LASER MODIFIED FUNCTIONAL CARBON-BASED COATINGS ON TITANIUM SUBSTRATE FOR CARDIAC TISSUE INTEGRATION AND BLOOD CLOTTING INHIBITION

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Abstract

The work focused on developing functional coatings on titanium substrates that would facilitate the integration with the cardiac tissue and with a specific form of connective tissue like blood. Surface modifications consisted in the laser evaporation of part of the biocompatible layer, thus creating a suitable environment for a particular tissue. For the myocardium integration, the metal surface was refined by biohemocompatible coatings. Such surfaces were the starting point for further modifications in the form of channels. The channeled surfaces enabled a controlled cell migration and proliferation. The interaction of endothelial cells with the material was highly dependent on the surface characteristics such as: topography, microstructure or mechanical properties. The controlled cellular response was achieved by modifying the surface to obtain a network of wells or channels of different dimensions via the laser interference lithography. This technique determined a high resolution shape, size and distribution patterns. As a result, it was possible to control cells in the scale corresponding to biological processes. The surface periodization ensured the optimal flow of oxygen and nutrients within the biomaterial, which was of a key importance for the cell adhesion and proliferation. The work attempted at producing the surface networks mimicking natural blood vessels. To stimulate the formation of new blood vessel the finishing resorbable synthetic coatings were applied on the surface to act as a drug carrier. Therefore, the initial trial to introduce factors stimulating the blood vessels growth was performed.

***Keywords**: thin layers, migration channels, laser ablation, microstructure*

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Introduction

Engineering of biomaterials requires a thorough understanding of the cell-material interaction. The aim of our project was to modify the material surface for application in the cardiovascular system regeneration, namely to solve a problem of the cardiac tissue integration with a metallic element. The main limitation in the *in vitro* tissue engineering is the lack of a sufficient blood vessel system — the vascularization [1]. *In vivo* a highly developed system of larger blood vessels which are subdivided into small capillaries supplies nearly all tissues with nutrients and oxygen. In the case of artificial materials, the spontaneous vascular ingrowth occurs after implantation, yet it is often limited to several tenths of micrometers per day. It means that the time necessary to complete the implant vascularization is measured in weeks. During this time, insufficient vascularization may lead to nutrient deficiencies or hypoxia deeper in the tissue. Moreover, nutrient and oxygen gradients are present in the outer tissue regions only, resulting in the non-uniform cell differentiation and integration. In consequence of all these phenomena the tissue functionality decreases. Therefore, additional strategies enhancing the proper vascularization are essential. Current strategies to create vascularized tissues are discussed in this review [1]. The paper describes the endothelial cells and their neoangiogenesis, i.e. the ability to form new vessels. The pre-vascularization techniques are compared to the biomolecules approach where growth factors, cytokines, peptides and proteins and cells, are applied to generate new vessels. Biomaterials are engineered to promote endothelial cell adhesion and proliferation. There are several explanations of the cellular response to the applied carrier based on the synergistic effect of the material mechanical properties and the presence of bioactive molecules. Metallic biomaterials are popular for cardiac usage due to their inertness and structural functions. The high strength and resistance to fracture promote their use for cardiovascular regeneration e.g., artificial heart valves, components of heart assist devices, vascular stents [2]. Commonly used biocompatible metals include titanium, stainless steel, gold and silver. The increased interest in titanium and its alloys as biomaterials comes from their high corrosion resistance and good mechanical properties. However, their main drawback is low potential to cellularization and low hemocompatibility. To solve this problem the surface modification of titanium scaffolds is required. According to the literature, modulation of surface parameters like topography, chemistry or microstructure have a direct influence onto the cellular response [1-4]. The architecture and design of a scaffold have a profound effect on the endothelial cell adhesion and the vascularization rate. Moreover, the topography of the scaffold is a critical determinant of the blood-vessel ingrowth. Druecke et al. showed that the vessel ingrowth was significantly faster in periodical structured scaffolds [3]. The latest reports have shown that effective vascularization in tissue engineering is inherently linked to the intelligent scaffold design [3]. For this purpose, the ability to maintain suitable oxygen tensions and nutrient diffusion throughout the scaffold is critical. One of the ways that this has been addressed is the use of channeled scaffolds. Alison P. McGuigan and Michael V. Sefton et al. [4] pointed out that endothelial cells are used in combination with biomaterials in a number of applications for the purpose of improving blood compatibility and host integration. Endothelialized vascular grafts are already used clinically with some success, while the endothelial seeding is being explored as a mean of creating the vasculature within engineered tissues. That's why surface engineering plays a significant role in the cell-material interaction. The details are presented elsewhere [5].

The interaction of cells and tissues with artificial materials designed for applications in medical biotechnology is governed by the physical and chemical properties of the material surface. Nanostructured substrates (i.e. substrates with irregularities smaller than 100 nm) are generally considered to be beneficial for the cell adhesion and growth, while microstructured substrates behave more controversially. According to the literature [6-15], micropatterned surfaces enable the regionally selective cell adhesion and directed growth, which can be utilized in tissue engineering, in constructing microarrays and in biosensorics. Nanopatterned surfaces are an effective tool for manipulating the type, number, spacing and distribution of ligands for the cell adhesion receptors on the material surface. As a consequence, these surfaces are able to control the size, shape, distribution and maturity of focal adhesion plaques on cells, and thus the cell adhesion, proliferation, differentiation and other cell functions. Control of cell phenotype involves a variety of signalling pathways and transcriptional regulators. This multifunctional signalling molecule is part of adhesion contacts in the endothelium and is able to translocate into the nucleus to activate genetic programs and control proliferation and the fate of the cells. Micropatterning can precisely reconstruct the spatial and temporal features of the cellular microenvironment. This technique represents one of the most effective, high-precision method to modify two main surface properties (topography and micro-structure) [16-18]. Laser interference lithography, consisting in the creation of organized periodical surfaces based on selective material ablation, offers the possibility to create 2D and 3D patterns on surfaces. This technique enabled the precise control of the pattern shape, size and distribution. Materials structuration has found application in the cardiovascular materials engineering field. The surface lithography permitting control of cellular behaviors at scales matching those of biological processes. Using this method Marczak et al [19] were able to control the cell behaviour on the DLC layer deposited onto the silicon substrate. Biomaterials dedicated for direct blood contacting purpose require the design of fully atrombogenic surface which do not adverse interact with any blood components [20]. This represents a really complex task due to a variety of processes occurring within this interface, including plasma protein adsorption, cell adhesion, and activation followed by thrombus formation [21]. Proteins mediate between the surface cell adhesion (e.g. fibrynogen) or can form a non-adhesive layer (e.g., albumin). Thus, controlling their selective adsorption to the surface is a key issue in hemocompatible materials design. A complete, tenaciously-adherent protein layer is formed within 5 seconds after the biomaterial exposure to blood flow [22]. Such a layer formation represents a dynamic and competitive process directly affecting the hemocompatible material properties because of different characteristics of specific proteins [22]. In literature, plasma proteins are divided into two main types: adhesive and non-adhesive ones [23-26]. The adhesive proteins (e.g., fibrinogen, fibronectin and von Willebrand Factor) tend to increase thrombosis by mediating platelet adhesion, while the non-adhesive ones (e.g., albumin, transferrin like proteins) can decrease subsequent thromboembolic events [24-26]. As the protein adsorption is the first step in a blood-solid state interaction, controlling the composition helps to improve hemocompatible properties of biomaterials. Over the past two decades, many studies have shown that the plasma protein adsorption followed by the platelet adhesion and activation is reduced on the albumin- coated biomaterial surfaces [25-26]. What is more, the adsorbed layer may minimize the adhesion of bacteria to biomaterials due to the lack of albumin/bacteria interaction [26]. This inspired researchers to develop new methods to obtain albumin-coated passive materials by selective albumin adsorption to the surface in a blood environment. However, it has not been described yet how the surface features affect the albumin adsorption from blood in dynamic conditions. The hemodynamic conditions cause superficial stresses near the vessel wall (shear stresses) caused by blood flow inside vessels. Shear stresses may lead to the blood components conformal changes and the cells aggregation and thrombus. This carries serious consequences including blood clotting followed by the unhindered flows and resulting in the implant failure [27]. For this reason, it is important to fabricate the self–assembling material surfaces which enable the spontaneous formation of an albumin layer from the blood.

Materials and Methods

As part of the task, titanium alloy substrates were modified with thin amorphous carbon coatings (a-C:H) and subsidized with nanoparticles of various elements to improve the hemophilic properties. All surface modifications were made in cooperation with MATERIALS - Institute for Surface Technologies and Photonics. The first stage consisted in optimizing the surface modifications in terms of interactions with blood. Coatings were deposited using the Physical Vapour Deposition (PVD) technique. Prior to deposition, the substrates were ultrasound cleaned with ethanol and then dried under vacuum. After the substrates were installed in parallel to the target surface at a distance of ~120 mm, a vacuum chamber was used to achieve at least 4×10^{-3} Pa. Before the coating was deposited, an anode layer ion source was used. It is a method enabling etching and used to clean the surface from oxide layers. Next, a Direct Current (80 kHz pulsed 80 kHz, 2000 W, pyrolytic carbon with 99.95% C as a shield from Schunk, Bad Goisern, Austria) was used to deposit the a-C:H and a-C:N coatings in Ar and Ar+N_2 at $3x10^{-1}$ Pa, respectively. The application of hemophilic thin film materials was carried out in several stages, successively improving their properties. The deposition parameters of the first group of materials are shown in TABLE 1.

Carbon-based coatings were deposited on silicon wafers by means of physical steam deposition. Silicon substrates were selected deliberately because of their smooth surface which allows for a thorough analysis of the surface effect of a thin layer on the cell-material interaction, with particular emphasis on red blood cells. The substrate dimensions for all the tests were 1.5 cm x 1.5 cm. For hydrodynamic tests, the substrates had 14.4 mm in diameter of and no more than 0.5 mm in thickness. The size of the tested hydrodynamic sample was matched with the geometry of the tester vessels. The deposition process was performed at room temperature using an industrially scaled vacuum coating (manufacturer: Pfeiffer Vacuum, Asslar, Germany). The deposition parameters of the second group of materials are shown in TABLE 2.

Endothelial cells play a major role in the complex mechanism that has evolved to ensure balance in the circulatory system. The endothelial layer acts as a dynamic interface that actively regulates inflammation, thrombosis and fibrinolysis. A number of undesirable reactions may occur in the case of the blood contacting the surface. The endothelium is commonly known as the most biocompatible in contact with streaming blood. Its combination with biomaterials can be used to prevent thrombotic and inflammatory reactions and improve the integration with artificial materials. One of the milestones in the task was to achieve the full endothelial cells confluence by controlling the surface structuring. The local environment is of key importance for the cells behaviour, shape, alignment and orientation.

The substrate chemical composition and topography influence such cell functions as: adhesion, growth, motility, gene expression and apoptosis. Controlling the biological environment through the appropriate substrate properties is crucial tissue engineering. Biocompatibility and mechanical strength support the growth and contraction of tissues. Appropriate physical and chemical properties promote the cell adhesion and growth. Channels and ridges in the surface structure lead to the proper migration and orientation of cells, ensuring the healthy tissue organization and its mechanical strength. Currently, the most popular techniques for preparing 3D scaffolding are photolithography, soft lithography, direct recording and laser ablation. Material and distribution constraints and high costs are disadvantages of photolithography, therefore the laser ablation method was used in the task to create migration channels. The advantages of this approach are: high resolution (up to 25 nm), non-contact interaction and applicability on any substrate. Thermal and mechanical propagation occurs during irradiation with nanosecond and longer laser pulses, causing melting and evaporation away from the absorption point and re-curing of the melting zone. The aim of the study was to structure the biomaterials surface with a laser beam to ensure the controlled migration of HUVEC (Human Umbilical Vein Endothelial Cells) cells. The presented results are of a cognitive nature in terms of the cell behavior depending on the substrate modification. This strategy has led to the generation of macroscopic pathways.

Migration channels were obtained via the laser ablation performed by the Optoelectronics Department of the Military University of Technology. Thin nanometric fragments of the 50 nm-long coating were removed to retain the half of the coating thickness. The process of ablation results from the interaction of the laser radiation (absorption and scattering) with the liquid ejected material. During the material surface treatment performed with the pulsed laser radiation of the proper power density (density of energy appropriate in time) the following phenomena occur: the radiation absorption and thermal or photochemical effects. The desired reflection requires a low level of radiation. Therefore, the excitation requires a large surface area of the laser beams intensity and low laser radiation absorption. The thickness of the evaporated layer depends on the material properties such as: optical, thermal and laser beam parameters, wavelength, power density, laser pulse duration.

Migration channels were formed as intersecting lines according to the scheme presented in FIG. 1. The task was performed in the Department of Optoelectronics of the Military University of Technology. The channels were designed especially to indicate the optimal pathways distance to the most effective overgrowth by endothelial cells. A diagram presenting the laser system used to make the channels is shown in FIG. 2.

TABLE 1. Deposition parameters of the first series of materials.

C225_2: 125 nm Si – a-C:H	
Deposition	
Target material	Si
Power [kW]	2.5
Voltage [V]	Start: 623; End: 624
Current [A]	Start: 4.00; End: 3.99
Gas flow [sccm]	45 Ar + 5 C_2H_2
Pressure [mbar]	$2.3*10^{-3}$
Duration of the process [min]	**7**
Voltage Bias [V]	50 (DC)
Current Bias [mA]	180 – 350
Resolution [nm/min]	18.1 nm/min
C225_2: 15 nm Si – a-C:H	
Deposition	
Target material	Si
Power [kW]	2.5
Voltage [V]	Start: 629; End: 627
Current [A]	Start: 3.96; End: 3.97
Gas flow [sccm]	45 Ar + 5 C_2H_2
Pressure [mbar]	$2.4*10^{-3}$
Duration of the process [min]	**1**
Voltage Bias [V]	50 (DC)
Current Bias [mA]	180 – 270
Resolution [nm/min]	18.1 nm/min
C225_6: 100 nm a-C:H	
Deposition	
Target material	C
Power [kW]	3.0
Voltage [V]	Start: 580; End: 573
Current [A]	Start: 5.16; End: 5.22
Gas flow [sccm]	40 Ar + 10 C_2H_2
Pressure [mbar]	$2.2*10^{-3}$
Duration of the process [min]	**19**
Voltage Bias [V]	off
Current Bias [mA]	-
Resolution [nm/min]	5.48 nm/min

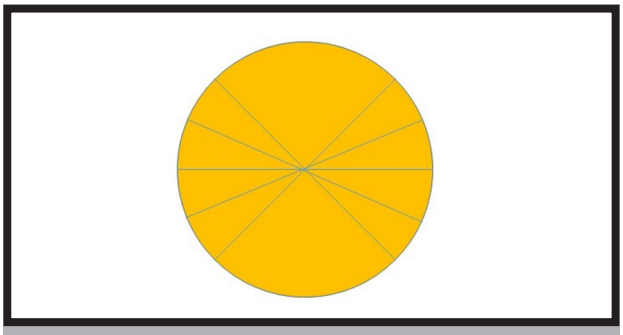

FIG. 1. Scheme of migration channels.

TABLE 2. Deposition parameters of the second series of materials.

Protokoll No. According to Table 1	Type of the coating			Thickness [nm]	Deposition process	Gas flow [sccm]
	a-C:H	a-C:H:N	a-C:H:Si			
Ti				0.0	sputter	-
C316_1	x			30	sputter	40.0 Ar + 10.0 C_2H_2
C316_2		x		15	sputter	40.0 Ar + 2.5 C_2H_2 + 7.5 N_2
C316_3	x			100	sputter	40.0 Ar + 10.0 C_2H_2
C316_4			x	125	sputter	24.0 Ar + 6.0 N_2

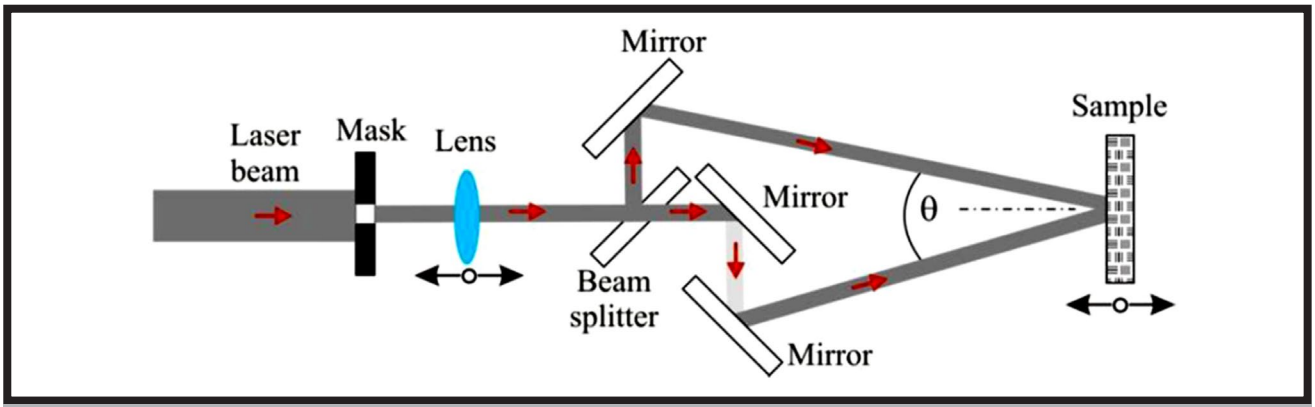

FIG. 2. Topography of migration channels at the lines intersection in the middle of the sample.

Results and Discussion

Characteristics of microstructure, chemical composition and mechanical properties. Studies on topography of migration channels are presented in FIGs. 3 i 4.

Microstructure

The self-inflicted stress distribution in the migration channels was performed using the X-ray diffraction method to measure the change of interplanar distance. The results of the stress distribution evaluation and the size of crystallites are presented in TABLES 3 and 4.

TABLE 3. The residual stress values in the designated places of the tested sample.

Area	Stress [MPa]	Standard deviation
Not modified	-6100.6	+/-300
Channel	-	-
Heat effective zone	-3500.5	+/-200
Area between the channels	-4600.2	+/-100

TABLE 4. The crystaline size values in the designated places of the tested sample.

Area	Size of crystallites Å
Not modified	96.4
Channel	116.3
Heat effective zone	96.0
Area between the channels	98.8

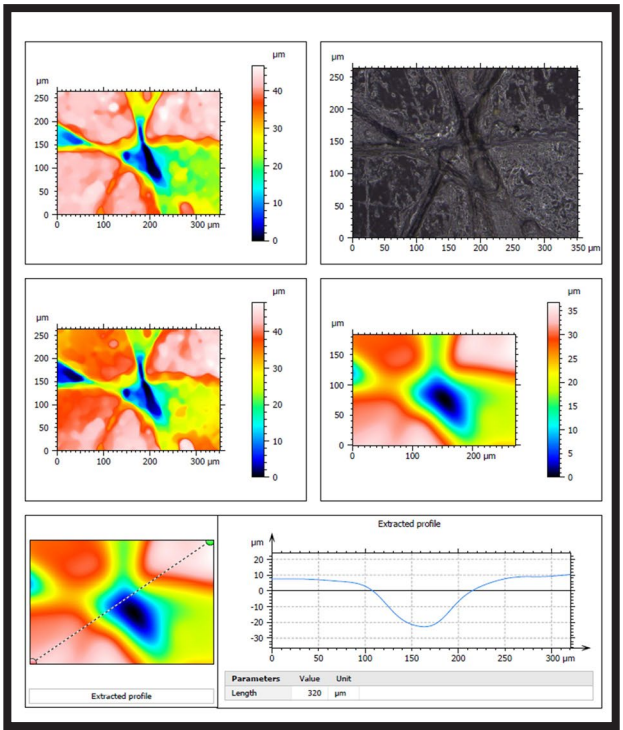

FIG. 3. Topography of migration channels at the lines intersection in the middle of the sample.

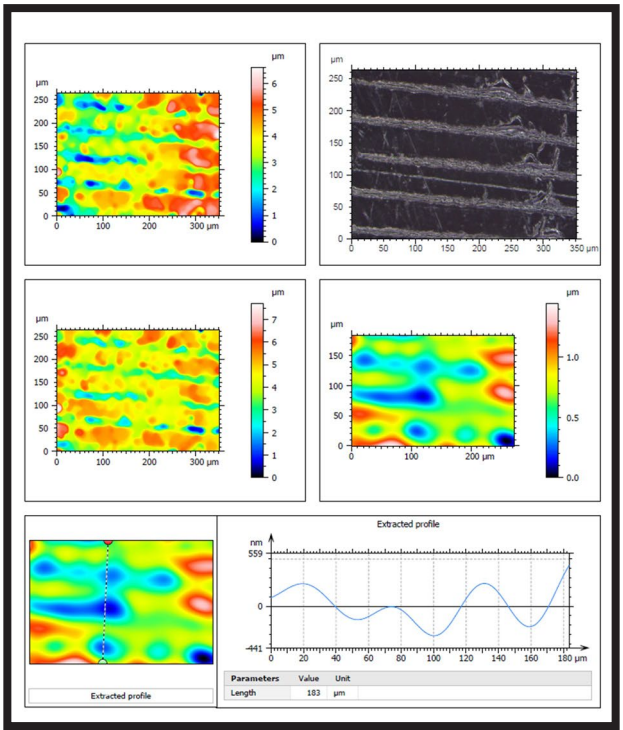

FIG. 4. Topography of migration channels at the edge of the sample.

The analysis of the migration channels structure was carried out using Transmission Electron Microscopy (TEM). For the TEM analysis thin films were prepared on the cross-section from the migration channel border to the unmodified surface. The platinum mask was used to differentiate the surface to be tested. The samples were made using the FIB (Focused Ion Beam Method) and the surface to be tested was marked on the SEM image. TEM was performed from the area at the tubule (FIG. 5) and in the tubule area (FIG. 6).

In vitro analysis

The influence of nano- and micro patterns on the adhesion, targeted growth and proliferation of endothelial cells was evaluated. The surface parameters were characterized to determine the proper formation of endothelial monolayer and blood vessel formation. The cell shape, contact surface, cell nucleus location, distribution of cytoskeleton elements (nucleus) and the number of adhesion molecules were compared. The expression of adhesion molecules, such as integrins, was transferred on the different stiffness and architecture materials. The cell migration process was observed on the structured surfaces. The hemophilic properties of surfaces and the protein adsorption to the substrates were analyzed using human blood. The analyses were carried out under conditions of high shear forces, simulating natural conditions in blood vessels. As a result of contact with the material, the degree of platelet and leukocyte activation in blood and the platelet aggregation were assessed.

Hemocompatibility test of a-C:H coatings

Hemocompatibility tests were carried out for the materials produced in the first phase of the project (TABLE 1). The aim of the experiment was to determine the biocompatibility in contact with blood and to select the materials for the final phase where the migration channels were made.

Two sets of tubes were prepared for each plate tested and for static control. Expression of platelet activation markers was determined by staining whole blood. In short, 5 µl of blood was gently mixed with monoclonal antibodies conjugated with fluorochrome: 5 µL FITC-PAC-1, 5 µL PE-CD62P and 4 µL PerCP-CD61 (all from Becton Dickinson, USA) in saline phosphate buffered (PBS) containing 0.2% bovine serum albumin and 2 mM calcium chloride (fi l. 35 µL). After 10 minutes of staining at room temperature, erythrocytes were lysed by adding 0.5 mL of lysing solution (FLS, Becton Dickinson, USA) and the plates were centrifuged (1,000 g, 6 min) and resuspended in the PBS buffer for further analysis with the cytometry flow. The samples were analyzed with the EPICS XL flow cytometer (Beckman Coulter Inc., Brea, CA, USA). The expression of platelet activation markers was measured on CD61 gated objects using PAC-1 antibody to change the conformational glycoprotein IIb/IIIa and CD62P for P. The activation marker was calculated as the product of the sum of geometric fluorescence averages and the percentage of marker-positive objects. The platelet aggregates were analyzed after the erythrocytes analysis by mixing 25 µL of blood with 0.4 mL FLS and then fixing them by adding 3.5 mL 1% paraformaldehyde in PBS. Cellular material was recovered by centrifugation (1,000 g, 7 minutes) and staining of active leukocytes (25 µL portions) with 4 µL PerCP-CD14 and 5 µL FITC-CD61 or 5 µL FITC-CD61 for 30 min at room temperature. The samples were then rinsed in PBS and subjected to the cytometric analysis. The percentage share of granulocyte platelet aggregates (leukocytes stained with CD61 platelet marker) was calculated using the forward/backward dispersion granulocyte gate and an additional CD14+ monocyte gate. The absolute number of platelets was calculated as the number of CD61 positive objects in relation to the total number of granulocytes.

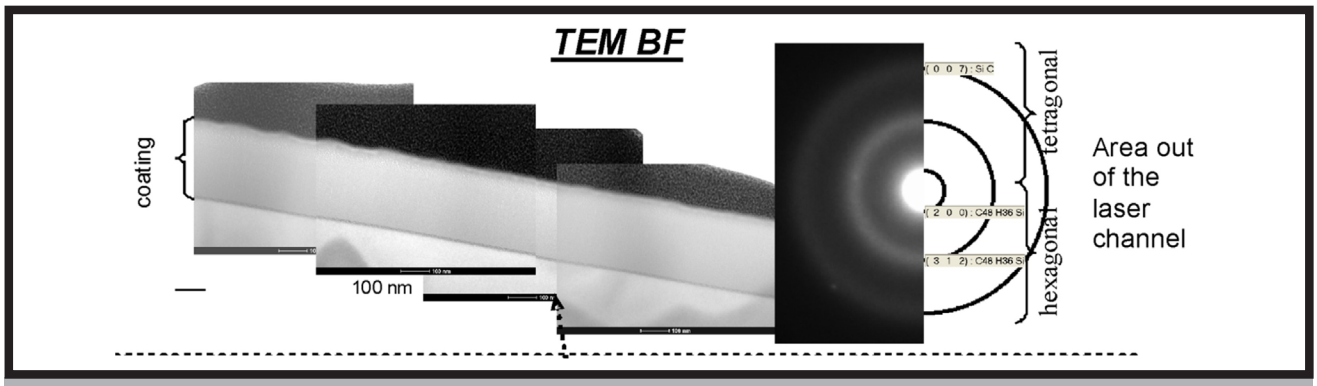

FIG. 5. The TEM microstructure of the area nearby the tubule channel.

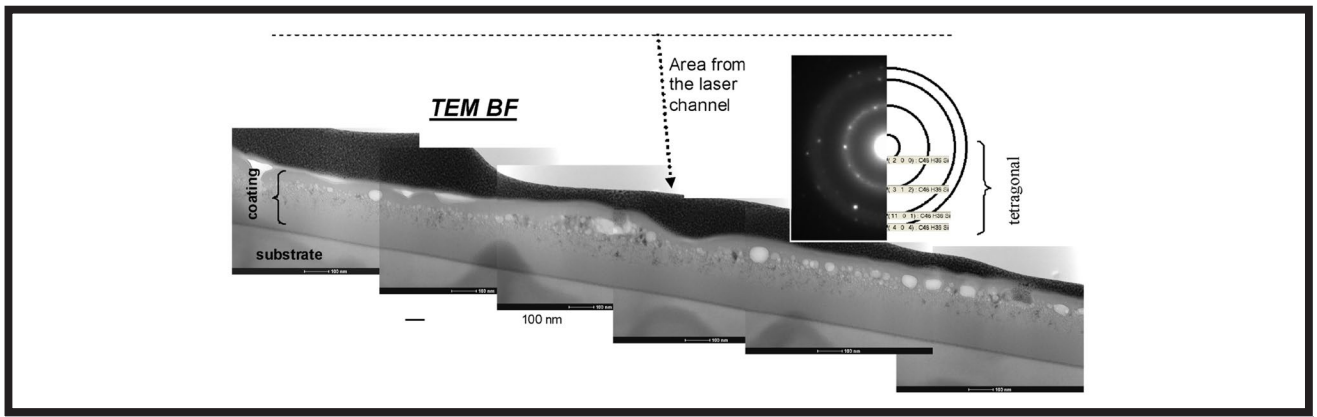

FIG. 6. The TEM microstructure in the tubule channel.

TABLE 5. Results of hemocompatibility tests.

seria		conversion into CD61+ objects (i.e. tiles and aggregates combined)				PLT % of all objects	expres-sion PAC-1 [PAC-1 %]	expres-sion P-selectin %+	Platelet leukocyte aggrega-tes (%)
	PLT count	PLT % of all objects CD61+	PLT-AGR % of all objects CD61+	SMALL PLT-AGG % of all objects CD61+	BIG PLT-AGR % of all objects CD61+				
bas	238.00	95.30	4.70	4.66	0.04	100.00	28.10	1.09	16.57
C225-2		93.87	6.13	6.04	0.09	89.69	30.83	4.42	72.60
C225-4		97.33	2.67	2.64	0.03	72.21	21.23	1.66	60.03
C225-6		97.00	3.00	2.97	0.03	72.26	20.03	1.82	72.43
Kontrola		86.84	13.16	11.24	1.92	67.99	36.07	5.60	39.40
ADP		62.99	37.01	20.84	16.17	20.07	97.47	74.23	16.73

TABLE 6. Tests of hemogliness of coatings of type a-C:H.

Series	Sample	Concentration nMx20
1	Bas (negative control)	8.0
1	C225 2+3	11.7
1	C225 4+5	16.3
1	C225 6+7	9.1
1	Ti	24.9
1	ADP (Positive control)	8.7
2	bas	29.1
2	C225 2+3	38.5
2	C225 4+5	35.9
2	C225 6+7	43.6
2	Ti	42.5
2	ADP	27.5
3	bas	15.4
3	C225 2+3	35.5
3	C225 4+5	20.4
3	C225 6+7	60.0
3	Ti	28.4
3	ADP	18.8

Small and large platelet aggregates were calculated using forward/backward scattering gates for CD61 positive objects. All other chemical compounds were obtained from Sigma-Aldrich. The results of the hemocompatibility studies show the degree of activation and aggregation (TABLE 5).

The thrombogenic potential of blood plasma was measured with the Zymuphen MP activity ELISA test (Hyphen Biomed, Er-agny, France), according to the manufacturer's instructions. This test is based on capturing phospholipid-rich microparticles from cell membranes using immobilized annexin V and then reconstituting the thrombin activity with a solution of calibrated clotting agents. The proteolytic activity of generated thrombin against the chromogenic medium closely correlates with the concentration of microparticles present in blood plasma. The test results are presented in TABLE 6.

Cytotoxicity assessment

Cytotoxicity testing was carried out on the materials selected from the first group and presented in TABLE 2. The tests were performed using human skin fibroblasts (NHDFNHDF) as model cells (purchased from Promocell) which continuously secrete various extracellular matrix components. Depending on their origin and physiological state, fibroblasts may exhibit different morphological phenotypes and different functional properties. Skin fibroblasts are isolated from the dermis of adolescents and adults.

Genotoxicity studies consisted in assessing the effect of the material on the lactate dehydrogenase level by means of the colorimetric method and analysis of microcellular nucleus formation by Luminex.

Cytotoxicity analysis – LDH

Lactate Dehydrogenase (LDH) is an enzyme that is found in the human cells and is involved in glucose metabolism. It easily penetrates the blood serum due to cell death, blood imbalance or increased cell membrane permeability. Its increased level is caused by the cell damage. By measuring the level of the released enzyme, the degree of cell lysis was determined using Lactate Dehydrogenase Activity Assay Kit (purchased from Sigma-Aldrich).

The results of cytotoxicity tests were based on the level of lactate dehydrogenase (LDH) and mutagenic effects on genes encoding characteristic control proteins responsible for the normal cell cycle. The results are shown in FIG. 7.

The given NeA negative control (set) consisted of HeLa cells treated with lambda phosphatase. Lambda Phosphatasephosphatase (Lambda PP) is an Mn^{2+} dependent protein phosphatase with activity against phosphorylated serine, threonine and tyrosine residues. Two positive controls were prepared for the experiment in order to observe the dynamics of activity derived from all possible proteins:
a) Positive control 1 (set) - Jurkata cells treated with 25 μM anisomycin.
b) Positive control 2 (set) - A549 cells treated with 5 μM of camptothecin. The cytotoxicity tests results carried out via the LDH release indicate a high level of safety of the tested materials. The results slightly exceed the control level.

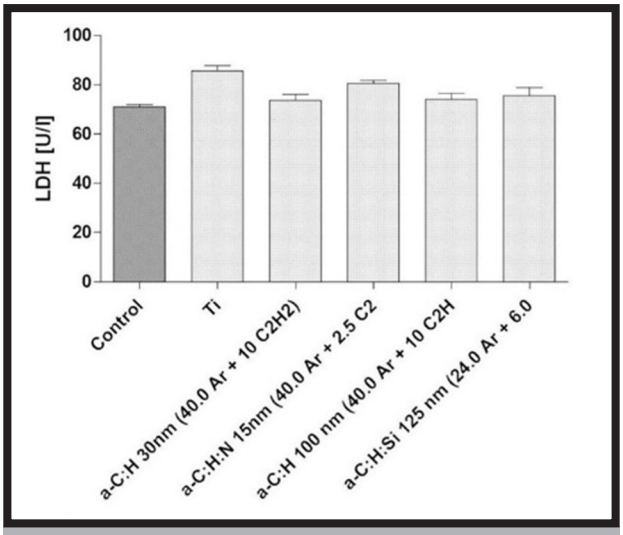

FIG. 7. Results of cytotoxicity assessment lactate dehydrogenase method.

Luminex genotoxicity analysis

MILLIPLEX® MAP is based on Luminex xMAP® technology (purchased from Merck) - one of the most widely accepted multiplexing technologies. This technology is used in life sciences and allows various biological tests, including immunological tests, to be performed on the surface of fluorescent encoded beads, called MagPlex®-C microspheres. Luminex uses its own techniques for internal coding of microspheres using two fluorescent dyes. Due to the precise concentration of these dyes, 100 individually colored sets of beads can be created, each coated with a specific interceptor antibody. After the sample is captured by the analyte, the biotinylated detection antibody is introduced. The reaction mixture is then incubated with streptavidin-PE conjugate, a reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres are illuminated and the internal colorants fluoresce, which means they indicate the set(s) of microspheres used in the test. A second light source induces PE phycoeritrin, a fluorescent dye on the reporter molecule. Fast digital signal processors identify each microsphere and quantify the biological test result from the fluorescent signals of the reporter.

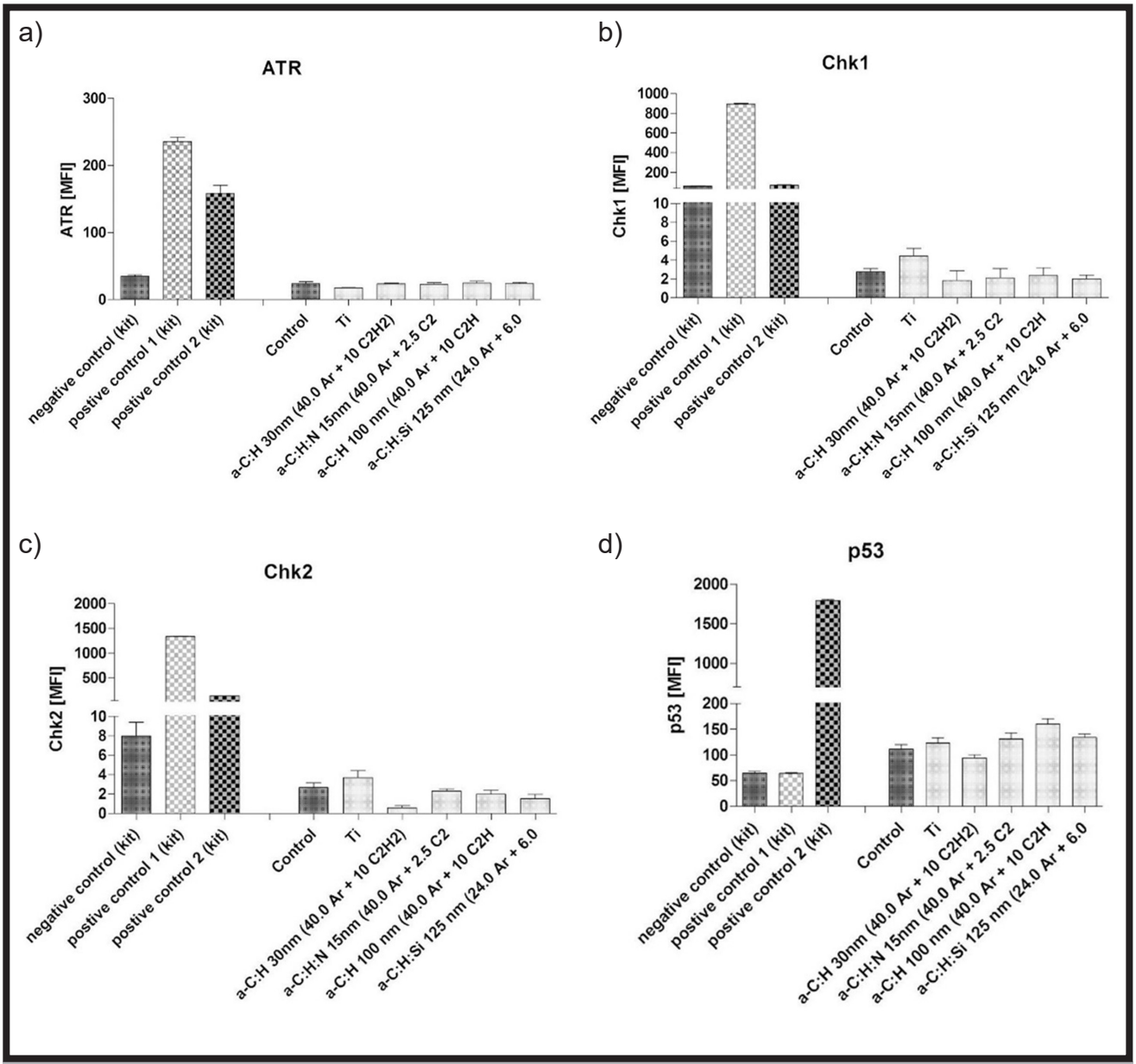

FIG. 8. Results of genotoxicity testing of selected surfaces.

The ability to add multiple conjugated beads to each sample gives the possibility to obtain multiple results for each sample. The open architecture xMAP® technology allows multiplexing of many types of biological tests, reducing time, labor and costs, compared to traditional methods. The following analytes were tested: RANTES, PDGF-AA and PDGF-AB/AA.

Chromatin binding and ataxia telangiectasia and Rad3-related activation (ATR) were observed in the cells treated with replication inhibitors. ATR recognizes replication abnormalities associated with the presence of DNA damage, such as replication forks blocked at the site containing the degradation products. The results are shown in FIG. 8a. No activation of the gene responsible for encoding the ATR protein was found.

Checkpoint 1 kinase, commonly called Chk1, is a human specific serine / threonine / throne kinase encoded by the CHEK1 gene. Chk1 coordinates the DNA damage response (DDR) and cell cycle checkpoint responses. Activation of the CHK1 gene initiates cell cycle checkpoints, stopping the cell cycle, DNA repair and cell death to prevent damaged cells from passing through the cell cycle (FIG. 8b).

The human gene encoding the CHEK2 protein is an effector kinase involved in DNA repair. CHEK2 is an anticoncogen; its protein product, interacting with the P53 protein, among others, stops the cell cycle. The results are shown in FIG. 8c. A low level was observed for all tested materials, at the control level.

The results of P53 activation are shown in FIG. 8d. In all the diversity and variety of proteins present in living organisms, one protein obtained a special status. The P53 protein, which is also sometimes called “genome guard”. P53 is a protein with transcription factor activity that binds to the DNA in the promoter region and can modulate the expression of many genes, so its effect on activation is crucial. The results of the study indicated the low level of activation of the gene encoding P53 protein.

Surface functionalization via ducts in porous coatings

Migration channels were created on the materials presented in TABLE 2. For cutting out the channels the III harmonic from 355 nm wavelength was used. The laser was coupled with a galvanometer scanner with a telecentric lens of 160 mm focal length. The scanning speed was constant in all the cases and equaled 1 mm/sec. The pulse duration was about 70 ps and the repetition frequency was 1 kHz. The energy of laser pulses ranged from 9 to 10 uJ. The surface functioning was based on the application of coatings on the tubules using the electrostatic influence method. Pol electrolytes were used for surface modification.

Multilayer coatings from polyelectrolyte were made using the so-called “layer by layer” method. In the first stage, a surface charge was generated at TPU to enable anchoring of the first layer of the first polyelectrolyte. The substrate was activated with 10 M NaOH for 25 min and was rinsed with pure Milli-Q water to remove NaOH. Such chemical etching resulted in a negative charge on the substrate surface. At the same time, polycation, i.e. poly-L-lysine (PLL) and polyanion, i.e. hyaluronic acid (HA), were dissolved in 400 mM solution HEPES/0.15 M NaCl with the concentration of 0.5 and 1 mg/ml respectively. The pH of the solutions was adjusted to 7.4 by adding 0.5 M NaOH. After each stage of the deposition, in order to remove excess polyelectrolyte, the samples were rinsed in 0.15 M NaCl buffer solution with pH 7.4. The process was repeated until the desired number of double-layers was reached. PLL was always the outer layer. Finally, the samples were rinsed and stored at 4°C in 400 mM of HEPES/0.15M NaCl buffer solution with pH 7.4.

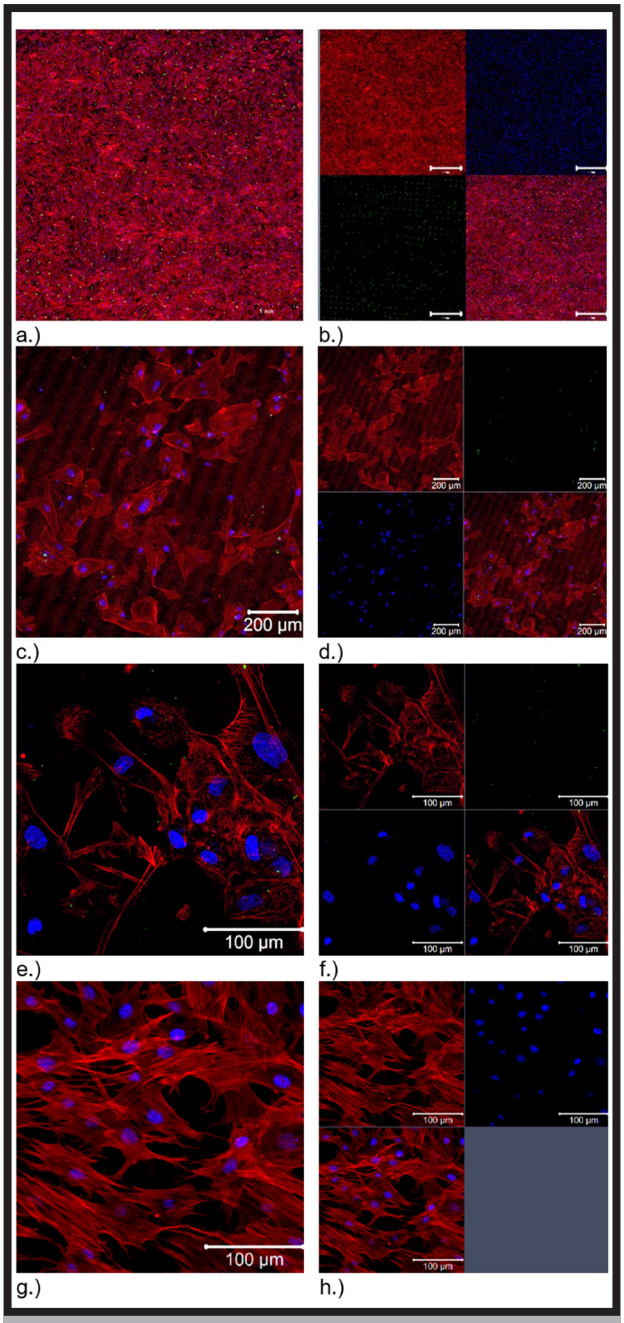

FIG. 9. Cell-material interaction on the surface without structuring.

HUVEC migration studies

Local adhesion and adhesive plaques are membrane bound complexes that serve as nucleation sites for actinic fibres and as connections between cells. They are also places of signal transduction, initiating path signaling in response to adhesion. The Focal Adhesion Staining Kit (FAK100 Catalogue Number) is a very sensitive immunocytochemical tool that contains fluorescent labeled falloidin (conjugated with TRITC) to map the local orientation of actin fibres in a cell and the monoclonal antibody against vinulin, which is very specific for staining focal contacts in cells. The kit also includes DAPI for fluorescent core marking. The results of the tests of the non-structured materials are shown in FIG. 9.

The results of the tests on the structured materials are presented in FIG. 10.

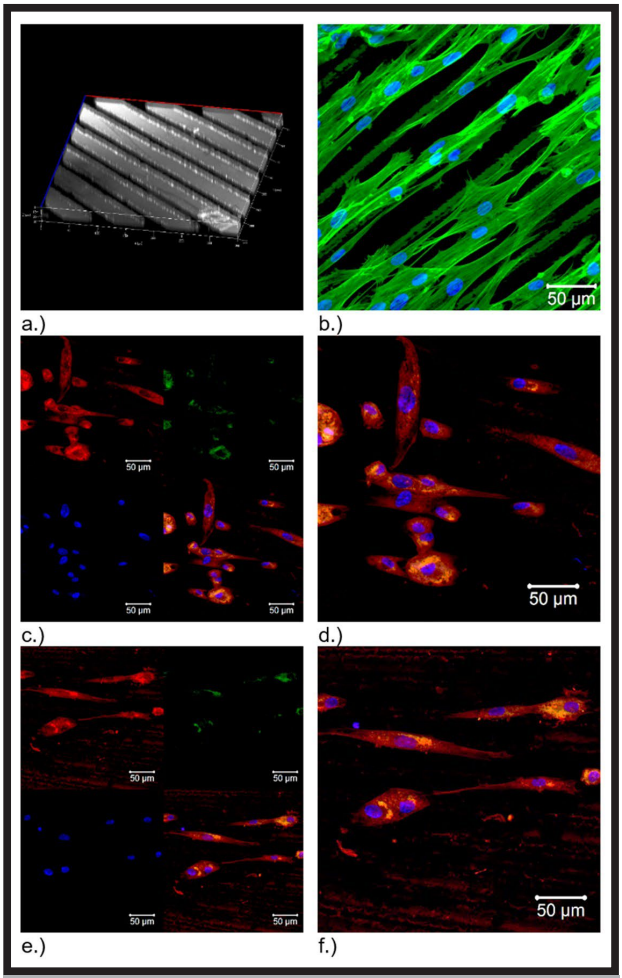

FIG. 10. Cell-surface interaction after surface structuring; a) Topography of migration channels; b) Staining of cytoskeleton actin (green, excitation 488 nm) and nuclei (blue excitation 405 nm) of HUVEC cells on migration channels; c) I place, SPLIT image - Staining: Vinculine monoclonal, maple 7F9, TRITC-guided phloidyne, DAPI; d) II place, full image, FAK 100; e) staining, Second place, SPLIT image - staining: Vinculina monoclonal, 7F9 clone, TRITC-guided phloidyne, DAPI; f) II place full image, tinting FAK 100.

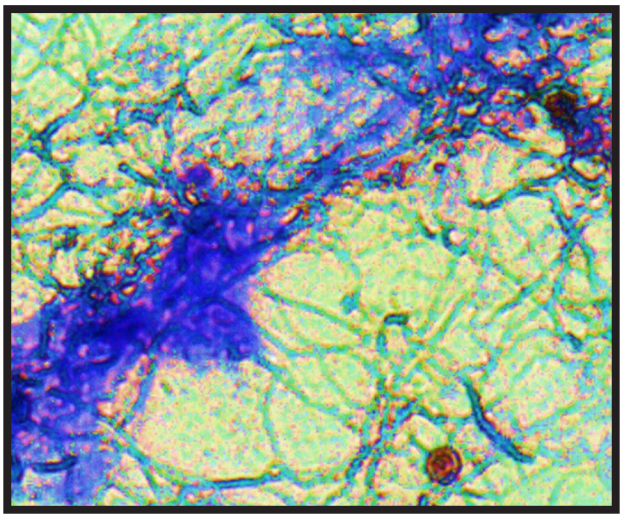

FIG. 11. Preliminary results of revascularization tests.

Discussion

The aim of this work was to develop the material surface facilitating integration with the tissue. The project focused on the surface integration with cardiac tissue and integration with a specific form of connective tissue such as blood. The surface modifications for both issues was performed via laser evaporation of a part of the biocompatible layer, creating a suitable environment for a specific tissue. The interaction of the cells with the material is strictly dependent on such surface properties as: topography, microstructure or mechanical properties. In the case of integration with the heart endothelium, the metallic surface was enriched with bio- and hemocompatible coatings based on amorphous, hydrogenated carbon. The prepared coatings were additionally modified with silicon and nitrogen. These surfaces underwent further modification - patterns made by laser nanolithography. This technique determines the high precision of the shape, size and distribution of the patterns. Periodisation of the surface provided an optimal surface and pattern suitable for cell adhesion and proliferation processes. In the project, an attempt was made to produce normal blood vessels on the network surfaces. We attempted to produce the network of blood vessels through polymeric polyelectrolyte coatings and to control proteins and proangiogenic factors. The conducted experiments gave positive results, however, it was not possible to obtain the appropriate repeatability. Further studies are recommended.

Conclusions

The following goals were achieved in the course of the work:
- ultra-thin amorphous carbon coatings (a-C:H) on Ti and Ti6Al4Vo substrates of varying thickness, applied using physical techniques from the gaseous phase
- description of the influence of coating application parameters on the hemocompatibility of the surface
- specific properties of the materials in terms of cytotoxicity and genotoxicity
- structured surface with different architecture (2D and 3D structures) and topography obtained and optimized by means of direct laser interference lithography
- characteristics of morphology, topography and mechanical properties of surfaces
- evaluation of protein adsorption (albumin) to the substrate and influence of the protein layer on inhibition of coagulation processes - description of the influence of surface periodisation on endothelial cell response and blood vessel formation
- the valuable influence of surface nanostructuring on targeted cellular growth and production of adhesion molecules -the revascularization attempt was not fully successful. Repetitive results were not achieved despite the introduction of VEGF growth factors into polyelectrolyte structures (FIG. 11).

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