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The robust bio-immobilization based on pulsed plasma polymerization of cyclopropylamine and glutaraldehyde coupling chemistry

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ABSTRACT

The performance of immunosensing devices crucially depends on the methodology of antibody or antigen immobilization on the sensor surface. Hence, the stable intermediate layers capable of specific and reproducible binding of antibodies are required. Herein, we introduce the amine rich (NH_x concentration of 6 at.%) layers prepared by pulsed plasma polymerization of cyclopropylamine (CPA) for functionalization of the quartz crystal microbalance (QCM) surface by the antibody specific to human serum albumin. In these layers the amine groups serve as anchor for the antibody binding. The sensitivity of QCM sensors prepared in this way surpasses the one for the previously reported sensors functionalized by the thiol-based self-assembled monolayers by the factor of 2. Our results thus show that CPA plasma polymers have a significant potential for further development of the active layers for biosensing applications.

1. Introduction

The immobilization of either antibody or antigen onto the sensor surface (often referred to as an interfacial design) is a key step in the fabrication of most immunosensors, including those based on quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) [1]. Immunosensors have been extensively developed and applied for biomedical and environmental studies and analytical assays which led to the establishment of the commercially available, compact real-time monitoring sensor devices [2,3].

The QCM detection is based on the piezoelectric effect, i.e. measuring the QCM resonant frequency f using oscillator circuit and frequency counter. This frequency decreases when mass is added to the surface of the sensor electrode. The sensitivity to very small mass changes (~1 ng) and simplicity of QCM sensor fabrication, portable use and reusability are important factors for its commercial applications as real-time detection of the specific binding events, including antibody-antigen interactions [2].

The high density of the bound antigen/antibody and the suppression of non-specific binding of antigen to the surface are essential for achieving a high sensitivity for the target analyte [4,5]. QCM sensors usually have gold electrodes and the immobilization can occur via either a direct physical adsorption onto the gold surface or formation of new covalent bonds [2]. Although there is a number of techniques for the immobilization of biomolecules (e.g. wet chemistry, electrochemistry, chemical vapor deposition), two main groups can be identified: site-oriented and random [4,6,7]. It is generally assumed, that the site-oriented immobilization based on the adsorption or grafting of protein-A or protein-G combined with the subsequent attachment of the antibody leads to a higher efficiency of the biorecognition [4–6,8]. Nevertheless, the generally higher density (absolute amount) of the immobilized antibodies in randomly oriented strategies leads to comparable performances of both types of biosensors [5]. However, the higher density of

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the immobilized antibodies not always induce the increase of the immunosensor response and both positive and negative effects of the antibody densities were reported depending on the nature of the biomolecule [7,9]. The latter effect can be related to the steric hindering of the biorecognition process if the density of the immobilized antibodies is too large [7].

Both site-oriented and random immobilization strategies require suitable surface groups such as NH_2, COOH or OH that serve as anchor for linking to the biomolecules via a coupling agent (e.g. glutaraldehyde, carbodiimide) [10]. Various functionalization strategies for the deposition of reactive functional layers have been employed for the immobilization of biomolecules. The most popular approaches are based on wet chemical treatments, such as a growth of self-assembled monolayers (SAMs) of alkanethiols or disulfides, polyethylenimine (PEI) layers and silanization [1,10–12]. Nevertheless, these approaches suffer from several drawbacks. Regarding the SAM, the formed S—Au bond is stable in water and air but decomposes under UV irradiation and at temperatures above 70 °C. Furthermore, although dense monolayers assemble quickly, well-ordered layers can take several days to organize [13]. The PEI layer exhibited very poor stability in water leading to a high level of noise and unstable baseline of the measured immunosensor frequency [4]. As an alternative, the surface functionalization by plasma processes is successfully employed [14–19]. The plasma polymerization has already been successfully applied to the deposition of thin films containing carboxyl [20], amine [21] or anhydride groups [22] and it is generally accepted that the essential chemical and morphological stability of the plasma polymer layers can be easily achieved by tuning the plasma parameters [23].

Thanks to the reactivity of primary amine groups, amine-rich plasma polymers are extensively employed for numerous biomedical and environmental applications [24,25] including the immobilization of the biomolecules [26]. Nowadays, amine plasma polymers are already being employed for biomolecules immobilization strategies for QCM and SPR biosensors [26–29], enzyme electrochemical sensors [30,31] and protein immobilization [32]. The fundamental investigation in the field was performed by Nakanishi et al. [33]. They applied ethylenediamine plasma polymers for the development of QCM immunosensors specific to human serum albumin (HSA) antigen and compared the performance of this sensor with those functionalized by conventional liquid phase methods [33]. It was demonstrated that plasma functionalized sensors exhibited a lower noise and higher sensitivity compared to the conventional immunosensors functionalized by PEI layers. Later Saber et al. [9] studied the influence of the density of the anti-HSA antibody on the performance of the QCM sensor modified by a similar method. It was found that HSA immunosensor response (the change of the resonant frequency, Δf) was increasing with the amount of the immobilized antibodies [9]. In the flow regime the Δf reached −50 Hz for the 64 μg/mL solution of HSA antigen and only −20 Hz for the 100 μg/mL bovine serum albumin (BSA) solution. Hence, the developed immunosensor was relatively specific to the HSA antigen, although improving the selectivity still seemed to be necessary. Nevertheless, the employment of the amine plasma layers for the QCM biosensing application was not very advantageous, probably due to the low amine concentration in the ethylenediamine plasma polymers which did not exceed 1–2 at.% [34].

Another widely used monomer, allylamine, was studied for many years because of the double bond in its structure that enables free radical polymerization. However, the amine-rich films obtained from allylamine plasma polymerization showed a significant decrease in nitrogen concentration (nitrogen/carbon ratio decreasing from 0.22 to 0.06) [35,36] and film thickness loss up to 90% after an immersion in water [37]. In order to improve the properties of amine-rich films, different research groups have studied polymerization of various amine precursors: butylamine [38], heptylamine [39], acetylene/ammonia mixtures [40], cyclopropylamine (CPA) [41]. Recently we have shown that pulsed radio frequency capacitively couple plasma (RF CCP) polymerization of cyclopropylamine can be tuned to deposit amine-rich layers carrying 9 at.% of NH_x functions and exhibiting only 20% thickness loss after immersion in water for 48 h after which they became stable and no further thickness loss occurred [42–44].

In this work, the cyclopropylamine pulsed plasma polymerization is employed to deposit stable amine-rich thin films on the surfaces of QCM sensors. The antibody against HSA is attached to the surface via crosslinking obtained by an intermediate reaction with glutaraldehyde. All steps of the bio-immobilization are controlled by X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared (FT-IR) spectroscopy to characterize the surface and layer chemistry. The stability of the deposited coating during immobilization and within biosensor operation is controlled by scanning electron microscopy (SEM) imaging and QCM measurements. The selective and efficient detection of HSA antigen is confirmed by flow test with HSA antigen and the negative response to the ovalbumin. The response of the immunosensor functionalized via CPA plasma polymerization shows at least 2 times better performance compared to the standard sensor employing SAM as the intermediate layer.

2. Experimental

2.1. Materials

Cyclopropylamine – monomer for plasma polymerization (purity of 98%, used without any further purification), glutaraldehyde (25% aq. solution), cysteamine, ovalbumin and HSA were purchased from Sigma–Aldrich. Anti-HSA monoclonal antibody (clone AL01, purified immunoglobulin G, IgG) was obtained from Exbio, Prague. Argon with a purity of 99.998% was supplied by Messer. Double-side polished single crystal silicon (c-Si) wafers ⟨111⟩ (N-type phosphorus doped) with the resistance of 0.5 Ω cm were supplied by ON-Semiconductor, Czech Republic and were used for the optical characterization. Round shape QCMs (AT-cut, 14 mm in diameter, resonant frequency of 10 MHz) coated by Au with a Cr interlayer were purchased from Krystaly, Czech Republic. All substrates were cleaned by sonication in isopropanol (Penta, 99.8%) for 10 min.

2.2. Plasma polymerization set-up

A detailed description of the CPA plasma polymerization set-up is given in our previous paper [42]. The process was carried out in a capacitively coupled RF discharge (13.56 MHz) fed by CPA/Ar gas mixture. The discharge was ignited in a tubular reactor with side flanges serving as the electrodes. The substrates (c-Si and QCM) were placed on the glass holder positioned in the middle of the tube. They were at the floating potential.

The substrate surfaces were cleaned in the Ar discharge for 10 min prior to the deposition. All the plasma processes, the surface cleaning and CPA plasma polymerization, were carried out in square-pulsed mode with the on-time power of 20 W and pressure of 120 Pa. The plasma duty cycle and pulse repetition function was set to 33% and 500 Hz, respectively. It corresponded to 660 μs plasma on-time and 1340 μs off-time. The flow rate of Ar was set to 28 sccm whereas the flow rate of CPA vapors was 0.3 sccm. The deposition time was tuned to achieve the film thicknesses of 40 and 120 nm on QCM and Si wafer, respectively. The 40 nm thick samples were used for XPS analyses and immunosensor tests, while 120 nm thick films were used for FT-IR studies.