# Nanoscaled Functionalization of Scaffolds for Cardiovascular Prosthesis

Boguslaw Major

*Institute of Metallurgy and Materials Science; Polish Academy of Sciences, Cracow, Poland*

## Abstract

The main topic in novel biomaterial science is the replacement of injured or disease tissue with materials of non-biological or even biological origin. The main research target of the contribution is focused on external and internal blood circulation devices. Blood contacting materials could initiate several processes, which can endanger the life, like the formation of thromboemboli. The performed research activity in the frame of integrated national- and international activity was focused on fabrication and diagnostics of materials characterized by reduction or erasing of thrombogenicity. Development of surfaces that both favor endothelial cell monolayer reconstruction and prevent platelets aggregation and binding was under examination. Research study was performed on soft polyurethane surfaces (PU) by application of thin inorganic coatings. Porous and semi-porous materials, with the functionalized surface, have been performed to study a contribution of surface morphology and pore size to protein adsorption. Experiments have also been performed on surface functionalization with application porous materials produced by electrospinning and of multilayers produced using polyelectrolites which influence the protein adsorption Thin films were produced using magnetron sputtering in direct current, unbalanced mode and migration channels by laser ablation. Advanced microstructure examinations were carried out using SEM and TEM on thin foils prepared by focused ion beam (FIB) and confocal laser scanning microscopy (CLSM) was used for complex examination of biological systems.

Keywords: **film deposition, microstructure, cell adhesion, biocompatibily, hemocompatibility, scaffolds, tissue analog**

## 1. Introduction

Research activity of new tissue biomaterials is mainly focused on blood contact aspects for external and internal blood circulation support devices. Replacement of injured or disease tissue with materials of non-biological or even biological origin is the main target. Properties of new designed materials could minimize influence the human organism. For blood contact, the biocompatibility requirements are of the highest level of all biomaterial applications, which is due to the continuous blood flow and the high reactivity of blood molecules and cells. Blood contacting materials could initiate several life-endangering processes such as formation of thromboemboli, even in the presence of anti-clotting agents. Thus, our research in the frame of integrated national- and international activity is focused on fabrication and diagnostics of materials characterized by reduction or erasing of thrombogenicity.

In the last years of material development, lower thrombogenicity was primarily tried to reach by modifying existing material surfaces. Despite the success in reducing protein and cellular deposits on some materials, this approach do not target to a truly non-thrombogenic surface. Recently, attempts are starting to create self-assembling layers. Such approach is realized in our research activity by the multidisciplinary, international work, based on three-steps modus operandi: (1) Materials topographic texturisation for controlled pseudointima growth, (2) Biologically modified hybrid materials with colonized endothelium cells, and (3) in-situ sterilization by pharmacologically-modified hybrid surfaces.

The 1st step is based on immediate clotting on the biomaterial in in-vivo blood contact. The aim – a growth control of the natural pseudointima layer by the biomaterial's surface topography/texture – is the inhabitation of thromboembolism development. The 2nd step focuses on the cellular interaction of adsorbed adhesion proteins with biomaterials. It supports the first step by protein modified surfaces in-between the nanostructured biomaterial surface and the flowing blood. Finally, the 3rd step aims at new in-situ sterilizing bioactive thin film materials, deposited by novel vacuum coating techniques (matrix-assisted pulsed laser evaporation, atomic layer deposition, plasma polymerization, etc.). Such anti-thrombogenic drug-eluting materials are deposited as nanoscaled multilayers and multi-components thin films.

In conclusion, the performed research work for design of new biomaterials is focus not-only on the platelets-biomaterial interaction, but also on the lymphosites T- and B-cell answer. Thus, it will make biomaterials

safer for patients and reduce disease by novel treatments. The best blood-contacting materials seem to be endothelial cells. Thus much work is done on development surfaces that both favor endothelial cell monolayer reconstruction and prevent platelet aggregation and binding. Our strategy is to develop thin film coatings on soft polyurethane surfaces (PU) which is widely accepted as a material suitable for a contact with the human body. Furthermore, the design system should have elastic properties close to that of natural tissues, so as to match the compliance of blood vessels and containing compartments.

Eestimation of the cell–material interaction plays an important role in the biomaterial design. Titanium and carbon basis biomaterials, such as diamond-like carbon (DLC), titanium (Ti), and stoichiometric titanium nitride (TiN) as well as titanium carbo-nitride (Ti(C,N)), seem to be good candidates for future blood-contact applications. These materials were deposited as thin films by the hybrid pulsed laser deposition (PLD) technique to examine the influence of such surfaces on cell behaviour. The cell-material reactions were examined in static conditions and then subjected to a dynamical test by application of a radial flow chamber specially design to observe the cell detachment kinetics. For a given cell, detachment occurs for critical stress values caused by the applied hydrodynamic pressure above a threshold which depends on cell size and physicochemical properties of the substrate. Tests revealed differences in behaviour in respect to the applied coating material. The strongest cell-biomaterial interaction was observed for the carbon-based materials compared to the titanium and titanium nitride. Biocompatibility was measured with application of fibroblasts. Hemocompatibility test of medical materials aims to detect adverse interaction between artificial surface and blood, which can activate or destruct blood components. In arterial flow conditions, due to a high shear stress, the platelet is the cell critical for the hemocompatibility compliance. A classical instrumentation for the dynamic test of hemocompatibility involves a flow chamber with a contact surface between a blood stream and tested plate. A simplified model of the whole blood shear stress, based on a cone and plate rotational viscometer was applied. Several indices of platelet activation were analyzed, including platelet- and granulocyte-platelet aggregates, platelet activation markers and platelet-derived microparticles.

Research was performed on scaffolds for precursors of tissue analogs and on cell migration channels. Migration channels were fabricated by means of the laser ablation. As an advantage one could find the method to observe and investigate a chemotaxic effect of the substrate into cells. The paper presents the original research results.

## 2. Concept of the design

The concept of design is to reconstitute the structure of the natural vascular tissue on an artificial material scaffold. The appropriate scaffold, cells and the signal stimulating cells for the efficient growth in the biomaterial design would greatly reduce the invasiveness after implantation. Consequences of an inadequate nutrient supply to implanted cells are the major challenges to a successful scaffold design. The novel solution considers the collagen base vascular bed. The generally known properties of endothelium cells (EC) acts as a barrier between the blood and the underlying bio-material as well as actively inhibit thrombosis. There are proposed the functional co-culture human umbilical vein endothelial cells (HUVEC) and umbilical vein smooth muscle cells (UVSMC). Regarding tissue analogues, it is crucial to point out the necessity to fulfill all the regulatory mechanisms. The research work of the authors which is in progress is focused on similar aspects of the novel biomaterial design. Following relatively old, but still valid approach found in literature [1, 2], we propose to incorporate fibronectin, as a main component of the extracellular matrix (ECM), introduced under the endothelium. The ECM is not just a static scaffold, it is a dynamic, information-rich source of inputs for cells. The idea of the basal lamina reconstruction is based on three aspects: (i) porous scaffold, (ii) adsorbed fibronectin (FN) as an endothelium bed and (iii) adsorbed collagen on the other side of the porous material under the muscle cell. FN is a prominent constituent of the ECM around and beneath many cells, and FN-rich matrices provide substrates for the cell adhesion and migration during development, wound healing, and other situations, as well as affecting many cellular functions including proliferation, survival, and differentiation. Collagen is a group of naturally occurring proteins which constitutes 1 to 2pct of muscle tissue, thus it has been chosen for experiments.

Research is in progress on porous and semi-porous materials, with the functionalized surface, performed to study the contribution of the surface morphology and pore size to the protein adsorption. Experiments have also been performed on surface functionalization with application of porous materials produced by the electrospinning and semi-porous multilayers produced using polyelectrolites. Studies of influence on the protein adsorption are in progress.

## 3. Materials and method of examinations

A part of the work is focused on a new generation implantable heart valve. We consider surface functionalisation. It should lead to the appropriate cell behaviour giving the designer an opportunity to have an easy control on the cell activation or deactivation. Our aim is to produce the endothelium layer on the surface dedicated to a blood-material interaction. Extracellular matrix (ECM)-like biomimetic surface modification of cardiovascular implants, is a promising method for improving hemocompatibility. The idea was to influence on the cell adhesion and proliferation. Thin coatings were deposited using a hybrid technique, based on physical processes [3, 4]. The idea was to reconstitute the structure of the natural vessel where the endothelium is formed

as the thin layer of cells that join the interior surface of blood vessels, forming an interface between the circulating blood in the lumen and the rest of the vessel wall. The substrate is prepared to stabilize the polymer from which the valve is made of, and to create the surface conditions for the protein adsorption. Therefore, initially, the physical methods like plasma etching, plasma deposition of inorganic films and plasma grafting with functional molecules (e.g. amine, carboxyl groups) of coating, based on the evaporation of the target, were used for both the above mentioned solutions.

### 3.1 Thin inorganic coatings

Films were fabricated by means of the surface modification dedicated to the blood-material interaction purposes. Materials were elaborated by a magnetron sputtering in direct current (DC), unbalanced mode. Titanium targets (medical grade) were used for titanium coatings (in neutral atmosphere), titanium nitride coatings (in nitrogen atmosphere) and titanium oxide coatings (in an oxygen-argon atmosphere).
To ensure a homogenous film thickness over the entire coated surfaces, substrates were rotated during deposition at a rate of 5.4 cm $s^{-1}$ through the plasma plume. The detailed description of the deposition arrangement is given elsewhere [4]. There were applied industrially up-scaled coating processes at JOANNEUM RESEARCH Forschungs-GmbH, MATERIALS UNIT Leoben, Austria, which allow coating of polymers at the room temperature and 3-axis substrate/planetary rotation. One of the main success was to elaborate ceramic layers exhibiting an elastic behaviour. The unusual and unique material properties are coming from the appropriate structure and the proper mechanism of the thin film nucleation from the gas phase [5-10]. The microstructure of the cross section of the titanium nitride coating is presented in Fig.1.

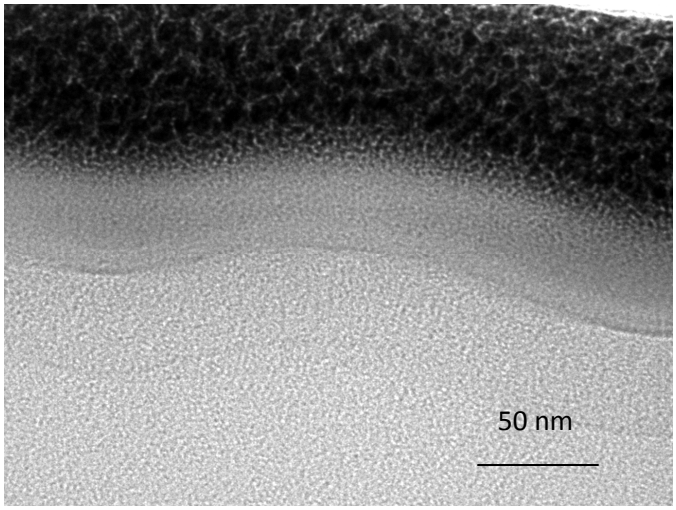


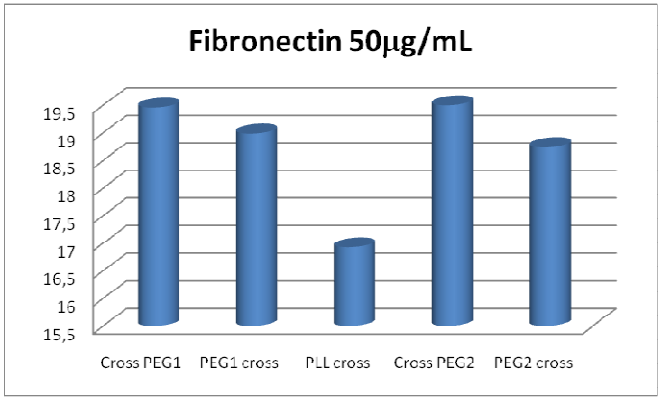

Fig. 1. High resolution microstructure (HREM)- thin ceramic TiN coating with elastic properties, cross section

### 3.2 Porous coatings; polyelectrolytes

Because hyaluronic acid (HA) is a biopolymer, it unlike other glycosaminoglycans does not create a covalent binding with proteins, and so cannot form part of a typical proteoglycan. It may, however, be at the center, which involves other proteoglycan form with them, "proteoglycan aggregate". The papers on "Layer-by-layer deposition of hyaluronic acid and poly-L-lysine (PLL) for patterned cell co-cultures" by Ali Khademhosseini et al. [11] and Zhiyong Tang et. al. [12] described a method to pattern two cell types on a surface by using the cell resistant properties of haluronic acid (HA). They demonstrated the feasibility of the approach to immobilize PLL and fibronectin (FN) on glass substrates. Their experiments showed that the adhesion of bovine serum albumin (BSA), immuneglobuline (IgG), and fibronectin (FN) was significantly reduced on haluronic acid (HA) compared to the glass controls. The other authors pointed out that physically adsorbed and chemically coupled FN were found to be distributed only at the top of the (PLL/HA)+PLL coating [13, 14]. They indicated that FN rapidly complexes with PLL with little or no FN penetration into the bulk substrate. Covalent fixation of FN to PLL/HA coatings proved necessary to maintain densely to grow (Fig. 2).

Protein adsorption on solid surfaces is a widespread phenomenon of large biological and biotechnological significance [15-18]. The idea of the surface functionalization should give possibilities to bind specific proteins and to prepare the surface for the cellular colonization. The main protein of the extracellular matrix is fibronectin, thus the surface was functionalized by fibronectin dissolved in the lyophilizate with sterile tissue grade water to a final concentration of 1 mg/ml. Diluted an appropriate aliquot with sterile PBS or basal medium to a final concentration of 50µg/ml. Pipetted 100 µl of this solution (50 µg/ml) per 1$cm^2$ surface area to be coated (5 µg/$cm^2$). Proteins were incubated for about 45 min at +15 to +25°C subsequently. and removed from

the edges without touching the surface area with the pipette. Results of the protein adsorption are presented in Fig. 2.




**PLL**- Poly-l-lysine
**Cross**- cross link reaction
**PEG**- poly etylen glycol

Fig. 2. Protein adsorption on the porous coatings.

Normal Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in optimized media. To label mitochondria, cells were simply incubated with MitoTracker probes, which passively diffuse across the plasma membrane and accumulate in active mitochondria. MitoTracker probes eliminate some of difficulties of working with pathogenic cells because once the mitochondria are stained, the cells can be treated with fixatives before the sample is analyzed. The results are presented in Fig. 3.

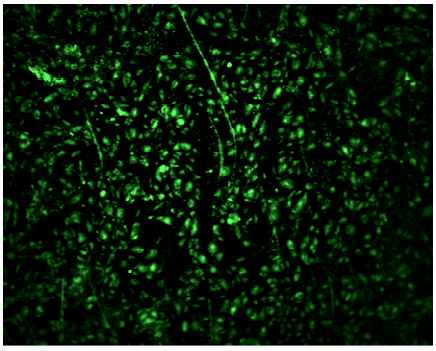


Fig. 3. Fluorescence analysis of the endothelium cells deposited on the porous coatings

### 3.3.1 Blood-material interaction

Hemocompatibility tests were done for PLL/HA cross linked and modified with PLL with HUVEC cells supported with fibronectin. These analysis, focused on medical materials, aim to detect adverse interaction between artificial surface and blood, which can activate or destruct blood components [19]. In arterial flow conditions, due to a high shear stress, platelet is the cell critical for the hemocompatibility compliance. A classical instrumentation for the dynamic test of hemocompatibility involves a flow chamber with a contact surface between blood stream and tested plate. In the current study we investigated a simplified model of the whole blood shear stress, based on a cone and plate rotational viscometer. Several indices of platelet activation were analyzed, including platelet- and granulocyte-platelet aggregates, platelet activation markers (integrin receptor IIb/IIIa) and selectin P. There are the appriopraate receptors on the surface of the platelets, which take participation in the adhesion and aggregation. There are mainly selectins, responsible for the initial stages of the adhesion process. Selectin P which is a typical for the blood platelet is the glicoprotein, which is accumulated in the platelet granules and transported to the membrane after platelet activation. The Impact-R test is a novel device for testing platelet function under close to physiological conditions. The device tests platelet adhesion and aggregation in an anti-coagulated whole blood (citrate buffer tubes) under arterial flow conditions. Furthermore, it provides a quick method for monitoring the response to various antiplatelet drugs (Fig. 4).

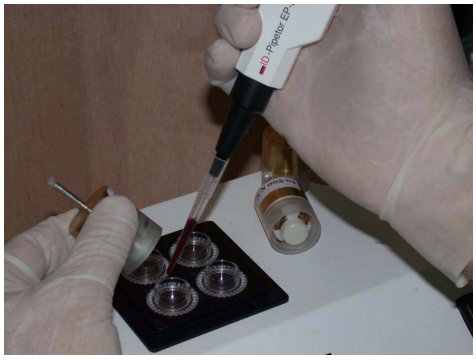
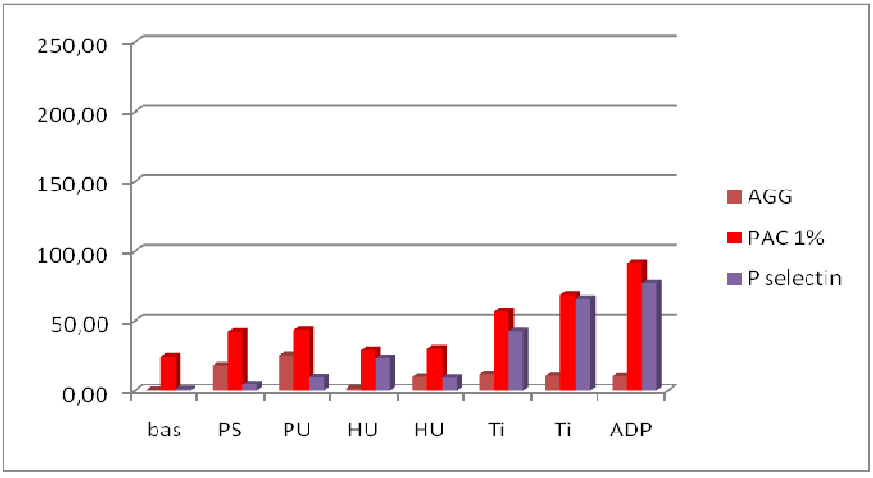


Fig. 4. Impact-R test

A blood- material interaction was performed for porous coatings without HUVEC cells deposition. It was observed a rapid clot formation. It was impossible to take the blood sample for the further examination (Fig. 5).

Fig. 5. Quartz crystal microbalance (QCM)

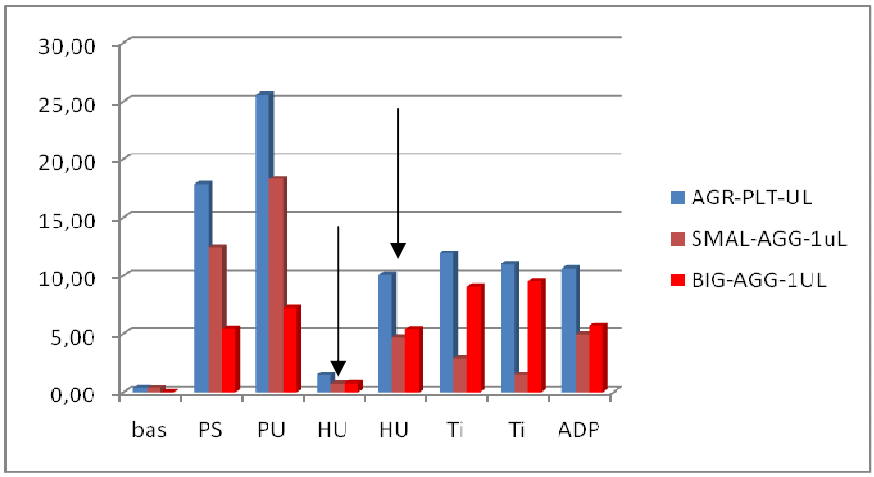
The layer of the endothelium cells deposited on the top decreases the probability of the clot formation. Fig. 5. illustrates the amount of platelet aggregates, activated cells with receptor IIb/IIIa and with selectin P is less than for inorganic titanium films.




**AGG-** platelet aggregates
**PAC 1%-** activated platelets with IIb/IIIa receptor
**P selectin-** activated platelets with P selectin receptor

Fig. 5. Blood- material interaction; platelet aggregates, activated platelet with receptor IIb/IIIa, activated platelet with receptor P selectin- as a function of the analyzed material

It was surprising to observe the variations between the same samples containing HUVEC cells (Fig. 6).




**AGR-PLT-** platelet aggregates; **SMAL-AGG-** small aggregates=2 platelets; **BIG-AGG-** big aggregates **>2platelets**; **PS-** reference substrate- polystyren; **PU-** polyurethane; **HU-** HUVEC deposited on the porous coatings; **ADP-** control adenosyno- tri phosporane- platelet activation

Fig. 6. Blood- material interaction; platelet aggregates, small aggregates, big aggregates- as a function of the analyzed material

The answer was found in fluorescence observations. Not all surface was covered with cells. There are some discontinuity which probably influenced on the rapid clots (Fig. 7).

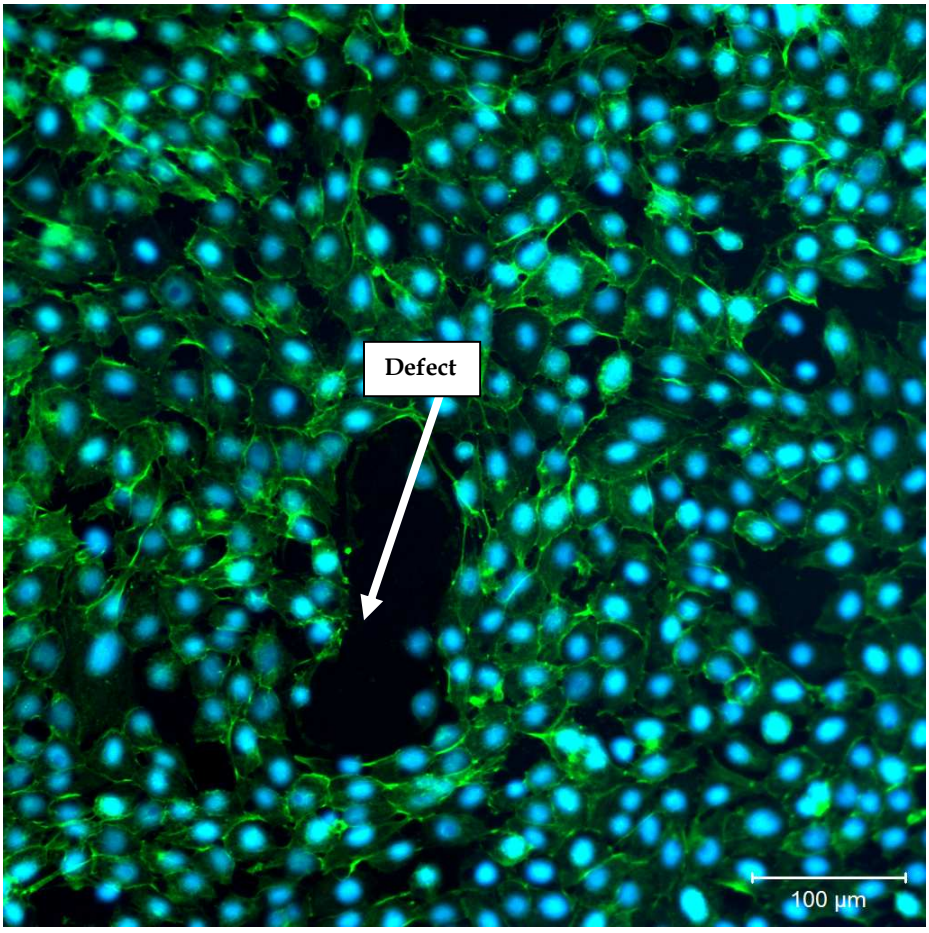



Fig. 7. Fluorescence analysis of the HUVEC cells deposited on porous coatings; defect in the structure

### 3.3.2 Additional observations

There is one contact point between the rotor and investigated plate in the impact-R design. It was expected that the cells would be removed from this part (Fig. 8).

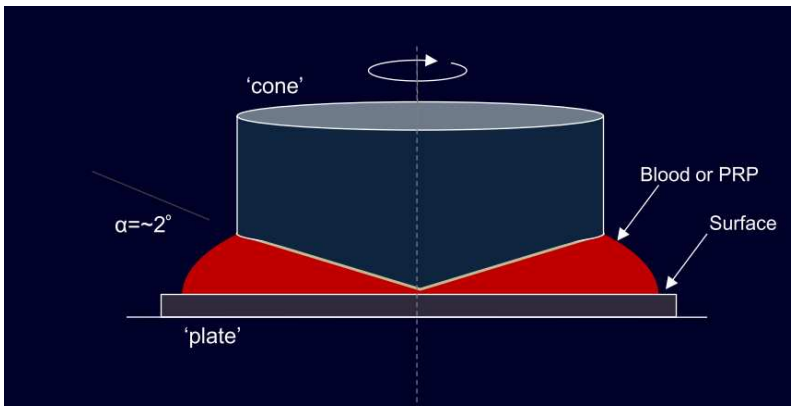



Fig. 8. Impact-R test- blueprint

Histological and fluorescence analysis showed the net of the vessel- like structures (Figs. 9, 10). It seems that the elaborated materials influence the angiogenesis. The detailed experiments, which are in progress, are focused on the explanation to prove if the net of the vessel like structure would appear once again for the similar structures or not.

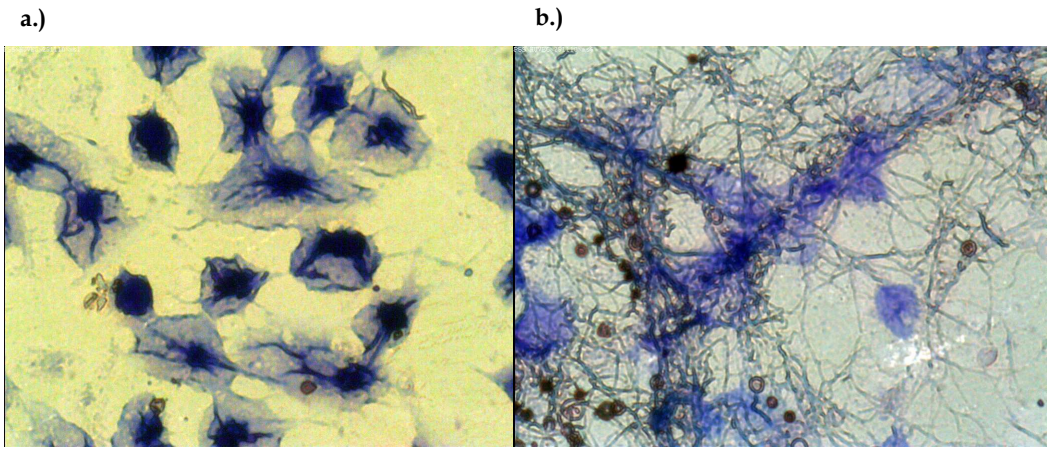



Fig. 9. Histological observations:
a.) HUVEC cells deposited on the porous structures b.) Net of the vessel- like structures

Vascular endothelial growth factors induce angiogenesis, which ensures proper oxygenation of the tissue. In addition, the angiopoietins stabilize the interaction between endothelial cells and supporting cells. They are therefore needed for both vascular remodeling and long-term vasculatur stability.

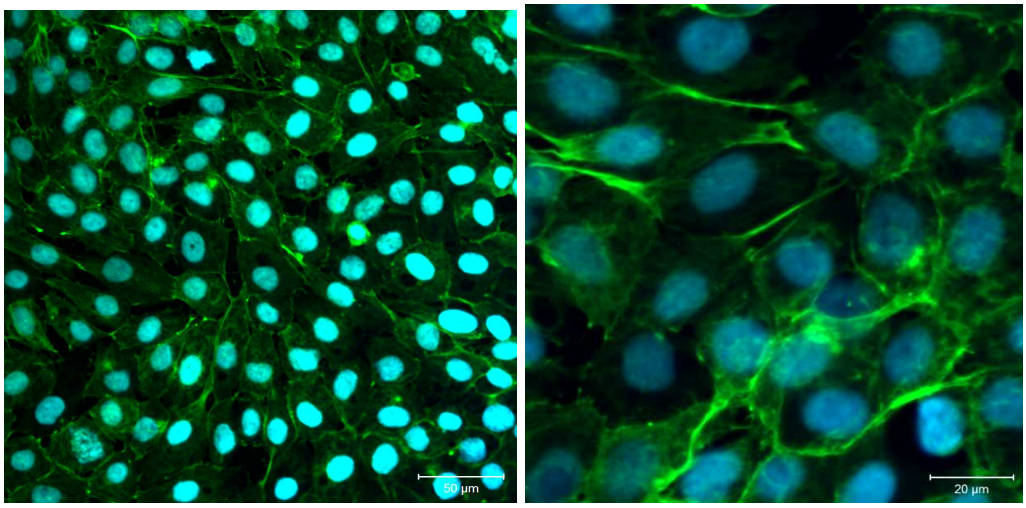



Fig. 10. Fluorescence observations:
a.) HUVEC cells deposited on the porous structures b.) Interconnections between the cells

### 3.3.3 Ongoing analysis

It is remaining task to answer why we have observed protein adsorption and cell attachment on PLL/HA cross linked and modified with PLL PEG while PEG should prevent nonspecific adsorption. The tests shown the strong attached cell number reduction comparing the structure when PEG was deposited before and after cross linking process. The possible explanation is that the concentration and deposition time were improperly adjusted. As suggested by the authors [18, 19] PLL-g-PEG polymers should be dissolved to 1 mg/mL concentration in autoclaved 10 mM PBS, pH 7.4. Before use, the polymer solutions should be remaining for three minutes at 37 °C in a water bath and equilibrated at experimental conditions (22 °C) for two minutes.

### 3.3.4 New solutions

Poly-ethylene glycol (PEG) is the material of choice for imparting protein resistance to surfaces [20]. By forming brush-like structures, PEG creates a structure which repels proteins, cells and bacteria [14]. The authors confirmed that it is a well known material for preventing nonspecific adsorption of proteins as well as its biocompatibility and low toxicity.

In our experiments we have performed the layer-by-layer (12 bilayers) deposition according to the following blueprint (Table 1) (Fig. 11):

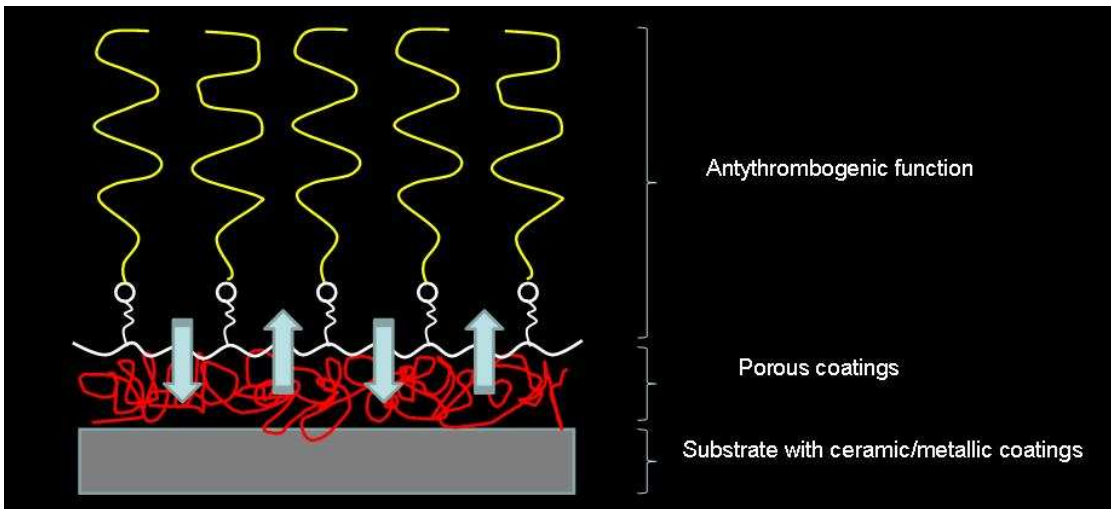
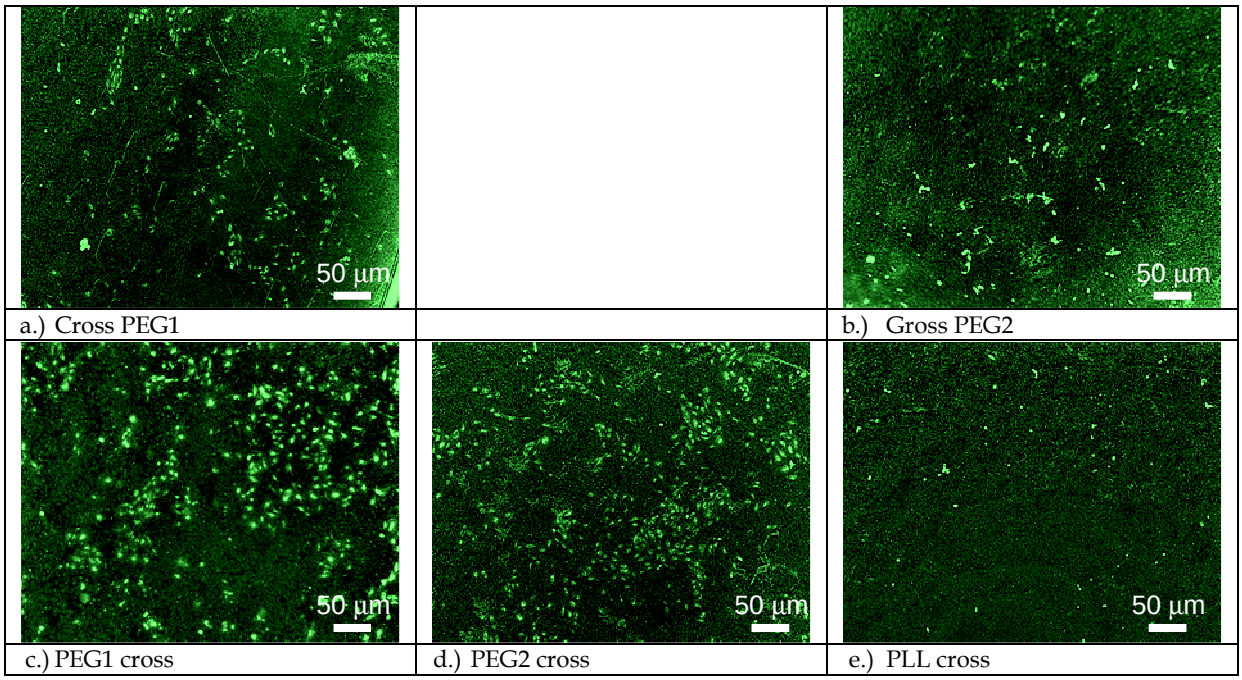



Fig. 11. New model of the layer design

Table 1. Experimental blueprint- program of the deposition experiment

| | | | | |
|---|---|---|---|---|
| Cross link and PEG 1 (PLL/HA)x12+ cross link+PEG 1 | Cross link and PEG 2 (PLL/HA)x12+ cross link+PEG 2 | PEG 1 and cross link (PLL/HA)x12+ PEG 1 +cross link | PEG 2 and cross link (PLL/HA)x12+PEG 2 +cross link | No PEG and cross link (PLL/HA)x12+PLL+ cross link |

Results of the surface morphology influencing the cell adhesion are presented in Fig. 12




a.) Cross PEG1 | b.) Gross PEG2

c.) PEG1 cross | d.) PEG2 cross | e.) PLL cross

Fig. 12. Human Umbilical Vein Endothelial Cells (HUVEC) deposited on polyelectrolyte layers

Following the information found in literature and the specific material's properties, the results seemed to be improper. The further literature study allowed to explain the observed phenomena. In publications which was citied at the beginning [11], the authors mentioned about the diffusive character of the coatings deposited by "layer-by-layer" technique. It was observed that cells adhered to PLL treated HA coatings at significant higher values than HA coated controls. The further and more specific information was found in publications written by C. Picart at al. [17] and L. Richert et al. [18]. They proved the "in" and "out" diffusion process of PLL through the whole film during each PLL deposition step. Richert et al. used photobleaching (fluorescence recovery after photobleaching- FRAP) experiments CLSM on un-cross-linked and cross-linked (PLL/HA) PLL-FITC films. For not-cross-linked films they observed a partial recovery of the fluorescence in the bleached zone, whereas for the cross-linked films, no recovery was found. This indicated the absence of PLL-FITC diffusion in the cross-linked films. Similar complimentary experiments were performed by Pickart et al. and results are presented in the elaboration cited [17]. In this article, they proved the existence of PLL diffusion process into the interior of the film by using fluorescent-labeled PLL. They underlined that exponential growth regimes are due to a similar in and out diffusion process of at least one of the two polyelectrolytes used in the film construction.

In our experiments "layer-by-layer", film growth was analyzed by a quartz crystal microbalance (QCM). QCM consists of measuring changes in the resonance frequency $\Delta f$ of a quartz crystal. A decrease in $\Delta f$ visually associated of the mass coupled to the quartz. The results of the QCM are shown in Fig. 13.

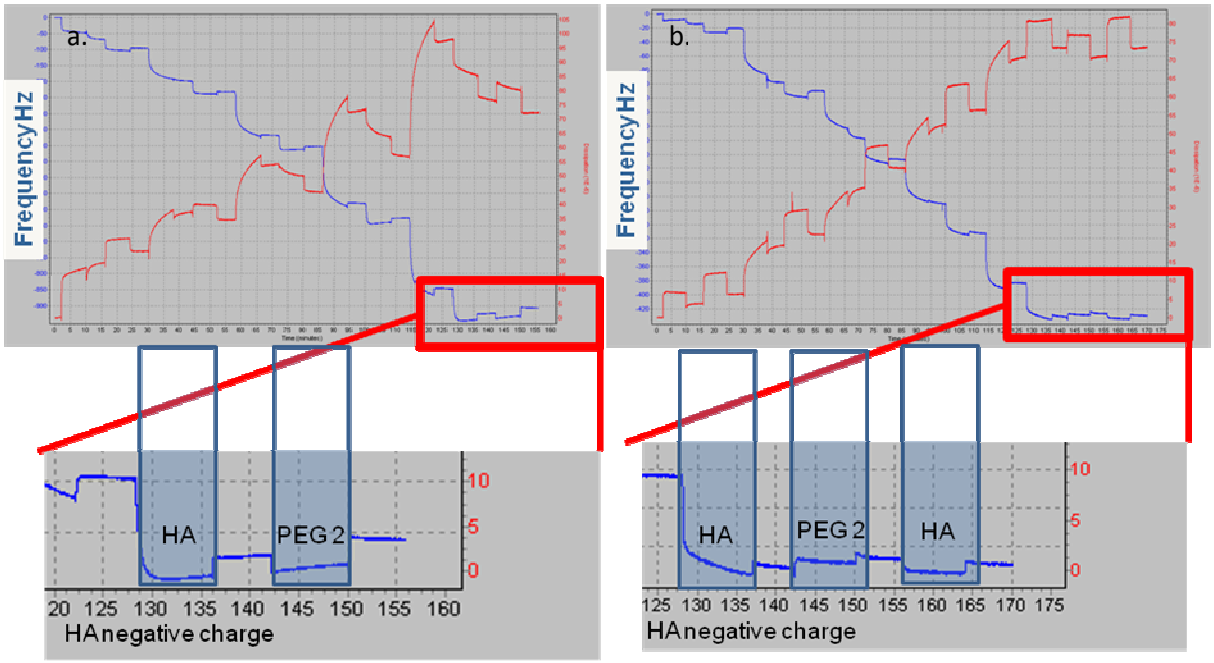
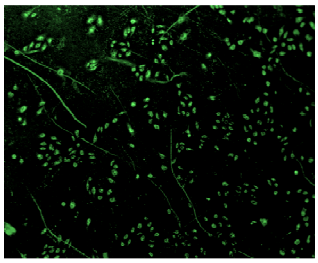


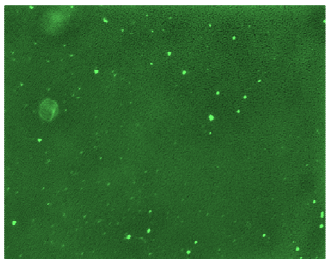

Fig. 13. Quartz crystal microbalance (QCM):
a.) no HA introduced in the end b.) HA introduced

It was observed the unique influence on the frequency after the PEG introduction into the structure (Fig 13a). It was postulated that difficulties associated with the efficient PEG incorporation were caused by the diffusion character of the underlying PLL/HA material. We decided to perform the experiment once more and hyaluronic acid deposit as a final layer. No frequency change was observed. We rather did not increase the mass of all "layer-by-layer" structure. Concluding, it was probable that the last layer before PLL PEG diffused into the structure. Difficulties with the PEG anchoring came from the fact that in practice we were trying to deposit PLL PEG onto PLL which was physically impossible. The further literature study has to be necessary to find the possible explanation. Experiments using fluorescently labeled PLL (PLL-FITC) exhibited in the work of Richert et al.[18] confirmed phenomena which we observed. For $(PLL/HA)_{20}$ cross linked film PLL-FITC coating was deposited. When there is no cross link reaction, no green surface fluorescence is observed. It commands the diffusion. After cross linking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfo-succinimide (sulfo-NHS) (NHS/EDC) they observed the light excitation. This strongly suggested that PLL diffusion was reduced. Moreover they proved that the zeta potential of the films becomes negative after the cross linking. This finally indicated why we observed no cells deposited on (PLL/HA)+PLL cross linked films and a lot attached on (PLL/HA)+ PEG cross linked films.

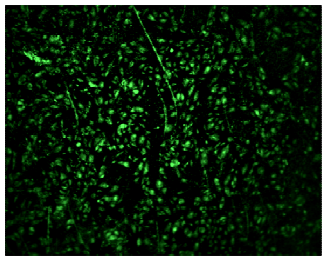
Several ways to create protein-resistant surfaces have been proposed. Among them, the most popular approach is based on the use of polyethylene glycol PEG. The protein resistance of PEG modifying surfaces is attributed mainly to entropic repulsion and the high water content of PEG chains. For the analysis PLL (see Fig 14a- just after cell deposition on the top, Fig. 14c after 3 days incubation) and fluorescent labeled PEG (see Fig 14b- just after cell deposition on the top, Fig. 14d after 3 days incubation) were deposited as a final layer after cross linking process.



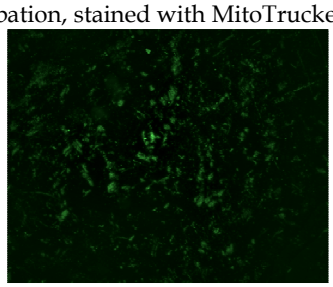
a.). HUVEC deposited on the PLL- before incubation, stained with MitoTrucker



b.). HUVEC deposited on the fluorescent labeled PEG- before incubation, stained with MitoTrucker



c.). HUVEC deposited on the PLL- after 3 days of incubation, stained with MitoTrucker



d.). HUVEC deposited on the fluorescent labeled PEG- after 3 days of incubation, stained with MitoTrucker

Fig. 14. New model of the layer design

## 4 Conclusions

The porous structures for the surface modifications plays the significant role for the proper scaffold preparation. The cross-ling reaction for the system stabilization influence on the diffusion process in the layer layout, which could cause the problems of the following layer deposition after the structure stabilization.

The novel biomaterials science combine materials science and biology because of the fact that the best choice for the restoration of the diseased human organs are the nature-mimic materials. The more natural-like, the better. For the porous and semi-porous (with the functionalized surface) materials it was observed the surface modification effect into the protein adsorption. For some materials surface fictionalization using proteins had a significant effect for the endothelialization process, although that the cracks were visible on the surface. From the material science point of view titanium nitride exhibit the most promising properties. Finally the initial cell density and pore size has a visible effect on full confluence.

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