Bioengineering methods in designing materials for the cardiovascular system regeneration

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Abstract

Laser techniques find its application in a variety of science branches. The work describes the laser application in the bioengineering analysis. To observe changes in the cell viability and morphology after cell-material interactions (attachment) as well as cells migration in channels the confocal microscopy was applied. This instrument enables to investigate material in so called optical sections of the observed specimen. It analyzes the light coming from the single plane. The system rejects the light coming from the planes above and below the focal plane. Thus, the resolution and the contrast is improved. It gives an opportunity of the three dimensional image constructions. The second analytical instrument which was designed on basis of the laser technique is flow cytometry. In the work this instrument was used for the haemocompatibility analysis of the blood-material interaction where platelet activation mechanism was studied. A laser was also used to fabricate migration channels by laser ablation and subsequent confocal microscopy analysis of cell migration in respect to the substrate type. The main idea of the design in both instruments is similar. The source of the light is laser system of lenses to keep the beam and direct it into the appropriate point and light detection system. The work introduces into the confocal microscopy and flow cytometry technique as well as laser ablation and presents their real application in the biomaterial engineering.

Keywords: confocal microscopy, migration channels, laser ablation, flow cytometry, endothelial cells

1. Confocal microscopy

The concept of confocal microscopy was founded in the 50s of the last century by Marvin Minsky. In 1961, this concept was patented. Late 60th brought a solution for multi-beam confocal microscopy. The solution presented by David Egger and Mojmir Petran. In 1979, Fred Brakenhoff built the first working confocal microscope. However, the first commercially available confocal microscopes appeared in 1987 [1]. Most appropriate, reflecting its work and construction, the name of the device should be: “microbiospectromultifluorimetry”. Confocal microscope makes it possible to carry out observations with a well defined optical plane. System eliminates the light coming from above and from the plane of the optical image sequences. Submission of individual planes, enables the construction of three-dimensional objects surveyed. In this microscopiy, point light source, illuminated point of the product and its image are in the focal lens (lie in confocal planes) and hence the name of this microscopy. The light that is triggered at points beyond the focus of the system is eliminated by special apertures and does not take part in the process of creating the image (Fig.1).

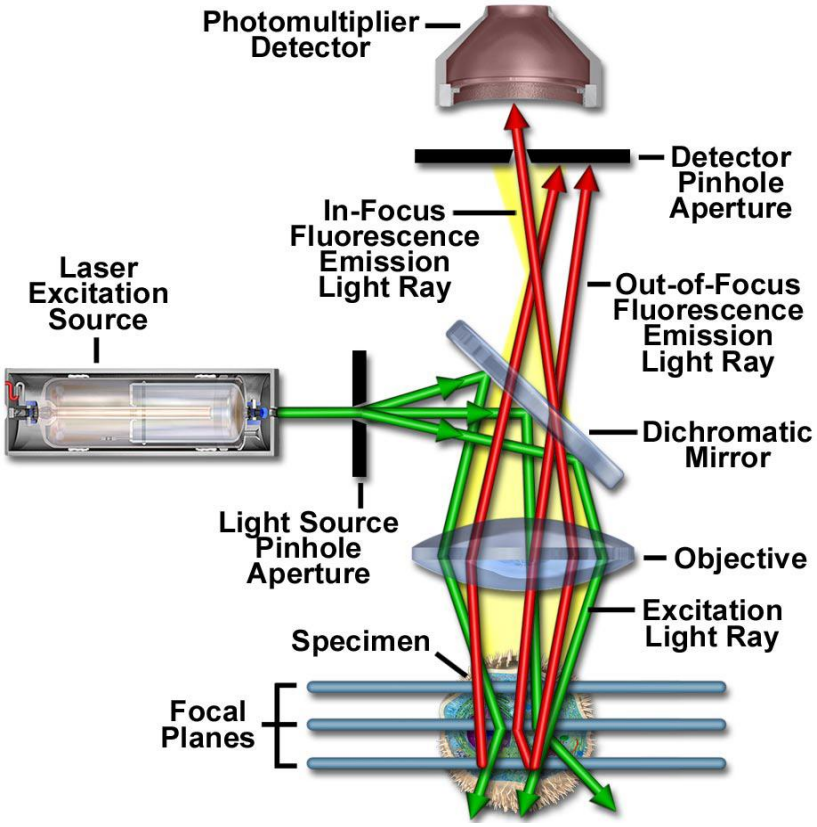

Fig. 1. Confocal laser scanning microscopy scheme [1]

In a conventional microscope, the transformation of the object image is performed simultaneously and in parallel for all points on the object. For confocal microscopy, laser and samples are irradiated in a point, i.e. the series. Physics of the interaction between the laser light and the sample (e.g. fluorescence) is measured point by point. Therefore, in order to obtain information about the sample, the laser beam must be conducted through the sample. This process is called scanning. In practice, the fluorescence analysis, using a confocal microscope based on the following stages: the coherent laser light (excitation source) passes through the diaphragm aperture, set in the confocal plane and laser light point is scanning on the sample. The second aperture is located at the entrance to the detector. The laser beam is reflected from the dichromatic mirror and scans the sample in a defined focal plane. Secondary fluorescence emitted from the sample points (in the same focal plane) passes through the dichromatic mirror and is focused as a confocal detector aperture. A significant amount of fluorescence from points above and below the focal plane is not confocal with aperture and formed so-called “Airy disks” (Fig. 2).

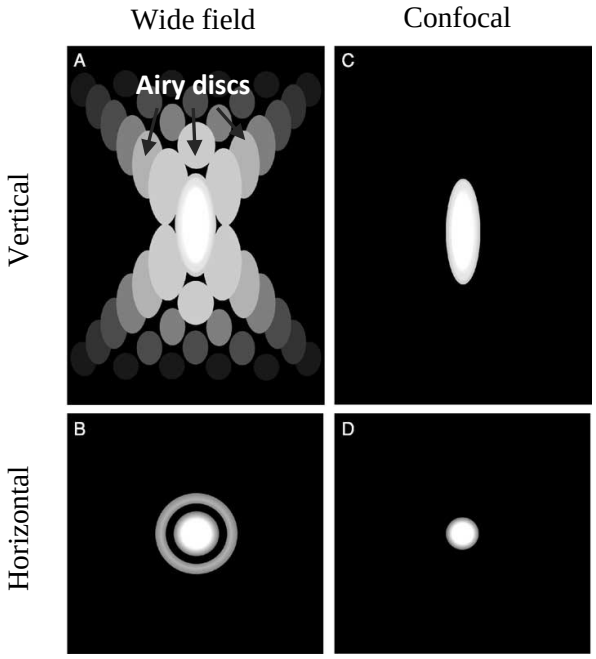

Fig. 2. Vertical and horizontal images comparison between wide field microscopy (A, B) (Airy discs formation A), and confocal laser scanning microscopy (C, D) [1]

The scanning head is a heart of the confocal microscope system responsible for collecting photon signals. For confocal microscopy, the maximum magnification is not a parameter that describes the resolving power of the microscope. Resolving power is the ability to distinguish between two small objects that are in close proximity. Confocal microscopy analyses the fluorescence patterns of the sample [1-4]. In recent years, lasers have become a popular research tool commonly used in biomedical engineering. In this contribution, application of lasers is presented as light source in confocal microscopy and flow cytometry in examinations of functional blood contacting materials.

1.1 Fluorochromes

Fluorochromes are categorized and described according to their absorption and fluorescence properties. One of the most useful parameters characterizing the quantitative absorption spectra is the molar extinction coefficient which measures the ability of molecules to absorb light. Most sources used to excite the fluorescent dyes are the broadband sources, such as mercury, halogen bulbs. They produce white light, which has peaks of varying intensity across the spectrum. In contrast, a laser excitation source provides one or more well-defined peaks, allowing more selective illumination of the sample. Best results are obtained when the excitation maximum of the dye is close to the wavelength of the laser light. The most commonly used lasers are 488 nm blue-green argon laser, 543 nm helium-neon green laser and a 633 nm helium-neon red laser. The mixed gas lasers, such as krypton-argon laser can output different laser lines and, therefore, optical filters are introduced to achieve selective excitation [5].

1.2 Fluorescent Dyes

For determination of cellular structures by fluorescence microscopy there are used specific fluorescent dyes divided into three groups:

Table 1 Fluorofores classification

Group	Type
I	- small molecule - fluorescent proteins
II	- specific - nonspecific
III	- penetrating the integral cell membrane - not penetrating the integral cell membrane

Non-specific dyes are often coupled with antibody and are used in immunofluorescence marking. One of the most common techniques for the visualization of cells using confocal microscopy is application of fluorescence molecules such as conjugated with antibodies fluorochromes. In these studies, the antibody is added to a sample containing cells. The antibody then is combined with specific molecules on the cell surface or inside. Finally, the laser light is directed at the sample, followed by excitation and emission of light, which is processed in the detection system of the microscope.

The first stage of immuno-histo-fluorescence cell staining is their fixing and / or permeabilization of cell membrane. These steps are designed to allow easy penetration of antibodies in both individual cells and intracellular proteins.

Examples of specific fluorescent dyes:

• dyes bind to nucleic acids (TOTO, YOYO, DRAQ5, ethidium bromide, DAPI, Hoechst)

• lipophilic dyes, coloring lipid membranes (DIO, DII)

• dyes stored in mitochondria, the potential to mitochondrialnemu (rhodamine 123, TMRE, JC-1)

• pH indicator dyes SPECIES (BCECF)

• dyes SPECIES concentration ratios of Ca + + (Fura-2, Indo1, Fluo-4)

The advantages of confocal microscopy are as follow:

• Ability to obtain optical sections (0.5-1.5 mm) with fluorescent sample thickness up to 50 mm

• The information received in the form of the image comes from a well defined plane

• Reduction of background fluorescence

• Non-invasive tomographic imaging technique that allows the analysis of living cells and established

1.3 Limitations of fluorescence microscopy

• Cytotoxicity- introduction of a fluorescent dye into the cell will lead to changes in cell physiology. Be sure to use the most neutral colors for the cell and the smallest concentrations that enable even imaging.

• Photobleaching- excited dye molecules react with components of the cell and change the chemical properties, it leads to a loss of fluorescence. The most frequently oxygen is involved in the process and the singlet oxygen produces reactive oxygen species
Fluorescence quantum yield tells how many photons absorbed take part in photobleaching. The ratio of fluorescence quantum yield to photobleaching quantum yield tells you how many photons emitted are subjected to photobleaching. The method uses the phenomenon of photobleaching is FRAP (Fluorescence Recovery after Photobleaching).

2. Flow cytometry

Flow cytometry is a laser based, biophysical technology employed in cell counting, sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus [6-8]. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in basic research, clinical practice and clinical trials. The device analyzes thousands of cells by passing the laser light.
The main elements of flow cytometry are as follow:
- Fluid system, by means of which the individual cells are introduced into the laser light
- Lasers, the light source for the analysis of the light scattered and fluorescent analysis. The most commonly used lasers are 488 nm blue-green argon laser, 543 nm green helium-neon laser and the 633 nm red helium-neon laser.
- Optics, which allows you to keep the light
- Detectors
- Electronics
- Peripheral electronic devices that convert the signal from the detector to a digital signal.
The so-called interrogation point is the heart of the system. It is a place of cross between the sample and the laser light. For analysis by flow cytometry, it is important that at any given time a single molecule or a cell passes through the point of testing. Most cytometers are equipped with a system of injection. Then, the construction is so arranged that the capillary light with flowing medium is tightening to the diameter of a single cell. The cell input into the capillary light in the analysis of single cells or particles is called hydrodynamic focusing. When a single cell appears on the path of the laser light, the light is refracted or scattered in all angles. The so-called. direct scattering or scattering at small angles is the amount of light that is scattered in the direction and phrase complies to the direction and phrase of the laser beam. The size of the dissipated beam is approximately proportional to the size of the analyzed cells. Therefore, with this type of analysis, the test can estimate the size of the particles at a given time. The collection of scattered light is using a system of detectors, where the intensity of the light is converted to a voltage. In most cytometers, a barrier, also known as blackout barrier detector is positioned directly in front of scattered light and it preventing getting too much light from the laser to the detector.

3. Examples of the researches executed in the area of the heart disease regeneration

There is still no consensus about whether hydrophilic or hydrophobic behavior of a surface is advantageous. These differences stem from a lack of complete understanding of the

biological responses caused by the interactions of tissue with artificial materials. In the development of materials with lower thrombogenicity, researchers have primarily focused their efforts on modifying surfaces. Unfortunately, a truly nonthrombogenic surface does not exist. Thus, surface modification plays a significant role. Surface modification methods for increasing hydrophilicity and electrically charging generally quite hydrophobic biomedical polymers include wet chemical (acid, alkali), dry (ozone, corona, or plasma), and radiation (ultraviolet/ozone and laser) treatments. The most versatile technique is plasma modification, which is attractive for biomaterials due to: (a) its ability to change and control the surface chemistry and properties of materials without altering their bulk, (b) its inherent sterility, and (c) the possibility of scaling up to industrial sizes and throughput with "real" substrates (e.g., webs and fabrics). The next step in efficient biomaterial design is the recreation of natural behavior.

3.1 Migration channels

3.1.1 Preparation

Migration channels were prepared by the laser ablation. Thin, nanometer scale parts of the coating of 50 nm were removed in the half deepness of the thickness. The process of ablation occurs during the laser pulse by an interaction between laser radiation (absorption and scatter) and the ejected material in the liquid form. During treatment of material surface with a pulsed laser radiation of sufficient energy density over time (power density), the following phenomena take place: absorption of radiation and thermal or photochemical effects. The desired reflectance needs low radiation. Hence, excitation requires a large area of the laser beams with intensities and small depth of absorption of laser radiation. The thickness of the evaporated layer depends on material properties like: optical, thermal and laser beam parameters, wavelength, power density, laser pulse duration time.

Process parameters were properly matched to enable execution of the half of the layer thickness. The selected migration channels were prepared in the form of single wells (Figs. 3a, 3b), as well as in the form of lines (Figs. 4a, 4b). For both patterns two characteristic zones were observed: laser evaporated zone and heat affected zone.

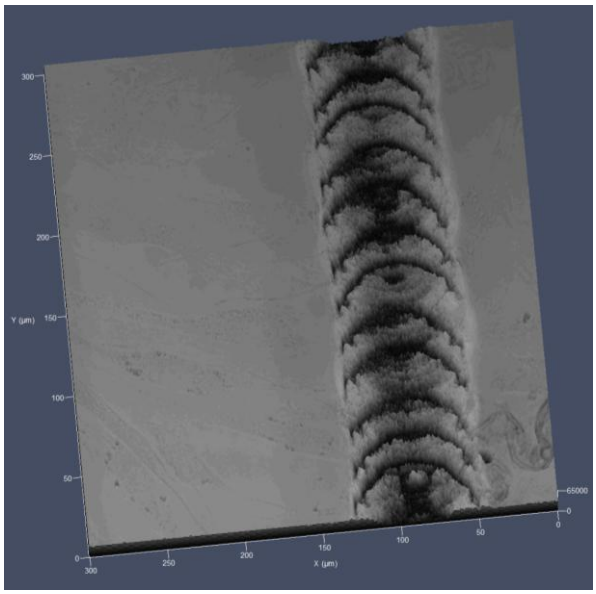

Fig. 3a. 2.5D line-like migration channel topography analysis using confocal microscopy

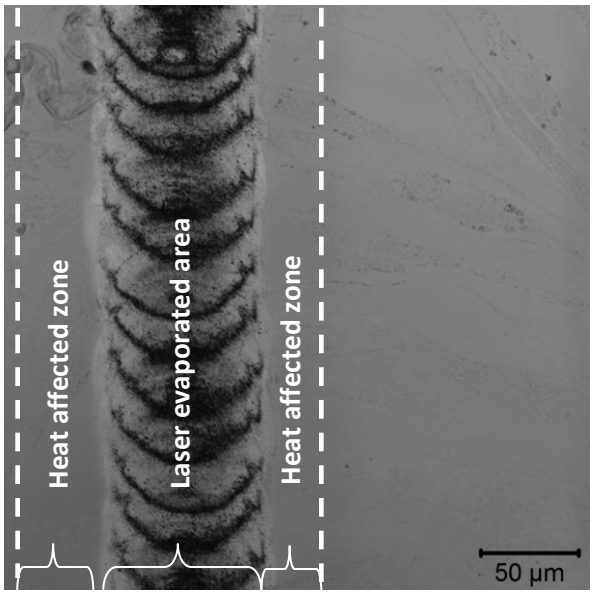

Fig. 3b. Surface analysis using confocal microscopy

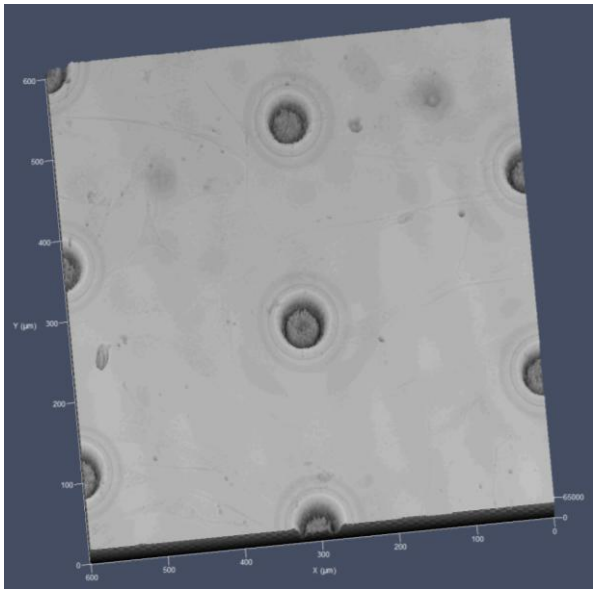

Fig. 4a. 2.5D point-like migration channel topography analysis using confocal microscopy

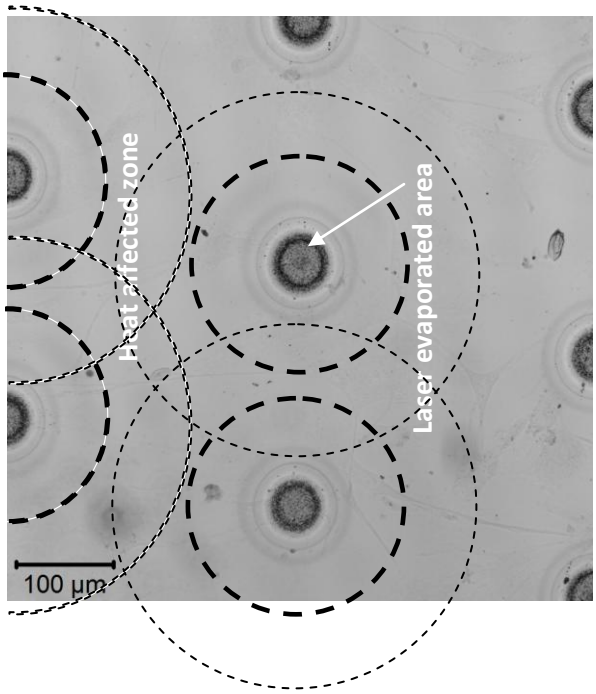

Fig. 4b. Surface analysis using confocal microscopy

3.1.2 Smooth muscle cells interaction with fabricated substrate

The fabricated grid template on a silicon wafer covered with titanium carbonitride (Ti(C,N) layer was used as migration channels for smooth muscle cells. External signals from the substrate (e.g. topography, physicochemical structure diversity) may induce an assembly of cytoskeleton through actin polymerization which could influence the cell locomotion. The cell migration undergoes series of characteristic events: extension of one or more lamellipodia (mobile edge of the cell) from the leading edge, adhesion to the substrate, forward movement,

re-tracting of the cell body. Cells fixed during the movement should be elongated and flatten as distinct from a stationery state.

The cells were kept in the incubation conditions: 5% CO_2 100% humidity, 37 ^{0}C for four days. After this time cells were fixed in 4% solution of paraform(aldehyde). Mitochondria, cytoskeletons and nuclei were stained with green carbocyanine-based MitoTracker Green, Alexa Fluor 488 and DAPI, respectively, and observed under a confocal microscope LSM 5 EXCITER. Transmission Electron Microscopy (TEM) measurements enabled detail analysis of the microstructure of the substrate.

Confocal microscope observations showed the relation between a surface topography: laser evaporated area and heat affected zone and cells preferences during migration on the surface. On flat surface the locomotion was not oriented. Cells did not proliferate on the laser evaporated zone. They formed interconnections through (Fig. 5). Quite different situation was observed for the well like shaped migration channel. Cells adhered, proliferated on the as deposited part and on the evaporated well. They did not like the area of the heat affected zone. The observed reaction possibly comes from the additive effect which took place during heat effected zone formation (Fig. 6).

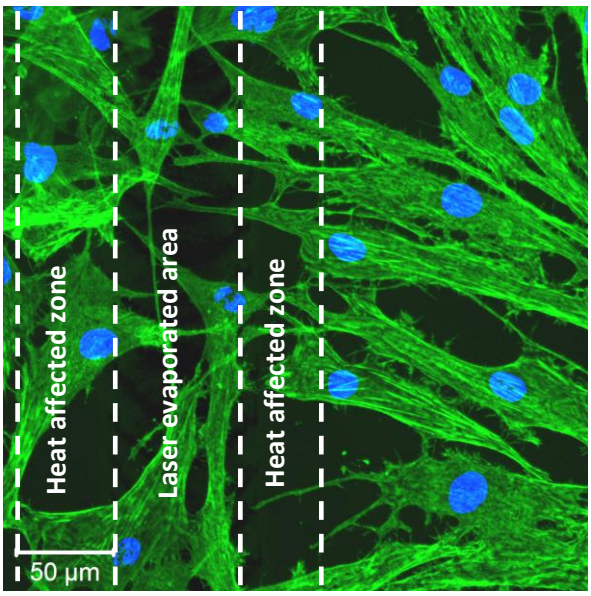

Fig. 5. Confocal laser scanning microscopy analysis of the cell (smooth muscle cells)-material interaction

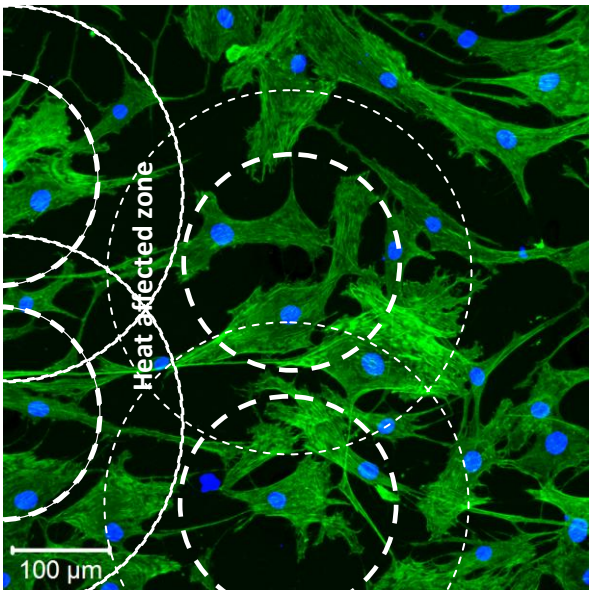

Fig. 6. Confocal laser scanning microscopy (CLSM) analysis of the cell (smooth muscle cells)-material interaction

3.2 Antithrombotic protection with tissue analogue precoursor coating

In general, the more a biomaterial surface resembles a natural one, the better the surface is. The ideal non-thrombogenic surface for vascular grafts/artificial hearts will consist of an intact luminal endothelial cell layer. Because of the decreasing amount of donors in conjunction with the simultaneously increasing amount of circulation problems, blood contacting materials development is of the highest importance. Porous materials (full porous and semi-porous) are the key issue.

3.2.1 Cell culture

Two cell types were used for these experiments, namely, human umbilical endothelial cells and blood cells. Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza. Each vial had a concentration of 500,000 cells/ ml. The cells were stored in liquid nitrogen until use. We plated 100,000 – 125,000 cells in a 25 cm^2 flask. From each vial, it was possible to prepare 4 or 5 flasks. Cells were resuspended in an endothelial cell culture basal medium mixed with cell growth and survival supplements (bullet kit growth mixture purchased from Lonza, including cell growth promoting serum, vitamins, and antibiotics). Before adding cells, the medium was warmed in a 37 ºC water bath. Cells were taken from the liquid nitrogen container and placed for 2-3 minutes into a 37 ºC water bath. Under the laminar air flow chamber, a maximum of 1 mL of medium mixes with the bullet kit was added. Everything was then pipetted into a 15 mL Falcon tube and diluted to 4 or 5 mL, to receive 100,000 or 125,000 cells / flask, respectively. The resuspended cells were taken in the

amount of one millilitre from the Falcon tube and introduced into a 25 cm^2 cultivation flask. Then each flask was filled in up to 6 mL of medium with supplements.

For the haemocompatibility experiments, blood from two sources was used. For the Impact-R test, performed in Collegium Medicum, blood was collected from volunteers and dedicated to experimental research. For the aortic blood flow analysis, blood was purchased from the Centre for Blood Donation. An anticoagulant consisting of sodium citrate, citric acid, glucose, sodium di-phosphate, and the amino acid adenine was added to the blood samples. Experiments on large models known as "artificial patients" were carried out using pig blood collected during slaughter. This blood was also treated to prevent coagulation.

3.2.2 Surface functionalisation, PEG incorporation

The biomimetic surface structure was prepared using two different methods. The first was the construction of a porous coating, consisting of 12 bi-layers of polyelectrolyte (PLL/HA), stabilised with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulphosuccinimide (NHS). An EDC/sulpho-NHS mixture (EDC at 400 mM and NHS at 100 mM were mixed v/v) was freshly prepared in a 0.15 M NaCl solution at pH 5.5. The coupling chemistry is based on the reaction of activated carboxylic sites with primary amine groups. The film-coated substrate was put in contact with the mixed EDC/NHS solution for 12 hours. The final coating consisted of PLL. After the polyelectrolyte coating preparation, the endothelium-appropriate protein, fibronectin (FN) was introduced into the porous coating structure. The fibronectin was prepared with a final concentration of 50 μg/mL. HUVEC cells were deposited on the surface of the selected, porous, extracellular-like material (Fig. 6). The second method was also based on constructing a porous, polyelectrolyte-based coating. The difference was in the final coating, which was deposited after the cross-linking process. The final coating consisted of poly(L-Lysine)-*graft*-poly(ethylene glycol) (PEG). PEG was prepared with a concentration of 0.5 mg/mL in 1x PBS. This mixture was stored for 12 h at -22 ºC. Immediately before deposition, the PEG mixture was warmed at 37 ºC for 30-45 minutes.

By forming brush-like structures, PEG -creates a “stealth effect”, repelling proteins, cells and bacteria. Biofunctional ligands, such as small peptide sequences, can be added to the (PLL-*g*-PEG) chains to induce specific interactions between cells and the surfaces of the biomaterial (Fig. 7).

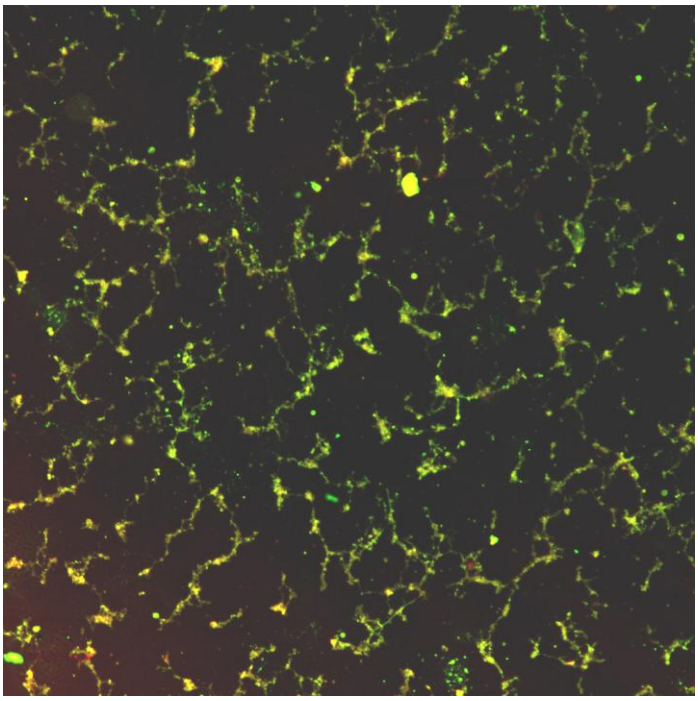

Fig. 7. Top view of the polyelectrolyte fictionalized surface. Polyethylene glycol deposited on top rejected all types of cells

3.2.3 Active peptide sequence (arginine-glycine-aspartic acid)RGD incorporation as a functional group to promote HUVEC cell growth

A cell-adhesive RGD was immobilised onto the carboxylated surface in 100 mg of EDC and 10 mL of phosphate-buffered saline (PBS) for 24 hours with gentle shaking at 0–4ºC following the instructions presented in literature [10]. The samples were subsequently immersed in 1 mg of RGD and 10 ml of a PBS solution for 36 hours with mild stirring at 0–4ºC. The endothelial cells were cultured as described in paragraph 2.4. After 3-4 days, the colony was generally confluent. Because endothelial cells are adhesive, it was necessary to detach the cells from the bottom of the culture dish. For this reason, trypsin EDTA 1x, diluted in 1x PBS was used. Trypsin was later utilised by the endothelial basal medium. Before cell deposition, the surfaces previously incorporated with RGD were washed several times with 1x PBS. Cells were generally cultured 3-4 days before being used in haemocompatibility tests. The functional surfaces without and with RGD incorporation are presented in (Fig. 8)

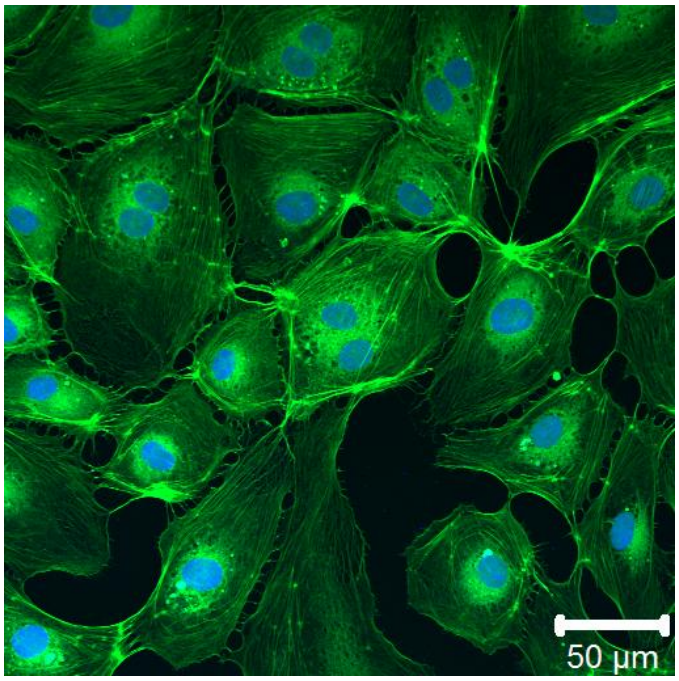

Fig. 8. CLSM analysis. Top view of the polyelectrolyte fictionalized surface. Polyethylene glycol deposited on top supported with small RGD molecular domains caused selective surface properties towards mezenchymal origin cells interaction.

3.2.4 Haemocompatibility testing

A classical instrumentation for the dynamic test of haemocompatibility involves a flow chamber with a contact surface between blood stream and tested plate. In the current study a simplified model of the whole blood shear stress was investigated, based on a cone and plate rotational viscometer. Applying laser based flow cytometry several indices of platelet activation were analyzed, including platelet- and granulocyte-platelet aggregates, platelet activation markers and platelet-derived microparticles. A common variation was to physically sort particles based on their properties, so as to purify populations of interest. The main result from the blood-material interaction are presented in Fig. 9.

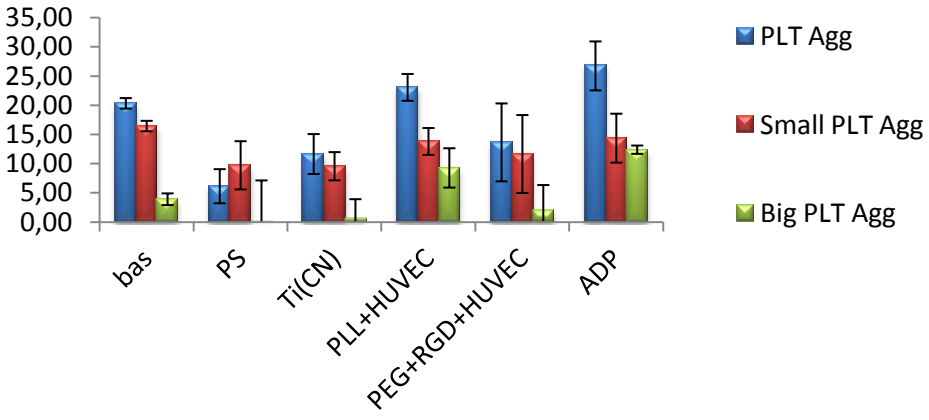

Fig. 9. Flow cytometry analysis of the aggregates formation. Small aggregates- 2 platelets together, Big aggregates- more than 2 platelets together

As presented on the figure, three tested material were taken under consideration: titanium carbonitride as a pure coating deposited on polyurethane, extracellular-like porous coating with endothelium (human umbilical vascular endothelium cells) simulating the luminal side of the vesels, and RGD functionalysed PEG supported with HUVEC. ADP- means adenosine di-phosphorane- it was used to acticate platelets for the positive control, bas- base line, PS- polystyrene- material of the well where the samples were investigated. There is no significant differences between the sample. The RGD modified surface is a complicated challenge. We were successful with RGD absorption, now the coating has to be adjuced for the long term application.

3.3 Antithrombotic protection of the tube-like elements for cardiovascular regeneration with ceramic coating

3.3.1 Surface preparation

To modify the inner surfaces of polymer tubes, the glow discharge technique was used in the coating process (Diener Electronics GmbH), which was performed under vacuum (pressure during deposition <100 Pa). This setup is similar to a tubular fluorescence lamp with flat electrodes on both ends of the tube-like substrate. To simplify characterization, after the system was pumped to vacuum conditions (10 Pa), plasma etching (activation) and plasma polymerization were performed using low-frequency pulsed DC glow discharges with a 535 Hz pulse frequency and a duty cycle of approximately 25%. The substrate was plasma-modified by igniting the glow discharge in a controlled gas atmosphere. The gas was supplied through the cathode and pumped out at the anode position. Hexamethyl disoloxane (HMDSO, $C_6H_{18}OSi_2$) was used as a liquid precursor and was evaporated at 25°C. The HMDSO pressure was measured using a Pirani gas flow controller and adjusted using a manual vacuum valve. Other process gases (argon, oxygen, C_2H_2) were controlled using mass flow meters and regulated to achieve stable deposition conditions. The coatings presented in Table 1, with various thicknesses and chemical compositions, were chosen for biomedical investigations.

After the system was evacuated to the required vacuum conditions, the surface was activated in an O_2-containing plasma to enhance coating adhesion.

Acronym	**HMDSO pressure [Pa]**	**Process gas**	**Film thickness [nm]**
A0/A1	1	O_2	110
A2			60
A3			220
B	1	Ar	200
C	1	C_2H_2	140
D	1	N_2	150

Table 1. Thin coatings and their parameters.

Microstructure analysis was performed using transmission electron microscopy (TEM) on a TECNAI G^2 F20 (200 kV FEG) microscope. Thin foils for TEM observations were

prepared using the focused ion beam technique (FIB). TEM observations were performed on the cross- section of the SiC coating deposited on the polymer.
The film exhibits elastic properties on its cross-section. Most of the irregularities in the substrate did not produce cracks in the surface (Fig. 10).

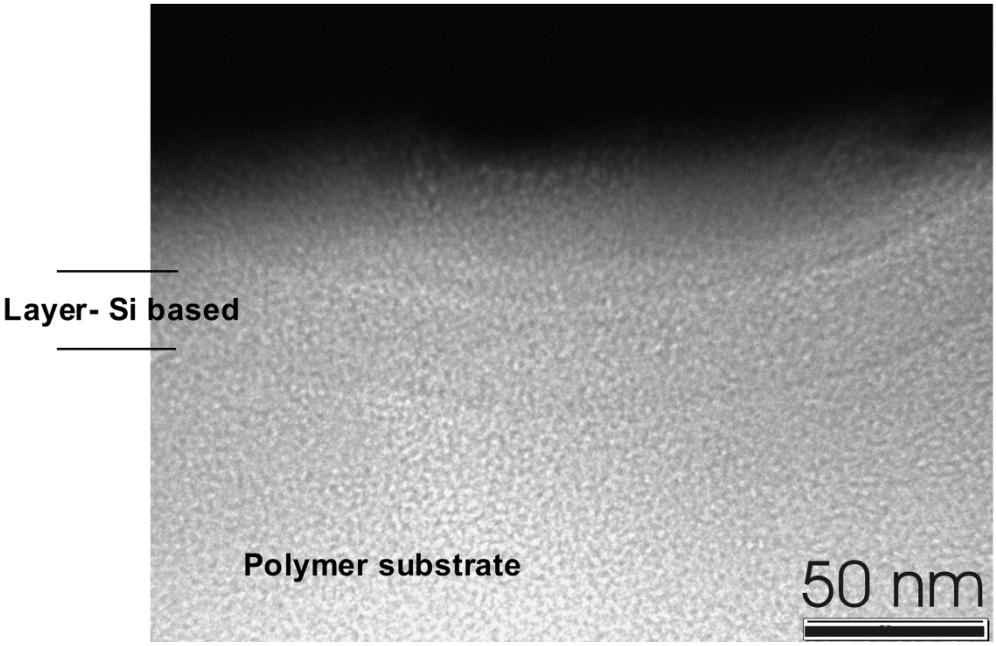

Fig 10 TEM (transmission electron microscopy) microstructure

Quantitative chemical analysis of the coatings was performed using the EDS technique (EDS-Energy Dispersive Spectroscopy), and the results are presented in Table 2.

Element	Weight %	Atomic %	Uncert. %	Detector Correction	k-Factor	Absorption Correction
C(K)	95.4	97.9	2.0	0.3	3.9	0.6
Si(K)	4.6	2.0	0.2	0.9	1.0	0.9
Pt(K)	0.0	0.00	100.0	0.7	81.2	1.0

Table 2. Quantitative EDS (Energy Dispersive Spectroscopy) chemical analysis of selected silicon carbide based coating.

3.3.2 Preparation of the cell culture

Blood from two sources was used in the experiments. For studies using an aortic blood flow analyser, the blood was purchased at the Centre for Blood Donation. The agent citrate phosphate dextrose adenine (CPDA-1) acted as an anticoagulation agent in the blood samples. CPDA-1 is a solution containing citric acid, sodium citrate, monobasic sodium phosphate, dextrose and adenine and can be used to preserve whole blood and red blood cells for up to 35 days [11]. CPDA-1 extends red blood cell survival by providing the adenine needed to maintain the cells' ATP levels.

Research using the large model termed the "Artificial Patient" was conducted using pig blood collected during slaughter. This blood was also subjected to anticoagulation protection using CPDA-1.

2.3 Haemocompatibility tests

Haemocompatibility tests evaluate the effects of blood-contacting medical devices or materials on blood and blood components [12]. The International Organisation for Standardisation (ISO) provides several test categories for haemocompatibility evaluation: thrombosis coagulation, platelet haematology, and immunology. The presented work addresses evaluating the blood-material interaction under dynamic conditions. ISO developed guidelines for testing medical materials that will be placed in contact with circulating blood [13], but they do not provide the exact test methods or evaluation criteria. The authors of the cited work presented a list of various suggestions.

The haemostatic mechanism is designed to stop bleeding from an injured blood vessel [12]. The blood-material interaction and the blood clothing cascade activation depend on the following elements: surface morphology, platelets, and coagulation proteins. Blood platelets are critical to vascular haemostasis, as they readily activate upon contact with the exposed components of the vessel wall. The blood platelets are roughly twenty times less abundant, and the platelet diameter is only one-fifth of that of erythrocytes. The primary haemostatic function of platelets could lead to thrombosis.

Thus, the most important definition of haemocompatibility is ”*A haemocompatible material must not adversely interact with any blood components*”.

2.3.1 Arterial flow conditions

Newly designed elements of a heart support system must be tested under conditions similar to their intended applications. Thus, it was necessary to design and build systems to allow for the analysis of novel solutions in realistic environments. From a biological point of view, the understanding of the molecular mechanisms, i.e., understanding the conditions under which cells adhere, roll, or slide on passive or reactive substrates, is critical. Many functions performed by living cells depend on these properties. From the physico-chemical point of view, bio-adhesion involves cells, a solid substrate, and a liquid medium. The relevant properties of the microorganisms are their hydrophobic properties, the charge of the cell surface, the cell size, and possession. A great deal of experimental and theoretical work has been dedicated to understanding cell adhesion [14, 15].

The haemocompatibility test is designed to detect adverse interactions between the artificial surface (which may activate or destroy blood components) and blood [16]. Under aortic flow conditions, because of the strong shear forces, the platelets play a crucial role in the blood-material interaction 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 tested sample of blood is introduced and a rotor with the normalized roughness of the active surface (Fig. 11).

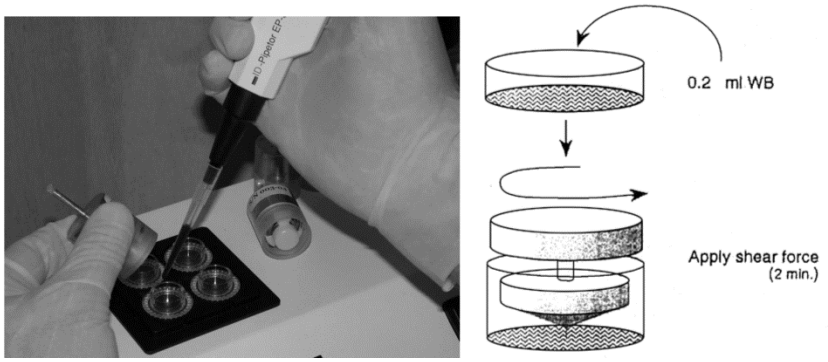

Fig 11 Impact R test scheme

Unfortunately, the test was designed for use with whole blood, and it is only possible to adapt this system for flat-shaped samples. More details are presented elsewhere [16-19**]**.

Materials were analysed under arterial flow conditions. The tube-like elements were analysed using similar assumptions as those applied in the Impact-R test. Testing conditions similar to those described in [20-23] were ensured during the experiments. A new device was designed by the author to test the tube-shaped elements under arterial flow conditions. The arterial flow simulator was designed and developed as a point-of-care method for real-time blood cell function testing. A method was developed to analyse 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 (Figs. 12, 13).

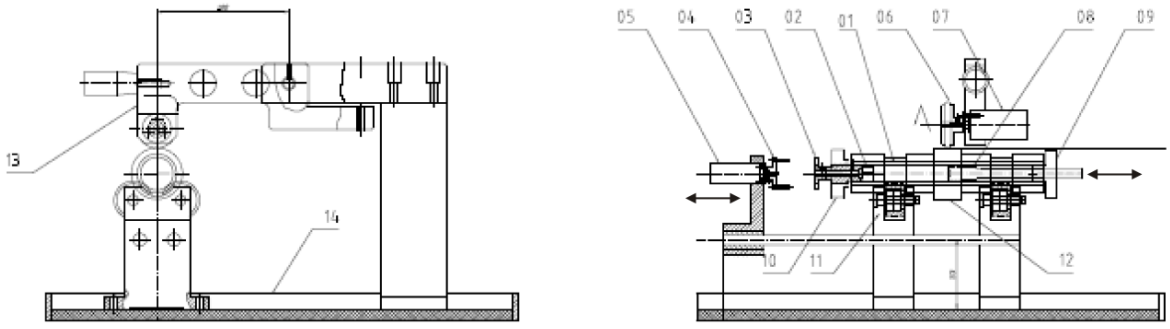

Fig 12 Arterial flow condition simulator- own concept design

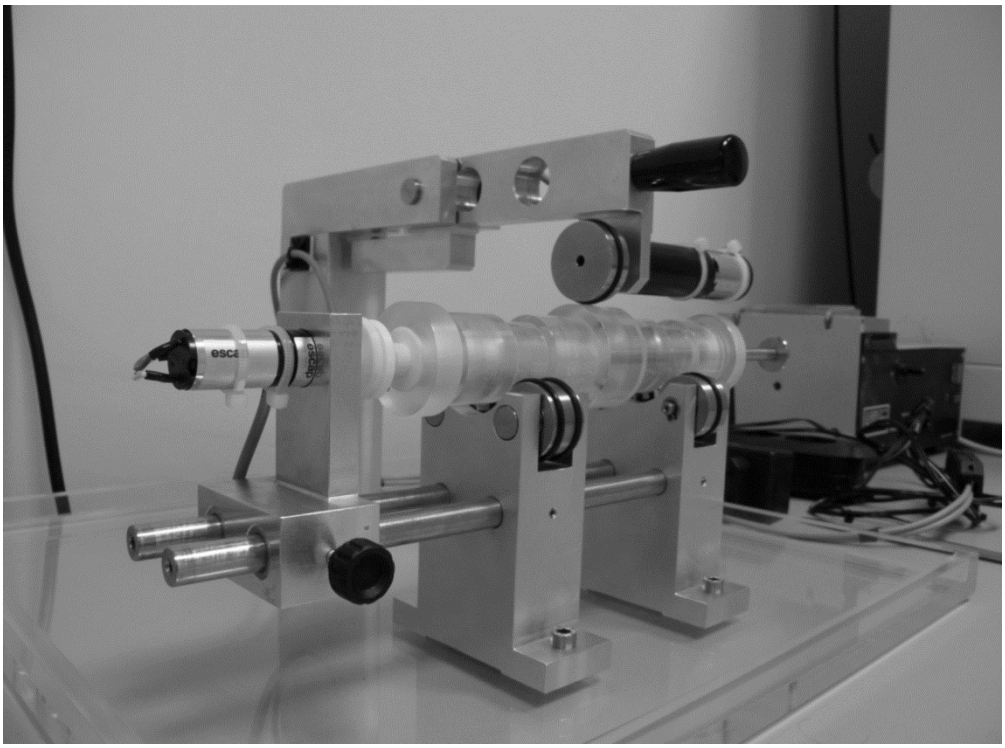

Fig 13 Arterial flow condition simulator

The system is composed of the following: 01- holder for the samples with the modified inner surfaces; and 02- rotor equipped with the appropriate surface roughness. The rotor's properties are based on the rotor used in the impact-R test. The other system components are the following: 03- clutch plate; 04- circular clutch; 05- high-speed engine to move the rotor; 06- drive roller; 07- motoreducer of the drive sleeve; 08- volume-adjusting punch; 09- stopper-leading plunger; 10- rotor-leading cap; 11- rotation roll; 12- rotating sleeve; 13- drive arm; and 14- bath.

Blood flow was induced by a rotating cylinder for two hours. Similar to the Impact-R test, the distance between the rotor and the analysed surface was held constant. The rotor was introduced along the height of the region of the tube under study. The blood-material interaction was analysed based on fluorescent techniques using confocal microscopy (CSM), flow cytometry and low-vacuum scanning electron microscopy (ESEM). After the test, the blood from above the surface was taken for further analysis, and the cells adhered to the surface were analysed using fluorescently labelled antibodies.

A simplified model was used to induce shear stress in the blood and simulate physiological vascular blood flow conditions. The purpose of these experiments was to analyse the dynamic interactions between the blood cells and the investigated surface. A volume of 1000 ml of human blood was introduced into the tube. The appropriate volume was adjusted using the special punch. After the test, 100 ml of blood was taken from above the surface and marked with fluorescently labelled antibodies. The appropriate receptors were present on the surfaces of the platelets that participated in the adhesion and aggregation. The content of the platelet surface was primarily selectins, which are responsible for the initial

stages of the adhesion process. Selectin P is a glycoprotein, and it is accumulated in platelet granules and transported to the membrane after platelet activation.
The tubes were cut into samples and analysed using confocal and scanning electron microscopes. The simultaneous analysis allowed for extraction of information about the amount of activated platelets and aggregates (Fig. 14).

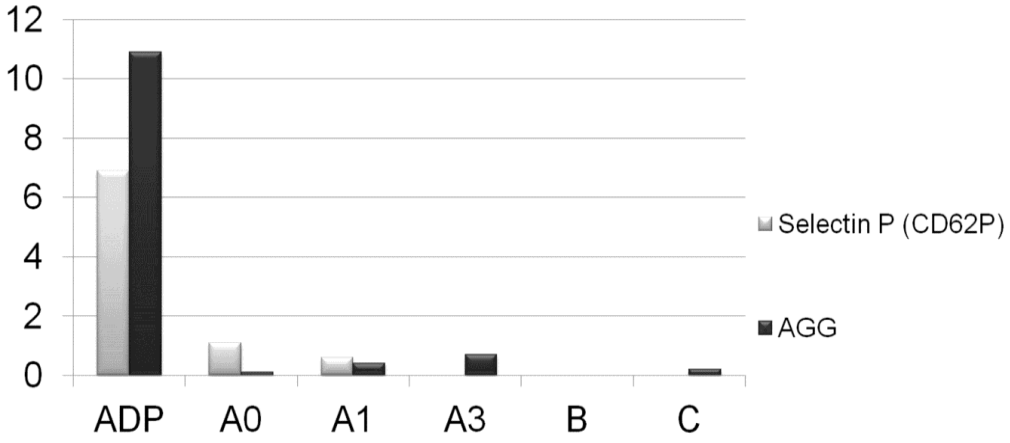

Fig 14 BMI (blood-material interaction) simulator

The antigen anti selectin P (also called anti CD 62 P) was used in this analysis (analysis of activated platelets). The size distributions of the objects observed on the surface after the test were analysed using the AxioVision 4.8 program equipped with the "Auto Measure" and "Auto Measure Plus" packages. (Fig. 14). The surface analysis after the test for sample A and B were performed using scanning electron microscopy (Figs. 15a,b).

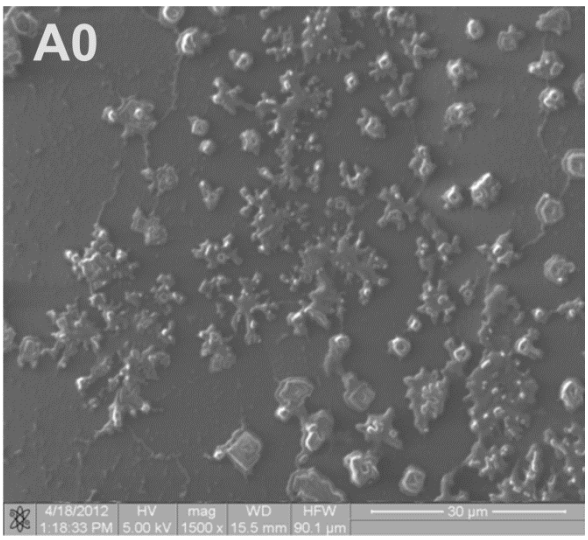

Fig 15a BMI- deposited cells on the surface of the sample A0 (the legend of the symbols is presented in table 1) SEM (scanning electron microscopy)

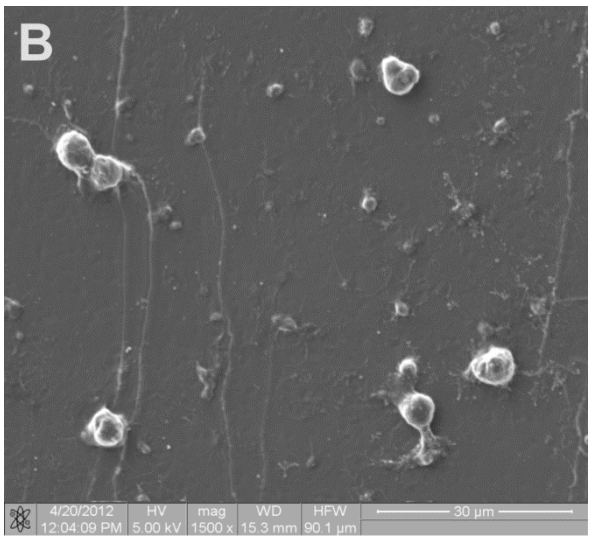

Fig 15b BMI deposited cells on the surface of the sample B (the legend of the symbols is presented in table 1) simulator B SEM (scanning electron microscopy)

2.3.2 Artificial patient

Testing of vascular prosthetic elements in *in vitro* conditions requires a model of an extracorporeal circulation system that simulates the flow conditions of the target systems [24]. The structure of the system needs to enable the following:

• analysis of the blood-biomaterial interactions;

• analysis of the mechanical strength of the layers under dynamic blood flow conditions.

The flow conditions were analysed in a target object, and two models of extracorporeal circulation were utilized: pulsate (Fig. 16a) and continuous flow (Fig. 16b).

The test system modelling continuous-flow circulation consisted of an impeller, a centrifugal pump compensating reservoir with a 650 ml capacity, a connected drain and two sections of tubing coated with the tested material. The individual system components were connected using Tygon tubing with inside diameters of 1/2" and 3/8". Probes were installed to monitor the flow rate and pressure in the system (Fig. 5). The blood flow conditions in the system were as follows: flow rate of 4.5 L / min, pressure of 120 mmHg, and temperature of 37°C.

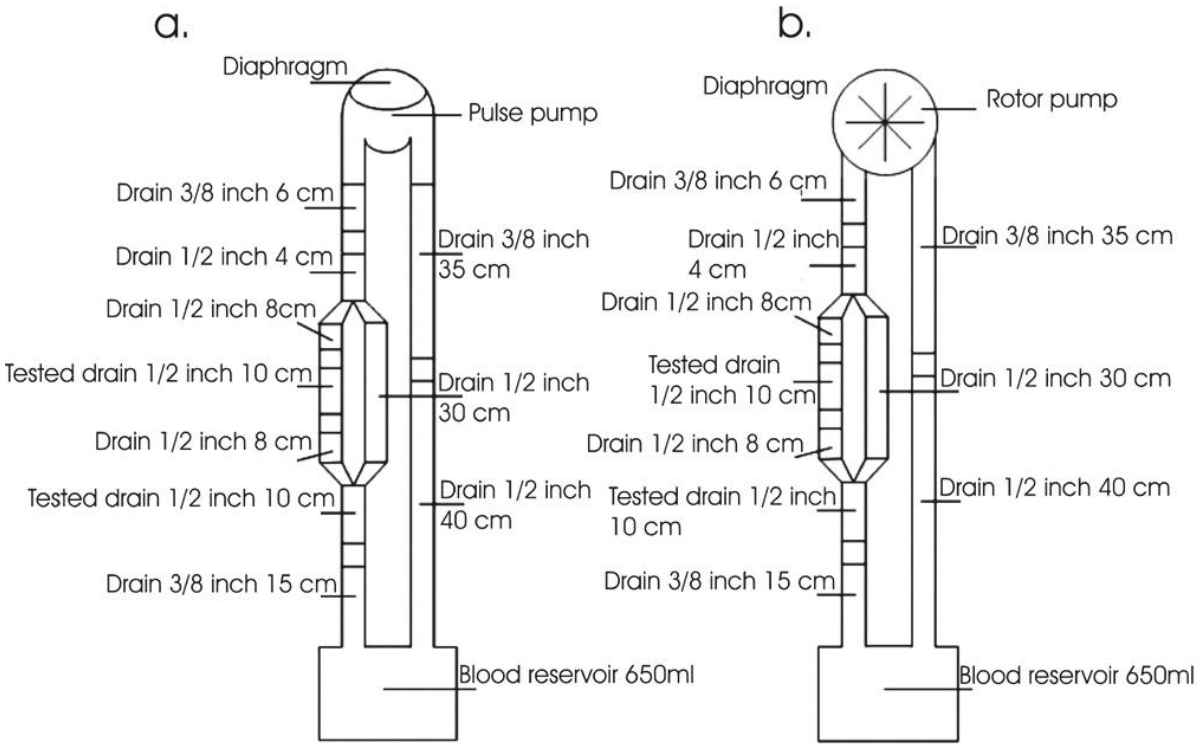

Fig 16 Artificial patient- the concept; a.) pulsed blood flow, b.) continuous blood flow

In the pulsed circulation test of the forced-flow extracorporeal cardiac support, a POLVAD-PED type pump was used. The detailed description of the POLVAD-PED pump is available on the web pages of its designer: *http://www.pwpss.pl* and *www.frk.pl*. The other parameters of the system were the same as those of the continuous-flow test.

Platelet activation was measured by flow cytometry using the following antibodies: CD62PPE (directed against activated platelets, conjugated with phycoerythrin) and CD45-

FITC (directed against the active cells of the leukocyte system, conjugated with fluorescing isothiocyanate). Immunofluorescence was performed for 0, 15, 30 and 60 minutes of circulation. In addition, sections of the studied fragments of the tubing were collected for surface analysis with an inverted fluorescence microscope using the antibodies mentioned above.

The measurements of the morphological parameters of the blood are normalized to the measurements conducted at 0 minutes of blood circulation.

This presentation of the results allows for a comparison of the dynamics of the processes in the systems with pulsed and continuous circulation independent of the initial values of the measured parameters. The following haematological parameters were determined in these experiments:

- amount of aggregates
- activated platelets

The primary haemostatic function of the platelets could lead to thrombosis. Fig. 17. present a comparison between the blood-material interactions under continous and pulsate blood flow conditions.

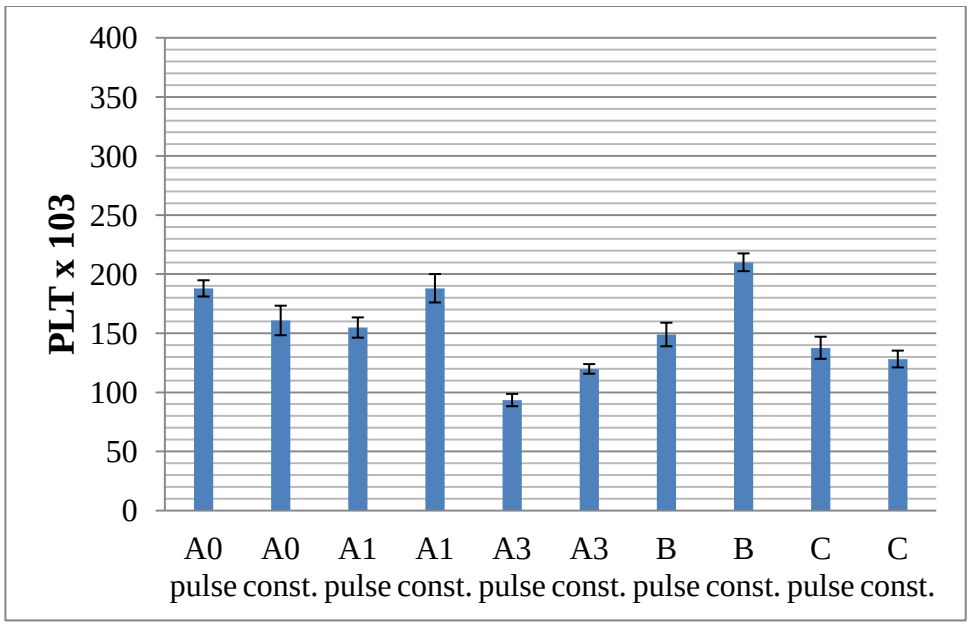

Fig 17 BMI-Artifficial patient (the legend of the symbols is presented in table 1)- pulse- pulsed mode. The scheme of the experiment is shown in fig. 16a; const- continuous mode. The scheme of the experiment is shown in fig. 16b

Two aspects of the blood-material interaction were studied. The first aspect was thrombus formation. Activated platelets were visualized, and the average amount of platelet aggregates was calculated. The second aspect was the immunoresponse, and the systemic activation of activated leucocytes was analysed to study this phenomenon. Confocal microscopy and flow cytometry are common ways to study cellular characteristics. More detailed description is provided through following the web page:

http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html. These methods involve the use of fluorescent molecules such as fluorophore-labelled antibodies. In these experiments, the labelled antibody was added to the cell sample. The antibody then became bound to a specific molecule on the cell surface or inside the cell. Finally, when laser light of the right wavelength was applied to the fluorophores, a fluorescent signal was emitted and detected. We used monoclonal antibodies directed against the antigen CD 62 conjugated with FITC (to visualise the active platelets) and CD 45 conjugated with R-PE under the optimal conditions indicated by the instructions [25]. Anti CD62P associated with FITC reacts with the human form of the 140 kDa membrane glycoprotein, which is a selectin-P membrane activation molecule. This glycoprotein is accumulated in cellular granulates when the platelet is not activated. When the platelet is activated, selectin-P is transported into the membrane. Selectin P is formally known as "platelet activation-dependent granule external membrane protein" or "granule membrane protein" [26**].**

4. Discussion and Conclusions

Modification of the inner surface of polymer tubes for biomedical applications is of crucial importance from the material, technological and biomedical points of view.

The presented study investigated new materials for coating cardiovascular implant surfaces applied in forced blood circulation systems, considering relevant parameters in design, processing and testing. New materials for these devices with improved blood-material interactions are urgently needed. The use of modern processing techniques can improve the quality of the applied materials, which could enable more frequent and more effective usage in a wider range of applications. However, the described innovations are new and very challenging, and there exist other areas of research surrounding blood circulation systems. Developing improved tube-shaped elements can play a significant role in the progression of these systems. The presented test demonstrated the significance and the difficulties of designing biocompatible blood-contacting surfaces, especially the inner surfaces of tubular elements. Because appropriate commercial systems are unavailable, the author's own design was adopted and successfully utilised to select materials. The work also revealed some disadvantages of *in vitro* analysis, including the problem of ensuring appropriate gas exchange. The performed research study provides some promising solutions, and the author will continue to investigate the possibility of advanced soft tissue systems with compatible implant surfaces and modified artificial cardiovascular tubes with non-thrombogenic surfaces.

The performed research work allows the following conclusions:

- The inner surfaces of tube-like elements can be modified using a glow discharge method to apply materials based on silicon carbide, silicon oxide and silicon nitride
- Modification of these inner surfaces with the appropriate architecture improves the inhibition of the blood clothing cascade activation
- Elaborated modifications could be applied in advanced biocompatible cardiovascular artificial implants
- New diagnostic methods were developed to examine tube-like elements under arterial flow conditions

- The experiments revealed that coatings based on silicon carbide exhibit potential for further applications

Variety of the laser possibilities makes the researchers being possible to design new materials and investigate them with the high resolution. Biomaterial engineering is a challenging duty. It does not bring rapid progress, because of the huge amount of parameters which have to be checked. Laser application in confocal microscopy and flow cytometry presented in the work, influenced on the fluorofores development. Both technique are described as the techniques which provide the opportunity to look into the cellular structure. There is no question what is the best diagnostic method of the newly developed material. The alive organism will give the most reliable answer whether it would like to interact with it or not. The obtained results bring sometimes surprising answer, sometimes it brings the researcher mad, but the final satisfaction is doubled when we finally come into the common conclusion with the cells.

Acknowledgments

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Literature

[1] J. B. Pawley, Handbook of Biological Confocal Microscopy, Springer(2006)
[2] S. W. Paddock, Principles and Practices of Laser Scanning Confocal Microscopy, Molecular Biotechnology Vol. 16, (2000)
[3]J. W. Dobrucki, Confocal Microscopy, Quantitative Analytical Capabilities Methods in Cell Biology, Vol. 75 Copyright (2004), Elsevier Inc
[4] http://www.invitrogen.com/site/us/en/home/support/Tutorials.html
[5] R. P. Haugland, A Guide to Fluorescent Probes and Labeling Technologies (2005)
[6]U.T. Seyfert, V. Biehl and J. Schenk "In vitro hemocompatibility testing of biomaterials according to the ISO 10993-4", *Biomol. Eng.* 19 (2-6), 91-96 (2002).
[7] M. Otto, C.L. Klein, H. Koehler, M. Wagner, O. Roehrig and C.J. Kirkpatrick, Dymanic blood cell contact with biomaterials: validation of a flow chamber system according to international standards, J Mater Sci Mater Med 8 (3), 119-129 (1997).
[8]U. Streller, C. Sperling, J. Huebner, R. Ranke and C. Werner, Design and evaluation of novel blood incubation systems for in vitro hemocompatibility assessment of planar solid surfaces, J. Biomed. Mater. Res. B Appl. Biomater. 66B (1), 379-390 (2003).
[9] J.M. Lackner, Industrially-scaled hybrid Pulsed Laser Deposition at Room Temperature. Orecop sc. Krakow, 2005.

[10]S.S. Hyun, M.K. Yeong, W.S. Jae, K.L. Yun, K. Joong-Ki, C. Dong-Lyun, Characterization of bioactive RGD peptide immobilized onto poly(acrylic acid) thin films by plasma polymerization. Applied Surface Science 257, 596-602 (2010).
[11] O'Toole M T. Miller-Keane Encyclopedia & Dictionary of Medicine, Nursing & Allied Health. In by Saunders, an imprint of Elsevier. New Jersey; 2005. ISBN: 978-1-4160-2604-4.
[12] Anderson J M, Schoen J. In vivo assessment of tissue compatibility. In: Ratner B D, Hoffman A S, Schoen F J, Lemons J E, editors. Biomaterials Science. An introduction to materials in medicine. San Diego: Elsevier Academic Press; 2004. p 360-366.
[13] Seyfert U T, Biehl V, Schenk J. In vitro Haemocompatibility testing of biomaterials according to the ISO 10993-4. Biomolecular Engineering 2002 ; 19 : 91-96.
[14] Decave E, Brechet Y, Bruckert F, Fourcade B. Peeling model for cell detachment D. Garrivier, Eur. Phys. J. E 2002 ; 8 : 79–97.
[15] Demilly M, Brechet Y, Bruckert F, Boulang´e L. Kinetics of yeast detachment from controlled stainless steel surfaces. Colloids and Surfaces B: Biointerfaces 2006 ; 51 : 71–79.
[16] Sanak M, Jakieła B, Węgrzyn W. Assessment of hemocompatibility of materials with arterial blood flow by platelet functional tests. Bulletin of the Polish Academy of Sciences, technical sciences 2010 ; 58(2) : 317-322.
[17] Lackner J M, Waldhauser W, Major R, Major B, Bruckert F. Hemocompatible, pulsed laser deposited coatings on polymers. Biomedizinische technik 2010 ; 55 (1) : 57- 64.
[18] Lackner J M, Wilczek P, Sanak M, Jakiela B, Stolarzewicz B, Kowalczuk M, Sobota M, Maksymow K, Spisak M, Major B. Functional cardio-biomaterials. Advances in Materials Science 2011 ; 11(2) : 5-25.
[19] Major R. Material science in the heart disease treatment. In Wilczek P, Major R, editors. Nanostructural materials for implants and cardiovascular biomedical devices. Zabrze: M Studio; 2011. p 67-90.
[20] Dardik R, Savion N, Gal N, Varon D. Flow Conditions Modulate Homocysteine Induced Changes in the Expression of Endothelial Cell Genes Associated with Cell-Cell Interaction and Cytoskeletal Rearrangement. Thromb Haemost 2002 ; 88 : 1047-1053.
[21] Germanier Y, Tosatti S, Broggini N, Textor M, Buser D. Enhanced Bone Apposition Around Biofunctionalized Sand-blasted and Acid-etched; Titanium Implant Surfaces. A histomorphometric study in miniature pigs. Clinical Oral Implants Research 2006 ; 17(3) : 251–257.
[22] Shenkman B, Inbal A, Tamarin I, Lubetsky A, Savion N, Varon D. Diagnosis of thrombotic thrombocytopenic purpura based on modulation by patient plasma of normal platelet adhesion under flow condition. British Journal of Haematology 2003 ; 120 : 597–604.
[23] Wang X, Dorsam R T, Lauver A, Wang H,. Barbera F A, Gibbs S, Varon D, Savion N, Friedman S M, Feuerstein G Z. Comparative Analysis of Various Platelet Glycoprotein IIb/IIIa Antagonists on Shear-Induced Platelet Activation and Adhesion. The Journal of Pharmacology and Experimental Therapeutics 2002 ; 303(3) : 1114-1120.
[24] Kustosz R, Gawlikowski M, Gorka K, Jarosz A. Structure of research stand and evaluation method of blood – biomaterial reaction and mechanical strength of coating. In Wilczek P, Major R, editors. Nanostructural materials for implants and cardiovascular biomedical devices. Zabrze: M Studio; 2011. p 91-100.
[25]http://www.bdbiosciences.com/external_files/pm/doc/tds/human/live/web_enabled/31255X_555483.pdf.
[26]http://www.bdbiosciences.com/external_files/pm/doc/tds/human/live/web_enabled/31794X_555523.pdf.