

Development of a Piezoelectric Immunosensor for Matrix Metalloproteinase-1 Detection

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Abstract— Monoclonal antibodies were immobilized onto the surface of quartz crystals for the development of a piezoelectric biosensor by means of the Layer by Layer self assembly technique (LBL). The immobilization of immunoglobulins specific to the matrix metalloproteinase-1 (MMP-1) was investigated. To this purpose multilayered ultra-thin films composed by precursor layers of cationic poly(dimethyldiallylammonium) chloride and anionic poly(styrenesulfonate) followed by a monolayer of antibodies were assembled by LBL. A quartz crystal microbalance was used to monitor and optimize the assembly process and to test the immunological activity of the deposited antibody molecules. Atomic force microscopy was used to characterize the surface roughness of the multilayers before and after the deposition of the immunoglobulins. The obtained results confirmed the successful deposition of the proposed immunosensor and demonstrated its high potential for the measurement of analytes of clinical interest.

I. INTRODUCTION

BIOSENSORS are devices characterized by the presence of a biological sensing component which is responsible for the specific interaction with the analyte of interest. This sensing element is put in intimate contact with a physico-chemical transducer where the interaction with the target molecules is converted into quantifiable electrical signals. Different kinds of biological components can be used as sensing elements such as enzymes, antibodies, cell membrane receptors, tissue slices or microbial cells [1]. In the last years, biosensors have found applications in different fields such as environmental monitoring, food industry and above all in medical diagnostics where the possibility to carry out a fast, easy and reliable determination of specific analytes represents a valid alternative to classical analytical techniques [2]. To this regard different commercially available biosensors have been already developed for clinical applications such as for the measurement of blood glucose levels [3]. The possibility to set up biosensors for the measurement of other analytes of clinical interest, such as biomarkers and pharmacologically active compound is then extremely attractive. To this purpose, since antibody molecules can be developed against

almost any substance the use of antibody-based biosensors (immunosensors) is the most promising [4]. As relates to the choice of the transducer system mass-sensitive transducers such as piezoelectric quartz crystals have attracted considerable attention since many important physical and chemical processes, including the binding of an antigen to its specific antibody, can be followed by observing the associated mass changes. Specifically, any mass increase due to the binding of an analyte by the antibodies immobilized onto the surface of a quartz crystal decreases its oscillation frequency which is the output signal from such transducers [5].

The immobilization of the antibody molecules represents the most critical step in the design of a piezoelectric immunosensor. To this regard the development of a stable and bioactive surface onto which the antigen of interest can interact in a reproducible way is pivotal. The reproducibility and stability of the whole sensor largely depend on this step. Among immobilization techniques, the LBL meets these requirements. It is simple and versatile and allows the fabrication of multilayered ultra-thin films by the sequential adsorption of oppositely charged macromolecules from solution [6], [7]. An advantage of LBL is that nanostructured films with desired architecture can be deposited onto substrates of any shape. Moreover, a large variety of molecules, including synthetic polyions, biopolymers, nanoparticles and proteins can be incorporated into the multilayers. These structures exhibit a large thermal and mechanical stability and bioactive molecules eventually included in their thickness are not denatured by the deposition process and present enhanced functional stability [6], [8].

In this work the use of the LBL technique was investigated for the development of a piezoelectric immunosensors specific to the human matrix metalloproteinase-1 (MMP-1). Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that mediate the degradation and the remodeling of the extracellular matrix thus mediating a wide range of physiological and pathological processes such as tissue reshaping, wound healing, inflammation, organ development, tumour metastasis formation, atherosclerosis, abdominal aortic aneurysms, and heart failure. MMPs are divided into different groups, based on their substrate specificity. MMP-1 is a collagen protease, which has been shown to be expressed in inflammatory atherosclerotic lesions and is known to be implicated in the vascular re-

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modeling events preceding plaque rupture (the most common cause of acute myocardial infarction) [9]. Within the EU project CardioWorkbench MMP-1 has been selected as a biomarker of interest to be monitored in the culture media of human cardiovascular cells isolated from patients affected by coronary heart disease. In the present paper we report on the immobilization of antibodies specific to MMP-1 for the development of piezoelectric immunosensors of clinical interest.

II. MATERIALS AND METHODS

Cationic poly(dimethyldiallyl ammonium chloride) (PDDA, Aldrich) and anionic poly(styrenesulfonate) (PSS, Aldrich) were used as precursor layers at a concentration of 2 mg/ml in 0.01M Phosphate Buffered Saline (PBS) solution. Anti-MMP-1 (Sigma) IgG monoclonal antibodies were used at a concentration of 20 µg/ml in PBS as anionic (working pH 7.4) polyelectrolytes. Human MMP-1 (Sigma) was used to test the immunological activity of the developed biosensors at different concentrations (35, 75 and 150 ng/ml in PBS). Bovine Serum Albumin (BSA) (Sigma) was used to prevent non-specific interactions at a concentration of 50 µg/ml [6]. The experiments were carried out at physiological pH (7.4). The water employed for all the experimental procedures was purified by means of a Milli Q water purification system with a resistivity of 18 MΩ·cm. The assembly working conditions were optimized on the surface of quartz crystals (AT-cut, 5 MHz) by means of a Quartz Crystal Microbalance instrument working in liquid environment (QCM-Z500, KSV Instruments, Helsinki, Finland).

A. Assembly procedure

Prior to use the quartz crystals were washed in sulfuric acid, rinsed with water and then dried in nitrogen flux. At this point the architecture (PDDA/PSS)₃/(PDDA/anti-MMP1) was assembled on their surface. A Teflon liquid chamber with a capacity of 2 ml was used in the experiments. Specific polyelectrolyte solutions were alternatively introduced into the measurement chamber and were left in contact with the quartz crystal for total 10 min. After each polyelectrolyte adsorption step, PBS was purged into the chamber and left in contact with the crystal for 1 min in order to remove the unabsorbed polyelectrolyte. Data analysis was performed using the QCM Impedance Analysis software (KSV Instruments, version 3.11). Each experiment was carried out in triplicate. The measured changes in terms of frequency, and adsorbed mass were expressed as the average of these three replicates.

B. Quartz Crystal Microbalance Measurement (QCM)

The QCM-Z500 instrument-measuring principle is based on impedance analysis [10]. The adsorbed layer and bulk liquid phase create mechanical perturbations in the quartz crystal and alter its electrical characteristics. The QCM-Z500 instrument allows one to record the impedance of the

5-MHz crystal and the adsorbed layers at several overtone frequencies. Thus, by measuring the impedance over the crystal and using equivalent circuit analysis, one can relate the electrical impedance of the quartz crystal to the mechanical properties of the adsorbed layer and contacting liquid phase. If the layer adsorbed on the crystal surface is rigid and homogeneous, the normalized frequency changes recorded for all overtone frequencies superimpose on each other and the frequency shift is proportional to the mass of the adsorbed material. Under this situation, the Sauerbrey equation derived for rigid films can be applied [11]. For adsorbed layers that do not fulfill the above requirements, there is a deviation from the Sauerbrey equation because the added mass is soft and/or not properly attached to the underlying surface; therefore, it is not fully coupled with the crystal oscillation. In such cases, measuring the electrical properties of the quartz crystal and the added layers at multiple overtones is useful to determine the mechanical properties of the adsorbed layer. By using equivalent circuit modeling, one can then access the mechanical properties of the added layers on the quartz crystal such as mass, density, thickness, and the viscoelastic properties [12] – [14].

C. Atomic Force Microscopy (AFM)

Silicon oxide supports, with a RMS (root mean square) of 0.3 nm, were at first cleaned in concentrated sulfuric acid for 10 minutes at 120 °C and then washed in ultra pure water. Subsequently the assembly procedure optimized by QCM was repeated onto their surface. The roughness of the developed multilayers was investigated before and after the deposition of the antibody molecules by AFM in air in non-contact mode.

D. Immunoassay procedure

The prepared immunosensors were used to detect MMP-1 and the response of the antibody–antigen reaction was characterized. As a first step, the modified quartz crystals were exposed for 30 min to a BSA solution (50 µg/ml in PBS) in order to block non specific binding sites during immunosensing [6]. The quartz crystals were then rinsed, and the frequency shift was recorded. As a second step, the modified quartz crystal were exposed to MMP-1 solutions at different concentration (35, 75 and 150 ng/ml) for 60 minutes. A calibration curve of the frequency response versus MMP-1 concentration was thus obtained. As a further step the QCM frequency–time profiles for the interaction of MMP-1 with immobilized anti-MMP1 was characterized. To this purpose different quartz crystals were at first exposed to BSA for 30 minutes and then to a solution of MMP1 at the concentration of 150 ng/ml at room temperature. The frequency shifts due to the specific interaction of the antibody molecules with MMP1 were monitored for a total of 60 minutes.

III. RESULTS AND DISCUSSION

In order to characterize the assembly of the

immunosensor the resonance frequencies of the crystals monitored in real time. The corresponding mass of the deposited layers was then determined. The frequency shifts obtained for the architecture (PDDA/PSS)₃/PDDA/anti-MMP1 are shown in Fig. 1. The corresponding ones obtained for the mass and for the surface density (density of the deposited mass at each assembly step) are shown in Fig. 2 and 3.

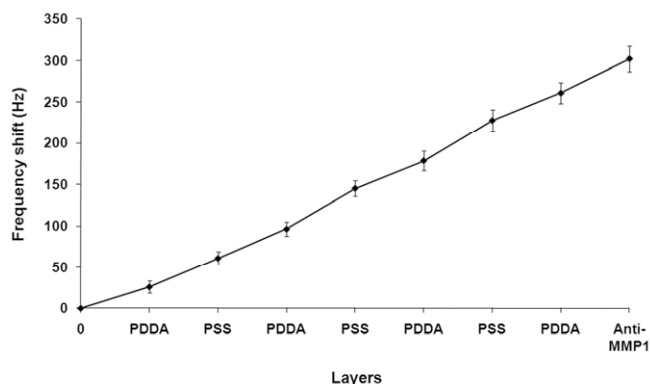


Fig. 1. QCM monitoring for the architecture (PDDA/PSS)₃/PDDA/anti-MMP1

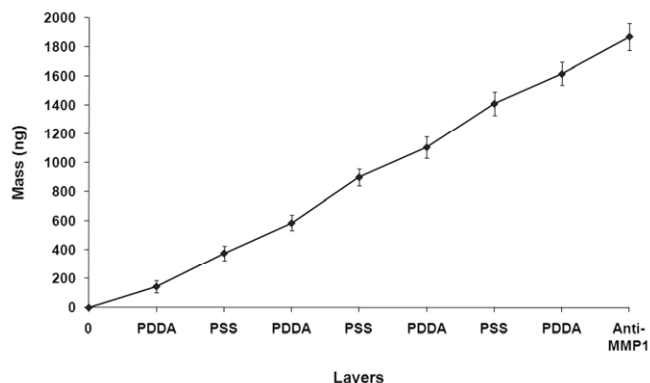


Fig. 2. Cumulative mass calculated for the architecture (PDDA/PSS)₃/PDDA/anti-MMP1

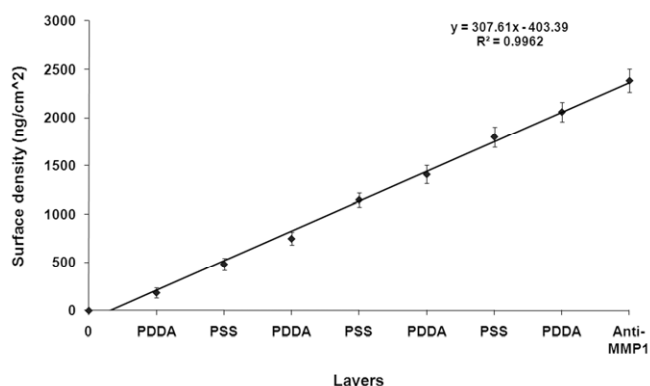


Fig. 3. Surface density of each assembly layer for the architecture (PDDA/PSS)₃/PDDA/anti-MMP1

As shown in Fig. 1, 2 and 3 film growth resulted to be reproducible at each deposition step, putting in evidence a

successful stepwise deposition procedure. As relates to the deposition of PDDA and PSS an average frequency shift of 32 $\pm$ 2 and 51 $\pm$ 5 [Hz] was respectively obtained. This resulted in an average mass increase of 192 $\pm$ 12 ng for PDDA and of 295 $\pm$ 29 ng for PSS which corresponds to an average surface density shift of 235 $\pm$ 14 [ng/cm²] and of 376 $\pm$ 34 [ng/cm²]. As relates to the deposition of the antibody molecules the assembly of the anti-MMP1 layer resulted in an average frequency shift of 40 $\pm$ 4 [Hz]. This corresponds to an average mass increase of 220 $\pm$ 22 [ng] and to an average surface density shift of 280 $\pm$ 28 [ng/cm²]. As reported in literature, the saturation value for a monolayer of side-on IgG is approximately 300 ng/cm², whereas for a monolayer of end-on IgG is about 1.5 μ g/cm² [15]. Comparing these values with the ones we obtained it can be assumed that anti-MMP1 molecules were deposited mostly in a side-on fashion.

The deposited architectures were further characterized by AFM to investigate their surface roughness. The calculated RMS resulted to be higher after the deposition of the antibody molecules. Specifically, the RMS calculated for the architecture (PDDA/PSS)₃/PDDA and (PDDA/PSS)₃/anti-MMP1 resulted to be respectively 3,5 $\pm$ 0.3 nm and 6.1 $\pm$ 1.3 nm. Taking into account that the dimensions of an IgG molecule, calculated from crystallographic data, are about 10 nm $\times$ 14 nm $\times$ 5 nm [7], the obtained results further suggested the hypothesis that the antibodies molecules were deposited in a side-on fashion.

At this point, the developed immunosensor was used to detect MMP1 and the response of the antibody–antigen reaction was characterized. As a first step, modified quartz crystals were exposed for 30 min to BSA to block non specific binding sites during immunosensing. Quartz crystals were then rinsed, and the frequency shifts recorded. As a second step, the modified quartz crystals were exposed to a solution of MMP1 at different concentrations (35, 75 and 150 ng/ml) for 60 minutes. Fig. 4 shows the calibration curve of the frequency response versus MMP-1 concentration. As shown by the regression coefficient an almost linear relationship of MMP1 concentration versus frequency shift has been obtained. The deviation from linearity could be due to the saturation of the biosensor surface (see Fig. 5) which could have been also responsible for the decrease of results reproducibility at high MMP1 concentration. The frequency–time profiles for the interaction of MMP1 with the immobilized anti-MMP1 was determined (Fig. 5) for a MMP1 solution having a concentration of 150 ng/ml and for a total reaction time of 60 min. The total frequency shift resulted to be of -195 ± 18 Hz. As a second step, in order to evaluate the influence of non-specific interactions, an IgG non-specific to MMP1 was immobilized on the surface of quartz crystals and used to perform the immunoreaction. The non-specific quartz crystals were then brought into contact with the MMP1 solution (150 ng/ml) and after 60 min rinsed. In this case,

the average frequency shift resulted to be of -28 ± 3 Hz. Assuming a similar non-specific adsorption for both the systems, the difference between the obtained frequency shifts corresponds to the specific binding. On the base of this premise, the mass of the detected MMP1 was found to be 117 ± 4 ng. After 1 h of reaction, the developed biosensor appeared to be almost saturated.

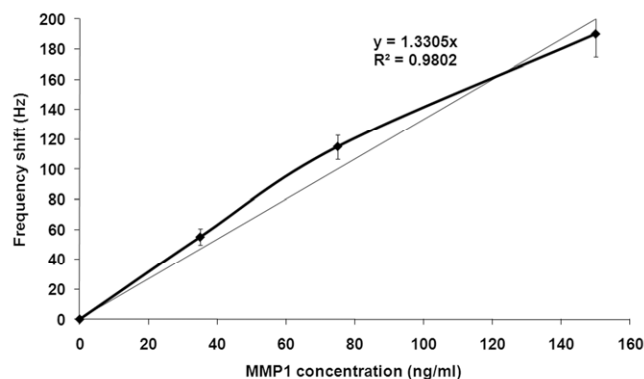


Fig. 4. Calibration curve of the immunoreaction.

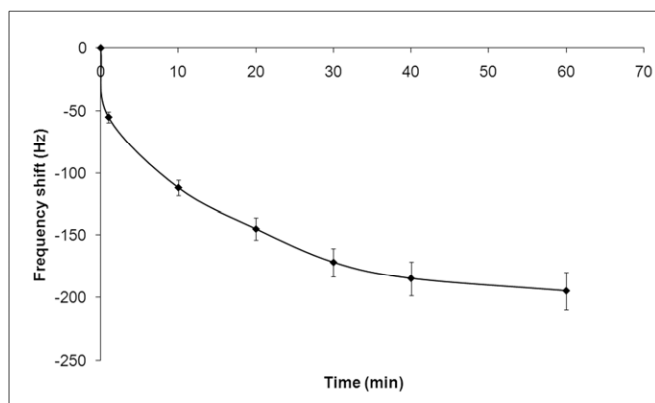


Fig. 5 QCM frequency-time profiles for the interaction of MMP1 with the immobilised anti-MMP1

IV. CONCLUSIONS

The obtained results demonstrated the reliability of the developed immunosensor, the conservation of the bioactivity of the immobilized antibodies and the specificity of the detected interactions. Finally, they put in evidence the high potential of the developed immunosensor for the detection and quantification of biomolecules of clinical interest in a fast and reliable way directly on aliquot of culture media and/or on cell extracts. Further work is in progress to better evaluate and optimize the range of the detectable MMP1 concentrations.

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