Silver nanoparticles influence on the blood activation process and their release to blood plasma from synthetic polymer scaffold

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Abstract. In the present work, blood and blood plasma interaction to silver stabilised polyelectrolytes was investigated in vitro. The designed materials are dedicated for regeneration of the cardiovascular system. Silver nanoparticles were introduced into the polyelectrolyte structure in order to reduce the risk of bacterial biofilm formation. The introduction of Ag nanoparticles occurred by deposition at high vacuum by magnetron sputtering. The analysis of blood-materials interactions were performed by using commercially available tester, Impact-R (Diamed). The assessment of silver ion nanoparticles release into the plasma consisted in determining the Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT). Unmodified surface of polyelectrolytes is a strong activator for blood elements. The introduction of silver nanoparticles resulted in a significant reduction in the probability of clotting. The extrinsic pathway of coagulation determined on the basis of the PT and the intrinsic and common pathways of coagulation measured by the APTT did not indicate the danger out of range. Microstructure was studied using TEM on thin foils prepared from the cross-section of samples subjected to biomedical treatments. The observations revealed hetero- interface between two different crystalline solids.

1. Introduction

In the frame of the work, biologically mimicking coatings were considered. The key issue was to express the minimal impact on blood activation process. The designed coatings form the scaffolding of cell repopulation. Tissue analogs allow better and longer use in the treatment of cardiovascular diseases. The work concerns the implementation of porous coatings and surface nano-functionalization.

A fundamental problem that affects the field of cardiovascular surgery is the paucity of autologous tissue available for surgical reconstructive procedures [1]. Although the best results are obtained when an individual's own tissues are used for surgical repair, this is often not possible as a result of pathology of autologous tissues or lack of a compatible replacement source from the body.

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Extracellular matrix (ECM) structure can be easily mimic by the synthetic, degradable polymers [2,3]. Degradable scaffold materials are advantageous in tissue engineering applications because they provide initial biomechanical integrity as cells adhere, mature to the desired phenotype, and secrete natural ECM, and, most important, they are designed to be replaced by the neo-tissue. Polymers that have been extensively used in tissue engineering applications are poly glycolid acid (PGA), polylactic acid (PLA), and poly-E-caprolactone (PCL) [4-6]. They are widely used because of their biocompatible degradation properties. Hydrolysis of ester bonds in situ is thought to release nontoxic monomers that are excreted or absorbed by surrounding tissues. A release of acidic degradation products can be a source of toxicity that causes adverse local and systemic reactions that could result in implant rejection.

An introduction of the nanoparticles of precious metals created an antibacterial function [7].

Binding anions and proteins in biologic systems, silver ion (Ag+) avidly binds to cell surface receptors of bacteria, yeasts and fungi [8]. Silver cations also strongly bind to electron donor groups of biologic molecules containing sulphur, oxygen and nitrogen. The binding of silver ion to sulfhydryl groups and proteins on cell membranes appears to be critical to the antimicrobial action. The ionizing capacity of various silver compounds is critical in comparing the antimicrobial activities.

Nanotechnologies allow an efficient exploitation of the antimicrobial properties of silver by using silver in the form of nanoparticles [9].These are used in applications such as preservatives in cosmetics, textiles, water purification systems, coatings in catheters and wound dressings. The anticipated widespread exposure to silver nanoparticles in the near future has prompted governmental bodies and the public to raise questions about the safety of such applications.

An understanding of protein adsorption and macromolecular interactions is crucial for the control of surface initiated coagulation, the humoral immune response, cell binding, activation of inflammation, and wound healing [10-12]. Such processes are of large interest for currently expanding technological areas in biosensors and the controlled drug release, but may be of even larger significance for the in vivo behaviour of artificial surfaces in the cardiovascular system during short to medium long contact times (minutes to hours) [13, 14]. The dynamics of plasma protein adsorption, exchange and degradation at solid interfaces are still obscure phenomena during physiological conditions. The time course of adsorption and displacement of fibrinogen in plasma is called the Vroman effect [15]. Its kinetics depends on surface chemical properties, where negatively charged hydrophilic surfaces are suggested to facilitate the displacement of adsorbed proteins and hydrophobic surfaces to retain them over extended times in plasma [16]. Positively charged hydrophilic surfaces (with point of zero charge (p.z.c.) on the other hand, are not at all understood in this context. The research activity was focused on studies of displacement of pre-adsorbed human serum albumin (HSA) by buffer and blood plasma, and the activation of the intrinsic pathway of coagulation and complement on silver nanoparticles introduces.

2. Materials and methods

2.1. Polyelectrolyte and Ag nanoparticle preparation

Polyelectrolyte multi-layered films were deposited by a “layer by layer” technique [17]. Substrates were activated by 10 M NaOH and washed with pure Milli-Q water. PLL and HA were dissolved in 400mM HEPES/ 0.15M NaCl solution with concentrations of 0.5 and 1mg/ml, respectively and pH of the solutions was set at pH 7.4 by adding 0.5M NaOH. Films were manufactured by an automatic dipping machine using an alternately immersing substrate in solutions of PLL and HA for 8 min each. After each deposition step, the substrate was rinsed in 0.15 M NaCl solution buffered at pH 7.4 to remove excess polyelectrolyte. The process was repeated until a desired number of 12 bilayers were obtained. Multilayers were further processed for the cross-linking. Then additional layer of PLL was

adsorbed. Afterwards, the samples were subjected to final rinsing steps and stored at 4 °C in 400 mM HEPES/0.15M NaCl solution buffered at pH 7.4 until measurements were performed.

After the evacuation of the tube to 10 Pa base pressure by a fine vacuum pump through the mesh anode, the process gases are delivered to the polymer tube through the mesh cathode. The introduction of Ag nanoparticles occurred by deposition at high vacuum by magnetron sputtering from pure Ag targets (99.9%) at extremely high gas pressure. Therefore, the dried polyelectrolyte samples were introduced to the vacuum chamber, which was afterwards pumped down to 3x10-5 mbar. Afterwards, sputtering occurred at 1x10-2 mbar Ar pressure at 150 mm distance between the Ag sputter target and the substrates. The deposition was stopped after 30 s.

2.2. Microstructure

The microstructure characterization of the deposited coatings was performed using a TECNAI G2 FEG (200kV) transmission electron microscope (TEM) equipped with a field emission gun (FEG). Thin foils for TEM observation were prepared for one sample in every group of materials using the focused ion beam (FIB) technique. For FIB preparation a Quanta 200 3D DualBeam was used. A detailed description of the TEM microstructure analysis is presented elsewhere [18]. Phase analysis was performed using electron diffraction patterns and was confirmed through identification of high-resolution images (HR-TEM). All thin foils were cut as a cross- section of investigated coatings.

2.3. Blood-material interaction

The analysis of blood-materials interactions were performed by using commercially available tester, Impact-R (Diamed) [18]. Expression of platelet activation was evaluated based on glycoprotein IIb/IIIa, and using CD62P for P-selectin.

Receptor IIb/IIIa is the characteristic receptor to assess the degree of activation. It is an integrin complex found on platelets. It is a receptor for fibrinogen and von Willebrand factor and aids in platelet activation. The complex is formed via calcium-dependent association of gpIIb and gpIIIa, a required step in normal platelet aggregation and endothelial adherence [19, 20]. Platelet activation by ADP leads to the aforementioned conformational change in platelet gpIIb/IIIa receptors that induces binding to fibrinogen.

P-selectin is a protein that in humans is encoded by the SELP gene [21]. P-selectin functions as a cell adhesion molecule (CAM) on the surfaces of activated endothelial cells, which line the inner surface of blood vessels, and activated platelets. In inactivated endothelial cells, it is stored in granules called Weibel-Palade bodies. Platelet activation (through agonists such as thrombin, Type II collagen and ADP) results in "membrane flipping" where the platelet releases α- and dense granules and the inner walls of the granules are exposed on the outside of the cell. The P-selectin then promotes platelet aggregation through platelet-fibrin and platelet-platelet binding [22, 23].

Integrated fluorescence of the activation marker is calculated as a multiplication total of geometric mean fluorescence by percentage of marker-positive objects. Aggregates of platelets are analysed after erythrocyte lisps by mixing 25 μL of blood with 0.4 mL FLS and subsequent fixation by addition 3.5 mL 1% paraformaldehyde in PBS. The cellular material is recovered by centrifugation (1 000 g, 7 minutes) and immunostained (25 μL aliquots) with 4 μL PerCP-CD14 and 5 μL FITC-CD61 or 5 μL FITC- CD61 alone for 30 minutes at room temperature. Samples are then washed in PBS, and analyzed by the flow cytometer. The percentage of granulocyte-platelet aggregates (leukocytes stained with platelet marker CD61) is calculated using granulocyte forward/side scatter gate and additional monocyte CD14+ gate. Absolute number of platelets is calculated as the number of CD61 positive objects in reference to total granulocytes count. Small and big platelet aggregates are counted using forward/side scatter gates for CD61 positive objects in the flow cytometer analysis. Samples were washed in PBS solution three times and analyzed by using confocal laser scanning microscopy Carl Zeiss Exciter 5. The specific monoclonal antibodies were used in order to analyze the adhesion of active platelets by using two antibodies; P-selectin conjugated with fluorescein isothiocyanate (FITC)

and (platelets with the active receptor selectin P), human monoclonal antibodies CD45 PE conjugated with PE-Texas Red (active leucocytes).

2.4. Silver release into blood plasma

In the assessment of nanoparticles of silver ion release into the plasma, the main parameters for their evaluation were taken under consideration, Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT).

PT is used to assess the extrinsic coagulation. Its value is dependent on the concentration of blood plasma coagulation factors such as factor II, factor V, factor VII, factor X, and fibrinogen. Tissue factor (TF) is added to the citrated plasma to activate factor VII. The binding of calcium ions by the citric acid causes that the activation of factor X does not take place by VIIa (activated factor VII) [24]. After incubation, the time when calcium ions are introduced is measured.

APTT measure of the activity of plasma coagulation factors. Kaolin-activated partial thromboplastin time- one of the indicator of blood clotting, since it is a measure of the activity of plasma clotting factors XII, XI, IX and VIII. They form the intrinsic system of prothrombin activation. Kaolin-activated partial thromboplastin time should remain in the level of 26.0 to 36.0 seconds.

For the analysis 22.5 mL of blood was taken on citrate (Vacutainer, 5x 4.5 ml). Blood was poured into one tube of 50 ml Falcon to ensure homogeneity of the experiment. 1 ml of blood was taken to the eppendorf and centrifuged 4000g/8min./4 °C. After centrifugation the plasma was transferred into 20 ul of appropriately signed eppendorf (0 bass) and frozen - 80 °C to determine the

concentration of input value of micro-particles. The rest of the plasma (~370 ul) was transferred to a new tube (0 bass) and measured for prothrombin time (PT) and partial activated tromboplastin time (APTT). Three plate wells were selected with the appropriate substrates coated with silver and having discs on the bottom of the test which was rinsed once with sterile physiological saline solution. For each testing well 3.5 ml of whole anticoagulated blood was added with sodium citrate and mixed in the incubation conditions at 37°C with shaking (120 rev./ Min.) for 1, 3 and 6 hours respectively. After appropriate incubation the wells were collected aseptically, taking 1 ml of blood and centrifuged at 4000g/8 min/ 4 °C. Then taken after 20 ul of plasma was taken of each sample (to measure micro-particles) and frozen at - 80 °C. 370 ul of plasma was transferred to a new tube for the determination of PT and APTT using BCS XP Siemens.

3. Results

3.1. Microstructure characterization

Analysis of the coating microstructure with incorporated silver nanoparticles is shown in figure 1. The investigated coating was uniform, without the effect of delamination. Silver nanoparticles were located in an orderly manner in the amorphous matrix. It was ascertained on the basis of high resolution transmission electron microscopy (Figure 2a,b). Interplanar distances were measured in pictures obtained by inverse fast Fourier transform (IFFT) of atomic planes to obtain better contrast (Figure 2c). Figure 3a shows hetero- interface between two different crystalline solids. The lattice mismatch was possibly adjusted by edge dislocation or by strain (Figure 3b).

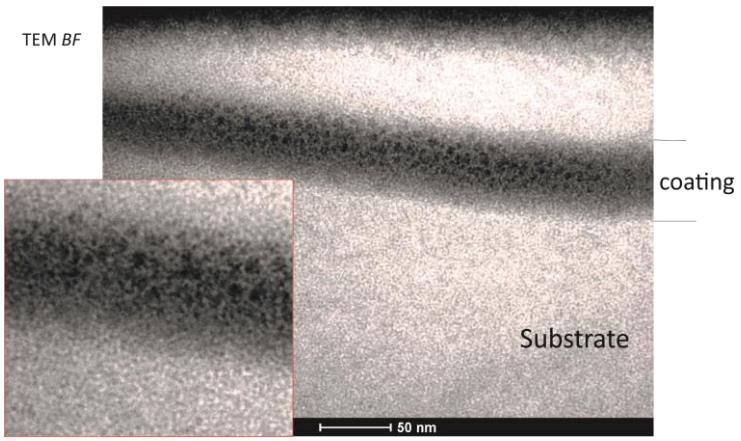


Figure 1. TEM bright field mode microstructure analysis from the cross section of the investigated coating..

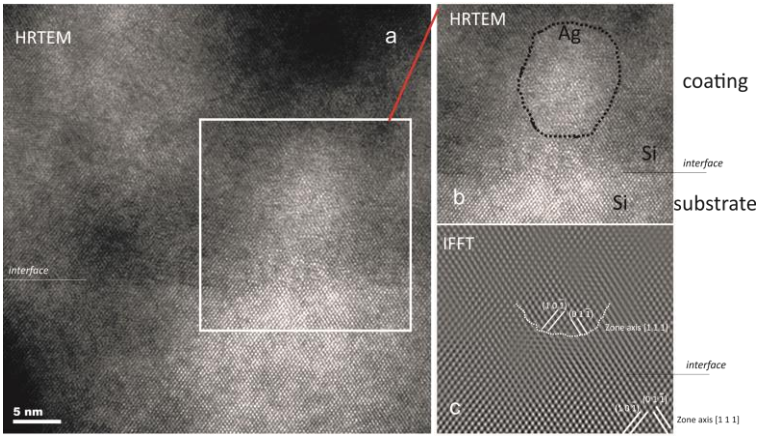


Figure 2. High resolution transmission electron microscopy analysis of the Ag nanoparticles introduced into porous synthetic coating a.) HRTEM, the general characteristics; b.) HRTEM, region of interest; c.) Inverse fast Fourier transform (IFFT) analysis of atomic planes.

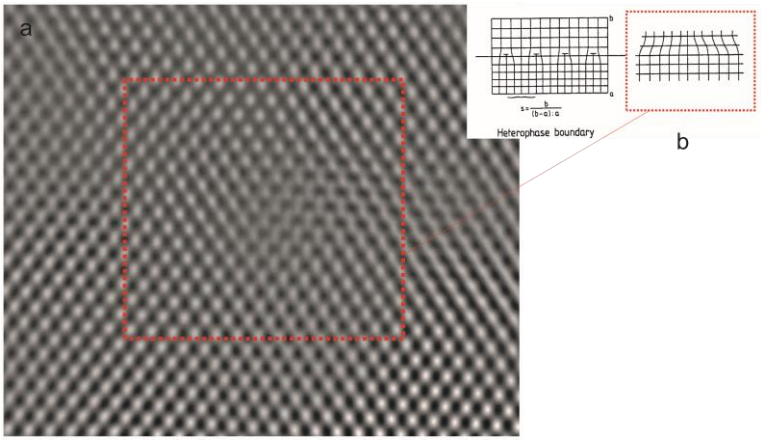


Figure 3. Hetero- interface between two different crystalline solids; b.) Theoretical interpretation of the lattice mismatch caused by edge dislocation.

3.2. Blood-material interaction in the dynamic conditions

Activation of platelets and its aggregation in blood collected after dynamic test and adhesion of active platelets were analysed. Analysis of the quality of blood through the techniques of flow cytometry is presented in figure 4.

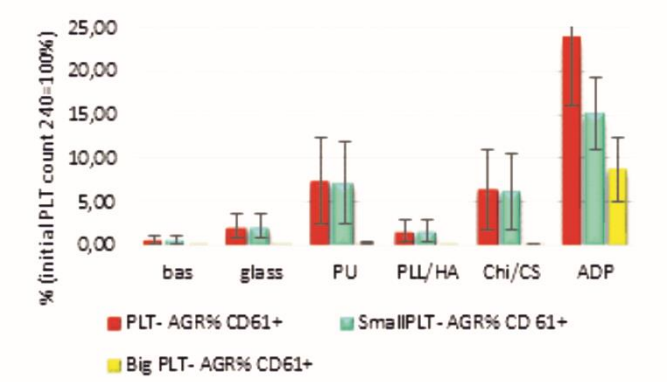


Figure 4. The probability of the platelet aggregates formation (CD61+) in a function of the tested material; bas- negative control, ADP- adenosine-di- phosphate activated platelets- positive control.

In the case of platelet aggregates, aggregates were distinguished as small and large once. Small units defined as two plates connected together. Big aggregates were defined as a conglomeration of more than two platelets.

The reference substrate was clinically used polyurethane. In the case of surface modification using polyelectrolyte-modified with silver nanoparticles, for all cases, hemocompatibility was improved. Better blood- material interaction was observed for PLL/HA modified with silver both for small and big platelets aggregates.

Analysis of platelet activation related to the analysis of membrane receptors, P-selectin and IIb / IIIa receptor.
Expression of platelet activation markers is measured on CD61 gated objects using PAC-1 antibody for conformational change of glycoprotein IIb/IIIa, and using CD62P for P-selectin (Figure 5).

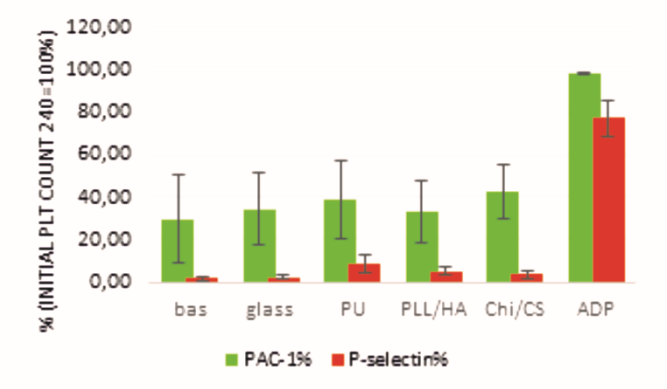


Figure 5. The amount of platelets with active receptors IIb/IIIa and selectin- P respectively in a function of the tested material; bas- negative control, ADP- adenosine-di- phosphate activated platelets- positive control.

In this case, considerable differences between the modified polyurethane with a polyelectrolyte with silver and a non-modified polyurethane were not observed. All materials showed the activation on the negative control level which means heparinized blood. However, in the case of pure, non-modified polyelectrolytes PLL/HA and Chi/CS without silver ions, the followed reaction concerns a very fast and strong activation and aggregation of blood on the surface (Figure 4).

3.3. Silver release into blood plasma
The coatings tested did not affect the blood clotting factors. Prothrombin time (PT) was marked. It increased with a decrease in fibrinogen, prothrombin, factors V, VII, and X (extrinsic coagulation pathway) (Figure 6).

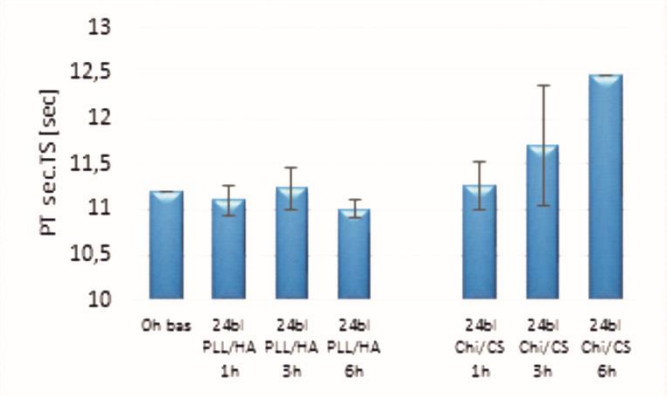


Figure 6. Prothrombin time (PT), analysis of extrinsic coagulation pathway V, VII and X.

APTT (partial activatet tromboplastin time), measures the extrinsic path and evaluates shortage activation of the contact pathway. It is able to further demonstrate the usage of factor VIII, IX, XI and XII. There are the factors form the intrinsic system of the activated prothrombin (Figure 7).

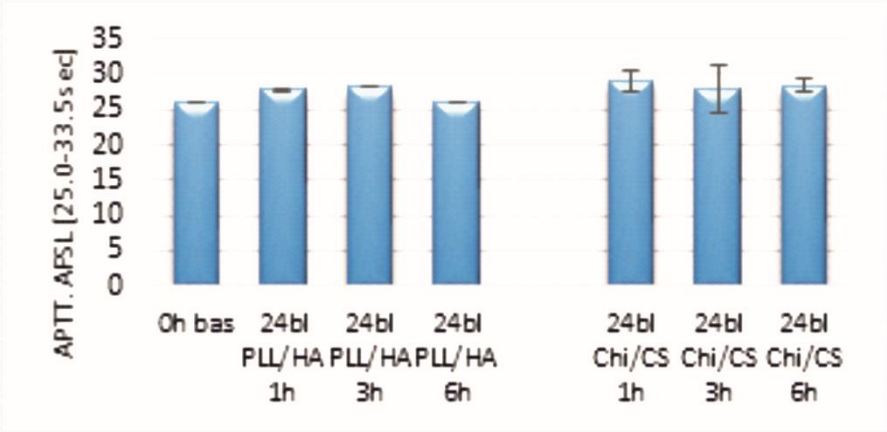


Figure 7. APTT (partial activatet tromboplastin time), demonstrating the usage of factor VIII, IX, XI and XII.

The second coagulation factor, it is a glycoprotein which is contained in the plasma, produced in the liver with the support of vitamin K. Prothrombin is a protein soluble in the serum, and is converted to insoluble thrombin. It is built of about 600 amino acids containing four oligosaccharide chains. Glycosylation of prothrombin occurs in the liver and is determined by the participation of vitamin K. APTT is also dependent on factors involved in the thrombin formation (prothrombin, factor X and V) and the conversion of fibrinogen to fibrin. Kaolin-activated partial thromboplastin time should remain in the range of 26.0 to 36.0 seconds. During our experiment the incubation time had to be reduced up to 6 hours because the mixed blood showed hemolysis.

4. Conclusions

The best hemocompatibility express materials mimicking tissue structure and its properties. For this reason extracellular like polymer structures were prepared.

Extracellular membrane is produced by the cells, filling the space between them, which is assembling part of the tissues.

Under normal conditions, the surface of polyelectrolytes causes severe thrombosis on the surface. A similar phenomenon is observed on the surface of pure extracellular matrix, excluding the vascular endothelial cell in the blood vessels.

Introduction of silver nanoparticles into the structure of the synthetic coating in order to stabilize the polyelectrolyte film, reduced the activation processes of blood without the adverse ion release into the body.

A specific type of intercellular substance is blood plasma. In this case, considerable differences between the modified polyurethane with the polyelectrolyte comprising silver and the non-modified polyurethane were not observed. It can be considered that the polyurethane surface modification is not necessary. There is another phenomenon that must be taken into account. The polymers in tissue environment change characteristics over their physical and mechanical properties. They become stiffer what could effect on the increased risk of clotting on the surface. Synthetic materials have a fundamental drawback. In a tissue environment they are biodegradable. This is not a significant problem when the materials dissolve into simple compounds. The real problem arises when synthetic

materials decompose into the complex compounds. Then a toxic effect on surrounding tissue is observed. Therefore, first of all, the appropriate polyelectrolyte selection is necessary.
Another important point was an influence on time of biodegradation and maintain the control over the process. For this purpose, the silver nanoparticles were proposed, introduced into the porous structure of the synthetic, porous, tissue-like coating. Silver is commonly known antibacterial material. It protects perfectly against the formation of biofilm. Another factor that was investigated was an influence of nanoparticles to processes of thrombosis and the release into the blood plasma.
It has been found that nanoparticles introduced into the porous polymer structure reduces coagulation processes and their release into the plasma falls within the limits of acceptability. The overall conclusion is as follows, the silver nanoparticles may be used to control the biodegradation of extracellular matrix- mimicking coatings designed for the cardiovascular therapy.

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