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Short Communication

QCM biosensor for Prostate Specific Antigen assay using antibody – gold particle conjugate

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Prostate Specific Antigen (PSA) is a key marker for prostate cancer screening by standard laboratory tests based on blood, plasma or serum analysis. The current laboratory methods for PSA level determination are based on various chromatographic, immunochemical and other instrumental analyses that has to be done in the specialized laboratories. Experiments in this work are focused on construction of a Quartz Crystal Microbalance (QCM) biosensor as a simple device suitable for point-of-care diagnosis based on PSA concentration determination in body fluids. The QCM sensors were covered with antibody against PSA and gold nanoparticles modified with specific antibody against PSA were prepared in a separate step. Change in oscillation frequency was measured as the immunocomplexes QCM biosensor – PSA – modified gold nanoparticles were formed. Calibrating for standard solution of PSA, limit of detection equal to 0.054 μg/l and limit of quantification equal to 0.18 μg/l were figured. The biosensor was not sensitive to interference of other plasma proteins or to matrix effect and fully correlated with the Enzyme-Linked Immunosorbent Assay (ELISA) as a standard method. In a conclusion, the biosensor appears to be a simple and reliable tool that can be used in standard laboratory and point-of-care testing.

Keywords: affinity; antibody; cancer; biosensor; gold nanoparticles; immunosensor; marker; piezoelectric; point-of-care; prostate specific antigen; quartz crystal microbalance

1. INTRODUCTION

Prostate Specific Antigen (PSA) is a key marker for prostate cancer screening by standard laboratory tests. Despite PSA has a limited specificity as a marker, it remains as the major target for the blood screening and prostate cancer diagnosis [1,2] . PSA is typically determined in blood, blood plasma or blood serum and it can occur in the form of free PSA or bound on blood proteins like alpha-1-antichymotrypsin and macroglobulin [3,4]. In the health men, PSA is produced by prostate epithelial

cells and it is responsible for fluidizing of semen and its level in blood is quite low, but the level is increased as it can be also released from prostate cancer cells [5,6]. Level of PSA in blood or blood serum is under 2.5 µg/l in the health men with age under 50 years. The expected concentration is increasing with age. Men in the range of age 50 – 60 years have PSA level up to 3.5 µg/l, the older one has 4.5 µg/l and even more than 6.5 µg/l for age above 70 years. In some works, value 4 µg/l is considered as a threshold for mild risk of cancer and high risk is considered for PSA concnetration above 10 µg/l [7-9]. Percent of PSA bound on the aforementioned proteins correspond with severity of prostate pathologies. While low percent (0 – 15 %) of free PSA from the whole PSA in blood is warning of prostate cancer and prostatic cells become malignant, higher percent (above 20 %) means that there is a benign prostatic hyperplasia.

The current analytical methods for PSA assay are based on various principles. The immunochemical methods like Enzyme-Linked Immunosorbent Assay (ELISA), electrophoresis, chromatography and mass spectrometry can be mentioned as the standard platforms suitable for the PSA determination [10-12]. In the recent years, small bioanalyzers and biosensors are considered as suitable tools for PSA diagnosis [13]. The standard platforms for PSA assay are reliable and exact enough to cover any demands of a hospital but they are not suitable for point-of-care diagnosis. The current effort is focused to fill this gap and offer methods that are sensitive enough to measure physiological levels and easy and cheap enough to be applicable at home conditions at the same time.

This paper is focused on the construction of biosensor that is suitable for the measurement of PSA out of laboratories and even adaptable for point-of-care testing. Piezoelectric sensor platform was chosen and standard 10 MHz Quartz Crystal Microbalances appear to be good physico-chemical transducers for the biosensor's construction because of good sensitivity, ability to easily record affinity interactions, low price of the sensors and measuring devices, and no necessity to apply specific chemicals or manipulate with harmful reagents. This idea is supported by the conclusions from cited papers [14-21].

2. MATERIALS AND METHODS

2.1. QCM biosensor

Standard commercial QCM sensors with circle shaped AT cut quartz crystal with diameter 19 mm, thickness 0.166 mm, and gold electrodes on the opposite sites with diameter 7 mm were bought from company Krystal (Hradec Kralove, Czech Republic). The bought QCM sensors were washed by ethanol and then dried under standard ambient temperature and pressure. The electrodes were modified to construct biosensor and steps described in the following text were made. All incubations were made in a wet chamber. In the first step, 50 µl of cysteamine 50 mg/ml was injected on one electrode and let to incubate five hours followed by washing in deionized water. Application of glutaraldehyde (50 µl, 10 % w/w, solved in water) followed and it was let to incubate for another five hours. The QCM was washed and dried again. After that, anti-PSA monoclonal antibody mouse type (50 µl, concentration 1 µg/ml in phosphate buffered saline pH 7.4 – PBS; purchased from Merck; Kenilworth, New Jersey, United States) was given on an electrode and let incubate for one day. In the last step, bovine serum albumin (Sigma-

Aldrich; Saint Louis, Missouri, United Sites) in a concentration 10 mg/ml was applied in an amount 50 µl and let to incubate for 12 hours. Finally, the newly prepared biosensors were rinsed by PBS and stored in a fridge until use.

2.2. Preparation of conjugate gold nanoparticle-antibody

Spherical gold nanoparticles with diameter 100 nm were purchased from Sigma-Aldrich and they were modified with cysteamine 50 mg/ml: 50 ml cysteamine solution was given per one gram of the spherical nanoparticles, mixed together and let to incubate for five hours. After the incubation, the mixture was centrifuged at 9,000 ×g for 10 minutes, excess of cysteamine was sucked out and the particles washed by deionized water, centrifuged again and then resuspended in 50 ml of 10 % w/w glutaraldehyde and let to incubate for 5 hours. After the incubation, the suspension was washed with water and the particles isolated by centrifugation again. Application of an anti-PSA polyclonal antibody produced in rabbit (Merck) was applied in a solution with volume 10 ml and antibody concentration 100 µg/ml. One day incubation followed and then the suspension was centrifuged, particles resuspended in 100 ml of PBS, shaken, centrifuged and isolated particles were mixed with 50 ml of bovine serum albumin 10 mg/ml and let to incubate for 12 hours. In the final step, the particles were washed with PBS (50 ml), centrifuged, isolated and finally resuspended in 10 ml of PBS and stored in a fridge until use.

2.3. PSA measurement by QCM biosensor

PSA from human semen with purity above 95 % (Merck) was solved in PBS up to demanded concentration. Pure PBS served as a blank and solvent for tested interferents (human serum albumin, human hemoglobin and human immunoglobulin M, all by Sigma-Aldrich).

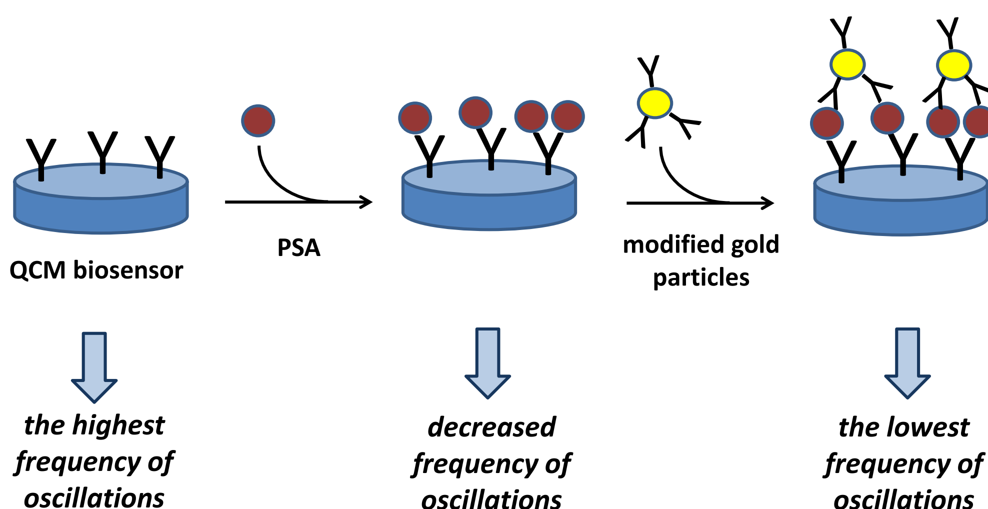

Figure 1. Principle of the PSA assay by QCM biosensor.

Oscillation frequency of QCM biosensors was initiated by ICM Lever Oscillator for 10 MHz QCMs (ICM, Oklahoma City, Oklahoma, USA) that was plugged into frequency counter UZ 2400 (Grundig, Nuremberg, Germany) which served for the actual frequency recording. Oscillation frequency f_0 of every biosensor was measured in the beginning. After that, 50 µl of sample was spread over electrode and let to interact for 20 minutes. In the further step, surface of the biosensor was rinsed with PBS, dried and oscillation frequency f_1 was measured. In the final step, suspension of modified gold particles was intensively shaken and 50 µl of them was applied per one electrode, let to incubate for 20 minutes, rinsed with PBS, dried and oscillation frequency f_2 was measured. The tested samples were measured in pentaplicates. Differences of frequencies $\Delta f_1 = f_1 - f_0$ and $\Delta f_2 = f_2 - f_0$ were calculated for each assay. Principle of the assay is depicted as figure 1.

2.4. ELISA for PSA

Beside assay by biosensor, the samples containing PSA were analyzed by a standard commercially available ELISA kit (Human total PSA ELISA kit; Merck) based on 96 wells microplates. The ELISA kit performance was done in compliance with instructions provided by the kit manufacturer. The manufacturer guarantee sensitivity 8 pg/ml and applicable range 10 – 2500 ng/l.

3. RESULTS AND DISCUSSION

The biosensor was tested for the detection of PSA in a concentration range 0 (pure PBS), 0.0244, 0.0488, 0.0977, 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25.0 and 50.0 µg/l which means two-fold dilution raw starting the upper concentration (50.0 µg/l). The chosen calibration solutions covered all expected physiological and typical pathological concentrations of PSA described in the introduction section. When used antibody modified gold nanoparticles and figuring of Δf_2, limit of detection and limit of quantification were calculated according rule signal to noise ration S/N equal to three (limit of detection) respective ten (limit of quantification). Using the calibration plot, limit of detection equal to 0.054 µg/l and limit of quantification equal to 0.18 µg/l were figured out. The achieved calibration curve is depicted as figure 2. It is important that the limits of detection and quantification for the PSA assay by the QCM biosensor are deeply under the threshold limit for men with age under 50 years: 2.5 µg/l which is the lowest plasmatic level that can be considered as the warning one. The other threshold limits (other age groups) are above the 2.5 µg/l and still in the range of calibration curve. Beside the use of gold nanoparticles, the biosensor was also used in a label free mode and calculated Δf_1 was considered as the final signal. Unfortunately, such approach was not successful and limit of detection was quite high (approximately 55 µg/l) which make the label free mode unsuitable for clinical testing as the limit of detection was higher than the expected physiological concentrations of PSA. Just the use of gold nanoparticles modified with antibody make the QCM biosensor applicable for clinical use.

Interference tests were performed in order to judge possible false positivity of the results. Human serum albumin solved in PBS up to the concentration 50 g/l, human hemoglobin in PBS in the

concentration 50 g/l, human immunoglobulin M solved in PBS up to the concentration 50 g/l and pure PBS were separately analyzed in five repats like the other samples. PSA in the concentration 6.25 µg/l served as a positive control for the assay. Results from the interference testing are depicted in figure 3. The signal provided by PBS was not significantly differing (P = 0.05; ANOVA test) from the signal provided by the human serum albumin 50 g/l, human hemoglobin 50 g/l and human immunoglobulin M 50 g/l.

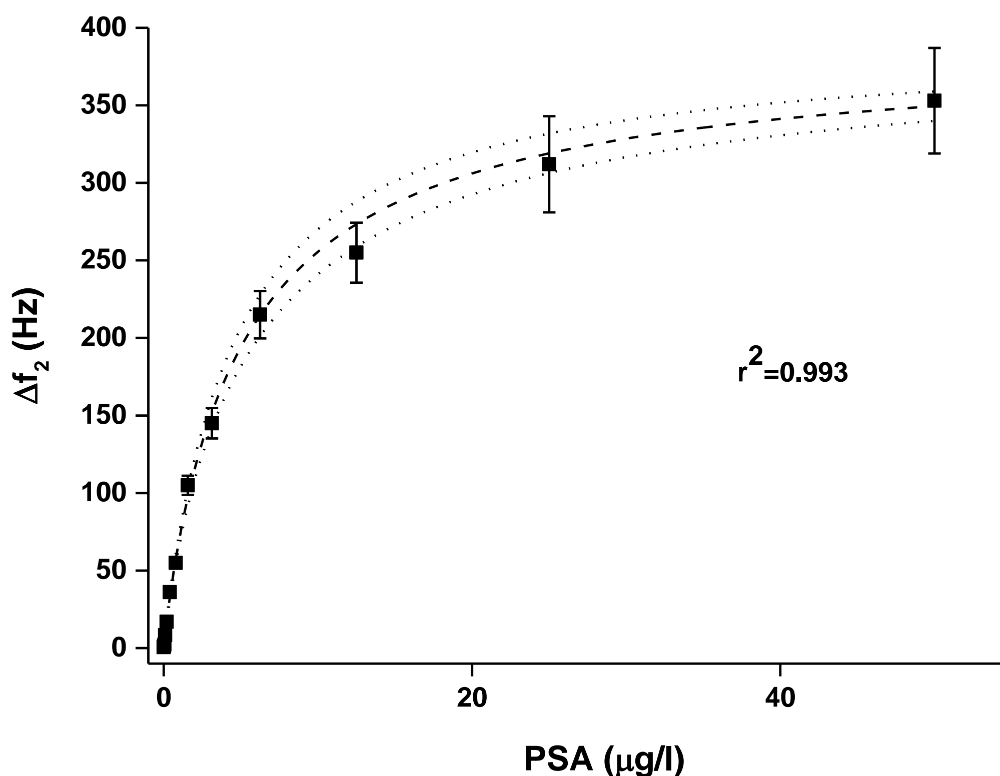

Figure 2. Calibration curve for PSA using QCM biosensor. Error bars indicate standard error of mean for five repeated measurements, dotted lines delimit confidence interval for P = 0.05.

Contrary, all the tested interferents and signal by pure PBS was significantly (P = 0.01; ANOVA test) differing to the signals provided assay of sample containing PSA in concentration 6.25 µg/l. The interference testing can be concluded by a statement that the QCM biosensor does not tend to provide false positive results and can be indicated as a reliable assay. The chosen concentrations of human serum albumin, human hemoglobin and immunoglobulin M are above expected physiological levels in blood plasma or serum hence no interference means that there is not expected an interference due to a pathological process related to this basic plasma and blood proteins.

The assay of PSA by QCM biosensor was validated to standard ELISA. The same samples used for calibration purpose were measured by the both QCM biosensor and ELISA kit. Results from the validation experiment are depicted as figure 4. The methods mutually correlated and reached coefficient of determination was equal to 0.996 which can be concluded by a statement that the assay by QCM biosensor provided comparable results like the ELISA kit. On the other hand, assay by QCM biosensor

is much easier as only one reagent (modified nanoparticles) was added beside the sample and the whole assay by biosensor was finished within approximately one hour while ELISA assay was more complicated, instable reagents (hydrogen peroxide) were necessary and the whole assay taken one day.

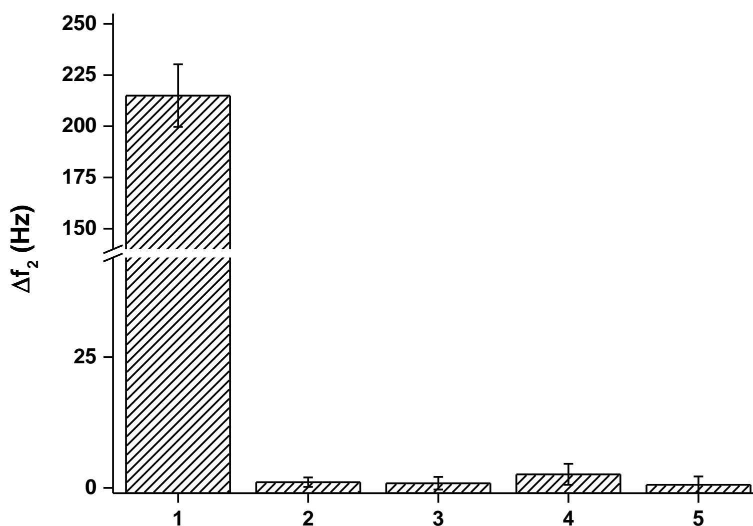

Figure 3. Interference testing for assay by QCM biosensor. Column 1: PSA (analyte) 6.25 µg/l, column 2: human serum albumin 50 g/l, column 3: human hemoglobin 50 g/l, column 4: human immunoglobulin M 50 g/l, column 5: pure solvent (PSB). Error bars indicate standard error of mean for five repeated measurements.

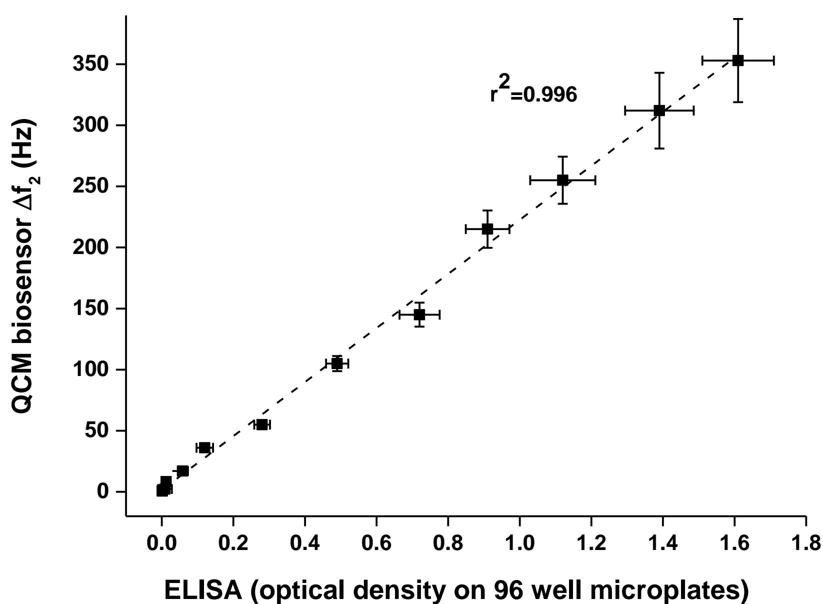

Figure 4. Validation of assay by QCM biosensor to standard ELISA. The same samples of PSA were used for validation purpose like for the calibration. Error bars indicate standard error of mean for five repeated measurements.

Matrix effect was searched testing standard female pig plasma spiked with human PSA. Three final concentrations of PSA were achieved by the spiking into pig plasma: 1.56, 3.13 and 6.25 µg/l and the prepared samples were analyzed by ELISA and QCM biosensor and signals were recalculated to the concentration using the aforementioned calibration plots. Results from the matrix effect testing are depicted as figure 5. The concentrations found by QCM biosensor and ELISA were mutually insignificant (P = 0.01; ANOVA). It appears that matrix effect by basic body fluids like plasma is not a problem and the QCM biosensor provides nearly the same results like the standard ELISA.

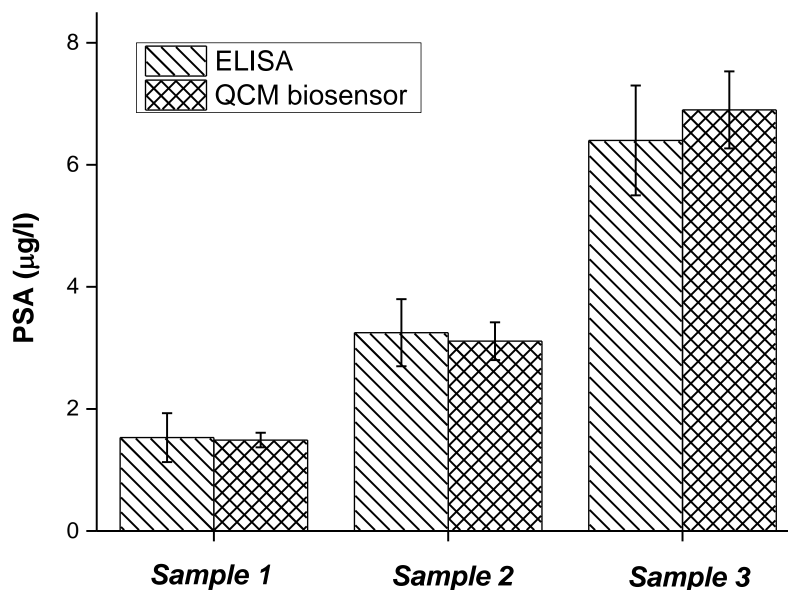

Figure 5. Testing of QCM biosensor and ELISA for pig plasma spiked with human PSA. Error bars indicate standard error of mean for five repeated measurement.

The effort to construct a functional biosensor for PSA correspond with the current directions in the both basic and applied research for developing of new tools for point-of-care diagnosis and related techniques. Other types of biosensors are known from the recent literature. Every of them has its pros and cons and deserves attention. Electrochemical impedance immunosensor exerting limit of detection for PSA 0.01 ng/ml [22], photoelectrochemical fuel cell aptasensor with limit of detection 1.3 pg/ml [23], electrochemical immunosensor based on glassy carbon electrode with limit of detection for PSA 0.5 pg/ml [24], and iron oxide nanoparticles based electrochemical sensor with limit of detection 1.9 pg/ml [25] can be exampled as the recently introduced biosensors. The exerted sensitivity is comparable or slightly better than the here presented biosensor; on the other hand, the here presented biosensor is sensitive enough to prove PSA highly under threshold limit. Overall simplicity of the assay, low price of reagents and reliability of the biosensor to prove PSA in plasma samples are major advantages of the introduced QCM biosensor. Survey of basic specifications for PSA assay by the presented QCM biosensor is given in table 1. The proposal of make a PSA biosensor correspond with the general principle to introduce new types of devices for a point-of-care diagnosis of various pathological states or detection of pathological factors [26-33].

Table 1. Basic specifications of QCM biosensor for PSA assay

Limit of detection for PSA	0.054 µg/l (54 pg/ml)
Limit of quantification for PSA	0.18 µg/l (0.18 ng/ml)
Time per one assay (including incubation and drying)	1 hour
Coefficient of determination (r^2) for correlation to ELISA method	0.996
Interference by human serum albumin, human hemoglobin, human immunoglobulin M	not significant for expected physiological concentrations (up to at least 50 g/l)

4. CONCLUSION

Point-of-care tests for fast diagnoses of various pathologies are highly demanded and the current research on them is progressively developing. The presented QCM biosensor represents such device and reflect demands on point-of-care tests. The biosensor is sensitive enough to cover expected physiological levels of PSA and simple for manufacturing and practical performance outside of laboratories. Reliability of the biosensor make it resistant to interferences by the other proteins or matrix effect. Though the biosensor cannot be performed in a label free mode because of necessity to use the label to reach physiological concentrations, the measuring protocol is still simple to be suitable for unskilled workers. All these facts make the QCM biosensor a highly competitive analytical device.

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