CYCLOPROPYLAMINE PLASMA POLYMER SURFACES IN QUARTZ CRYSTAL MICROBALANCE AND SURFACE PLASMON RESONANCE IMMUNOSENSING

MAKHNEVA Ekaterina[1], FARKA Zdeněk[2], SKLÁDAL Petr[2], ZAJÍČKOVÁ Lenka[1]

[1] RG Plasma Technologies, CEITEC - Central European Institute of Technology, Masaryk University, Brno, Czech Republic, EU
[2] RG Nanobiotechnology, CEITEC - Central European Institute of Technology, Masaryk University, Brno, Czech Republic, EU

Abstract

Immunosensors with highly sensitive and rapid detection capabilities of various biomolecules are of a great demand in the field of biomedicine and environmental control. The two mostly employed transducer principles are either optical surface plasmon resonance (SPR) and mass sensitive quartz crystal microbalance (QCM). The first task encountered in the immunosensor development is the preparation of a coating matrix suitable for the immobilization of antibody on the golden layer of SPR or QCM. The most popular approaches are based on wet chemical treatments such as self-assembled monolayers (SAMs) of alkanethiols, disulfides, or polyethylenimine (PEI). However, these approaches suffer from several drawbacks such as a poor stability, long time preparation, unstable baseline or a high level of noise. As an alternative to SAMs, the deposition of thin functional coatings by plasma polymerization can be employed. The plasma polymerization has already been successfully applied to the deposition of thin films containing carboxyl, amine, anhydride groups. In this work, pulsed plasma polymerization of cyclopropylamine is employed to deposit stable amine-rich thin films on the surfaces of QCM and SPR chips. The AL-01 antibody, specific to human serum albumin (HSA), was attached to the QCM and SPR chips surfaces via glutaraldehyde activation. For the developed immunosensors the stable baseline was recorded. Selective and high response was achieved during the reaction with the solution of antigen. The results confirmed that the introduced methodology have a great potential for biosensing applications.

Keywords: Plasma polymerization, cyclopropylamine, stable amine-rich films, quartz crystal microbalance, surface plasmon resonance, immunosensor, label-free detection

1. INTRODUCTION

Immunosensors with highly sensitive and rapid detection capabilities of various biomolecules are of a great demand in the field of biomedicine and environmental control [1],[2]. The two mostly employed transducer principles in label-free immunosensors are the mass sensitive quartz crystal microbalance (QCM) and the optical surface plasmon resonance (SPR). The most popular approaches for the preparation of a coating matrix onto the gold surfaces of QCM crystals and SPR chips are based on wet chemical treatments such as formation of polyethylenimine (PEI) or the self-assembled monolayers (SAMs) of alkanethiols and disulfides [3],[4]. However, these approaches suffer from several drawbacks such as a poor stability, long time preparation, unstable baseline or a high level of noise [5].

As an alternative to the above mentioned chemical methods, the plasma enhanced chemical vapor deposition (PECVD) of thin functional coatings can be employed. It has already been successfully applied to the preparation of plasma polymers (PPs) containing carboxyl [6], amine [7] or anhydride groups [8]. The essential chemical and thickness stability of the plasma layers can be achieved by tuning the plasma parameters [9].

Amine PPs are well known for the successful bioapplications [10]. The most studied PP processes used allylamine monomer. The PPs from allylamine exhibit high level of cell adhesion and proliferation [11], high

hemocompatibility [12]. In our work we use the cyclopropylamine (CPA) as a monomer, which is the non-toxic isomer of allylamine and performs slightly higher retention of the functional groups in the obtained PPs [13].

Previously, the optimum conditions for the preparation of stable CPA PPs were found [14]. In our recent studies, the two optimized films with a similar chemical composition deposited in two reactors with different geometrical parameters were studied and compared [15]. QCM-based immunosensors were developed using CPA PPs as matrix layers for immobilization of biomolecules via glutaraldehyde (GA) approach. The CPA PP prepared in tubular reactor performed high level of activity and three different immobilization approaches were tested. Stable baseline, selective and high response were recorded for the GA and sulfo-SMCC methods. With the aim to prove an efficiency of the developed sensors, the comparison between CPA PPs and cysteamine SAMs for QCM immunosensing was carried out [16]. It was shown that the CPA PPs are more stable in PBS than cysteamine SAMs and provided high level of response during measurements [17].

In this study the AL-01 antibody against human serum albumin (HSA) was attached to the gold surfaces of QCM crystals and SPR chips through GA activation. In case of QCM immunosensing the regeneration using 0.1 M solution of HCl was successfully tested and allowed 3 measurements with a single crystal (5, 10 and 20 µg/mL solutions of HSA antigen). In case of SPR chips two different AL-01 antibody immobilization procedures, online and offline, were utilized. The obtained sensograms during immunotests with 10 µg/mL solution of HSA were studied and compared.

2. EXPERIMENTAL

2.1. Chemicals and Materials

Cyclopropylamine (CPA) (98% purity, used without any further purification), glutaraldehyde (GA) (25% aqueous solution), human serum albumin (HSA) were purchased from Sigma Aldrich (USA). Sodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride for the preparation of phosphate buffered saline (PBS) with pH 7.4, were supplied from Penta (Czech Republic). Anti-HSA monoclonal antibody (clone AL-01) was obtained from Exbio (Czech Republic). Argon with purity of 99.998 % was supplied by Messer. Double-side polished single crystal silicon (c-Si) wafers from ON-SEMI (Czech Republic) were cut into 10×15 mm substrates. Round shape QCMs (AT-cut, resonant frequency of 10 MHz) coated by Au were purchased from Krystaly Hradec Králové (Czech Republic). MP-SPR (Multi-Parametric Surface Plasmon Resonance) Navi gold sensors, **SPR102 Au were purchased from** BioNavis (Finland). All the substrates were cleaned by sonication in isopropanol (Penta, 99.8%) for 10 min.

2.2. Plasma polymerization in tubular glass reactor

The plasma polymerization from CPA/Ar gas mixture was performed in capacitively coupled RF discharge (13.56 MHz). The discharge was ignited in a tubular glass reactor enclosed by aluminium flanges serving as the grounded and RF electrodes. The diameter of the electrodes and their distance were 80 and 185 mm, respectively. The substrates (c-Si, QCM crystals and SPR chips) were placed on the glass holder positioned in the middle of the tube at the floating potential. The substrate surfaces were cleaned in the Ar discharge for 10 min prior to the deposition. Both the Ar plasma cleaning and the polymerization, were carried out with the on-time power of 20 W and at pressure of 120 Pa. The flow rate of Ar was set to 28 sccm whereas the flow rate of CPA vapors was 0.3 sccm. A detailed study of the relation between plasma conditions and film properties in this polymerization set-up was reported previously [[14]]. All plasma processes, the surface cleaning and CPA/Ar plasma polymerization, were carried out in square-pulsed mode with the pulse repetition frequency of 500 Hz and 33 % duty cycle. It corresponded to 660 µs plasma on-time and 1340 µs off-time. The deposition time was tuned to achieve the film thicknesses of 40 nm - SPR, QCM substrates and 120 nm on Si wafers.

2.3. QCM Immunosensing

The surface of QCM electrodes with the deposited 40 nm thick CPA PP was activated by the reaction with GA (droplet of 3 % solution in PBS, 1 h, r.t.). Then the AL-01 antibody was covalently bonded (droplet of 100 µg/mL solution in PBS, 18 h, 4 °C). Free reactive groups were deactivated using bovine serum albumin (BSA) (droplet of 200 µg/mL solution in PBS, 20 min, r.t.). After the immobilization procedure, the QCM crystals were placed in a flow-through cell. The measurements were performed using the QCM analyzer (KEVA, Czech Republic) that served as both, the oscillator and the frequency counter. The flow of solutions was driven by the milliGAT pump (Global FIA, USA) and a selection valve (Valco Instruments, USA). PBS was used as a running buffer with a flow rate of 40 µg/mL. After baseline stabilization the samples of HSA solutions (5, 10, 20 µg/mL) were injected for 10 min. Regeneration was done by the 0.1 M solution of HCl, injected for 2 min.

2.4. SPR Immunosensing

SPR studies were carried out using the MP-SPR Navi gold sensors **SPR102 Au** and SPR Navi 210A system from BioNavis (Finland). PBS was used as a running buffer with the flow rate of 20 µL/min. The injections of different solutions were programmed using SPR-Navi software. The injection volume was 200 µL. The flow cell consists of two channels: binding and reference. The changes of resonance angle due to the interactions on the sensor surface were measured at two wavelengths: 670 and 785 nm. For the data evaluation the 670 nm wavelength results were used due to the higher sensitivity. The position (angle) of surface plasmon resonance peak was determined using build-in centroid fitting function.

The surface of SPR slides with deposited 40 nm thick CPA PP was activated by the reaction with GA (droplet of 3% solution in PBS, 1 h at room temperature) forming aldehyde groups. The activation was carried out offline to prevent binding of GA to the tubing of SPR system. Then, the chip was washed in PBS, sterile water, dried and inserted into BioNavis.

Two procedures were used for the antibody (Ab) immobilization. In the first approach the Ab immobilization was performed online. At first, every chip was exposed to the PBS flow (flow rate 20 µL/min) for approximately 40 min to reach stable baseline. Afterwards, the solution of antibody (anti-HSA, AL-01) in concentration of 100 µg/ml was injected to the binding channel at the flow rate of 4 µL/min during 54 min. In the reference channel only PBS was flowing at the same flow rate. Free reactive groups were deactivated applying 2 mg/ml solution of BSA at the flow rate of 10 µL/min for 20 min to both channels.

In the second procedure, the overnight offline Ab immobilization and BSA blocking was performed as follows. The surface of the SPR chip activated by the reaction with GA was washed and the Ab solution was applied overnight (droplet of 100 µg/mL solution in PBS, 18 h, 4 °C). After this step the SPR chip was again washed in PBS and sterile water and then the BSA solution was applied (droplet of 2 mg/mL solution in PBS, 20 min, r.t.). The chip was then washed in PBS, sterile water, dried and inserted into BioNavis.

After the immobilization of Ab and blocking non-specific sites with BSA the immunosensing was carried out using 10 µg/mL solution of HSA at the flow rate of 20 µL/min for 10 min.

2.5. FT-IR analysis

Fourier transformed infrared (FT-IR) spectra were obtained on the films deposited on the IR transparent c-Si substrates in the transmission mode using the Bruker Vertex 80v spectrophotometer. The measurements were performed with a parallel beam transmittance accessory in the spectral range from 370 to 7500 cm^{-1}. The data were collected at a pressure of 2.5 mbar with the resolution of 4 cm^{-1} and 500 scans. The transmittance of the films on Si substrate was divided by the transmittance of bare Si substrate for the thin film analysis. The FT-IR spectra are shown only in the range from 1400 to 3700 cm^{-1} because no significant absorption peaks belonging to the films were identified outside this range and the identification of weak absorption peaks below 1400 cm^{-1} was difficult due to the strong absorption peaks in c-Si that could not be reliably subtracted.

3. RESULTS AND DISCUSSION

3.1. Chemical composition and properties of CPA PPs.

In our recent studies it was shown that pulsed plasma polymerization of CPA, deposited at the floating potential, is an effective method for the preparation of amine-containing films that are proposed as a replacement of SAMs on the gold electrode of QCM sensors. The atomic composition of the 40 nm thick films was determined previously by XPS, the C:N:O ratios was 77:20:3 [15]. The amount of primary amine groups in the CPA PP was 1,3 %, which was determined by chemical derivatization with TFBA [18].

The structural fragments of the film were analyzed from the FTIR spectra of the as-deposited CPA PP film (**Figure 1**). Signal at 3350 cm^{-1} corresponds to the N-H stretching, which can be equally referred to the primary and secondary amines, imines or even amides. In the 2970 - 2870 cm^{-1} region the C-H stretching signals are observed, which correspond to methyl, methylene and methine groups presented in the polymer chain. The triple bonds signals are observed at around 2200 cm^{-1}, which were assigned to the nitriles and isonitriles, but these signals could correspond to other highly unsaturated structures, such as

-N=C=N-, -C=C=N- or -C≡C-. At around 1680 cm^{-1} there is the C=O stretching signal, but due to the low concentration of O in the film, this signal is hidden in the relatively high signal of C=N and C=C. C=O starts to be recognizable only after long times of immersion in water or buffer [14].

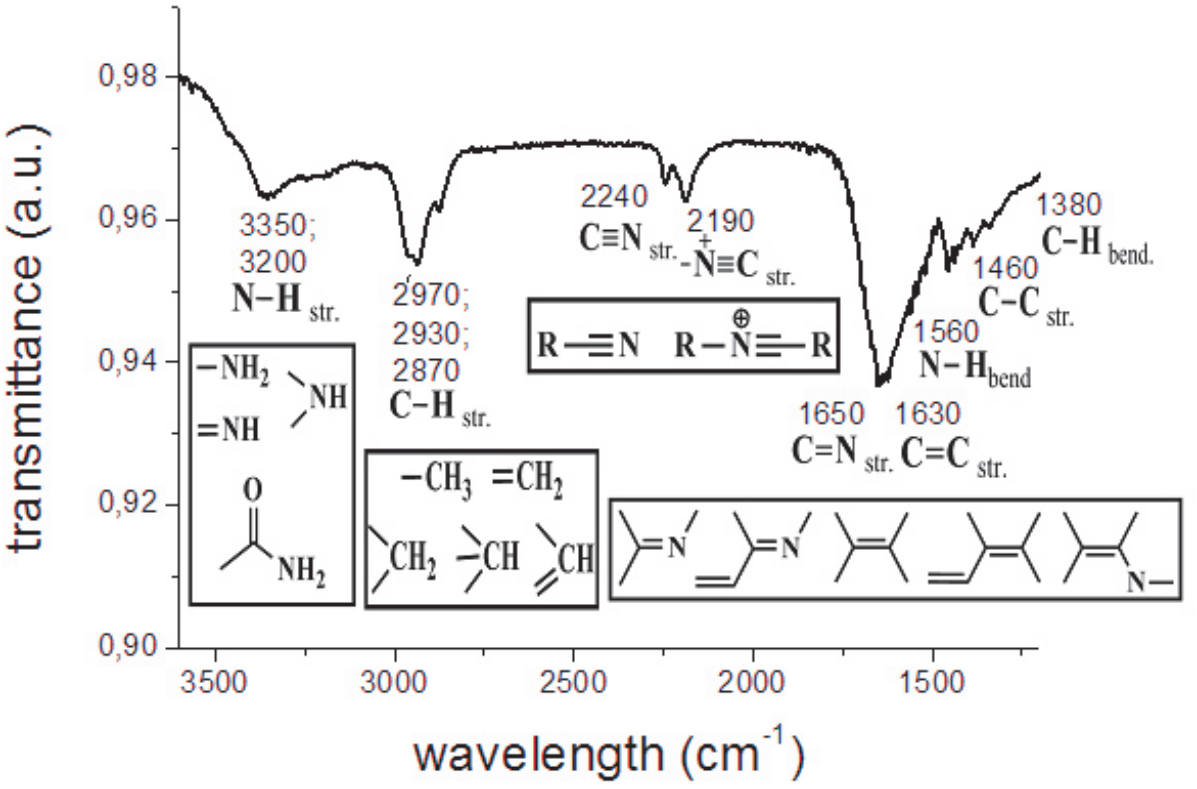

Figure 1 FTIR of CPA PP: the detected signals with the corresponding structural fragments

The GA activation is effective not only due to the reaction with primary amines. The binding should occur through all of the accessible active sites: primary and secondary amines, imines, amides [19]. Aldehyde, ketone and acid groups can also participate in the condensations with the GA. These groups appear in the film due to the oxidation and reactions with water in solutions and under air, but only after long times of immersion. At the moment of GA application the concentration of these chemical groups was still negligible.

3.2. QCM immunosensing

The performance of QCM and SPR immunosensors was evaluated by the immunoassay flow test probing the interaction of immobilized AL-01 Ab and free HSA Ag as a model. It is known that this pair provides a quite reliable immunocomplex under different conditions and assay formats [16].

In the QCM immunosensing, the GA activation was applied to as-deposited film, the AL-01 antibody was immobilized overnight and non-specific sites were blocked with BSA. Then, the tests with different solutions of HSA were carried out. Stable baseline and the selective response were recorded (**Figure 2a**). Each concentration was applied after regeneration using 0.1 M HCl (2 min), which allowed analyzing three concentration probes with a single crystal (**Figure 2b**).

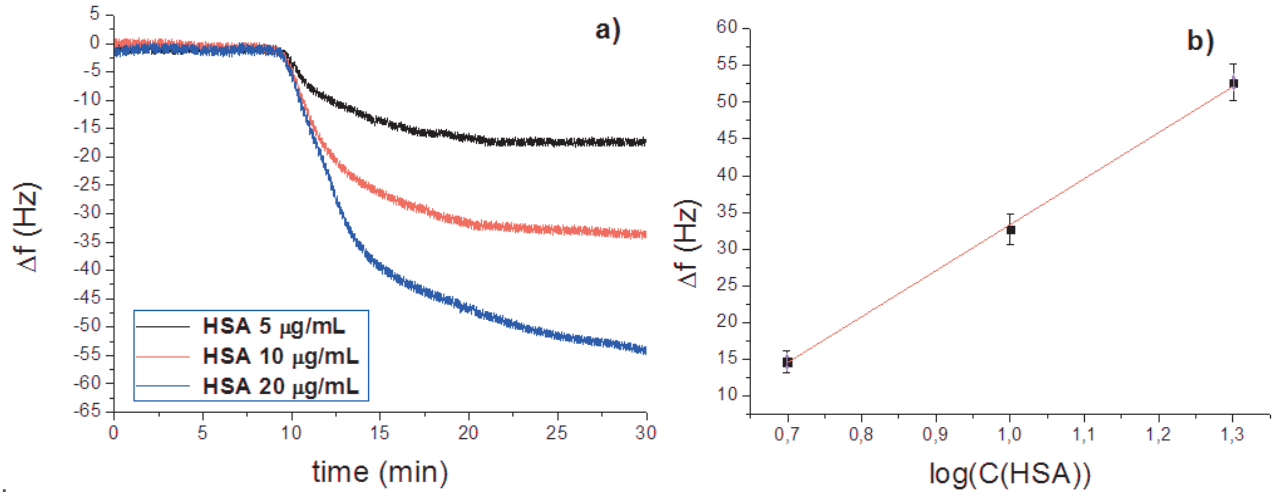

Figure 2 a) Immunointeraction between immobilized AL-01 antibody and solutions of HSA antigen, b) Calibration curve. Each concentration was applied after regeneration with 0.1 M HCl

3.3. SPR immunosensing

In case of the SPR chips, the GA activation was applied to as-deposited film in offline mode (droplet of 3% GA solution in PBS, 1 h, r.t.). During the first procedure (**Figure 3a**), after the GA step, the SPR chip was washed with PBS and water and inserted into system. The measurements were carried out in the angular scan mode. The AL-01 (binding channel only) and the blocking agent (BSA) (both channels) were successively immobilized onto the surface of a chip. After that the reaction with the 10 µg/mL solution of HSA was carried out. During these measurements the drift of the baseline was recorded, but no non-specific interactions were detected in the reference channel. The signal change due to the reaction can be approximately estimated as 20 mdeg.

During the second procedure (**Figure 3b**), after the GA step and washing, AL-01 was applied overnight onto the surface of a binding channel. Then the SPR-chip was washed and the BSA was applied offline to both channels (the same experimental procedure as in case of QCM). After that the chip was inserted into SPR system and after the baseline establishing the reaction with 10 µg/mL solution of HSA was carried out. During these measurements the drift of the baseline was significantly reduced, the non-specific interactions were detected, but at the low level - approximately 2 mdeg. The signal change due to the reaction can be approximately estimated as 8 mdeg.

The comparison of the responses leads us to conclusion, that the online immobilization is more effective, because then the sensor is more sensitive, but the drift problem is needed to be solved.

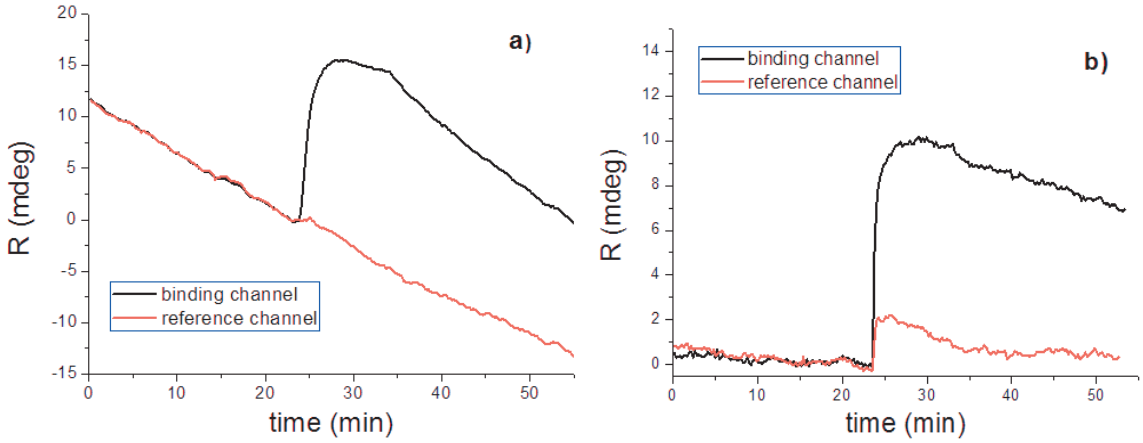

Figure 3 SPR immunotests with the 10 µg/mL solution of HSA: a) online AL-01 immobilization, b) overnight AL-01 immobilization

4. CONCLUSIONS

Pulsed plasma polymerization of CPA is an effective method for the preparation of amine-containing films that are proposed as a replacement of SAMs on the gold surfaces of SPR and QCM sensors. The GA coupling of AL-01 was employed for QCM crystals and SPR chips. The performance of immunosensors was evaluated by the immunoassay flow test probing the interaction of immobilized AL-01 and HSA as a model.

The label-free QCM immunosensor for a rapid detection of HSA was developed. The QCM immunosensors exhibited high-stability of the signal. Linear response was obtained in the range of the used concentrations. The regeneration by 0.1 M HCl was successfully applied allowing to carry out three measurements with the single crystal.

The possibilities in the SPR immunosensing were investigated through the AL-01 - HSA pair interaction, as well. In case of the online AL-01 immobilization, the response during SPR immunosensing of 10 µg/mL solution of HSA was approximately 20 mdeg, whereas in case of the overnight immobilization, the response was 8 mdeg. The online immobilization of AL-01 showed to be more effective but, in this case, the drift of the baseline was observed. The results confirmed that the presented methodologies for the grafting of biomolecules on the gold surfaces are very promising and have a great potential for biosensing applications.

ACKNOWLEDGEMENT

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

REFERENCES

[1] COOPER, M. A., SINGLETON, V. T. Review A survey of the 2001 to 2005 quartz crystal microbalance biosensor literature : applications of acoustic physics to the analysis of biomolecular interactions, *Journal of Molecular Recognition*, 2007, vol. 20, pp. 154-184.

[2] MALMQVIST, M. BIACORE: an affinity biosensor system for characterization of biomolecular interactions, *Biochemlcal Society Transactions*, 1999, vol. 27, no. 2, pp. 335-340.

[3] FORHLEROVA, Z., SKLADAL, P., TURANEK, J. Adhesion of eukaryotic cell lines on the gold surface modified with extracellular matrix proteins monitored by the piezoelectric sensor, *Biosensors and Bioelectronics*, 2007, vol. 22, no. 9-10, pp. 1896-1901.

[4] MARX, K.A., Quartz Crystal Microbalance: A Useful Tool for Studying Thin Polymer Films and Complex

Biomolecular Systems at the Solution, *Surface Interface*, 2003, vol. 4, no. 5, pp 1099-1120.

[5] PELUSO, P., WILSON, D.S., DO, D., TRAN, H., VENKATASUBBAIAH, M., QUINCY, D., HEIDECKER, B., POINDEXTER, K., TOLANI, N., PHELAN, M., WITTE, K., JUNG, L.S., WAGNER, P., NOCK, S. Optimizing antibody immobilization strategies for the construction of protein microarrays, *Analytical Biochemistry*, 2003, vol. 312, no. 2, pp. 113-124.

[6] DONEGAN, M., DOWLING, D.P., Protein adhesion on water stable atmospheric plasma deposited acrylic acid coatings Protein adhesion on water stable atmospheric plasma deposited acrylic acid coatings, *Surface and Coatings Technology*, 2013, vol. 234, no. January, pp. 53-59.

[7] CRESPIN, M., MOREAU, N., MASEREEL, B., FERON, O., GALLEZ, B., VANDER BORGHT, T., MICHIELS, C., LUCAS, S. Surface properties and cell adhesion onto allylamine-plasma and amine-plasma coated glass coverslips, *Journal of Materials Science: Materials in Medicine*, 2011, vol. 22, no. 3, pp. 671-682.

[8] MANAKHOV, A., MORENO-COURANJOU, M., BOSHER, N.D., ROGE, V., CHOQUET, P., PIREAUX, J. Atmospheric Pressure Pulsed Plasma Copolymerisation of Maleic Anhydride and Vinyltrimethoxysilane: Influence of Electrical Parameters on Chemistry, Morphology and Deposition Rate of the Coatings, *Plasma Processes and Polymers*, 2012, vol. 9, no. 4, pp. 435-445.

[9] HEGEMANN, D., HANSELMANN, B., GUIMIND, S., FORTUNATO, G., GIRAUD, M.N., GUEX, A.G. Considering the degradation effects of amino-functional plasma polymer coatings for biomedical application, *Surface and Coatings Technology*, 2014, vol. 255, pp. 90-95.

[10] COOLS, P., MORENT, R., DE GEYTER, N. Plasma Modified Textiles for Biomedical Applications in *Advances in Bioengineering (ed. P.A.Serra),* 2015, pp. 117-148.

[11] AZIZ, G., DE GEYTER, N., MORENT, R. Incorporation of Primary Amines via Plasma Technology on Biomaterials in *Advances in Bioengineering (ed. P.A.Serra)*, 2015, pp. 21-48.

[12] YANG, Z., WANG, J., LUO, R., MAITZ, M.F., JING, F., SUN, H., HUANG, N. The covalent immobilization of heparin to pulsed-plasma polymeric allylamine films on 316L stainless steel and the resulting effects on hemocompatibility, *Biomaterials*, 2010, vol. 31, no. 8, pp. 2072-2083.

[13] DENIS, L., MARSAL, P., OLIVIER, Y., GODFROID, T., LAZZARONI, R., HECQ, M., CORNIL, J., SNYDERS, R. Deposition of Functional Organic Thin Films by Pulsed Plasma Polymerization: A Joint Theoretical and Experimental Study, *Plasma Processes and Polymers*, 2010, vol. 7, pp. 172-181.

[14] MANAKHOV, A., ZAJICKOVA, L., ELIAS, M., CECHAL, J., POLCAK, J., HNILICA, J., BITTNEROVA, S., NECAS, D. Optimization of Cyclopropylamine Plasma Polymerization toward Enhanced Layer Stability in Contact with Water, *Plasma Processes and Polymers*, 2014, vol. 11, no. 6, pp. 532-544.

[15] MAKHNEVA, E., MANAKHOV, A., SKLADAL, P., ZAJICKOVA, L. Development of effective QCM biosensors by cyclopropylamine plasma polymerization and antibody immobilization using cross-linking reactions, *Surface and Coatings Technology*, 2016, vol. 290, pp. 116-123.

[16] NAVRATILOVA, I., SKLADAL, P., VIKLICKY, V. Development of piezoelectric immunosensors for measurement of albuminuria, 2001, vol. 55, pp. 831-839.

[17] MANAKHOV, A., MAKHNEVA, E., SKLADAL, P., NECAS, D., CECHAL, J., KALINA, L., ELIAS, M., ZAJICKOVA, L. The robust bio-immobilization based on pulsed plasma polymerization of cyclopropylamine and glutaraldehyde coupling chemistry, *Applied Surace. Science*, 2016, vol. 360, pp. 28-36.

[18] FAVIA, P., D'AGOSTINO, R., PALUMBO, F. Grafting of chemical groups onto polymers by means of RF plasma treatments: A technology for biomedical applications, *Journal de Physique IV, 1997,* vol. 7, no. C4, pp. 199-208.

[19] KLAGES, C.-P., HINZE, A., KHOSRAVI, Z. Nitrogen Plasma Modification and Chemical Derivatization of Polyethylene Surfaces - An In Situ Study Using FTIR-ATR Spectroscopy, *Plasma Processes and Polymers*, 2013, vol. 10, no. 11, pp. 948-958.