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Surface-Modified Metallic Biomaterials in Contact with Blood and Endothelial Cells

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Summary: The experimental procedures were undertaken in order to characterize of surface-modified metallic substrates. Results revealed that carbon layers coating the substrates made metallic surface generally more biocompatible and more resistant to biofilm formation. Titanium-nitride or titanium carbo-nitride layers on the titanium alloy (Ti6Al4V) allow for modulation of thrombogenity degree of the surface. Our investigations employed several methods: fluorescence and electron microscopy techniques, SPR-biosensor technology, 2D-electrophoresis and flow cytofluorymetry.

Keywords: biocompatibility; blood platelets; endothelial cells; metallic materials; surface modification

Introduction

There are several features of biomaterial surfaces that play a fundamental role in a successful use of implants in the surgery. A long term contact of blood components or endothelial cells with biomaterials can be crucial to a variety of biological processes. Response of blood platelets and endothelial cells to this contact may be a highly important factor in a case of such processes as inflammation, blood coagulation, angiogenesis, osteointegration and many others, in which endothelial cells and blood platelets play a key role. In the case of reconstructive arterial or cardiac surgery cells are in direct contact with the implant surface. Controlling the interaction of cells with the biomaterial surface is also fundamental to regenerative medicine. The usefulness of metallic materials for implant production is well documented. Metallic alloys are durable and exhibit excellent mechanical properties, however they undergo corrosion processes in contact with body fluids.[1,2] This electrochemical phenomenon results in metal ions release, and is responsible for toxic effects (metallosis)[3] or allergic response to metals.[4] Significant progress made in materials science, mainly in the technology of surface, allows for intentional modifications of metallic surfaces. Thin layer of modified surface separates metallic substrates from environment and prevents thus from corrosion.[5] However, a real opportunity to modulate the interaction of surface with surrounding tissues and body fluids, with respect to the anticipated application, seems to be even more important. This way, one can produce a metallic implant with strictly defined properties for specific applications. Our practical experience with modification of surface concerns 316L medical steel and Ti6Al4V titanium alloy. Both substrates were coated with thin layers of carbon (films of: diamond like carbon - DLC or nanocrystalline diamond - NCD). The titanium alloy was also subjected to glow discharge procedure resulted in the production of thin layers of TiN (titanium-nitride) or TiCN titanium carbo-nitride) on titanium alloy surface. Both modifications prevented the base materials from corrosion and made the alloy surface more durable.[6,7] Our recent investigations were focused on the cellular and molecular

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aspects of interaction between biological objects and modified surfaces of metallic biomaterials.The biological objects were observed directly on the surfaces of interest as well as away from the surfaces, after the contact of the objects with the studied surfaces.

The Biological Objects

All implants, earlier or later, are in contact with blood. So this tissue is especially interesting and important from the point of view of implantology. Blood is flowing in blood vessels, and blood hemostasis is very important to keep blood in the circulation system. But not less important is a local blood thrombosis and clotting. Native blood vessel consists of endothelial cells, which are neutral (inert) for contact with blood cells. There are also smooth muscle cells, collagen fibbers and other subendothelial matrix proteins. These are responsible for elasticity of the vessel. When wall of blood vessel is destroyed, blood can come out of the circulation system, and it is defined as bleeding. To stop bleeding, blood platelets come into the contact with biological structures creating vessel wall, i.e. with fibres of collagen and other subendothelial matrix proteins. This contact results in activation of blood platelets and release of substances necessary for further platelet activation and aggregation. During these events inactive form of platelet fibrinogen receptors is converted into the active one, which is able to bind fibrinogen – a fibrillar protein of blood plasma. During the same time, molecules of P-Selectin, originally located in alpha-granules of the native platelets, are moved to surface of platelet, where serve as procoagulant factor. Both proteins, active form of fibrinogen receptors and surface expressed P-Selectin are used as markers of blood platelet activation.[8,9] Of course it should be mentioned, that tissue factors, released from destroyed cells, activate the blood clotting cascade and convert prothrombin to thrombin. Thus, thrombin converts fibrinogen to fibrin, a form of active monomers, which spontaneously create network of fibrin stabilizing platelet clot. This way the clot can prevent the circulation system from bleeding. But the clotting process, if not stopped, can close the blood vessel resulting in stenosis, and necrosis of a tissue fragment supplied by the vessel. Fortunately, hemostasis contains opposite component - fibrinolysis. Any way, low platelet reactivity can be a reason of bleeding, whereas overactivity of blood platelets usually results in acceleration of arteriosclerosis. It is also well known, that implants removed from the human body, due to inflammations and resistant infections, are colonized by microbes.[10] Biomaterial, to be well adopted by the body, should be bio-, hemo- and thrombo-compatible, but should also exhibit resistance to microbial colonization and biofilm formation.

In the light of the above, the main biological objects of our interest are blood plasma proteins, blood platelets, endothelial cells and the model microorganism *E coli* cells.

Materials and Methods

Materials

For sample preparation 316L medical steel and Ti6Al4V titanium alloy were used. These metallic substrates were superficially modified by synthesis of thin carbon layers in the form of NCD or DLC. The carbon surfaces were synthesised in Department of Biomedical Engineering, headed by Prof. S. Mitura.[11] Titanium alloy was also subjected to creation of thin layer of titanium-nitride (TiN) or titanium carbonitride (TiCN). Those coatings were prepared on Faculty of Materials Sciences, Warsaw University of Technology, by Prof. T. Wierzchoń team.[12] The samples were discoidal in shape with diameter of 8 mm and thickness of 3 mm. Some experiments were carried out with the studied materials used in a powder form.

Fluorescently labelled antibodies (CD61, CD62P and PAC-1) were provided by

Becton Dickinson. Reagents, materials, instruments and software for 2D electrophoresis were from General Electric Healthcare (formerly Amersham Biosciences). Fluorescence dyes were supplied by Molecular Probes. All other chemicals and reagents came from SIGMA-ALDRICH.

Methods

Simple optical inspection of the studied surfaces and quantitative fluorescence intensity measurements were done with OLYMPUS GX71 microscope equipped with CCD camera. Blood plasma was isolated from the whole citrated blood by centrifugation (1000× g, 15 min, RT) and whole plasma proteins were applied for fluorescence labelling with fluorescein, according to the manufacturer method.[13] The adhesion of fluorescently labelled blood proteins to the surfaces were then quantified with the use of fluorescence microscope equipped with the suitable software. The quantitative and qualitative analysis of surface colonization with *E coli* cells were enabled by application of DNA sensitive fluorescence probe bisbenzimide.[14]

Scanning electron microscope HITACHI S-3000N was used for detailed observation of adhesion of blood platelets to the studied surfaces. The samples were incubated in contact to the whole citrated blood for one hour, and after that the surfaces were carefully washed out with PBS (phosphate buffered saline), pH 7.4, to remove not attached blood cells. The series of dilutions of ethanol were used for surface dehydration, and dried surfaces were coated with a thin layer of gold before the observations.[15]

Becton Dickinson flow cytofluorymetry instrument FACScann Calibur was employed for analysis of blood platelet aggregates formation in the whole blood. The same instrument was also used for quantitative analysis of surface expression of active fibrinogen receptors and P-Selectin molecules, both indicators of blood platelet activation. The blood samples after contact with the studied surfaces were prepared according to the manufacturer instruction[16] with use of the above mentioned fluorescently labelled antibodies. The antibodies were labelled with spectrally different labels, ant the instrument was able to detect the signal from each label simultaneously and independently. The antibody CD61, directed against GPIIIa molecule on the platelet surface, was used for specific platelet recognizing and signal gating. The antibody CD62P recognized only surface expressed P-Selectin, whereas the antibody PAC-1 specifically recognized the active receptors for fibrinogen on the platelet surface.

2D-electrophoresis equipment: MULTIPHORE II system, Image Scanner II and ImageMaster 2D Elite software, all products of GE Healthcare (formerly Amersham Biosciences), were used for separation of endothelial cells proteins.[17] The method allows for simultaneous electrophoretic separation of several thousands of proteins, extracted from the cells, according to their isoelectric point pI (the 1st dimension) and molecular mass (the 2nd dimension). The cells were lysed with lysing buffer containing urea, detergent and reductant of sulphate bridges. Proteins extracted from the lysed cells were purified by precipitation and used for the isoelectrofocusing separation (1D). After that, the set of proteins, separated according to their pI, was transferred onto the SDS slab gel and was separated in the second dimension. The two-dimensional map (2D) of separated proteins was then developed by silver staining and was converted into the electronic format with the use of optical scanner. Differences in proteome of endothelial cells, treated by the contact with variety of artificial surfaces, were then analysed with the use of the ImageMaster 2D software.

The Surface Plasmon Resonance (SPR) biosensor, working in the BiaCore X system, was used for the real time observation of blood plasma proteins adhesion to the biomaterial surface under flow conditions.The commercially available SIA sensor kit, containing ready to modify glass plates with thin gold film on the surface, was

used. Thin layers of DLC or titanium were deposited on the gold surface by RF PCVD or by sputtering method, respectively. Thickness of the layers did not exceed 10 nm.[18] The modified glass plates were mounted into the sensor envelope and used for measurements. Diluted blood plasma proteins (1:5000) were then injected into the system, and mass change on the sensor surface, corresponding to the mass of adhered proteins, was observed and recorded.

Endothelial cells (EAhy 926 cell line) were grown at the standard conditions, and confluent cells were used for experiments. The NCD or medical steel, in the powder form, was added to the culture for 24 hours. After that the cells were inspected with the optical and fluorescence microscope and the harvested (with the help of trypsin) cells were used for proteome analysis.[17]

Results

On Site Analysis

Our studies carried out with the SPR sensor coated with DLC or titanium films, and with gold film alone used as the reference surface, unequivocally indicate that DLC surface is much more resistant to protein adhesion and deposition than titanium is. It was shown, that blood plasma adhesion strongly depends on the flow rate, and the amount of adhered proteins increased with decrease of flow rate. This dependence suggests an importance of shave stress in protein adhesion to the surface. Amount of blood plasma proteins adhered to the DLC surface represented only about 4% of proteins adhered to the reference gold surface, whereas adhesion to titanium surface reached 10% of the reference one. The results obtained with the SPR biosensor technology were confirmed by quantitative fluorescence microscopy method. The fluorescein labelled blood plasma proteins exhibited significantly higher adhesion to medical steel and titanium alloy (the substrate metals) than to their modified surfaces. The studied surfaces can be arranged in respect to the increasing amount of adhered proteins as follows: non-polished steel coated with DLC, non-polished steel coated with NCD, electro-polished steel coated with DLC, electro-polished steel coated with NCD, titanium alloy coated with DLC, titanium alloy coated with NCD, titanium alloy alone, titanium alloy coated with TiN, electro-polished steel and non-polished steel.[19]

The next observation made with the use of scanning electron microscope revealed the medical steel surface as a place of massive blood platelet adhesion and aggregation, whereas in contrast to that, NCD surface was almost entirely free from adhered blood platelets.[15] It was also found, that titanium alloy alone as well as its modified surfaces were more susceptible to platelet adhesion than medical steel alone is. The studied surfaces can be arranged in respect to the number of adhered blood platelets as follows: medical steel coated with NCD or DLC, medical steel alone, titanium alloy coated with TiCN (16%C), titanium alloy alone, titanium alloy coated with TiCN (4%C), titanium alloy coated with TiN, and titanium alloy coated with NCD.[20,21] In reference to the medical steel, there is a positive correlation between the number of adhered blood platelets and the amount of plasma proteins deposited on surface, but in reference to the titanium alloy such the correlation was not valid, but it is necessary to note, that the roughness of titanium surfaces, even after modification, significantly exceeds the roughness of surfaces of medical steel. It suggests that both, presence of surface attached proteins and surface roughness can modulate a surface susceptibility to blood platelet adhesion.

The next set of experiments proved that the NCD surface coating medical steel is extremely resistant for microbial colonization when compare it to medical steel or titanium alloy alone.[22] Moreover, preincubation of sample surface in contact with proteins (bowine serum albumin (BSA) or blood plasma proteins) resulted in a very

significant increase in the number of *E.coli* cells found on the surface of all samples. It was threefold increase for medical steel and NCD, and four-time increase for titanium alloy. It means, presence of proteins at the surface is crucial for bacterial adhesion.[22] This finding is also important due to earlier introduced procedures of implant surface passivation with albumins in order to get more thrombo-compatible surface.[23] Unfortunately, surface friendlier to blood platelets is also more susceptible to bacterial colonization.

Another observation was made for endothelial cells. It was found, that neither medical steel alone nor medical steel coated with NCD were hostile to endothelial cells growth, however they were differently attractive for cultured cells.[17]

Post Contact Analysis

Using flow cytometry analysis we found significant difference in the number of blood platelet aggregates in the blood after contact with different surfaces. The number of aggregates found in the blood contacting NCD surface, coating electro-polished medical steel, was comparable to that of the control blood. It means, this surface does not activate blood platelets. In contrast to that, significant increase in the number of aggregates was observed after contact with NCD surfaces, made on non-polished medical steel, and on both NCD surfaces coating electro-polished and non-polished titanium alloy. The tendency to increase the number of aggregates created by the contact with non-polished surfaces was evident.[24]

Corresponding conclusions could be drawn from the analysis of P-Selectin expression on the platelet surface. It was found, that the presence of NCD layer on electro-polished medical steel reduces activation of blood platelets to the control level. Titanium alloy alone was found as the most prominent activator surface, and the presence of NCD coating on the titanium alloy reduced significantly this property, buy not to the level of control. Similar results were obtained with observation of expression level of the active fibrinogen receptors. The titanium alloy substrate, both alone and coated with NCD, caused strong expression of the receptor on blood platelets, whereas NCD layer on medical steel prevented this process.[24]

Another interest was focused on changes in protein profile caused by a short-term contact of endothelial cells with surfaces of medical steel and with NCD. It was found, that contact of endothelial cells with NCD powder particles resulted in a minor changes in profile expression (found a few changes in the protein map). In contrast to that, contact of endothelial cells with medical steel resulted in a quite dramatic increase in expression of more than 17 proteins,[17] which are currently under an identification procedure.

Conclusion

At present we know nothing about adaptative processes occurring during the cell contact with an artificial surface, but our results indicate that this kind of investigations on the cellular response and molecular level of gene expression and production of proteins should be considered in laboratories working on biomaterials with a preference.

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