IN VITRO HEMOCOMPATIBILITY OF THIN FILMS MATERIALS FOR DIRECT BLOOD CONTACT

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Abstract

When designing new biomaterials for tissue contact devices it is important to consider their architecture as it affects different cell response. Surface modification of tubular structures requires the use of different techniques than in the case of flat samples. Similarly, analytical techniques also need to be adapted to the specific shape of substrate. For blood contacting devices this issue is critical because of shear forces generated by fluid flow and responsible for blood components activation. This necessitates the use of diagnostic techniques dedicated for material analysis in dynamic conditions in order to simulate physiological conditions. In the frame of the work, the flat samples as well as tube like elements were considered. The flat samples were prepared for basic research. Based on the results of the basic research the thin coatings were selected for the internal side of the tube like elements which have been analysed in contact with blood using blood flow simulator. The cross section of the coating-substrate interaction was tested using transmission electron microscopy. The attachment of cells to coatings was determined by radial flow chamber. Hemocompatible analysis was carried out in two ways. The quality of the blood after the dynamic test was analysed using flow cytometry. In this case the aggregates formation, platelet consumption and apoptosis derived microparticles were considered. The amount of cells adhered to the materials surfaces was determined by confocal laser microscopy.

Keywords: *hemocompatibility, thin film, protein adsorption, shear stresses*

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Introduction

The development of regenerative medicine creates new challenges for scientists who search new techniques for the surface modification of biomaterials designed for direct contact with blood [1]. The variety of processes occurring after exposure of material to blood stream including protein adsorption, cell adhesion, and thrombus formation is complex [2-5]. This leads to serious consequences like decrease in peripheral blood pressure, hindered blood flow and can be a reason of implant failure. Because of lack of fully athrombogenic synthetic materials, there is still need to find new methods of materials modification in order to improve their compatibility in contact with blood.

Hemocompatibility is defined as the ability of material to prevent host response after exposure to blood contact [6-8]. International Standard Organisation (ISO) described the methods of the medical devices testing, dedicated for interactions with blood in 10993-4 standard [9]. According to this norm many aspects of blood activation like, protein adhesion, platelet activation, thrombus formation, should be taken into consideration. The tests should simulate clinical conditions as much as possible [10]. However, this standard describe, only list of the proposed diagnostic methods and does not impose the need to use the one specific dedicated for evaluation of biomaterials' hemocompatibility [11]. This is due to the complexity of the issues and the lack of a comprehensive method that would simulate natural conditions. The lack of strict instructions creates a need to develop new techniques that would enable an accurate description of the interactions of blood with artificial surfaces. There are static and dynamic blood-material interactions highlighted in literature [12-14]. Dynamic conditions differ from the static one by the presence of shear forces which are responsible for platelet activation as well as adhered cell detachment or blood components degradation. The forces are generated by the fluid flow and may reach different values depending on different shear conditions. Commercially available tester Impact-R (Diamed, Switzerland) is very popular for describing the influence of shear forces to blood components activation and its adhesion to surfaces [15,16]. Unfortunately, this technique allows to analyse only flat samples [17]. Based on the Impact-R tester main assumptions, for the tubular elements, like artificial cannulas the own blood flow simulator was designed and evaluated.

Interaction of blood with artificial material depends on bio-physical properties of implant. Mechanical and physical properties affect the materials strength and durability. However, the surface of biomaterial has a direct contact with blood and is responsible for its hemocompatibility properties as well. Surface features such as topography or wettability play a key role in blood activation process. Nowadays many works are focused on surface modification by application of ultra-thin coatings deposited by physical (PVD) and chemical (CVD) plasma assisted vapour based method. This technique exhibits high potential due to a wide range of application and wide variety of achieved films. The advantage of this method gives the possibility of metal as well as polymer substrates surface modification. The aim of the research are athrombogenic coatings for the internal side of tubular elements used in heart assist systems. The coatings were deposited firstly on flat substrates in order to determine their biomechanical properties. Based on achieved results the group of coating was selected and deposited on the internal side of poly(vinyl chloride) (PVC) tubes.

Materials and Methods

Flat surfaces preparation

Carbon based coatings were deposited on flat substrates using magnetron sputtering technique in direct current (DC), unbalanced mode. TABLE 1 presents the composition and thickness of the designed films. Coatings were deposited at room temperature in an argon atmosphere. Before deposition, substrates were cleaned ultrasonically with ethanol and dried afterwards in vacuum. After mounting the substrates parallel to the target surface in a ~120 mm distance, the vacuum chamber was pumped down to at least 4×10^{-3} Pa. Substrates were rotated during deposition at a speed of 5.4 $cm\cdot s^{-1}$ in order to ensure homogenous film thickness over the entire coated surface. A detailed description of the deposition arrangement is presented elsewhere [18].

TABLE 1. Thin coatings deposited on flat substrates.

Coating	Thickness [nm]
a-C:H	100
Si-DLC	500
Si-DLC	300
Si-DLC	200
Si-DLC	125
Si-DLC	15

Coating deposition for internal side of tubular structures

Thin coatings were deposited on a substrate made of clinically applicable in heart assist system, polyvinyl chloride (PVC). The goal of applying amorphous carbon (a-C:H) and silicon doped amorphous carbon (a-C:H:Si) coatings was to separate the polymer from the tissue environment. The applied coating setup for inner surfaces of tubes based on the gas discharge lamps. The detailed deposition parameters are listed in TABLE 2. The coatings were deposited by a industrially-scaled equipment for plasma polymerization of nanoparticles (Diener Electronik, Ebhausen, Germany). It consists of a cubic chamber in which the pressure is pumped by a dry vacuum pump. Start pressure was set to 5 Pa for all samples. The PVC substrates were mounted on a vertical rotating cage between square, vertically positioned electrodes. Gases argon (Ar) and acetylene (C_2H_2) and precursor hexamethyldisiloxane (HMDSO) were introduced into the chamber. A pulsed DC discharge (40 kHz frequency) was used. Thin coating depositions occurred in the "volume polymerization mode" at pressures of around 50 Pa in the capacitive pulsed DC discharge, whereby gas mixtures of 20 Pa hexamethyldisiloxane (HMDSO, 99.5%, Sigma Aldrich, Austria) as liquid precursor and 25 Pa argon (99.999%, Linde Gas, Stadl-Paura, Austria) were applied. The whole deposition process was performed at 32°C for controlled precursor vaporization conditions. The constant temperature of the substrate cage was set (<2°C) during nanoparticle deposition. The process of coatings deposition was described in detail elsewhere [19].

Microstructure analysis of coatings

An analysis of the deposited coatings was performed using transmission electron microscopy (TEM). Thin foils for the TEM analysis were prepared on cross-section by means of the focused ion beam method (FIB) on the QUANTA 200 3D device (using a focused gallium ion beam). The device is equipped with a micromanipulator OmniProbe, giving the film exactly the places of interest (defect or phase boundary). The platinum mask was used to distinguish the proper area for examination.

Tribology

Indentation tests were performed at a load of 1 and 2 mN using Berkovich indenter geometry to study microhardness and Young's modulus of the coatings. The rate of loading and unloading was 2 and 4 mN/min respectively. Holding time at maximum load was extended to 5 seconds. The adhesion of the coatings to the substrate was determined by scratch test using 200 μm tip radius Rockwell C indenter. Scratch tests were performed in the range of 0-25 N. The indentation load 25 N due to the cracking of the silicon substrate.

Protein adsorption

Adsorption of protein to flat and tubular materials was analysed using human whole blood. Before experiment, samples were cut into squares of 1 cm^2 and thoroughly cleaned and sterilized. Surfaces were covered by blood and stored at 37°C with mixing in order to prevent blood sedimentation. After 30 minutes of incubation, materials were washed three times in PBS solution. Adsorbed proteins were removed from the surfaces by sodium dodecyl sulfate solution (SDS, 10%). The protein concentration in received solutions was determined by Qubit Fluorometer 2.0 using Quant-iT Protein Assay Kit (Invitrogen).

TABLE 2. The parameters of coatings deposition.

		Starting pressure [mbar]	Gas-flow	Coating pressure [mbar]	Coating time [min]
Pre-treatment (for all Versions)		0.15	6 sccm Ar	0.27	3
Undercoating (for all Versions)		0.15	HMDSO (counter = 340); 3 sccm O_2	-	0.5
Top coating	Version A	0.15	4 sccm C_2H_2	0.60	2
	Version B	0.15	10 sccm C_2H_2	1.10	2
	Version C	0.15	HMDSO (counter=340); 4 sccm C_2H_2	0.95	2
	Version D	0.2	HMDSO (counter=440); 10 sccm C_2H_2	1.20	2
	Version E	0.2	HMDSO (counter=440); 16 sccm C_2H_2	1.6	2
	Version F	0.15	HMDSO (counter=340); 4 sccm C_2H_2	0.60	2

Cell-material interaction under hydrodynamic conditions: radial detachment test

Radial flow chamber was used to analyze the strength of cell-material interactions (FIG. 1). This technique allows to determine the influence of shear stresses on cell behavior on the surface [18,21]. It consists of two plates with a space between them (FIG. 1). In the center of the upper plate there is a hole through which fluids (PBS, pH 7.4) is pumped following gravity. Between the plates the analyzed material is inserted. Before the experiment the material surface is covered with red blood cell solution (diluted 400 times) and leaved for 10 minutes in order to lead cell adhesion (FIG. 2). After that time the shear stresses are generated by the fluid flows. At the beginning, in the center of the sample (within the hole) shear forces are applied perpendicular to the material and do not cause cell detachment. Along radius, shear forces act parallel to the surface and can cause cell detachment. The values of applied shear stresses can be calculated using equation $\sigma = 3D\eta / \pi r \cdot e^2$ where, D is the flow rate, which depends on the material, η is the dynamic viscosity of the fluid (10 g/(cm·s)), r is the sample radius, and e is the distance between the disk and the plate set at 150 μm. Under the influence of applied stresses cell detachment can be observed. The condition of the cell detachment from the substrate is the adhesive strength of the cell and the value of the applied stress. Cells remain on the surface not torn off when the value of the adhesive strength to the surface is greater than the applied shear forces. In another case, the 'one by one' or selective detachment is observed. Applied shear forces decrease along radius and near the edges of the disc are equal zero. Here we can observe only spontaneous cell detachment. This phenomenon occurs spontaneously and depends on the affinity of the cell to the substrate. After the experiment the surfaces were analyzed by scanning confocal laser microscopy. The number of adhered cells along radius was calculated by dedicated software (AxioVision, Zeiss).

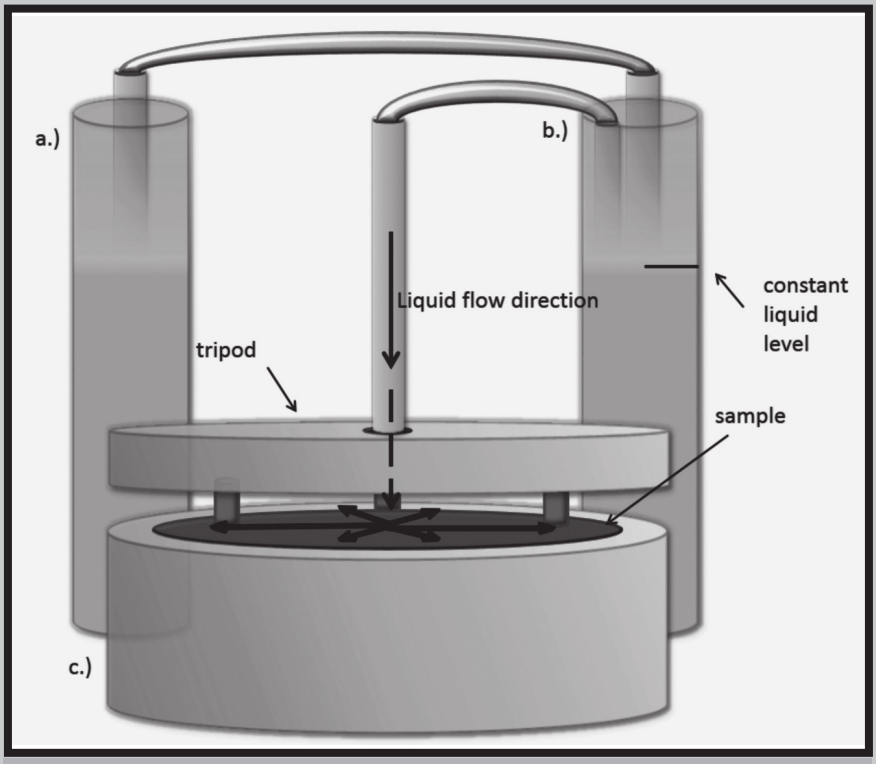

FIG. 1. Schematic illustration of radial flow test; Radial flow chamber assembly (a) working reservoir (b) stress determining reservoir (c) radial flow chamber with sample.

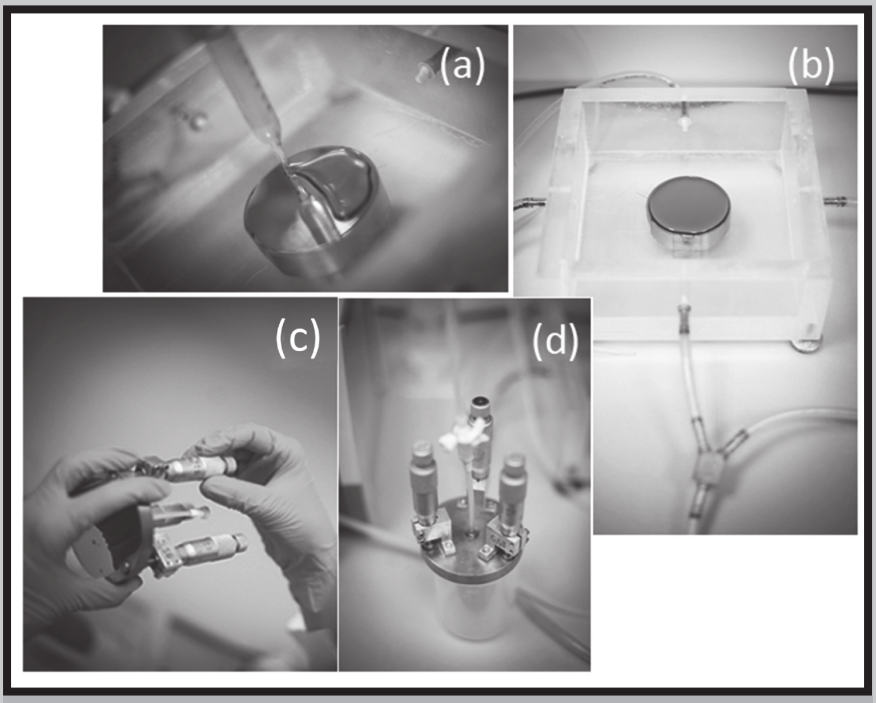

FIG. 2. Stages of the performed analysis using a radial flow chamber (a) cell deposition (b) cell seeding (c) disc adjustment (d) system assembling.

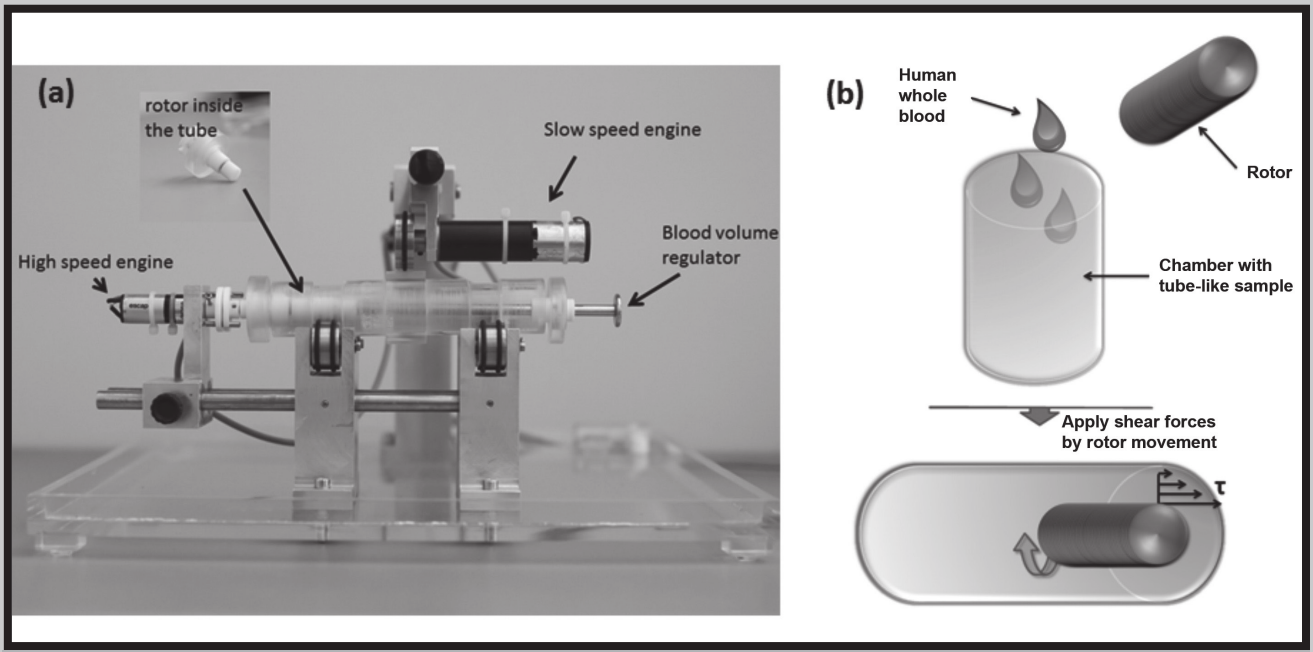

FIG. 3. Blood flow simulator for tubular structure analysis (a) and schematic illustration of experiment (b).

Hemocompatibility of tube-like elements

Hemocompatibility of coatings deposited on the internal side of polyvinyl chloride (PVC) tubes were determined by blood flow simulator (FIG. 3). The detailed description of this method was described elsewhere [20]. For analysis whole human blood collected from healthy volunteer was used. Experiment was performed according to ethic norm and the blood was purchased from the Blood Center in Krakow in accordance to the requirements of health and safety. Cooperation with the Regional Blood Center was specifically stated in the signed agreement. The aliquot of blood (2 mL for sample) was poured into the interior of the tube and the cone shape rotor was placed on the other side of the tube. The whole rotor was immersed in the blood by punch movements. Shear forces were applied by cone rotation for 20 min. After that time blood above sample was collected and analyzed by flow cytometry. Expression of platelet activation markers was measured on CD61 gated objects using PAC-1 antibody for conformational change of glycoprotein IIb/IIIa, and using CD62P for P-selectin. Integrated fluorescence of the activation marker was calculated as a multiplication total of geometric mean fluorescence by percentage of marker-positive objects. Tubes were cut into pieces with dimension 10 mm x 15 mm and analyzed by scanning confocal laser microscopy (CLSM Exciter 5, Carl Zeiss) and dedicated monoclonal antibodies. Cells adhered to material surfaces were fixed and permeabilised for 10 min with 4% paraformaldehyde (PFA). After washing the samples three times for 5 min with PBS, they were incubated with anti-human CD62P P-selectin antibody conjugated with fluorescein isothiocyanate (FITC), which is a dye that marks platelets with the P-selectin active receptor. After 30 minutes of incubation, the samples were washed three times with PBS and were covered by the human monoclonal antibody CD45 PE conjugated with Texas Red for 10 minutes. This secondary antibody marks active leukocytes on the material surfaces. Finally, the samples were rinsed three times in PBS and were immediately imaged. The cell densities on the surfaces were measured using the "colocalization" tool of the microscope software (Carl Zeiss Exciter 5 equipped with ZEN 2008). Colocalization analysis was done on the basis considering pixel by pixel analysis.

Results and Discussions

Microstructure

The microstructure (TEM) and distribution of the C, O, Si and Ti elements are shown in FIG. 4. The high-resolution analysis showed the presence of crystalline phases in the amorphous matrix. It is associated with the initial mechanism of the thin film nucleation from the gas phase. Two dimensional thin film nucleation allows achieving mechanical properties, unusual for ceramic coatings. The coating which has reached elastic properties was applied directly on the polymer.

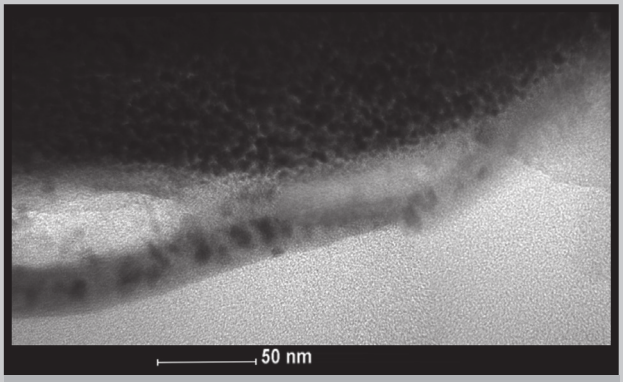

FIG. 4. The microstructure analysis of coating on silicon substrate.

Tribology

The mechanical properties of coatings deposited on flat, silicone substrates are presented in TABLE 3. The coatings have the hardness of 9-15 GPa and Young's Modulus of 129-155 GPa. With decreasing thickness of the coating, the hardness decreases due to the increased impact of the silicon substrate on the measurements. For the control sample (silicone substrate) is about H = 9 GPa and E = 120 GPa, which practically corresponds to the measurement for the thinnest film thickness 15 nm.

TABLE 3. Microhardness (HiT) and Young's modulus (E) of coatings at a load of 1 and 2 mN. Hmax represents the deepness of material deformation.

Material	Thick-ness [nm]	1 mN			2 mN		
		hmax [nm]	HiT [MPa]	E [GPa]	hmax [nm]	HiT [MPa]	E [GPa]
a-C:H	100	73 ±3	10785 ±1049	129 ±9	100 ±2	10101 ±426	138 ±4
Si-DLC	500	65 ±2	14618 ±1126	155 ±7	93 ±3	13075 ±819	143 ±7
Si-DLC	300	63 ±3	15570 ±1615	148 ±6	93 ±1	13001 ±790	146 ±4
Si-DLC	200	66 ±4	13487 ±1445	145 ±5	95 ±3	11225 ±988	143 ±7
Si-DLC	125	72 ±3	9731 ±1150	140 ±5	99 ±3	9648 ±842	136 ±6
Si-DLC	15	73 ±3	9186 ±614	132 ±7	99 ±3	8373 ±213	135 ±8

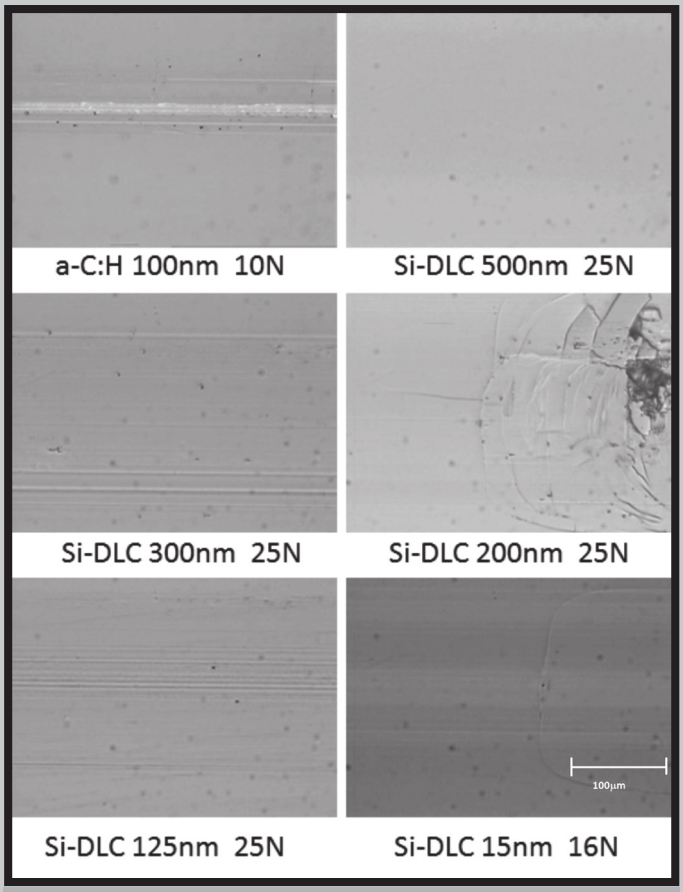

FIG. 5. Optical micrographs of scratch tracks of films on silicone substrates.

Optical micrographs of scratch tracks of films on silicone substrates are presented in FIG. 5. In the case of flat samples, the coatings are characterized by a very good adhesion to substrates. The last film has cracked at load 16 N but has not fall off the substrate despite the small thickness of this coating (15 nm)

Biomechanical properties of cell-material interaction for flat samples

The biomechanical interaction of red blood cells with coatings is presented in FIG. 6. The percentage of attached cells after the radial flow detachment test were calculated and presented as a function of a distance from the center of the sample. Typical washing-out valley can be observed. The narrower and shallower valley represents the stronger interaction of cells with the substrate. In the center of the sample shear forces have the strongest values resulting in significant eluting cells in this area. Due to the design of the tester, the stress is reduced along the radius, the amount of attached cells increases linearly until the plateau is reached. After that, small differences in the number of cells can by observed. These changes may occur due to the local displacement of cells within a sample under the action of static shear stress. The applied stress is too weak to cause cell detachment but strong enough to shift cells along the radius. It also explains the measured value of more than 100% detached cells. For the a-C:H:Si films with thickness of 125 nm and 200 nm almost complete cell detachment (≥99,5%) near the center of the sample is observed, which then decreases linearly. The weak cell-material interaction for these cells is confirmed by the small amount of cells at the edges of the disc where shear forces are close to zero. In the case of coatings type a-C:H:Si 500 nm and a-C:H 100 nm at the beginning, cell detachment reaches the value of 90% which shows a stronger interaction of these materials with cells. For a-C:H:Si 300 nm sample a characteristic double plateau shape of function can be observed. The slight (about 3 Pa) decrease in the value of applied shear forces caused a sudden, sharp decline in cells washout. For all samples the threshold stress values were calculated and presented in FIG. 7. These values represent a state where the probability of cell detachment from the surface is 50%. For a-C:H:Si 15 nm the value of threshold stress is the highest.

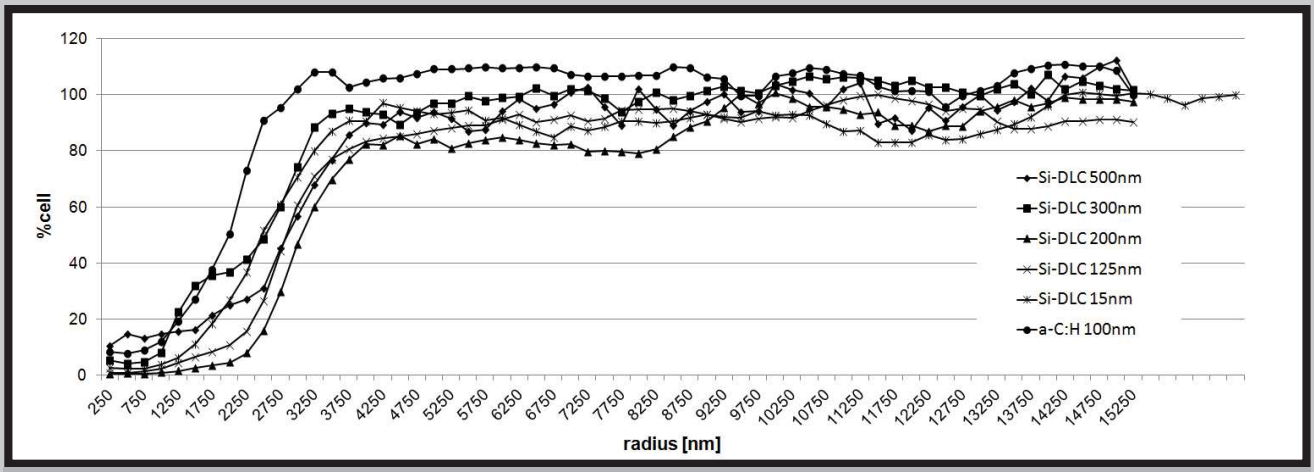

FIG. 6. The percentage of attached cells on a function of distance from the center of the sample after the radial flow detachmend test.

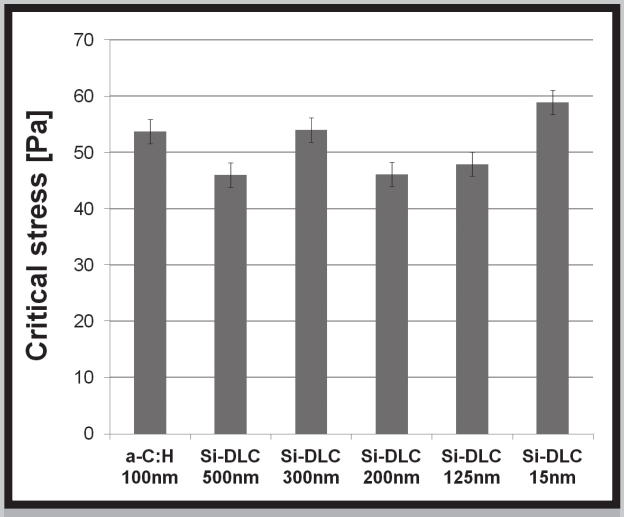

FIG. 7. Threshold stress values.

Protein adsorption

Adsorption of protein to tubular structure is presented in TABLE 4. The results were correlated to the most abounded blood protein – albumin. For two types of coatings (Version B and E) the highest values of protein concentration in comparison to control sample was observed. This indicates an increase in the affinity of these coatings to albumin captured from blood. Albumin is a blood protein which can decrease subsequent thromboembolic events [22-24]. The achieved results may indicate the formation of a densely packed albumin monolayer and may cause the weaker adhesion of platelets and leukocytes to the surfaces of these coatings.

TABLE 4. Protein adsorption from foetal bovine serum (FBS) solution.

Sample	FBS concentration [ug/ml]
control	2.69 ±0.13
Version A	1.96 ±0.04
Version B	3.46 ±1.4
Version C	0.925 ±0.14
Version D	1.99 ±0.014
Version E	3.545 ±0.68
Version F	2.59 ±0.48

Hemocompatibility

Blood-material interaction for tubular structures were analysed by blood flow chamber. The surfaces coverage by platelets and leukocytes were presented in FIG. 8. The relative number of cells was calculated in comparison to control sample and exchange rate per unit area (μm^2). For almost all the samples except sample indicated as Version F improvement in hemocompatibility properties is visible over the control sample. Promising results were obtained for coatings marked as Version B and Version F where a significant decrease in the number of platelets and leukocytes is observed. The surface modification by thin coating deposition has also an impact on cells activation in blood after exposure of materials to blood stream (FIG. 9). Expression of platelet activation markers decreases for all coatings except sample marked as Version D. For this sample the adhesion of platelets to surface is lower than for non-modified surface, but on the other hand, their activation in blood is much stronger. Opposite situation is observed for sample Version F. A small number of active platelets in blood can result from a high affinity of the surface of the film to cells resulting in their strong adhesion. Taking into consideration results obtained for surface and blood analysis, Version B and Version E seem to exhibit the most promising properties of the blood-material interaction.

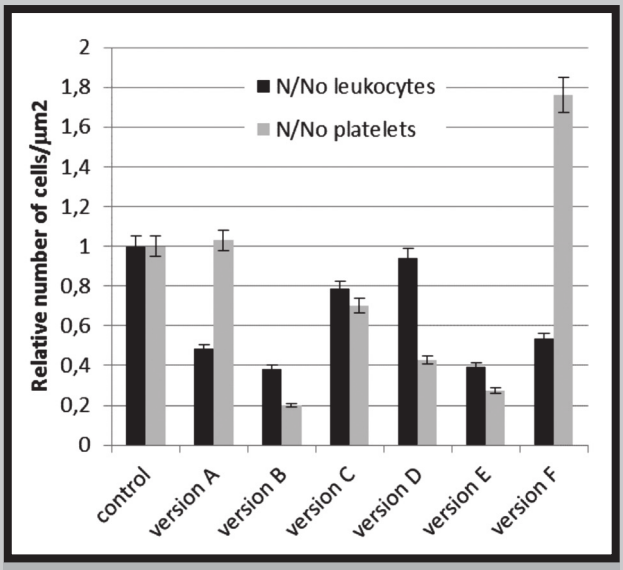

FIG. 8. The relative number of platelets and leukocytes on the surfaces of analyzed materials.

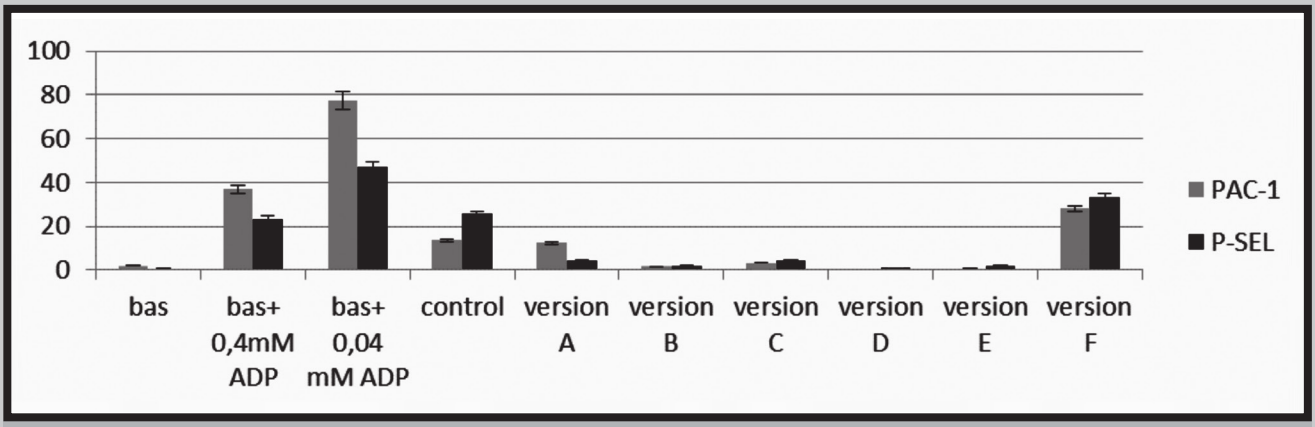

FIG. 9. The PAC-1 and P-SEL expresion in leukocytes after dynamic test.

Conclusions

While designing new materials for the direct contact with blood, one should consider their final application. Materials have to be tested in the conditions similar to physiological environment. For biomaterials dedicated for cardiovascular devices many aspects of blood-material interaction should be taken into consideration, like, biomechanical interaction of blood cells with material, protein adsorption, cell adhesion and activation. Untypical architecture of tube-like elements requires the development of novel deposition techniques of the thin coating and the following diagnostic methods. In the frame of the presented work the self-elaborated system dedicated for the analysis of blood-material interactions in dynamic conditions was properly designed and elaborated following 10993-4 rules. Blood flow simulation chamber based on commercially available tester (Impact-R) is dedicated to tubular structures. Using presented method the athrombogenic properties of a-C:H and a-C:H doped with Si coatings were analysed. As part of work the coatings were deposited on flat samples and tested for their biomechanical properties. Microstructure and tribology analyses were used to evaluate the coatings anchoring to the substrates and their hardness and elasticity. Research carried out for the films on flat substrates have enabled the selection of groups of materials, which in a later stage have served to modify the internal side of tubular structures. This has permitted multiscale diagnostics and allowed us to assess the different phenomena occurring on the cell-material interface.

Based on achieved results the following conclusions can be made:

• Ceramic coatings showed elastic properties. This is achieved by the dispersion of crystalline phase uniformly in the amorphous structure. The elastic properties of the ceramic coatings are dependent on the appropriate mechanism of thin nucleation from the gas phase. Such coatings could be applied for the soft polymer substrate modification.

• Materials showing low affinity to platelets attachment can cause their activation in blood.

• The coatings deposited with the high acetylene flow as well as with hexamethyldisilazane (HDMSO) and low acetylene flow in the reactive chamber demonstrated better hemocompatibility. On the basis of the dynamic analysis on blood, the decreased activation of the coagulation system and the immune response compared to the other analyzed coatings was observed. These characteristics prove the selected appropriate parameters.

• The coatings with the highest hemocompatibility properties exhibit high affinity to adsorption of blood protein - albumin. Albumin is known to inhibit clotting process which confirms hemocompatibility properties of these coatings.

Acknowledgments

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