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Piezoelectric Immunosensor for the Determination of Immunoglobulin G

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Immunoglobulin G (IgG) is an immunochemical marker with broad importance because of its employment in various diagnoses like chronic infections, some inflammatory diseases, hyperimmunization, multiple myeloma, leukemia or lymphoma. The IgG level in blood or blood plasma is typically measured by an instrumental method from which Enzyme-Linked Immuno-Sorbent Assay (ELISA) is the most important one. Simple method suitable for field or homecare is however missing. This paper is devoted to the construction of immunochemical biosensor based on Quartz Crystal Microbalance (QCM) platform for a simple determination of IgG. A polyclonal antibody specific to IgG was covalently attached to electrode and standard IgG was assayed and the resulted data were validated to ELISA method. The immunosenor was found to be a reliable platform and IgG was determined with limit of detection 9.7 μg/ml and the achieved calibration fully correlated with ELISA which is perceptible from coefficient of determination r^2 = 0.976. The immunosensor exerted long term stability and it was usable after at least two months. In a conclusion, the immunosensor appears to be a reliable tool for a fast, label-free determination of IgG without any necessity to have expensive equipment or skills.

Keywords: affinity; antibody; biosensor; biorecognition; immunochemistry; immunoglobulin; label free assay; piezoelectric; quartz crystal microbalance

1. INTRODUCTION

Total level of immunoglobulins (antibodies) is a marker of various pathological states and it belongs between the standard markers that are measured in most of clinical laboratories [1-3]. Increase of immunoglobulins level can be polyclonal or monoclonal which means that their level can be increased because of many B lymphocytes or only one clone of B lymphocyte. The polyclonal increase can be caused by the both acute and chronic infections and some inflammatory diseases or hyperimmunization. Multiple myeloma, leukemia or lymphoma can be mentioned as the monoclonal

cause of abnormal immunoglobulins increase. Immunoglobulins are presented in plasma or serum of adults in quite high concentration. Immunoglubulins G (IgG) can be expected in human plasma in a range 7 – 16 mg/ml. Immunoglobulin A (IgA) has lower concentration than the IgG: 0.7 – 4 mg/ml. Total level of IgM in serum of health people can be expected in a range 0.4 – 2.3 mg/ml. The aforementioned pathologies cause significant increase in immunoglobulins level. High level of immunoglobulins (hyperglobulinemia) up to 30 mg/ml [4] or IgG above 70 mg/ml [5] can be exampled as values from known case reports.

Immunochemical markers including specific antibodies and total level of antibodies are measured by standard methods like fluorescent or spectral immunoassays, immunochromatography assays and electrophoretic assays. Enzyme-Linked Immuno-Sorbent Assay (ELISA) is probably the most important method for the determination of immunochemical markers which is available as a method of the first choice in the most of clinical laboratories. This paper is focused on finding of an alternative to the standard analytical procedures used for the determination of immunochemical markers. In this study, a piezoelectric platform with Quartz Crystal Microbalance (QCM) sensor has been chosen for the purpose of IgG determination. The fact that the affinity interactions can be recorded without using any labeling or samples treatment is the major advantage of QCM. Physical principle of the assay can be learned from quoted papers [6-12]. In a brief description, QCM is a part of an oscillatory circuit and the quartz mechanically oscillates when alternating voltage put on it. The frequency remains unchanged until analyte interaction with the surface (electrode) of QCM. After that interaction, frequency drops proportionally to amount of the bound analyte. In this work, it is intended to make an immunosensor suitable for the label free detection of IgG. It is expected that this assay can provide analytical properties close to ELISA but it would be significantly simpler so better available for small laboratories or caregivers.

2. MATERIALS AND METHODS

2.1. Sensor and procedure of antibody immobilization

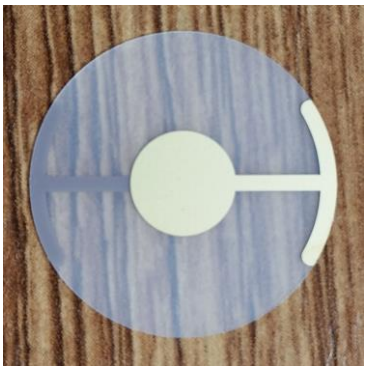

Figure 1. QCM used in the experiments.

QCM (Krystaly; Hradec Kralove, Czech Republic; http://www.krystaly.cz/en/) had basic frequency of oscillation 10 MHz. In a physical appearance, it was a quartz disc with wide 166 μm and

external diameter 19 mm. Gold electrodes (7 mm in diameter) placed on chromium interface were located on the both sides of quartz disc. Photography of used QCM is given as figure 1.

New QCMs were washed before immobilization procedure starting. Each QCM sensor was consequently washed by deionized water (prepared by Aqua Osmotic 02 device, Aqua Osmotic, Tisnov, Czech Republic) and pure ethanol (Litolab, Chudobin, Czech Republic) and let to dry. The immobilization procedure was adopted from previous works and the mentioned paper where cysteamine and glutaradehyde as reagents for self-assembled monolayer construction was chosen [12-15]. In a total 50 µl of cysteamine (Sigma-Aldrich; St. Louis, MO, USA) 50 mg/ml in deionized water was spread over each electrode and the electrodes were left in a wet box for 5 hours. The box protected from premature desiccation. In the end of reaction with cysteamine, the sensors were rinsed by phosphate buffered saline (PBS) pH 7.4 and dried. The rinsing was chosen for removing of physically adsorbed cysteamine not involved in self-assembled monolayer creation [16]. In the next step, glutaraldehyde (Sigma-Aldrich) was solved in deionized water up to concentration 5 % w/w and 50 µl of the solution was spread over each electrode placed into dark and wet box for at least 5 hours and then washed and dried again. Lyophilized antibody against mouse IgG produced in goat (Sigma-Aldrich) was dissolved in PBS in a ratio recommended by manufacturer – 1:1000. After that, 50 µl of the solution was given per one electrode and let incubate in a dark and wet chamber overnight (12 hours). The surface was then washed by a mild stream of PBS, dried and blocked by 5 mg/ml of bovine serum albumin (Sigma-Aldrich) for five hours in dark and wet chamber. The finished immunosensor was washed by a mild stream of by PBS with 0.1 % (w/w) Tween 20 prior to use. The electrodes were stored in dry state in a paper box at 4 °C until use in the experiments.

2.2. QCM immunosensors performance

The immunosensor worked on a label free principle. It means that no further reagent was needed and the immunosensor directly reacted with analyte and the reaction was measurable. In the beginning of assay, immunosensor was connected with ICM Level Oscillator 10.000 MHz (ICM; Oklahoma City, OK, USA) and the oscillator was plug into frequency counter UZ 2400 (Grundig; Nuremberg; Germany). Frequency was measured before sample application. After that, a sample sized 50 µl was spread over the electrode and then it was incubated for two hours. IgG or IgM from mouse serum and bovine albumin (all from Sigma-Aldrich) were dissolved in PBS and used as standard samples or interferents. IgG was used in a calibration range 0, 0.0390, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0 and 20.0 mg/ml. After two hours incubation, each electrode was washed by PBS with 0.1 % (w/w) Tween 20. Oscillation frequency was measured again after electrodes drying and difference of oscillation Δf was calculated.

2.3. Assay by ELISA

ELISA method was performed using the same samples and recognition antibody against IgG like the immunosensor. In the first step, 50 µl of the tested sample (PBS as blank, IgG or an

interferent) was mixed with 150 µl of PBS and get per a well of a 96 well microplate (flat bottom, MaxiSorp type, Nunc, Roskilde, Denmark) and let to incubate in a wet chamber overnight. The second day, the plate was washed with PBS and uncovered binding sites on the surface were blocked by 100 µl of 0.1 % (w/v) gelatin (one-hour incubation). Application of anti IgG from goat and secondary antibody labelled by horse radish peroxidase and specific against goat antibody (Sigma-Aldrich) followed. Each antibody was incubated for four hours and the plate was washed by PBS with 0.1 % (w/w) Tween 20. In the last step, o-phenylenediamine 0.5 mg/ml and hydrogen peroxide 5 mmol/l were injected per well and reaction was stopped by 100 µl of 2 mol/l sulfuric acid after one minute. Optical density was measured at 450 nm using ELISA reader Sunrise (Salzburg, Austria).

2.4. Statistics

Each sample was measured five times and the both mean as well as standard deviation were calculated from the data. Origin 8 (OriginLab Corporation, Northampton, MA, USA) software was used for graphs making and data fitting. Signal to noise equal to three (S/N = 3) was used as a rule for limit of detection calculation.

3. RESULTS AND DISCUSSION

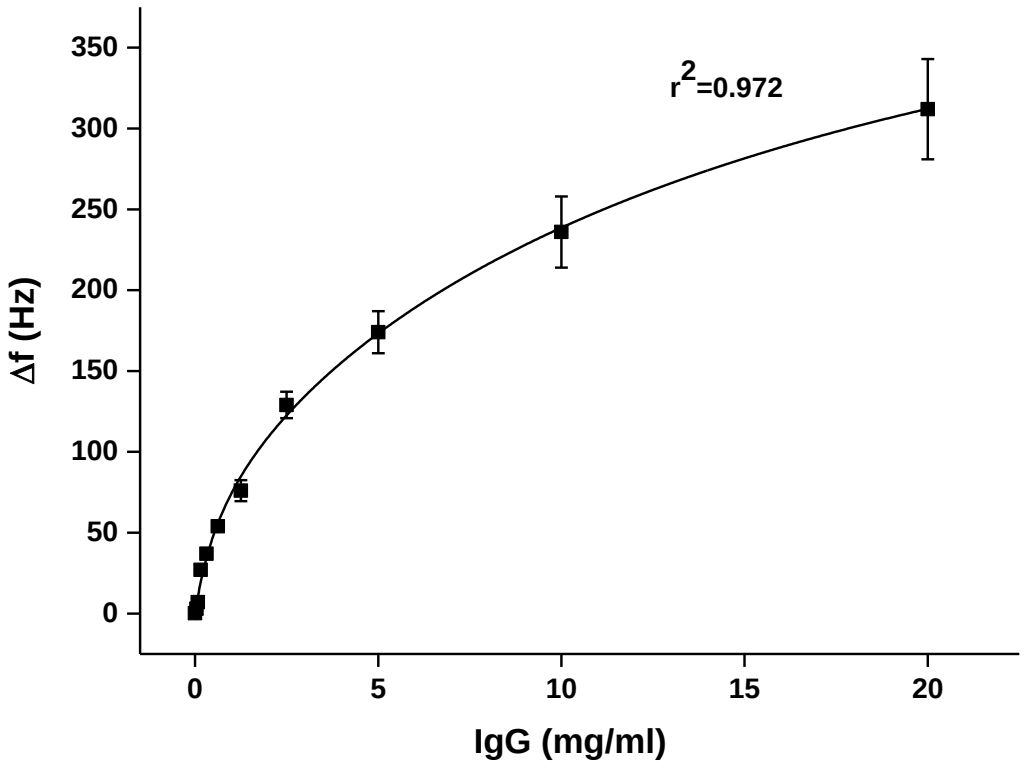

Figure 2. Calibration curve for IgG using QCM immunosensor. Error bars indicate standard deviation for n = 5.

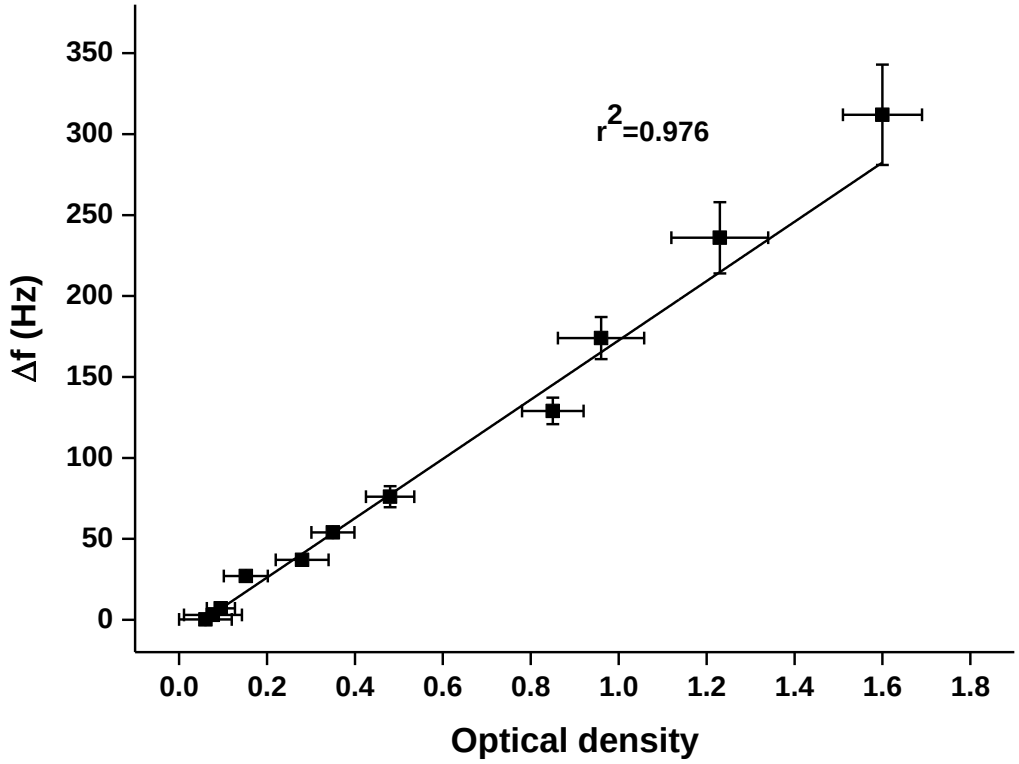

Figure 3. Validation of immunosensor (Δf expressed in Hz) to ELISA method (optical density on x-axis) as an outputting value. Calibration range of IgG 0.0390 – 20.0 mg/ml was used for the validation purpose. Error bars indicate standard deviation for n = 5.

The immobilization procedure and preparation of the immunosensor was also followed and difference of oscillations was recorded for each step. No oscillation change was observed when the self-assembled monolayer was formed. It is not surprising because the both cysteamine and glutaraldehyde are low molecular weight compounds and total weight of bound mass was too low to be recorded by the measuring device. Binding of the lyophilized antibody against mouse IgG produced in goat caused drop of frequency of oscillation equal to 512±45 Hz. The following washing procedure caused displacement of immunoglobulins not covalently bound and the oscillation frequency increased of 47±33 Hz. As a result of the immobilization, oscillation frequency dropped of 465 Hz.

The prepared immunosensors were tested for IgG and calibration plot was constructed and limit of detection was calculated from the plot. The calibration for IgG is depicted as figure 2 and validation to ELISA method as figure 3. The achieved limit of detection for IgG using immunosensor was equal to 9.7 μg/ml. It has to be emphasized that the achieved limit of detection is significantly lower than the expected physiological range of IgG which is 0.7 – 4 mg/ml for humans. The level of IgG variates because of pathological reasons or it can be increasing because of adaptive immunity initiation [17-21]. The used calibration range 0.0390, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0 and 20.0 mg/ml was wider than the physiological range is. Though lower sensitivity was observed in the range above 5 mg/ml, this concentration can be also covered or sensitivity improved by mild sample solution by saline or phosphate buffer. The assay based on immunosensor correlated with ELISA as a standard

method and quite good coefficient of determination r^2 = 0.976 was achieved. It appears that the immunosensors have neither better nor worse sensitivity to IgG when compared to ELISA. However, the assay by immunosensor was simpler regarding number of steps necessary to finishing the analysis and total number of used chemicals. All these factors make immunosensor assay suitable for routine use outside of laboratories. ELISA method is precise and applicable for many types of markers and analytes but not convenient for examination outside equipped workplaces [22-24].

IgM and albumin were tested as potential interferents which can be presented in tested samples. Concentration of the interferents was derived from their standard plasmatic levels which is 0.4 – 2.3 mg/ml for IgM and 35 – 55 mg/ml for albumin. Approximately two times higher than the upper physiological limit was used which is 5 mg/ml for IgM and 100 mg/ml for albumin. Neither IgM nor albumin caused fundamental interference. While signal for assay with albumin was equal to 0.4±0.2 Hz, IgM effected change in oscillation -0.2±0.3 Hz. An interference assay was also done with pooled human plasma. The pooled plasma caused shift of equilibrium frequency equal to 4±1 Hz. Though the pooled plasma has significantly higher impact comparing to the tested interfering solutions, the frequency shift is higher. The impact of the pooled human plasma can be indicated as a matrix effect rather than true interference. Interference would be expected when the used antibody does not have full selectivity to the target molecule (mouse immunoglobulin in this case). In this case, non-specific physical adsorption of plasma parts was observed. On the other hand, the effect is low and no malfunction of the immunosensor is expected. Such finding can be considered as a quite good result and interference in a routine assay appears improbable.

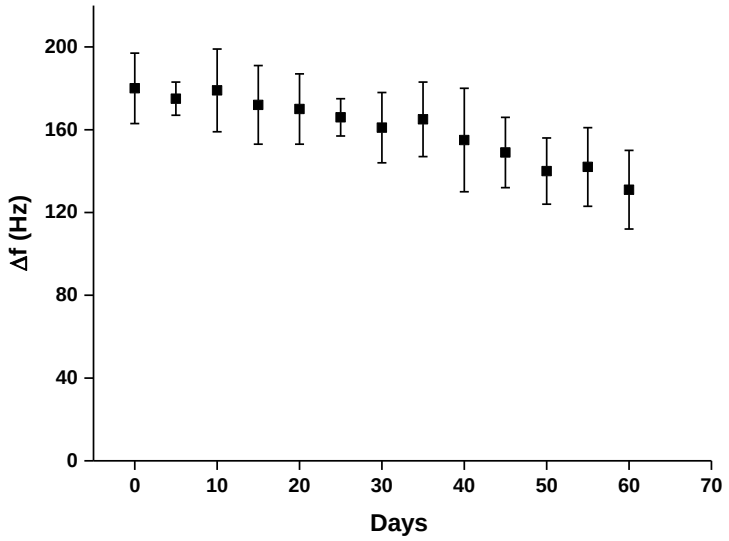

Figure 4. Long term stability of immunosensors presented as a repeated assay of IgG 5 mg/ml. Error bars indicate standard deviation for n = 5.

Long term stability was tested as the last from the examined parameters and the resulted values are shown in figure 4. Solution of IgG 5 mg/ml was chosen for the purpose of testing and time period of two months (60 days) was covered by the test. As can be learned from the figure 4, decrease of

sensitivity to IgG was lower scale during the first month. Approximately 90 % of the signal was recorded after 30 days when the signal is compared with the first day. Higher decrease of signal was observed from the second moth of this experiment. In the last day of this experiment, 73 % of signal was recorded when compared to the first day. The immunosensors were stored for the whole time in dark but no other protection like keeping in cold or special package was applied. Further improvement in the immunosensors packaging or surface stabilization by chemical way would prolong stability of immunosensors fabricated in high numbers [25]. In the case of production, reusability of the immunosensor can be considered. Because the device is considered as a simple tool for diagnosis outside specialized laboratories, the reusability is not expected here. On the other hand, QCM can be chemically cleared and the modification can be made again. Cleaning procedures were tested in some works and they appear to be promising [26].

4. CONCLUSION

The described piezoelectric immunosensor appears as a suitable alternative to the more elaborative ELISA method. Though the immunosensor does not provide significantly better analytical parameters comparing to the current laboratory methods, the assay is evidently simpler and readily to be performed in conditions of homecare. There is also minimal demand on laboratory equipment and simple oscillation circuit with frequency counter optimized for 10 MHz oscillators. Costs per one assay are comparable between ELISA and the immunosensor. This work is based on older studies where specific antibodies were assayed [27,28]. The developed immunosensor performed in this work is an ending procedure of the research on the immunosensor construction and it brings the opportunity to proceed further to reach the development phase for a diagnostic device. Practical impact of the findings presented here is expected.

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