mainly laser scanning confocal microscope. The fluorescence is particularly useful in studies of interaction between the material and the cells. It allows obtaining direct information of the impact on the cell surface of the biomaterial.

B. In vitro Analysis of Blood-Material Interaction – Impact-R Test

Haemocompatibility tests evaluate the interactions of blood contacting medical devices or materials with blood and blood components. The International Organization for Standardization (ISO) provides several test categories for evaluating haemocompatibility: thrombosis coagulation, platelet haematology, and immunology. The present work evaluates blood-material interactions under dynamic conditions. The ISO has developed guidelines for testing medical materials that will be placed in contact with circulating blood, but it has not provided exact test methods or evaluation criteria. The authors of the work cited above presented a list of various suggestions. The haemostatic mechanism is designed to stop bleeding from an injured blood vessel [34]. The interaction of materials with the blood and the activation of the blood clotting cascade both depend on the following elements: the surface morphology, platelets, and coagulation proteins. Blood platelets are critical for vascular haemostasis, as they activate readily upon contact with the exposed components of the vessel wall. Blood platelets are roughly twenty times less abundant than erythrocytes, and the diameter of a platelet is only one-fifth of that of an erythrocyte. The primary haemostatic function of platelets could lead to thrombosis. The most important definition for haemocompatibility is therefore as follows: “A haemocompatible material must not adversely interact with any blood components” [35]. Haemocompatibility tests are designed to detect adverse interactions between an artificial surface (which may activate or destroy blood components) and blood [36]. Under aortic flow conditions, as a result of strong shear forces, platelets play a crucial role in blood-material interactions and are relevant to haemocompatibility. A commercially available blood flow simulator, the Impact-R test, is equipped with a flow chamber, a well in which the sample of blood to be tested is introduced, and a rotor with a normalized roughness similar to that of the active surface (Fig. 1).

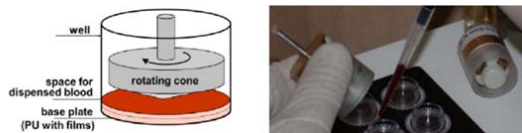


Fig. 1 The main assumptions of the Impact-R method

The test was designed for use with whole blood, and this system can only be adapted for use with flat-shaped samples. More details are presented elsewhere. [37], [38]. Materials were analyzed under arterial flow conditions. The tube-like elements were analyzed using assumptions similar to those applied in the Impact-R test. Testing conditions similar to those described in [39]-[42] were maintained during the experiments. A new device was designed by the authors to test the tube-shaped elements under arterial flow conditions. This arterial flow simulator was designed and developed as a point-of-care method for real-time testing of blood-cell function. A method was developed to analyze the modifications to the inner surfaces of the tube-like elements. This system was designed to test whole-blood platelet adhesion and aggregation on a thrombogenic surface under flow conditions. The design of the arterial flow simulator is presented in Fig. 2.

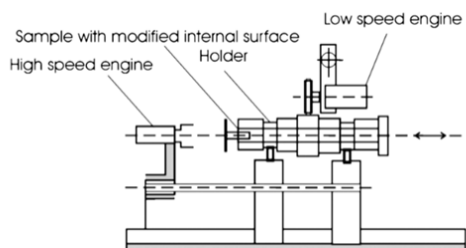


Fig. 2 The design of the arterial flow simulator

Fig. 3 presents the images of the elements of the final configuration.

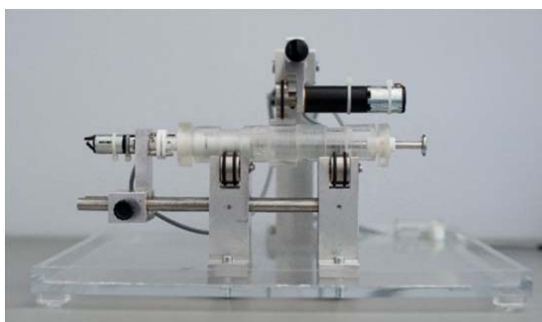


Fig. 3 The real appearance of the aortic flow simulator

The system is composed of a holder for the samples, with modified inner surfaces, and a rotor with the appropriate surface roughness, which provides a simulation of the aortic flow. The properties of this rotor are based on the rotor used in the impact-R test. The system is also equipped with two engines, one operating at a low speed to protect the blood against sedimentation and one connected to the rotor,

operating at the high speed, to provide aortic flow. Blood flow was induced by a rotating cylinder for two hours. Similar to the Impact-R test, the distance between the rotor and the analyzed surface was held constant. The rotor was introduced into the system along its longest dimension of the region of the tube under study. Blood-material interactions were analyzed based on fluorescent techniques using confocal microscopy (CLSM), flow cytometry and low-vacuum scanning electron microscopy (ESEM). After the test, blood that had not adhered to the surface was removed and retained for further analysis, and the cells that had adhered to the surface were analyzed using fluorescently labelled antibodies.

IV. DIAGNOSTIC METHODS OF MICROSTRUCTURE AND MECHANICAL PROPERTIES

The niche is a complex and dynamic structure that transmits and receives signals through cellular and acellular mediators. The niche factors include the regulatory factors such as oxygen, extracellular matrix (synthetic and decellularized), paracrine/autocrine signaling and physical forces (i.e., mechanical force, electrical force and flow shear). Individual stem cell niches use distinct combinations of signaling molecules to control stem cell self-renewal and proliferation. In all cases, both cadherins and integrins are required for stem cell – niche interactions [43]. Stem cells physically interact with the ECM including chemical and topographical cues at the micro and nanometer scale. Substrate properties influence the energetic of cell- niche interaction causing changes in cell shape and morphology. Fig. 4 presents three different models of the stem cells niche- like structure reconstruction. The work was mostly focused on the designing the surfaces as combinatorial signal mixtures (Fig. 4 b) and modular substrate stiffness (Fig. 4 c).

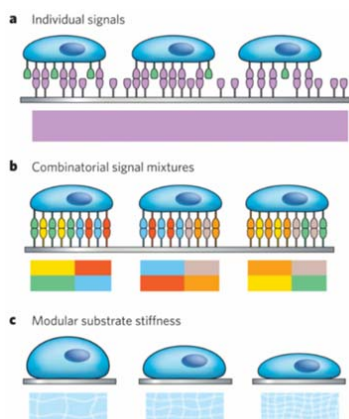


Fig. 4 Models of the stem cells niche- like structure reconstruction

A. Hydrogel Coatings – Reconstruction of the Combinatorial Signal Mixtures

The hydrogel coatings were deposited by the dip-coating method. The hydrogel coating preparation on luminal side of the tube like element made of polyvinylchloride was carried out in 2 stages using dip-coating method. The tubes were washed in 5% aqueous alcohol solution and then with water.

They were dried to constant weight at 40°C. In the first stage the covered tubes were immersed in a solution of hexane, containing 5% ethylene glycol dimethacrylate (EGDMA) and 3% Cumene hydroperoxide (CHP) for 5 min at 25°C. In a second step the samples were placed in an aqueous solution containing 0.1% FeCl₂, 1% ascorbic acid AA and 5% polyvinylpyrrolidone (PVP) for 15 min at 25°C. PVP is high hydrophilic, biocompatible polymer with low cytotoxicity. Therefore this material is an appropriate one for many medical applications such as artificial pancreas, wound dressing, artificial skin, and cardiovascular devices [44], [45]. The discs were immersed in an aqueous polymer solution of 0.1% Sodium dodecyl sulfate (SDS) and inserted on the shaker for 5 min to remove unbound PVP. The samples were washed in water, then transferred to phosphate buffered saline (PBS) and incubated at 37°C overnight. Films were chemically cross-linked by ethylene glycol dimethacrylate (EGDMA). The exact concentrations of reagents used in the preparation of hydrogel coatings are included in Table I.

TABLE I
THE EXACT CONCENTRATIONS OF REAGENTS USED IN THE PREPARATION OF HYDROGEL COATINGS

The step of using a solution	Chemical compound	PVP Content %
The first step	PVP	5.0
	FeCl ₂	0.1
	AA	1.0
The second step	CHP	3.0
	EGDMA	5.0

The process of the hydrogel coating formation consists of two steps. Step One: polymer substrates were immersed in a solution containing CHP and EGDMA in order to absorb these reagents to the outer layer and to form hydrogen bonds between the two groups -NH-CO -OOH and occurring molecules: substrate, CHP and EGDMA. Second step: immersing the samples in an aqueous solution containing FeCl₂ resulted in a redox reaction between cumene hydroperoxide and Fe²⁺ ions. CHP on the surface of the polymer substrates is in contact with iron ions and decompose to form radicals. Part of the hydroperoxide radicals is converted into hydroxyl radicals. The free radicals present in the system react with the polymer chains of PVP and a PCV, leading to the formation of covalent bonds between the base polymer and the modifying polymer.

With implemented double stages of the reaction of free radicals are formed mainly on the surface of polyvinylchloride tubes, which ensures the formation of high yield covalent bonds between the substrate and the coating polymer hydrogel. The biocompatible polyvinyl pyrrolidone (PVP) was used for the preparation of hydrogel coatings on luminal side of the PCV tubes according to the reaction of initiated polymerization. A Hydrogel layer of the PVP concentrations of 5% were obtained and was deposited by dip-coating. The coatings were chemically cross-linked with ethylene glycol dimethacrylate (EGDMA). PVP is a polymer of high hydrophilicity, biocompatibility, with low cytotoxicity. Consequently, this material is suitable for many medical

applications [44]-[48].

The high-resolution analysis showed the presence of crystallized elements in the amorphous matrix. It is associated with the initial mechanism of the thin film nucleation from the gas phase. Two dimensional thin film nucleation allows achieving mechanical properties, unusual for ceramic coatings. The coating which has reached elastic properties was applied directly on the polymer. Crystalline elements are arranged uniformly along the coating (Fig. 5).

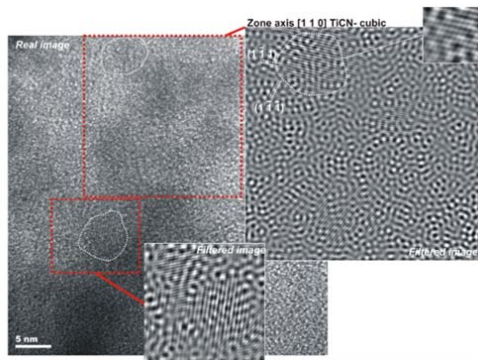


Fig. 5 TEM analysis of the hydrogel formed for haemocompatible purpose

In order to fabricate biologically functional coatings, the final top hydrogel layer was applied on the surface of the partially amorphous introducing the functional molecular groups. It was observed the presence of crystalline components irregularly distributed in the hydrogel.

A hydrogel layer applied on the inner side of the tubular element determines a constant level MNF and IPA for PAC-1 receptor (Fig. 6), regardless of the coating.

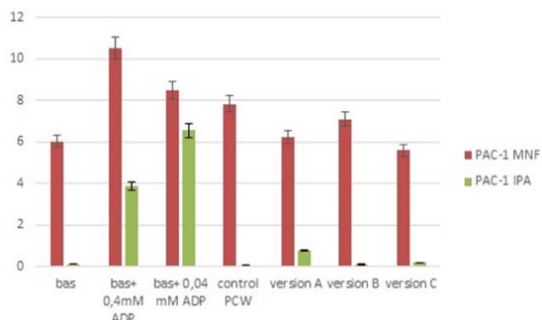


Fig. 6 Analysis of the activation of the coagulation system, made on the basis of the active platelet IIb / IIIa receptor collected from over the analyzed surface (Option A, B, C), after the dynamic test of blood; MNF- average amount, IPA- indicator of platelet activation

The second receptor, which activity was analyzed using flow cytometry was selectin-P (P-SEL). The results of the analysis of platelets collected from above the tested surface are shown in Fig. 7. The result of the analysis of P-SEL shows a similar dependence as for the analysis of PAC-1.

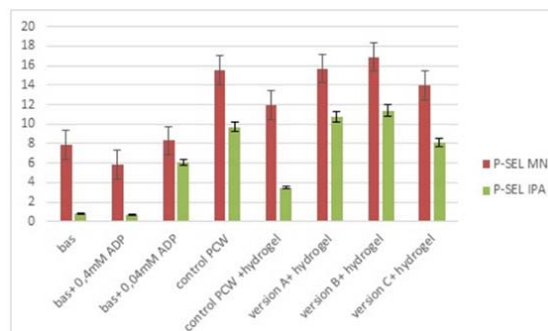


Fig. 7 Analysis of the activation of the coagulation system made on the basis of platelet active receptor P-selectin collected from over the analyzed surface (Option A, B, C) + hydrogel after the dynamic test of blood; MNF- average amount, IPA- indicator of platelet activation

B. Reconstruction of the Stem Cell Microenvironment: Thin Coating Deposition by Using PVD Technique

Films were deposited by physical method based on evaporation from plasma (PVD). A precise description of the method is published elsewhere [24]. Prior to deposition, we cleaned the substrates (PA 6 (Senova Zellamid™250), PC (Senova Senolex™), PI (Vespel® SP1), and PU (Advan-Source Biomaterials Chronothane™ P)) ultrasonically with ethanol and dried them afterwards in vacuum. After mounting the substrates parallel to the target surface in a ~120 mm distance, we pumped the vacuum chamber down to at least 4×10^{-3} Pa. Titanium, tungsten, carbon, gold, and silver thin films were deposited by pulsed laser deposition (PLD, Nd:YAG laser, 1064 nm wavelength, 10 Hz pulsing, and 10 ns pulse length) from pure (>99.9%) targets in argon atmosphere. TiN coatings were grown in mixed argon–nitrogen atmosphere reactively from titanium target. Growth of all coatings in different thicknesses from 5 nm to 0.5 μm was achieved at room temperature (25°C) with less than 5°C heating during deposition in plasma.

C. Surface Modification of the Controlled Residual Stresses

The glow discharge technique has been applied to modify the surface. The process is carried out at reduced pressure. The setup for this process is similar to the tubular flat fluorescent lamp electrodes. The system was evacuated to a vacuum (10-6 Pa) during plasma etching (activation) and the polymerization carried out using a pulsed plasma polymerisation of a low frequency. DC glow discharge was performed at a frequency of 535 Hz and a cycle of about 25%. The surface was modified by plasma induced glow discharge in a controlled gaseous atmosphere. The gas supplied by the cathode and anode pumped. Hexamethyldisiloxane (HMDSO C6H18OSi2) was used as the liquid precursor and evaporated at 25°C. The pressure was measured using HMDSO gas flow controller and controlled by manual latch vacuum. Other process gases, such as argon oxygen, C_2H_2 were controlled by mass flow meters and controlled in order to achieve a stable deposition conditions. Table I shows the parameters of the coatings. Fig. 5 shows the hierarchical manner of wrinkling. Such hierarchical structure occurs in the continuous process of

stress relaxation during film growth. Cracking occurs, if the tensile stress due to film bending exceeds ultimate film strength (Fig. 8).

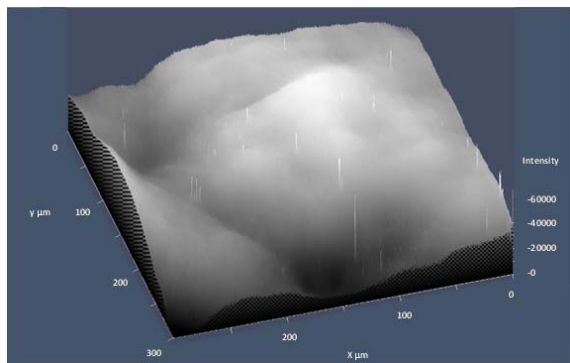


Fig. 8 Surface modification of the controlled residual stresses

The final verification of surface modification was made in direct contact with endothelial cells (Fig. 9).

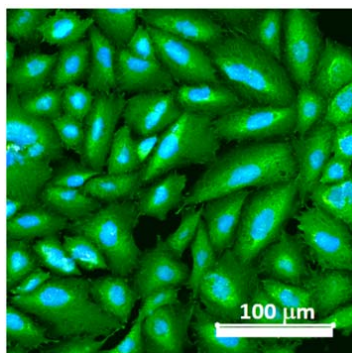


Fig. 9 Cell- material interaction- laser scanning confocal analysis of the endothelial cell proliferation on the investigated surface

The proposed coating should allow to uptake stem cells from whole flowing blood. Then, stem cells should be differentiated to endothelial cells in order to effectively inhibit the coagulation process. At this stage of the work, an attempt has already been trained on the cells in vitro studies to show that the proposed coatings have been designed correctly and might be used for in vivo analysis.

D. Analysis of Microstructure

A cross section analysis of the microstructure was carried out for the samples with the crystallized thin buffer titanium carbo-nitride coating (Fig. 10).

Thin foils for TEM observation were prepared directly from the place of interest using the focused ion beam technique (equipped with an in situ micromanipulator). Analysis of phase were performed by electron diffraction pattern and confirmed by identification of high resolution images (HR-TEM). HR-TEM techniques were applied for phase and lattice orientation analyses. TEM analysis was used to determine the detailed construction of a titanium carbonitride coating and its interaction with the PU substrate as well as with the hydrogel

final coating. The technique called, “bright and dark field observations” were used to determine the structure of the coating. The analysis of the phase composition was carried out using electron diffraction techniques as well as by high resolution image analysis. Maintaining the stability of the chemical composition of the coatings is very important aspect. High resolution transmission electron microscope was done to observe the structure of the coating in the atomic scale.

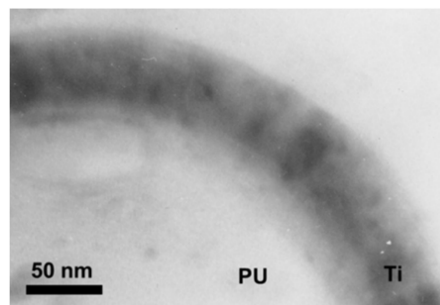


Fig. 10 Cross section analysis of the microstructure of the residual stresses controlled thin coating

V. CONCLUDING REMARKS

- Wrinkling is obviously the surface topography formation mechanism for bio- inspired thin films topography design. The idea is to use advanced materials surface to reconstruct niche like structures for the stem cell differentiation.
- Ceramic coatings showed elastic properties. This is achieved by the dispersion of crystalline particles uniformly in the amorphous structure. The elastic properties of the ceramic coatings are dependent on the appropriate mechanism of thin nucleation from the gas phase.
- The coating, deposited with the high acetylene flow in the reactive chamber demonstrated high haemocompatible properties. On the basis of the dynamic analysis on blood, the decreased activation of the coagulation system and the immune response compared to the other analyzed coatings was observed. These characteristics prove the selected appropriate parameters.
- The coating, deposited in the high acetylene flow, the same which demonstrated high haemocompatible properties, showed the strongest influence on the formation of microparticles. Despite the low activation of platelets and leukocytes, the number of membrane fragments is the greatest. The microparticles have an indirect effect on the coagulation process; hence it can be assumed that in the long-term use of this material in direct contact with the blood can lead to complications.
- The hydrogel coatings are highly hydrophilic. An additional layer of water is created on the surface that protects the surface from blood aggregation processes. The hydrogel coatings are very highly absorbing liquids, which affects the expansion of the material. The dynamic tests of the blood revealed a strong hemolysis.

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