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SPR Biosensor Technique Supports Development in Biomaterials Engineering

Bogdan Walkowiak et al.[*]
Department of Biophysics, Technical University of Lodz,
Poland

1. Introduction

Various biomaterials are presently employed in the production of a very wide spectrum of medical implants. The choice of biomaterial is of course determined by the medical application for which it is intended and to date no one biomaterial has been found to be fully biocompatible and biotolerant. Furthermore, it is a well known fact that quite often implants must be removed due to tissue reactions and resultant health problems (Khan et al. 2008; Schierholz& Beuth, 2001). The key role in implant tolerance depends on a very short period of time during which the biomaterial surface first comes into contact with body fluids. During this time, water molecules come into contact with the surface of the biomaterial and the results of this reaction determine the further course of events. Water molecule interaction is generally dependent on surface nanostructure and highly dependent on its energy and hydrophobicity. The next stage of interaction, which depends on the presence of water on the biomaterial surface, is the creation of a thin protein film on this surface. A hydrophilic surface will collect a large amount of hydrophilic proteins readily available in body fluids, however these proteins are weakly adsorbed and can be easily removed or replaced by other molecules. A hydrophobic surface will adsorb proteins by their hydrophobic regions often causing changes in protein structure and biological activity. The final stage, cellular attachment, adhesion and proliferation depends on the profile of the adsorbed proteins, their accessibility and a proper spatial structure which enables expression of biologically active sites. Thus, the type of protein present on a biomaterial surface seems to be crucial for biomaterial tolerance in the human body. The most common experimental models developed to characterize protein adsorption on biomaterial surfaces involve the incubation of proteins in contact with a studied surface and the estimation of adsorbed proteins by a variety of methods including electrophoretic, enzymatic or immunoenzymatic approaches together with a number of labeling techniques. The common disadvantages of these techniques is that it is not possible to observe protein adsorption as a kinetic process and protein quantification is strongly limited by the sensitivity of the methods used, which is usually limited to nanograms per square millimeter. Surface

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[*] Witold Szymanski[1], Jacek Szymanski[2], Marta Walczynska[1], Magdalena Walkowiak-Przybyło[1],
Piotr Komorowski[1], Wiesława Okrój[1], Witold Jakubowski[1] and Marta Kaminska[1]
[1]*Department of Biophysics, Technical University of Lodz,* [2]*CoreLab of Medical University of Lodz, BioTechMed*
Technology Centre Lodz, Poland

plasmon resonance (SPR) technology is a potent analytical tool for biomaterial surface study. This technology makes it possible to prepare a surface of interest (including polymers, metals, ceramics or carbon) and essentially make it the biosensor surface. Subsequently, the kinetics of molecule adsorption to the surface can be observed in real time, without the need for any labeling, together with an extremely high sensitivity of picograms per square millimeter. Moreover, this technique also allows for the identification and quantification of adsorbed molecules by use of specific antibodies. The aim of the present study was to develop conditions that enabled the measurement of plasma protein adsorption to a variety of biomaterials (including Parylene C, nanocrystalline diamond and titanium alloy) using commercially available glass plates pre-coated with gold. The preliminary results obtained regarding plasma protein adsorption were compared with blood platelets adhesion, *E. coli* and endothelial cells proliferation, as well as changes in proteome of endothelial cells grown on the surfaces of these materials.

2. SPR biosensor technique in biomaterials engineering

The SPR effect, as a convenient tool for surface investigation, was mentioned in the monograph describing usable analytical techniques for biomaterial surface study (Davies & Faulkner, 1996; Davies & Skelton, 1996). The following year a study concerning bovine serum albumin (BSA) adsorption by thiolated dextran layers present on metallic surfaces, monitored by SPR technique, was reported (Frazier et al. 1997). In subsequent years SPR sensors were used for kinetic studies of protein adsorption by polymeric surfaces (Green et al. 1997; Green et al. 1999) and degradation of polymer surface (Green et al. 2000). Papers describing SPR technique as a method of supplementing atomic force microscopy (AFM) in biomaterial studies have also been published (Vansteenkiste et al. 2000; Jung et al. 2009). Beside the most frequently studied polymeric biomaterials, SPR technique was also used to study nanocrystalline diamond surfaces and their interaction with plasma proteins (Walkowiak et al. 2002). Nevertheless, none of these reports describes the application of SPR sensors to the study of metallic biomaterials, other than substrate metals of the SPR sensor itself.

2.1 Background of SPR biosensor functioning

The first documented observation of surface plasmons was reported in 1902 (Wood, 1902). These observations concerned anomalies in the spectrum of light diffracted on a metallic diffraction grating. The first theoretical approach to these abnormalities was undertaken by Lord Rayleigh (Lord Rayleigh, 1907) and was continued by Fano (Fano, 1941), who proved that these anomalies result from excitation of electromagnetic waves on the diffraction grating. A complete explanation of this phenomenon was reported in 1968 in different studies that described excitation of surface plasmons (Otto, 1968; Kretschmann & Raether, 1968). Since that time the phenomenon of surface plasmon resonance (SPR) has found practical applications in modern optics, as a sensitive detector for monitoring molecular interactions in real time without needing to label interacting molecules. A historical overview and fundamentals of surface plasmon resonance can be found in numerous review articles and books (Tudos & Schasfoort, 2008; Kooyman, 2008; Homola, 2008). The most common geometry in which a surface plasmon can be found, is the structure of dielectric-metal interface. Analysis performed using Maxwell's equations with appropriate boundary

conditions, indicates that this structure can support only a single guided mode of electromagnetic fields i.e. a surface plasmon. Several configurations of SPR devices capable of generating and detecting SPR signals can be utilized for biosensor construction. These are: a) prism coupled total internal reflection (TIR) system, b) optical fibers, c) grating coupled systems, and d) optical wave-guide systems. Of these the most frequently used is the prism-based system, which was developed for the Kretchman configuration (Kretschmann & Raether, 1968) This refers to an arrangement where a metal layer is put directly on a top of a TIR surface (prism) enabling efficient plasmon generation. The second most commonly applied configuration utilizes core optical fibers coated with a thin metallic film. When light enters the fiber at certain discrete angles, the conditions for SPR generation and signal detection are fulfilled (Kanso et al., 2008). The last two configurations are rather less important for biosensor construction, however new systems that use these techniques have aroused great interest. In a grating coupled system light penetrates a flow channel and is angle-reflected onto diffraction grating. The effective refractive index depends on the concentration of particles within a flowing sample (Hoa, et al. 2009). An optical wave-guide system is a somewhat similar to the optical fiber based configuration, here a glass plate instead of an optical fiber is used (Suzuki et al., 2005).

Most commercially available systems are working in the Kretchman configuration. Put simply this SPR method can be described as a physical process taking place when plane-polarized light, propagated in a dielectric environment, hits a metal surface under total internal reflection (TIR) conditions. Assuming that the dielectric-metal interface consists of a transparent dielectric (glass prism) and a layer of metal of suitable thickness, we can consider an evanescent p-polarized electromagnetic field (light) penetrating the metal layer, which excite plasmon surface wave propagating within the conductor surface. For a non-magnetic metal such as gold, this surface plasmon wave is also p-polarized. Because the electric field of this wave also penetrates a short distance into the external environment, usually with a lower refractive index, the conditions for SPR are sensitive to the refractive index of the media at the gold surface. When the wavevectors for the photon and plasmon are equal in magnitude and direction, the resonance condition can be fulfilled. Thus, an increased refractive index of the medium (sample) penetrated by the plasmon increases the wavevector of the plasmon wave. Varying the angle of incidence or the wavelength of light, the wavevector of the light can be attuned to the plasmon wavevector. This enables resonant absorption of energy via the plasmon excitation (SPR) causing a characteristic drop in the reflected light intensity. For a fixed wavelength of incident p-polarized light, SPR is seen as a drop in the intensity of reflected p-polarized light at a specific angle of incidence. Biomolecular interactions occurring at the sensor surface affect the solute concentration and thus the refractive index. The SPR angle is therefore altered and the resulting angle shift is measured as a response signal. In general, different biomolecules have very similar contributions to the refractive index, thus SPR provides an extremely sensitive detector of mass change on the sensor surface. Moreover, it is very important for laboratory practice that the technique requires no labeling of the interacting molecules. A linear correlation between resonance angle shift and protein surface concentration determined via a radiometric method has been reported in the literature (Stenberg et al., 1990). The sensitivity of the mass change detection on the sensor surface depends on the instrument used, more precisely the type and resolution of the refractrometer, which can vary between 50 pg/mm^2 (Stenberg et al., 1990) and 1 pg/mm^2 (our own observations).

The geometric scheme of the measurement cell used in the BiaCore X instrument is shown in Figure 1. The prism and the glass plate of the SPR sensor are made of the same high quality glass and create one piece of a transparent dielectric. The other side of glass plate is coated with a thin gold film usually carrying a dextran matrix suitable for chemical immobilization of selected biomolecules. For our experiments we used a pure gold sensor surface instead of gold coated with dextran. The gold coated side of the sensor surface completes the flow cell of a flow channel and is a place where molecular interactions can be observed. P-polarized light comes from the monochromator and passes through the prism, the glass plate and reaches the gold film, where it excites a plasmon wave. The resonance of plasmon evanescent waves and light results in the energy deficit of the reflected light, which can be detected for specific resonance angles. Binding of flowing molecules (analyte) to the immobilized molecules (ligand) results in a shift in the reflected resonance angle.
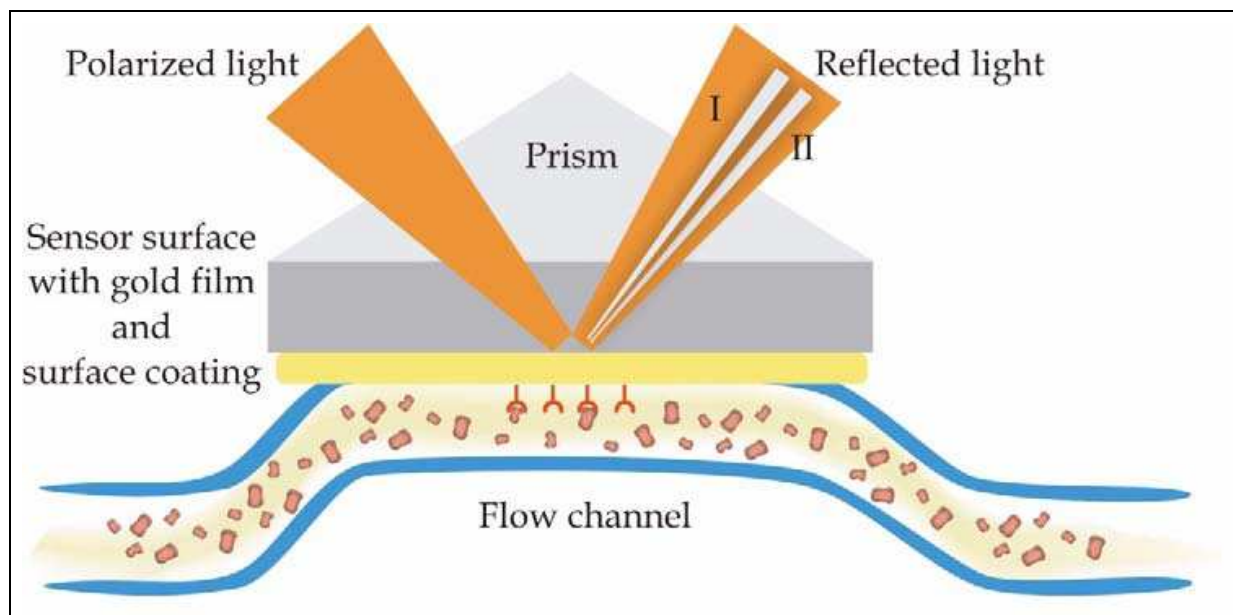

Fig. 1. The geometry scheme of the measurement cell in the BiaCore X instrument (the scheme was adopted from Surface Plasmon Resonance Technology Note 1, Biacore AB, Sweden).

A typical response produced by the SPR biosensor technique is presented in Figure 2. The response signal can be expressed as a shift in resonance angle (degree) or as a resonance unit (RU). The baseline represents the response attributed to the initial level of mass at the sensor surface. An injection of analyte over the immobilized ligand results in a two-component response. The first part, a bulk response, corresponds to the presence of a constant amount of mass flowing by the sensor surface during the injection interval. This subsequently drops to the level of the baseline when injection is finished. The second component, a binding response, corresponds to an increase in mass resulting from binding of analyte molecules, including nonspecific interactions. The response increases until binding saturation is achieved, which means an equilibrium between the number of associated and dissociated complexes is reached. This phase is considered as an association phase. When injection is stopped, the bulk response is rapidly switched off, and the dissociation phase of bound analyte is observed. The cycle can also be repeated with different analytes, for example enhancing specific antibody.
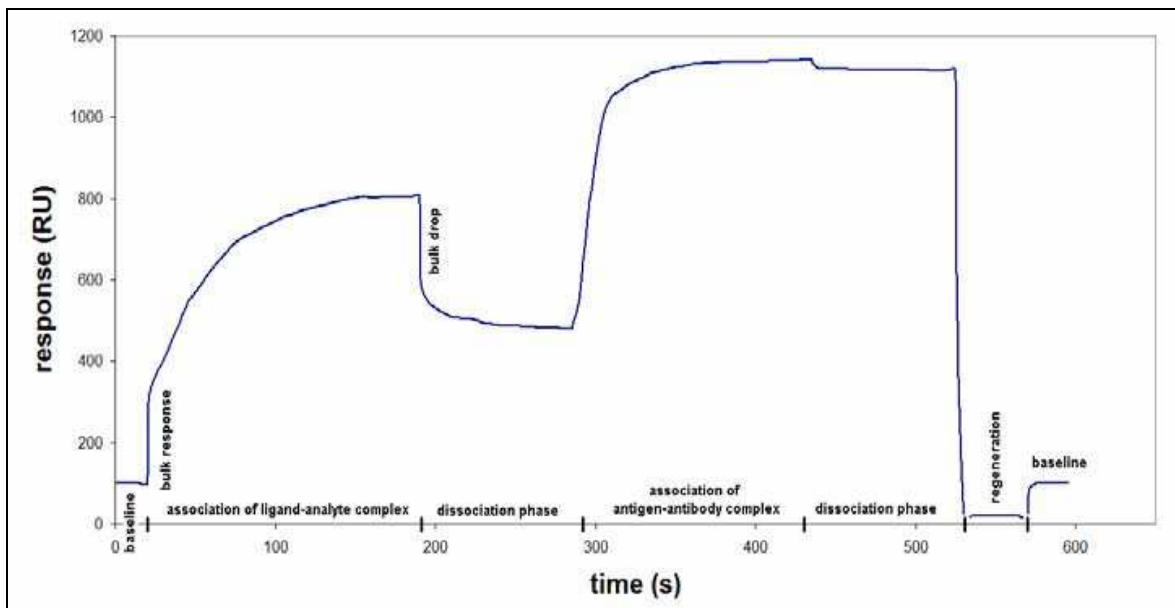
Fig. 2. Typical response produced by the SPR biosensor technique

Collected data can be used for analyte fishing and recognition, concentration estimation or kinetic analysis. Very useful surveys of literature, concerning commercially available SPR systems, containing a lot of interesting suggestions and comments, regularly updated since 1999 is accessible (Myszka, 1999; Rich & Myszka, 2010).

3. Materials and methods

Samples for the study of blood platelet adhesion, endothelial cell proliferation and bacterial biofilm formation were prepared as follows: a round bar (8 mm in diameter) of commercially available stainless steel (AISI 316 L) was cut into discs each 3 mm thick. These discs where then machined, polished and later coated with nanocrystalline diamond (NCD) or chlorinated poly(para-xylylene) (Parylene C). Titanium alloy samples were prepared as above using a Ti6Al4V round (8 mm) bar substrate. For blood plasma protein adsorption studies samples were prepared on commercially available pre-sensor glass plates precoated with gold (SIA Kit AU, BiaCore Life Sciences). A carbon layer was synthesized on the gold surface of the pre-sensor and characterized as described previously (Mitura et al. 1999; Okroj et al. 2006), with a slight modification that involved adjusting the duration of the process. The purpose of this alteration was to obtain a uniform carbon layer with a thickness of approximately 10 nm. Ten nanometer thick layer of Parylene C was deposited onto the gold surface of the pre-sensor by chemical vapour deposition (CVD) method in a manner that had been reported previously (Gazicki-Lipman 2007; Kaminska et al. 2009). Titanium alloy layer was prepared by magnetron sputtering of titanium substrate (Wendler et al. 2004) with process parameters tailored to achieve uniform and thin (10 and 20 nm) coatings. All sample surfaces were prepared at the Institute of Materials Science and Engineering, Technical University of Lodz, Poland, and were kindly provided by Prof. Stanislaw Mitura, Prof. Maciej Gazicki-Lipman and Prof. Bogdan Wendler.

Hydrophobicity of the studied surfaces was estimated by measurement of the contact angle of deionized water droplets. The values of the contact angle were determined using the commonly available software Image J.

Adsorption of blood plasma proteins on the surface of the examined samples, under flow conditions, was measured with a BIACore X system (BIACore AB, Uppsala, Sweden). The system temperature was set at 37°C. After sensor docking the system was primed with HBS-EP buffer containing 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20, pH 7.4. Before any measurements were carried out, each sensor was subjected to sensitivity assessment (Kaminska et al. 2005). For this purpose 20 µl of glucose solution in increasing concentration, up to 10%, was repeatedly injected. The procedure was performed at a flow rate of 60 µl/min. When sensor sensitivity was satisfactory, small portions (10 µl) of blood plasma, diluted in HBS-EP (1:1000), were then injected and adsorption of plasma constituents on the studied surfaces was recorded for a number of flow rates starting from 10 µl/min through 25 and 50 µl/min up to 100 µl/min. The system exhibits extremely high sensitivity in determination of mass change on the sensor surface - approximately one resonance unit (RU) corresponds to one picogram per square millimetre (1 RU ~ 1 pg/mm^2). Pure gold was used as a reference surface. Monospecific polyclonal antibodies specific for human fibrinogen were produced at the Department of Molecular and Medical Biophysics, Medical University of Lodz, Poland, according to a previously published procedure (Walkowiak et al. 1994).

SPM Veeco MultiMode V atomic force microscope (Plainview, USA), equipped with NanoScope 7.3 software, working in tapping mode with a type 15 scanning probe, was used for measurement of Parylene C coat thickness. For this purpose a piece of glass plate was partially coated with an adhesive tape and treated with the same process as used for the parylene coated sensor. Next, the adhesive tape was removed and an AFM device was used to estimate the Parylene C layer thickness.

The interaction of sample surfaces with blood platelets was studied using a standard method developed in our laboratory (Okroj et al. 2006). Blood samples used for these experiments were collected from healthy volunteers and approval for this study was obtained from the Bioethical Committee of the Medical University of Lodz (RNN/46/06/KB 21.02.2006). The donors had not been treated with any antiplatelet drugs for at least two weeks prior to the examination. The investigated surfaces were immersed in whole citrated blood at 37 °C for one hour. Blood was constantly kept in motion by gentle end-to-end mixing. Thereafter, the samples were rinsed twice in 0.1 M phosphate buffer, pH 7.4. The fixing procedure was carried out with glutaraldehyde and sample dehydration was achieved with ethanol applied in increasing concentrations. Finally, the surface was sputtered with a thin layer of gold (JEE-4X, JEOL, Tokyo, Japan). Quantitative analysis of SEM (HITACHI S – 3000N, Tokyo, Japan) images, obtained from thirty randomly selected areas, was carried out for each sample.

Endothelial immortalized cell line EA.hy 926 was used for the experiment (Jerczynska et al. 2005). Cells were cultured in tissue culture plastics (TPP, Trasadingen, Switzerland) using Dulbecso's modified Eagle's medium with high glucose concentration (4,5 g/l), containing 10% FBS supplemented with HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine) and antibiotics, at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were applied onto the examined surfaces immersed in the above mentioned culture medium and were grown for 48 hours. For the control, cells cultured in standard conditions were used. Cell proliferation and cytotoxicity were estimated with live/dead test using calcein-AM and ethidium homodimer (Molecular Probes, Eugene, USA) and GX71 fluorescence microscope (Olympus, Center Valley, USA).

For proteome analysis 2D electrophoresis technique was carried out. Harvested cells were disintegrated with a lysis buffer containing urea (7M), tiourea (2M), CHAPS (4%), IPG buffer (2%) and DTT (40 mM), and proteins were purified with a 2D-Clean-Up Kit. IEF separation (1D) was carried out with an IPGphor integrated isoelectrofocusing system using IPG strips (11 cm, pH 4-7). The second dimension was performed with a Multiphore II system using ExcelGel SDS 2-D Homogeneous 12,5%. Finally, gels were stained with silver, scanned using ImmageScanner II and analyzed with ImageMaster 2D Platinium 6.0 software. All instruments, materials and reagents used for 2D electrophoresis were sourced from GE Healthcare (Waukesha, USA).

E. coli cells (DH5α strain, 2×10^3 cells) were cultured on the surfaces of the examined samples. The culture was carried out for 24 h at 37°C in a medium containing NaCl (1%), bactopeptone (1%), yeast extract (0.5%) and pH 7.0. Next, the surfaces were extensively washed with deionized water and labeled by immersion in a fluorescent dye solution containing two dyes, bis-benzimide and propidium iodide, which made the visualization of both living and dead cells possible (Jakubowski et al. 2004).

Both F-Snedecor's test and unpaired Student's t-test or alternatively nonparametric ANOVA test with Bonferroni p-value correction were used for statistical analysis of the results. A value of $p < 0.05$ was considered as significant.

4. Results

4.1 Surface hydrophobicity

The measured contact angle for deionised water showed NCD and Ti6Al4V surfaces to be hydrophilic, whereas Parylene C surface was found to be hydrophobic. The differences were statistically significant. The results are shown in Table 1.

surface	contact angle (degree)	ANOVA test significance
NCD	66.34 ± 0.43	
Parylene C	96.42 ± 0.40	p<0.001
Ti6Al4V	76.28 ± 1.63	

Table 1. Hydrophobicity of examined surfaces expressed as the contact angle of water drop.

4.2 Adsorption of plasma proteins estimated with SPR biosensors
4.2.1 Sensor sensitivity

The sensitivity of sensors coated with thin layers of studied materials was assessed by sequential injection of glucose solution (20 µl) in increasing concentration (up to 10%). Figure 3 summarizes the crude results obtained for the reference (gold) sensor together with NCD, Ti6Al4V and Parylene C coated sensors. These results demonstrate, that with an increase in density of coating material the sensor response also increases, however sensitivity may decrease (see results for titanium alloy). It should be also noted that titanium alloy is a conducting material and can affect SPR phenomenon.

The responses normalized to the initial values and presented as a function of glucose concentration are shown in Figure 4. NCD and Parylene C coated sensors exhibited the same sensitivity as the reference sensor, however titanium alloy as more dense metallic

material caused a decrease in the response. The thinner layer lowered sensor response by 10-15 %, whereas the thicker layer of titanium alloy diminished the response by 85-90%. The sensitivity of the last sensor was too low to be included in any further investigations.
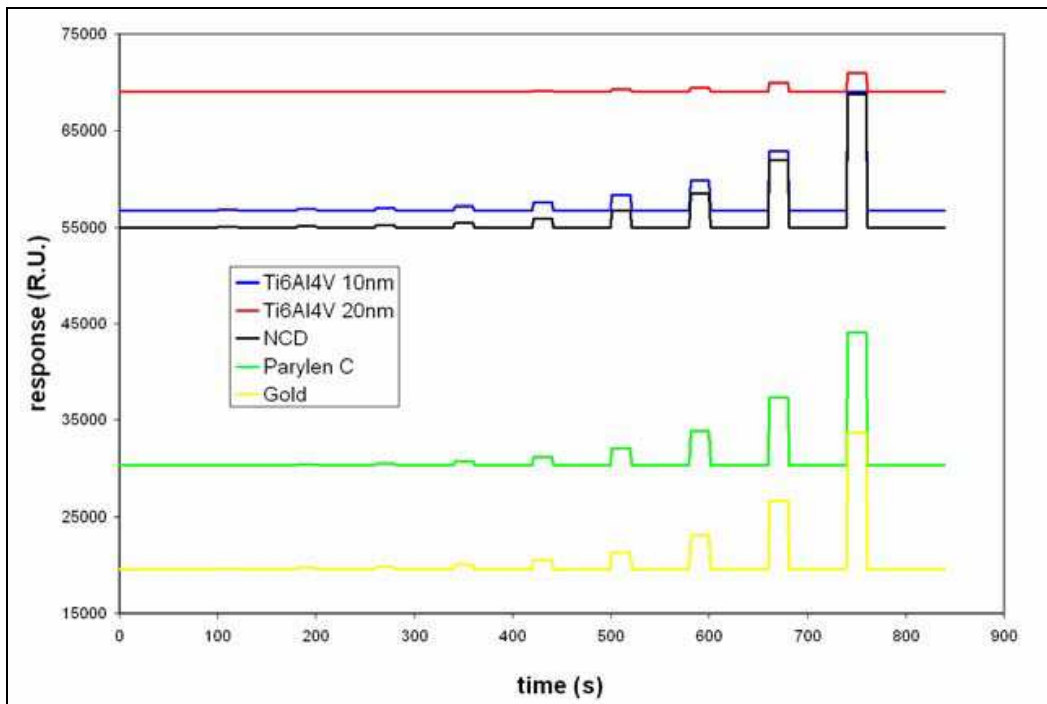

Fig. 3. Crude results of sensors response to the presence of increasing amounts of glucose. The glucose concentration varied from 0.04% up to 10%.
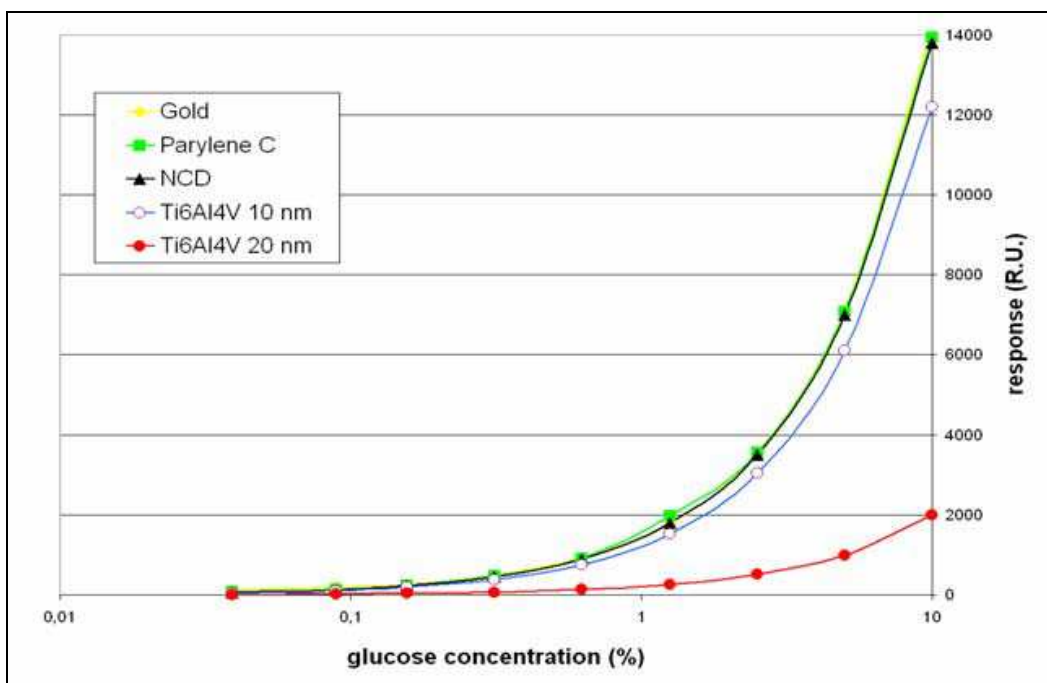

Fig. 4. Normalized to the initial values sensor responses as a function of glucose concentration.

4.2.2 Adsorption of blood plasma proteins to the sensor surface

The same volume (10 µl) of 1000 times diluted blood plasma was applied under variable flow rates starting from 10 µl/min through 25 and 50 up to 100 µl/min. It was found, that the amount of blood plasma proteins attached to the surfaces of interest strongly depends on the shear stress at the sensor surface. With higher share stress lower protein deposition was observed. En example of protein adsorption to Parylene C surface as a function of flow rate is presented in Figure 5. Figure 6 summarizes the results and shows a comparison of the amounts of adsorbed plasma proteins to different surfaces, including reference gold surface, for different levels of shear stress. It is evident, that for low shear stress, Parylene C adsorbs more proteins than other surfaces. However, with an increase in flow rate the amount of adsorbed proteins decreases and is similar to that of titanium alloy. NCD surface exhibited the highest resistance for protein adhesion for the entire range of flow rates applied.
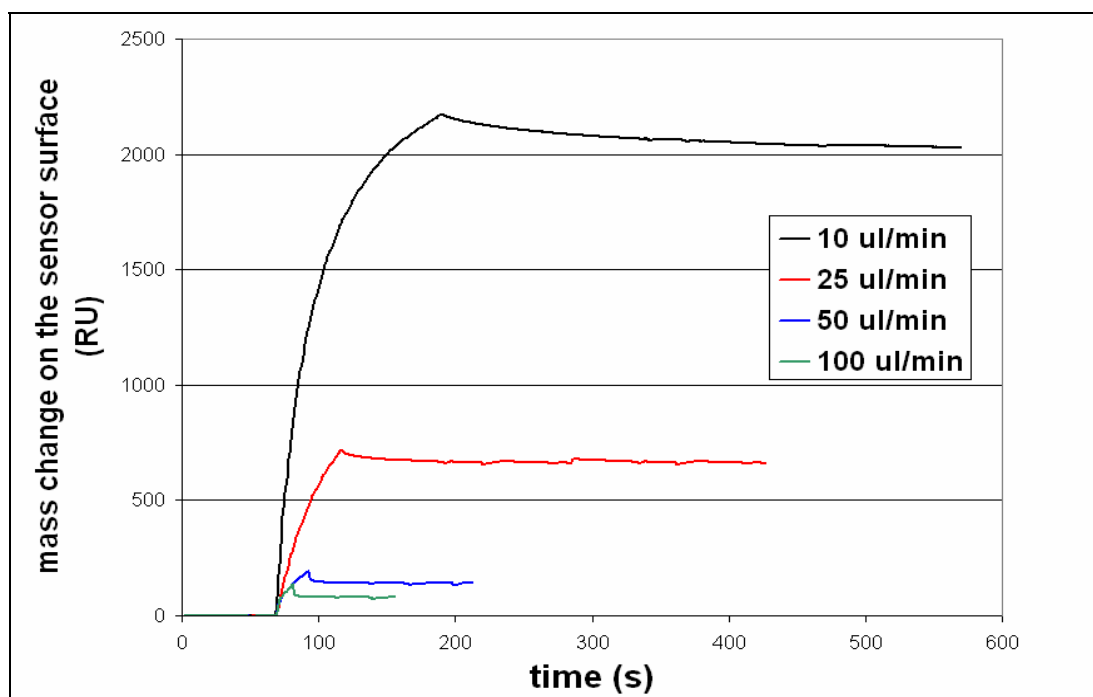

Fig. 5. Blood plasma proteins adsorption to the surface of Parylene C. Different flow rates results in different amounts of adsorbed proteins.

The following graph (Figure 7) presents example results of blood plasma protein adsorption to Parylene C and reference (gold) surfaces. In both cases curves were obtained for flow rates of 10 µl/min, and identical volumes (20 µl) of diluted plasma proteins were injected. However, the time intervals for buffer flow were twice as long for Parylene C. The arrows indicate time points of subsequent plasma protein injection. It is evident, that repeated injections initially cause an increase in the amount of adsorbed proteins, but within a short space of time the adsorption process becomes saturated. The forth injection resulted in almost no change to the mass of adsorbed proteins, moreover the desorption process was also significantly slower. The last injection, which was marked with anti-Fbg, contained rabbit anti-fibrinogen monospecific polyclonal antibodies. The observed increase in resonance signal resulted from binding of the antibodies to fibrinogen molecules present at the surface. This made it possible to quantify the amount of fibrinogen fixed to the surface. It

is worth noting, that although the response for the antibody used was different, this was to a lesser degree than the recorded responses to injected plasma proteins. This may indicate that the gold surface adsorbed relatively more fibrinogen molecules than Parylene C surface.
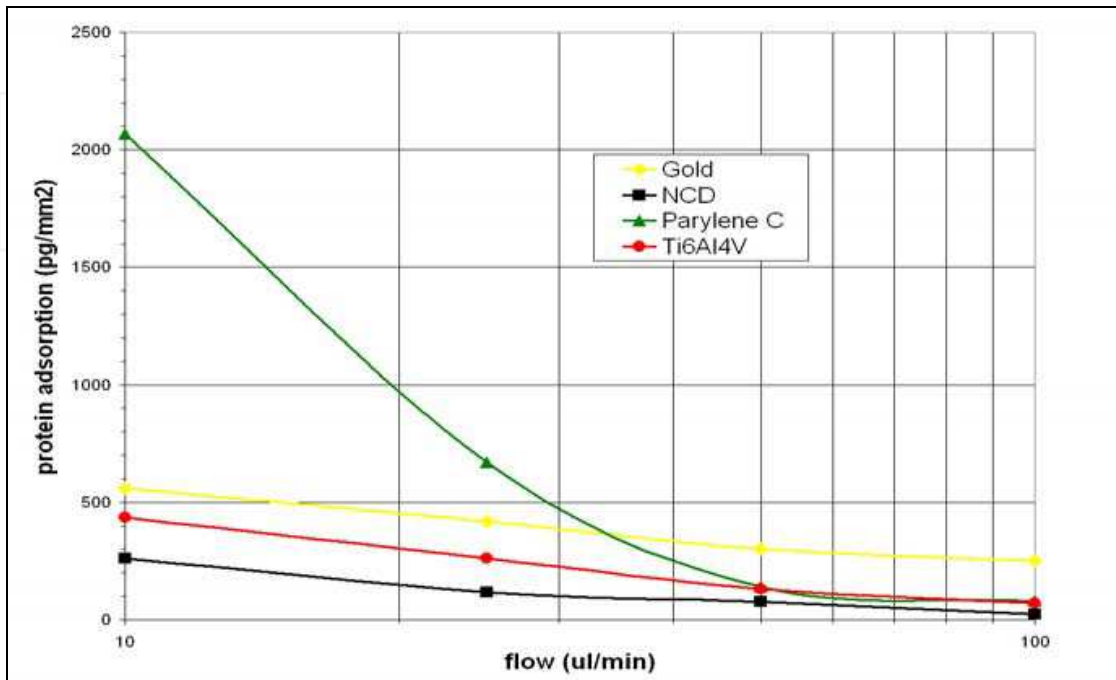

Fig. 6. Blood plasma proteins adsorption to the examined surfaces as a function of flow rate.
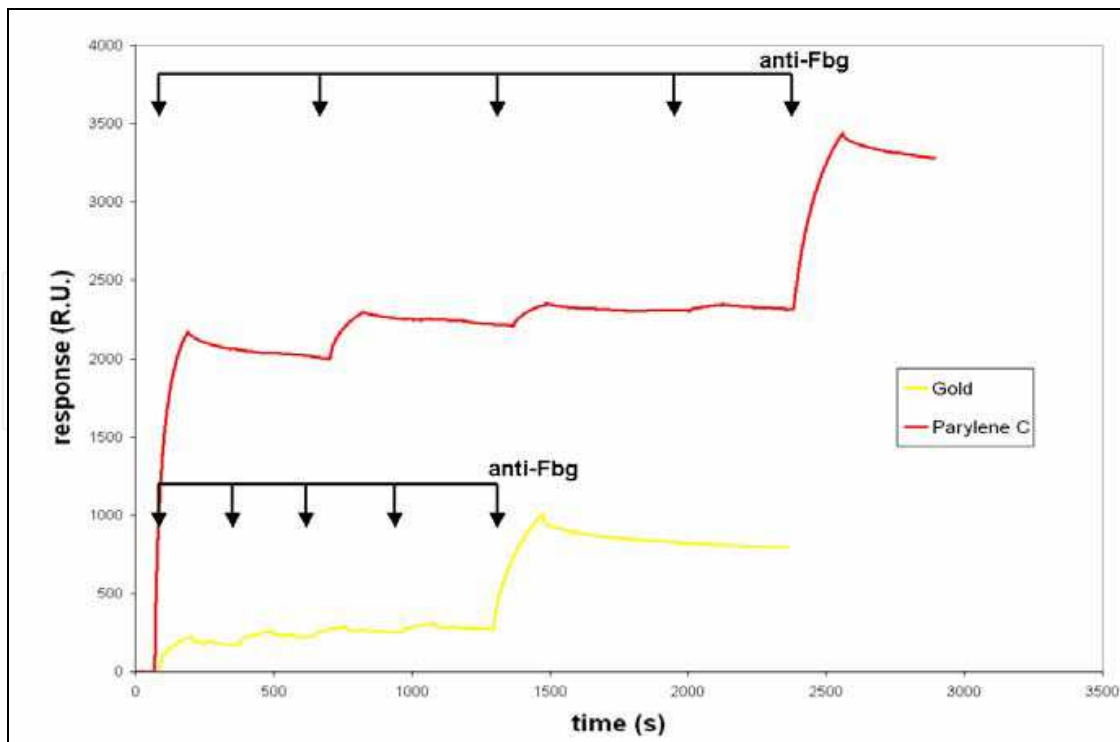

Fig. 7. An example of repetitive injection of diluted plasma proteins. The injection marked as anti-Fbg contained rabbit anti-fibrinogen monospecific polyclonal antibodies.

material	blood plasma proteins (ng/mm²)	anti-Fbg IgG (ng/mm²)	ratio IgG/plasma proteins
Parylene C	2.319	1.016	0.438
gold	0.276	0.614	2.225
ratio Parylene C/gold	8.402	1.655	

Table 2. Comparison of amounts of adsorbed proteins and IgG to the Parylene C and reference (gold) surfaces.

Table 2 summarizes the amount of plasma proteins attached to the surfaces and the amount of specific IgG molecules enhancing the signal. The ratio of IgG to plasma proteins is about 5 times higher for smooth, nonporous gold surface than for porous Parylene C surface. It also means that sticky, adhesive, large fibrillar molecules such as fibrinogen, adhere more easily to the gold surface than to the Parylene C. However, other smaller proteins must be trapped by porous Parylene C in very large amounts.

It is also important for future studies, using SPR biosensors, to know how to control the process of biosensor surface synthesis with regards to biomaterial film thickness. It must be known whether the thickness of any films of used materials correspond to the parameters we have assumed. Table 3 summarizes data of the initial responses recorded for each sensor used. If the specific density of the materials and their specific response are known, i.e. after subtracting the signal from the reference gold film signal, and assuming that 1 RU corresponds to about 1 pg/mm², it is possible to calculate the thickness of the films. For NCD we managed to achieve a layer that was exactly 10 nm thick. On the other hand, for Parylene C the estimated thickness was about 8,44 nm instead of the 10 nm that was assumed to present. For titanium alloy, where we assumed the layer thickness to be 10 nm, it was approximately 8.3 nm. Furthermore for the 20 nm layer it was only 11.2 nm. It is possible that for such a weak sensitivity, as was exhibited by the thicker titanium alloy, the recorded signal does not accurately represent the amount of titanium alloy on the sensor surface.

material	density (g/cm³)	response (RU)	specific response (RU)	segment mass (ng/mm²)	thickness (nm)
NCD	3.52	55 000	35 500	35.5	10.09
Parylene C	1.28	30 300	10 800	10.8	8.44
Ti6Al4V 10nm	4.42	56 000	36 500	36.5	8.26
Ti6Al4V 20nm	4.42	69 000	49 500	49.5	11.20
gold		19 500			

Table 3. Data used for estimation of thickness of the films of biomaterials. The specific response was calculated as the difference between the initial response recorded for the material of interest and the corresponding response for reference (gold) surface. The segment mass corresponds to the mass of the film falling on the flow cell surface.

Of course, it was necessary to confirm the above results with different method. For this purpose the AFM instrument was used. Figure 8 presents the surface of Parylene C film deposited on gold surface in the same process used to prepare the SPR biosensor. Prior to initiating this process, a small area of the surface was covered with adhesive tape. After the process was complete, the tape was carefully removed and AFM inspection was carried out. The resulting thickness of the Parylene C film was about 8 nm, which corresponded well with the 8,44 nm estimated from the SPR reading.
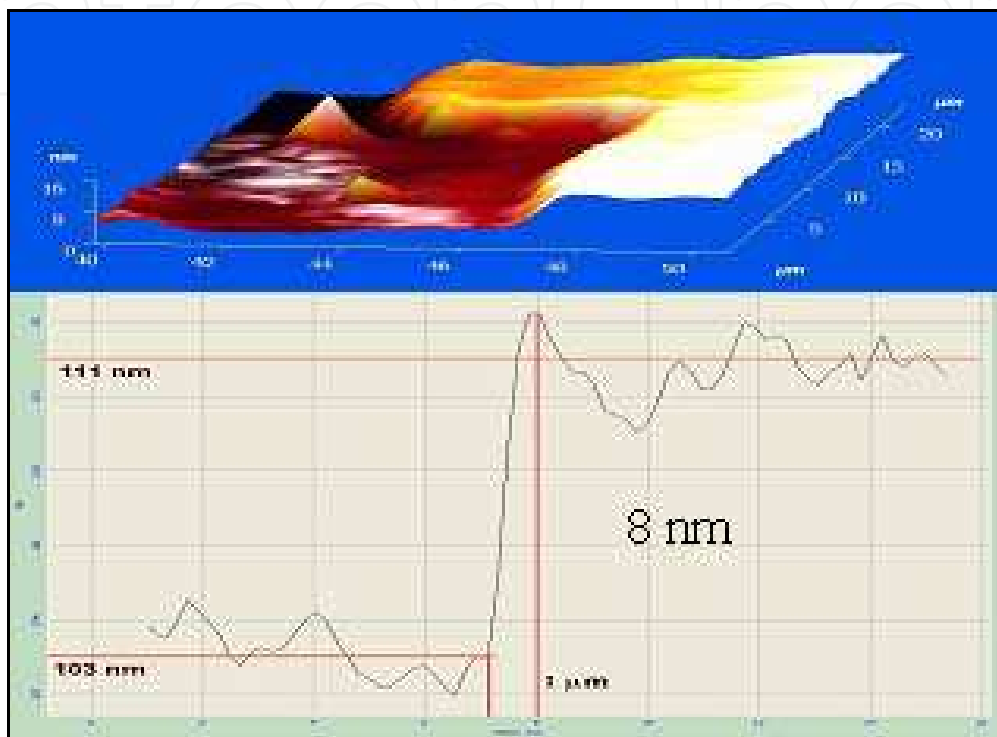

Fig. 8. Measurement of thicknes of the Parylene C film deposited on SPR sensor surface with use of AFM instrument.

4.3 Blood platelets adhesion

Blood platelet adhesion to the surface of any biomaterial strongly depends on the presence and exposure of adhesive proteins such as collagen, fibrinogen, fibronectin and others. Since plasma proteins are adsorbed by the examined surfaces, it was assumed that blood platelets would adhere to them. Figure 9 illustrates example photos of selected biomaterial surface fragments. The panels on the left side (lower magnification) were adequate for quantitative analysis, whereas the panels on the right were used to analyse the degree of activation of the adhered blood platelets. The lowest number of adhered platelets was found on the surface of NCD, a greater amount of platelets adhered to surface of titanium alloy, however the highest thrombogenic properties were exhibited by the Parylene C surface (Table 4). These differences were statistically very significant. Most platelets found on Ti6Al4V and Parylene C surfaces were in a similar dendritic-like form, but some of the platelets attached to Parylene C were also in spread form indicating a higher degree of activation. Platelets adhering to NCD were mainly in spherical form with short dendrites. This form is usually attributed to an initial level of platelet activation.
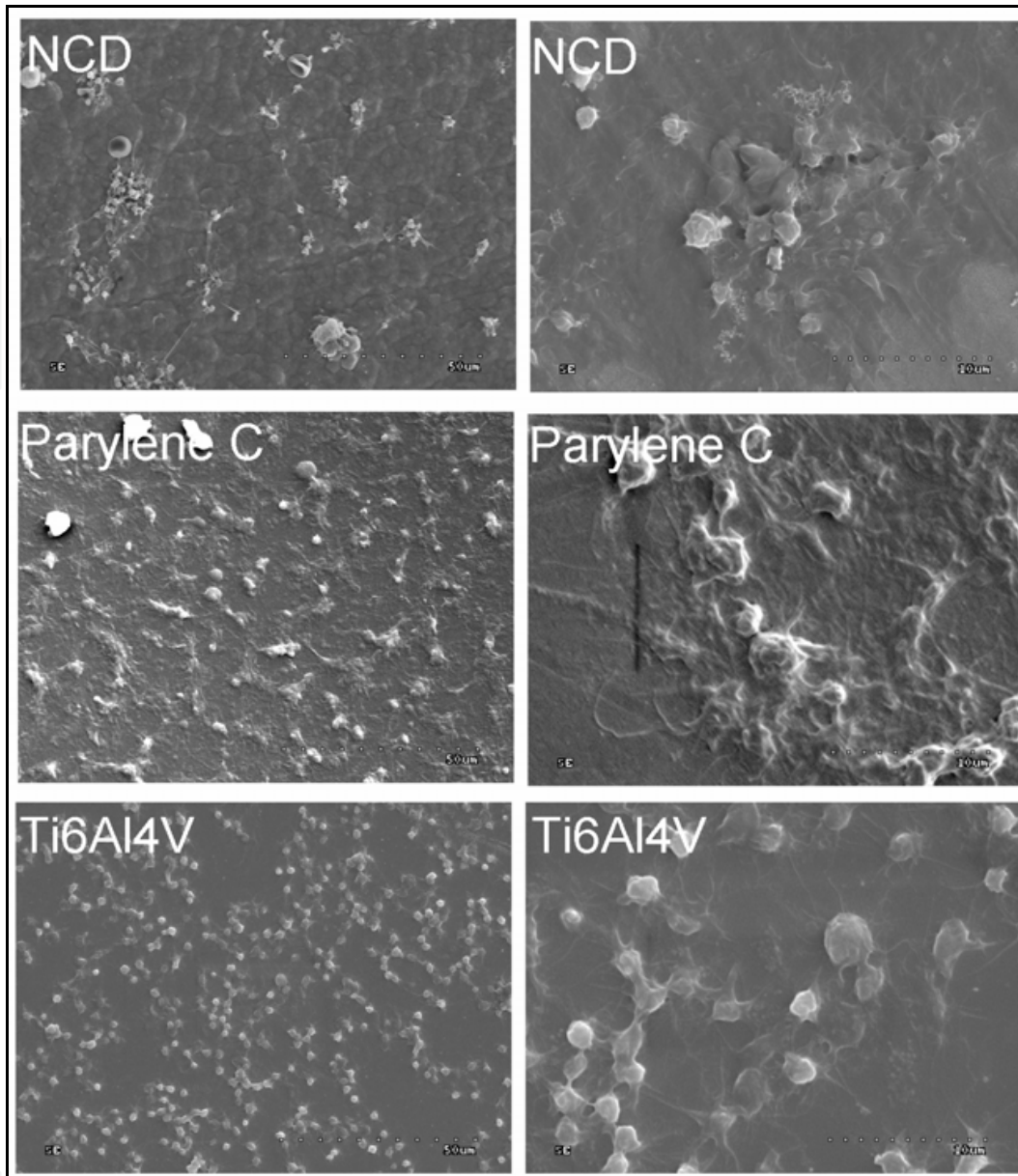
Fig. 9. Blood platelet adhesion to NCD, Parylene C and Ti6Al4V surfaces observed with SEM. Bars for left and right segments are 50 μm and 10 μm, respectively.

material	number of adhered platelets per 100 μm²	ANOVA test (significance)
NCD	0.9 ± 0.3	
Parylene C	3.8 ± 0.2	$p<0.0001$
Ti6Al4V	1.7 ± 0.3	

Table 4. Number of blood platelets adhering to the surfaces of examined materials. Surfaces exhibited statistically relevant differences in susceptibility to blood platelet adhesion. The data were collected from at least 10 separate readings. Significance for material pairs was as follows: NCD vs. Parylene C $p<0.001$, NCD vs. Ti6Al4V $p<0.001$, Ti6Al4V vs. Parylene C $p<0.001$

4.4 Endothelial cells proliferation and proteome analysis

A thin layer of endothelial cells line the interior surface of blood vessels, forming an interface between circulating blood and the rest of the vessel wall. Endothelial cells line the entire circulatory system from the heart to the smallest capillary vessels. Thus, any implant introduced into the human body, even for a short period of time, must come in contact with endothelial cells and the cell response to this material can be crucial to the final outcome of surgery. On the other hand, cells contact and interact with the implant surface via a thin protein film created from components of body fluids immediately after implantation. In our model immortalized endothelial cell line EA.hy 926 was used to observe cell proliferation and viability on the surfaces of NCD, Parylene C and Ti6Al4V.

material	living cells (% of control)	ANOVA test (significance)	dead cells (% of living cells)
NCD	158 ± 43		2
Parylene C	7 ± 3	p < 0.0001	59
Ti6Al4V	145 ± 9		2

Table 5. Proliferation and mortality of EA.hy 926 cells cultured for 48 hours on the surfaces of examined materials. Data were collected from at least 10 separate readings. Significance of differences between material pairs was as follows: NCD vs. Parylene C p<0.001, NCD vs. Ti6Al4V ns, Ti6Al4V vs. Parylene C p<0.001.
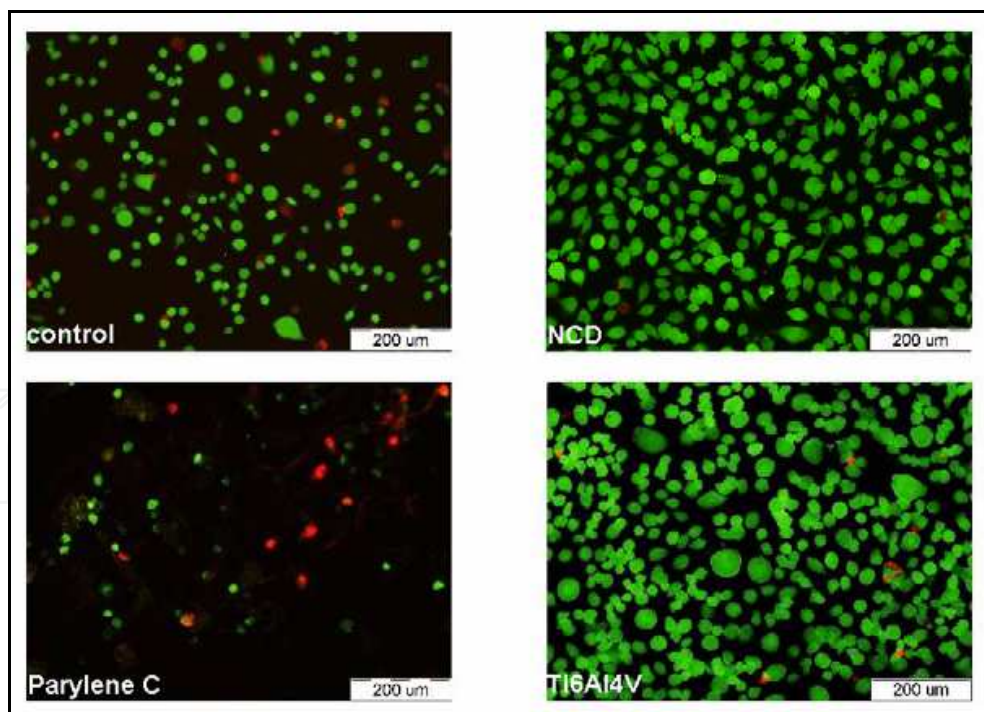

Fig. 10. EA.hy 926 cells grown (48 h) on surface of NCD, Parylene C and Ti6Al4V.

A control experiment was performed with cells grown in standard cell culture conditions described above in materials and methods. Living cells were stained green and dead cells red with calcein-AM and ethidium homodimer, respectively. It was obvious that both NCD

and Ti6Al4V surfaces caused a significant increase in EA.hy 926 cell proliferation, together with a rather negligible, comparable to the control, number of dead cells (approx. 2%). In contrast to this, the Parylene C surface exhibited cytotoxic effect towards EA.hy 926 cells (about 60% dead cells).
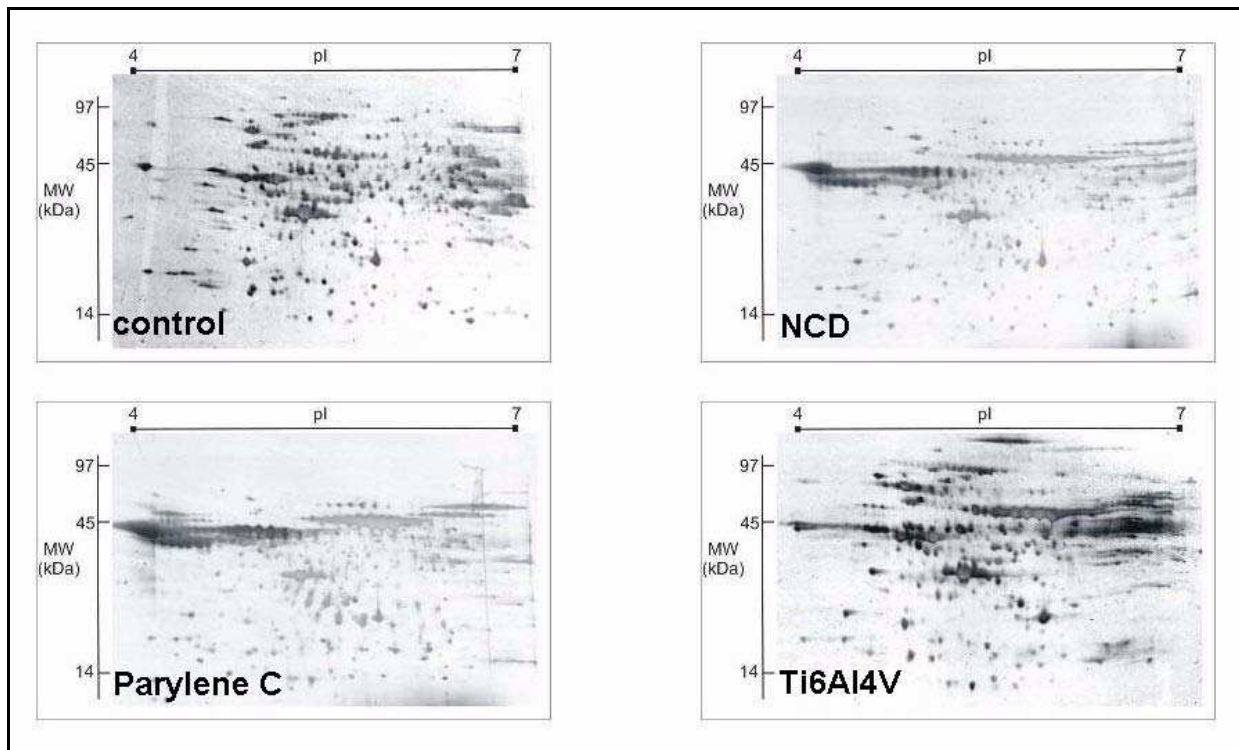

Fig. 11. Proteome analysis of EA.hy 926 cells with 2D electrophoresis.

material	detected spots (number)	matched spots (%)	up regulated matched spots (%)	down regulated matched spots (%)
control	326	100	0	
NCD	261	55.9	6.1	22.5
Parylene C	323	49.0	10.1	25.2
Ti6Al4V	256	63.2	4.9	3.8

Table 6. Quantitative analysis of EA.hy 926 cell proteome. Matched spots represent corresponding peptides present in both control and examined gels. Expression ratios higher than 1.5 or lower than 0.5, were taken as cut-off values for up or down regulated matched spots, respectively.

All the proteins extracted from the cultured cells (proteome) were separated using 2D electrophoresis (Figure 11). When compared to the control culture, the proteome pattern obtained for cells grown on all the studied materials differs significantly throughout the whole range of pI and MW. The results of simple visual inspection (Figure 11) and quantitative analysis (Table 6) indicate that Ti6Al4V titanium alloy causes the smallest changes in protein expression of endothelial cells. Although these cells expressed the lowest number of spots (peptides) in comparison with the control, the number of matched spots was the highest (63.2%). Furthermore, among the matched spots only 4.9% were up

regulated and 3.8% down regulated. The proteome patterns of cells cultured on both NCD and Parylene C surfaces were similar and both materials produced lower levels of matched spots (55.9% and 49%, respectively). These materials also had the highest degree of up and down regulated matched spots (Table 6).

4.5 Biofilm of *E. coli* cells formation

Implant infection caused by opportunistic microorganisms is a frequent and difficult to cure complication after surgical implantation. Materials with a high susceptibility to microbial colonization and biofilm formation should not be used in any surgical procedures or for manufacturing implants. Otherwise, the surface of these materials will serve as an uncontrolled source of opportunistic infections that is impossible to eradicate. Thus, estimating the resistivity of biomaterials to bacterial colonization is one of the most important tasks during the process biocompatibility evaluation. As for any other cells, bacterial interaction with a material is mediated by proteins present at its surface. As shown in Figure 12 and Table 7, the NCD surface is almost entirely free from *E. coli* cells, whilst Ti6Al4V is less resistive to microbial colonization. On the other hand, Parylene C is rather susceptible to colonization, although the percent of dead cells on its surface is the highest among the studied materials.

material	number of living cells (FOV)	ANOVA test (significance)	dead cells (%of living cells)
NCD	3 ± 1		1
Parylene C	112 ± 30	p < 0.0001	9
Ti6Al4V	31 ± 5		1

Table 7. The number of living and dead *E. coli* cells found in the field of vision (FOV). The data were collected from at least 10 separate readings. The significance of difference between pairs of materials are as follows: NCD vs. Parylene C p<0.001, NCD vs. Ti6Al4V p<0.01, Ti6Al4V vs. Parylene C p<0.001.
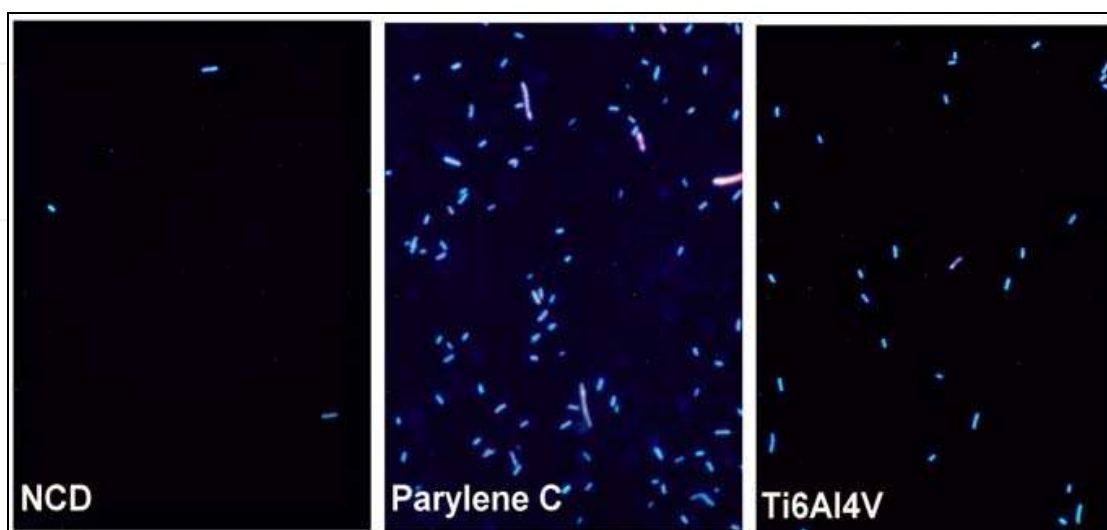

Fig. 12. *E. coli* cells detected on the surface of studied materials. The living cells (blue) were stained with bis-benzimide and the dead cells (red) were visualized with propidium iodide.

5. Conclusions

The SPR biosensor seems to be a very sensitive tool, that allows for remarkably sophisticated examination of biomolecule interactions with artificial surfaces of biomaterials. As was shown above, SPR biosensors can be used for a wide spectrum of materials, including dielectric carbon (NCD), polymers (Parylene C), and conductors (Ti6Al4V), although for the last class of materials we must be aware that the SPR effect could be disturbed by the presence of an additional layer of metallic material. Thus, a calibration procedure must be obligatory for any newly prepared biosensor surface. This strategy makes ensures that the biosensor is working properly in our experimental system. Kinetic study of biomolecule adsorption and desorption as a function of shear stress, real time observation of complex formation together with biomolecule identification without the need for any labelling, and easy experimental procedures make the SPR biosensor technique very useful and productive in numerous areas of research including biomaterials engineering. Our attempt to measure thickness of biomaterial layer indicates a possible application for assessing material aging or degradation manifested by a change of mass with time. In fact, the use of SPR phenomenon to monitor thin film deposition or removal (Woollam, 2007) and degradation of thin polymer film (Chen, et al. 1995; Green et al. 2000) has been previously reported. It is also worth noting the easy and specific recognition as well as quantitation of biomolecules adsorbed to the sensor surface. This makes it possible to monitor dynamic changes in the protein biofilm composition, which exists on the surface in contact with flowing fluid. And finally, the results obtained from SPR biosensor study correspond well with other observations. As mentioned above, cell interaction with any artificial surface is mediated by proteins attached to this surface. Our experiments showed a high amount of proteins adsorbed by Parylene C surface, and this correlates well with the elevated number of adhered blood platelets and attached *E. coli* cells to this surface. On the other hand, NCD surface gathers the lowest amount of proteins and this surface is resistant to platelet adhesion and microbial colonization. Unfortunately, this well organized arrangement is seriously disrupted by experiments with EA.hy 926 cells. Parylene C, despite very effectively adsorbing proteins, exhibits cytotoxic effects to endothelial cells. Another deviation can be seen also for NCD and Ti6Al4V alloys. Despite the limited adhesion of blood platelets and *E. coli* cells to NCD surface, which corresponds well to a low level of adsorbed proteins, endothelial cell proliferation is significantly elevated by this material. This may mean, that long term cell culture can adopt the surface to the cell needs, if this surface is not toxic to this cell. Further surprising results were obtained with 2D electrophoretic separation of EA.hy 926 cells proteome. Similarities were found between the patterns of 2D pictures obtained for NCD and Parylene C, despite the fact that NCD was able to stimulate cell proliferation and Parylene C was cytotoxic. The smallest difference was found between control cells and cells cultured on the surface of Ti6Al4V alloy. The initial impression resulting from the simple visual analysis of the gels is well supported by quantitative data from spot analysis and matching.

Summing up the above, SPR biosensor technique can significantly improve sensitivity and selectivity of tests applicable to material evaluation for biomedical use.

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New Perspectives in Biosensors Technology and Applications

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