

Short Communication

Piezoelectric Immunosensor for the Determination of C-Reactive Protein

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C-reactive protein (CRP) is a common biomarker of inflammatory processes and it typically serves for the distinguishing of bacterial and viral diseases. It is typically measured in the blood, plasma or serum by immunoassay like agglutination or enzymatic immunoassays. In this paper, a new immunoassay based on a piezoelectric biosensor was introduced. QCM with basic frequency of oscillations 10 MHz and gold electrodes served as a platform and it was modified with antibodies against CRP and silica nanoparticles. Separately, antibody functionalized silica nanoparticles were prepared and they further served for signal amplification. CRP was assayed with limit of detection equal to 0.080 mg/l. The assay was successfully validated to standard ELISA method and no significant interference by IgM, TNF α and albumin was observed. The biosensor-based assay appears to be practically applicable and can serve for routine diagnosis purpose.

Keywords: affinity; antibody; biosensor; CRP; C-reactive protein; immunosensor; infection; inflammation; label free assay; liver; piezoelectric; quartz crystal microbalance

1. INTRODUCTION

CRP is a well-known biomarker indicating inflammatory processes in the human body and it is secreted from liver to blood stream as a consequence of innate immunity initiation. It is typically increased because of bacterial infections and it well correlates with the other inflammatory markers typical for these diseases [1]. Beside bacterial infections, exposure to some toxins and hormonal disruptors [2], development of pancreatitis and liver injury [3] and some types of tumor [4] can cause initiation of CRP release. However, the most typical role of CRP in the general medicine is to distinguish between bacterial and viral infections and the increased CRP is an indicator for antibiotics prescription [5-7].

In the current analytical praxis, CRP is measured by a wide spectrum of immunochemical assays which selection depends on required number of samples which have to be analyzed, experience and

education of laboratory staff and equipment of the laboratory. Enzyme-Linked Immunosorbent Assay (ELISA) and various agglutination tests are the common tools for CRP detection in the current praxis. More advanced immunotests like lateral flow assay using quantum dots labeled antibodies [8], immunotests based on functionalized hydrogel microparticles [9] and impedance biosensors [10] can be exemplified as the recent innovations for CRP diagnostics.

Piezoelectric biosensors are a specific platform suitable for recording of affinity interactions and the assay can be arranged as a label free which means that interaction between biosensor surface and the analyte is recorded directly without application of any specific reagent or sample processing. Quartz is the typical material having piezoelectric properties which means ability to generate potential when mechanically squeezed or mechanically deformed when oriented dipole given on crystal surface [11,12]. For the purpose of biosensors and similar analytical devices construction, quartz in the form of Quartz Crystal Microbalance (QCM) appears as the most common sensor platform [13-15]. In some examples, the QCM biosensors were found to be effective for assay of thrombin [16], *Salmonella typhimurium* [17], and recording of cell surface interactions [18]. This manuscript is devoted to construction of a biosensor suitable for a simple diagnostic of inflammatory processes where a layer sensitive to CRP is located directly on a QCM. This approach would be easier to be performed in home care conditions or outside specialized laboratories but it is expected that it will keep similar analytical properties like the common method like ELISA.

2. MATERIALS AND METHODS

2.1. Manufacturing of biosensor

QCM with basic frequency of oscillations 10 MHz, two circled gold 7 mm sized electrodes on the opposite sites, quartz disc diameter 19 mm and thickness 0.166 mm were purchased from Krystalý (Hradec Kralove, Czech Republic). Prior to further processing the new QCM were washed by rinsing with pure ethanol (96 % v/v) and drying under laboratory conditions with standard ambient temperature and pressure (25 °C, 100 kPa, approximate humidity 50 %) and no direct sun light into the space where the biosensors were prepared. The drying procedure lasting approximately 2 hours under the conditions. Immobilization of antibody specific to CRP done in a wet chamber protecting from a premature desiccation followed. Particular steps of antibody immobilization are written here:

- 50 µl of cysteamine diluted in deionized water in concentration 50 mg/ml was applied per one electrode and let to incubate for five hours;
- electrodes were rinsed with deionized water and dried;
- 50 µl of glutaraldehyde 10 % w/w in deionized water was given per an electrode and let to incubate for five hours;
- electrodes were rinsed with deionized water and dried;
- protein A (Sigma-Aldrich; St. Louis, MO, USA) 1 mg/ml solved in phosphate buffered saline pH 7.4 (PBS) and applied in amount 50 µl was let to bind on the activated electrode surface for 12 hours
- electrodes were rinsed with PBS and dried;

- monoclonal mouse type IgG1 antibody against human type of CRP (Sigma-Aldrich) in concentration 0.4 $\mu\text{g}/\text{ml}$ in PBS was spread over electrodes in an amount 50 μl (total amount 0.02 μg per one electrode) and let to incubate for 12 hours;
- electrodes were rinsed with PBS and dried;
- mixture of 3-aminopropyl functionalized silica nanoparticles (Sigma-Aldrich) in concentration 3 % w/w in ethanol with glutaraldehyde 20 % w/w in PBS were poured together in a ratio 1:1 and the mixture was spread over electrode in an amount 50 μl , five-hour lasting incubation followed;
- electrodes were rinsed with PBS and dried;
- the freshly prepared biosensors were stored in a fridge with temperature 4 $^{\circ}\text{C}$.

2.2. Manufacturing of modified silica nanoparticles modified with antibody against CRP

3-aminopropyl functionalized silica nanoparticles 3 % w/w in ethanol were poured with glutaraldehyde 30 % w/w in PBS and polyclonal antibody against human CRP produced in rabbits (Sigma-Aldrich) diluted up 1 $\mu\text{g}/\text{ml}$ in a volume ratio 1:1:1. The mixture was gently shaken on vortex and let to react at laboratory temperature for 12 hours. After that, the solution was dialyzed using membrane tubes placed into fresh PBS. The silica nanoparticles modified with antibody (further modified nanoparticles) were stored in a fridge with temperature 4 $^{\circ}\text{C}$ until use.

2.3. Assay by piezoelectric biosensor

Human type recombinant CRP prepared from *Escherichia coli* (Sigma-Aldrich) was solved in PBS in a two serial fold dilution: 0, 0.0488, 0.0977, 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 100 mg/l (concentration 0 indicates pure PBS) and the solutions served as a sample for analysis. Frequency of oscillations was measured using ICM Level Oscillator 10.000 MHz (ICM, Oklahoma City, OK, USA) and frequency counter UZ 2400 (Grundig, Nuremberg, Germany). Oscillations were measured after sensor surface drying in laboratory conditions under standard ambient temperature and pressure (25 $^{\circ}\text{C}$, 100 kPa, approximate humidity 50 %). Measurement by the biosensor consisted from following steps:

- initial frequency of oscillations f_0 by a new biosensor was measured;
- 50 μl of a sample was applied per one electrode and let to incubate 20 minutes;
- biosensor was rinsed with PBS and dried;
- oscillation frequency f_1 was measured;
- 50 μl of a modified nanoparticle solution was given per one electrode and let to incubate 20 minutes;
- biosensor was rinsed with PBS and dried;
- oscillation frequency f_2 was measured.

The principle of the biosensor function depicted as a graphical abstract can be learned from figure

1.

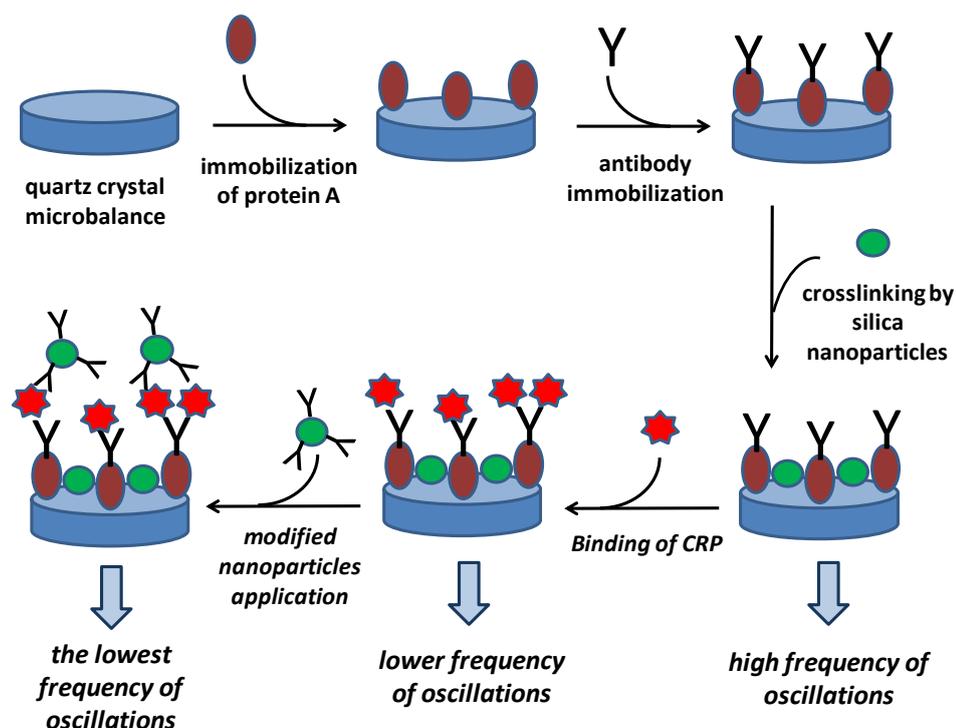


Figure 1. Principle of CRP assay by the proposed biosensor.

2.4. Reference assay of CRP

Standard ELISA assay kit for CRP was purchased from Abcam (Cambridge Biomedical Campus, Cambridge, United Kingdom) and the kit was processed in compliance with provided protocol. The same samples of CRP like used in the biosensor characterization were also tested by this ELISA kit.

2.5. Data processing

Every sample was measured five times and mean with standard error of mean were calculated. Frequency change was calculated for the both direct assay without use of modified nanoparticles: $\Delta f_1 = f_1 - f_0$ and for the use of modified nanoparticles: $\Delta f_2 = f_2 - f_0$.

Graphs were done in statistical software OriginPro 9.1 (OriginLab Corporation, Northampton, MA, USA). The rule that signal to noise ratio is equal to three ($S/N = 3$) served for limit of detection calculation, standard deviation of control assay was considered as the noise. Analysis of Variance (ANOVA) on probability levels $P = 0.05$ and $P = 0.01$ served for statistical comparison between sets of experimental data.

3. RESULTS AND DISCUSSION

The biosensor was performed for CRP assay using the samples prepared in calibration scale and the both Δf_1 and Δf_2 were determined. The chosen calibration range covered the expected CRP levels in health people and people suffering from inflammatory diseases. While health individuals have CRP level typically under 1 mg/l, people with suspected diseases associated with inflammation have CRP above 3 mg/l, the range of CRP above 1 mg/l and under 3 mg/l is considered as a risk factor [19-22]. Bacterial infectious diseases typically cause elevation of CRP above 10 mg/l [23]. When the Δf_1 used for calibration, limit of detection 41 mg/l was reached which is above physiological level for CRP and such method has no practical relevance. However, application of modified nanoparticles caused that the limit of detection was equal to 0.080 mg/l (80 ng/ml) which is significantly under expected physiological range and therefore suitable for clinical praxis. The calibration curve obtained by use of the both biosensor and modified nanoparticles is depicted as figure 2.

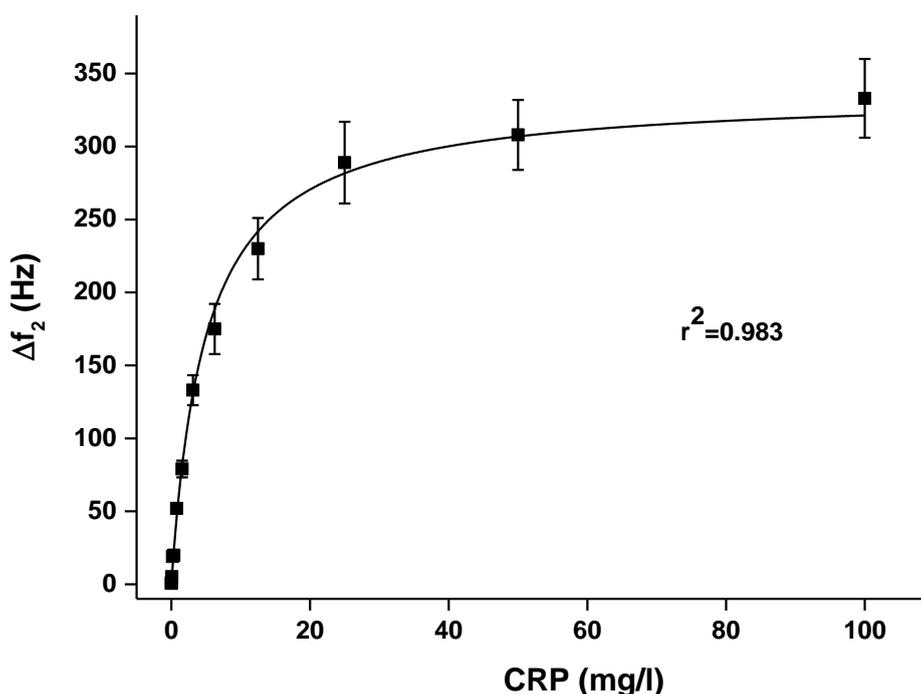


Figure 2. Calibration of CRP using piezoelectric biosensor in combination with modified nanoparticles as a label.

The calibration curve well covered expected physiological levels of CRP and the flat part of the curve was achieved for the CRP concentration which undoubtedly confirms inflammatory processes i.e. the level above 10 mg/ml. The exerted limit of detection was above the recently developed biosensors working on the principle of microfluidic chip with limit of detection 3.2 ng/ml [24], interferometric biosensor with limit of detection 2.1 ng/ml [25]; on the other hand, overall simplicity and low cost for manufacturing are the major advantages of the here described piezoelectric biosensor. The achieved limit

of detection is also lower than demanded for clinical praxis which make the piezoelectric immunosensor a promising diagnostic tool.

Interference testing was performed as another important parameter characterizing an analytical method. Tumor necrosis factor α (TNF α), human serum albumin and human type of immunoglobulin M (IgM) were chosen as potential protein interferents because they are widely presented in blood plasma and are relevant as immunity markers respectively have potency to widely interact with other structures. Concentration of the potentially interfering proteins was set on values exceeding expected physiological levels. All the interferents were solved in PBS, albumin in concentration 100 mg/ml, IgM in the concentration 50 mg/ml and TNF α 100 ng/ml. Bar graph representing the interferences in comparison with signal for CRP in concentration 12.5 mg/l (possible level under bacterial infectious) and PBS is shown as figure 3. In this experiment, silica nanoparticles served as a blocking agent so the potency to stop nonspecific interaction is due to the protecting from interferents penetration to the electrode and no ability to interact with it.

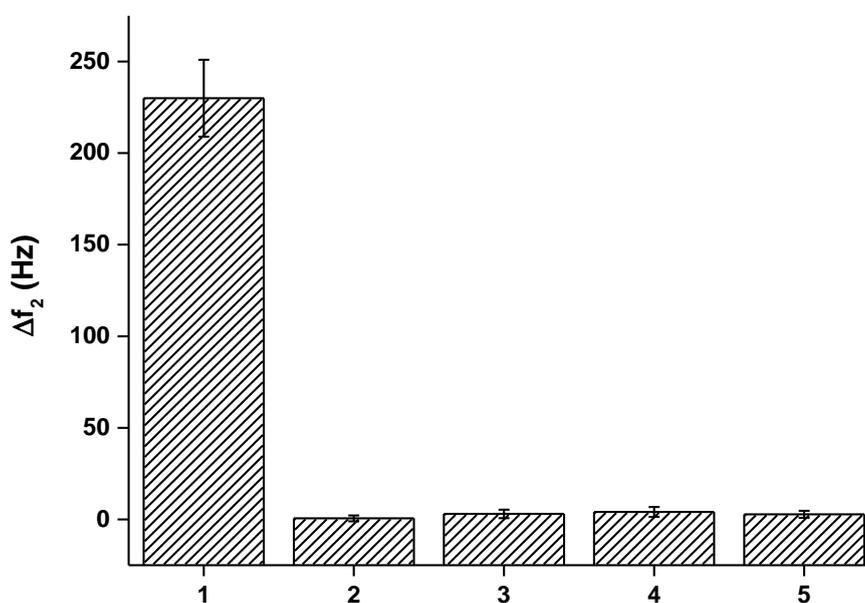


Figure 3. Bar graph describing effect of potential interferents albumin 100 mg/ml (bar 3), IgM 50 mg/ml (bar 4) and TNF α 100 ng/ml (bar 5) on biosensor serving for CRP assay. CRP in concentration 12.5 mg/l (bar 1) and pure PBS (bar 2) are given for comparison.

When compared the Δf_2 value for blank (PBS) with the potentially interfering compounds, they were mutually insignificant by ANOVA test. The value Δf_2 was significantly differing from the results achieved from assay of samples containing the potentially interfering compounds on probability level $P = 0.01$. The experiment on interference testing can be concluded by a statement that the biosensor is selective for given analyte. The selectivity is conditioned by the used antibodies which are crucial in this

regard and they are responsible for the affinity interaction. Replacement of the used antibodies by a less selective analogue can cause decrease of biosensor selectivity.

The samples used for calibration of biosensor were also measured by standard ELISA and the both assays were mutually correlated (figure 4). The biosensor assay exerted coefficient of determination 0.956 when compared to the ELISA. The both assays had approximately the same sensitivity to CRP. When compared the two methods, the biosensor represents the easier way how to analyze CRP because the whole assay can be finished within less than one hour with a use of limited number of reagents. Nevertheless, analytical parameters are not worse than the one for ELISA. Expected price for biosensor construction is also close to the cost per one assay by ELISA. Four real samples from human volunteers were also analyzed by the both ELISA and immunosensor (figure 5). The data also confirmed agreement between the both methods, the results from one method was insignificant to the results from the other. It is another confirmation of the immunosensor plausibility to determine CRP level.

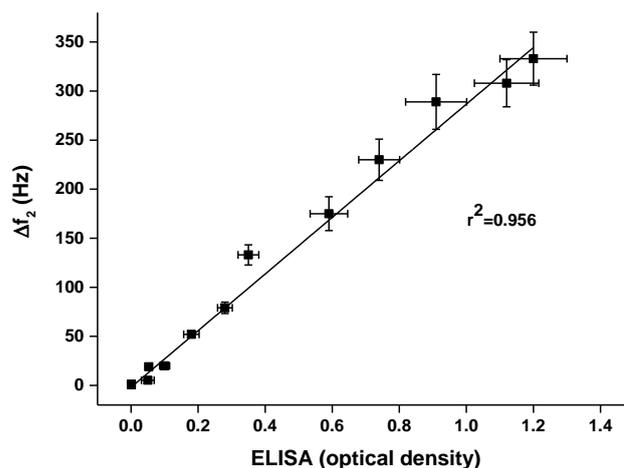


Figure 4. CRP assay by biosensor v to ELISA. The both methods were performed to assay of CRP solution in a range 0 – 100 mg/l.

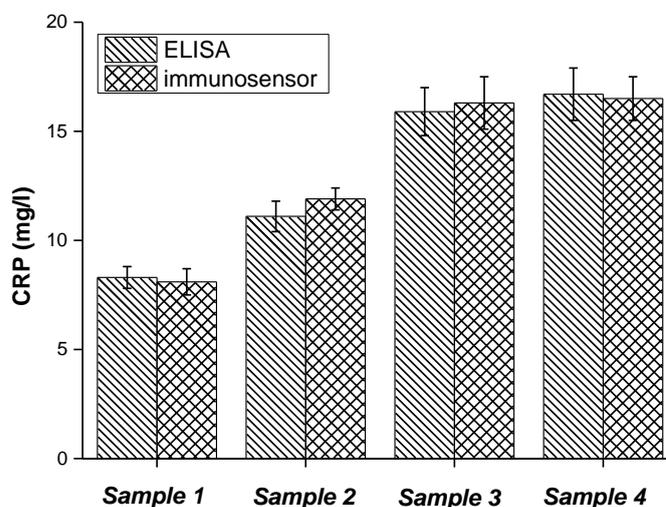


Figure 5. Testing of four real human samples by ELISA and by the piezoelectric immunosensor.

The constructed biosensor was compared with other devices belonging to the group of small analytical tools. Considering the recent research, biosensors and bioassays working on the principle of electrolyte-gated organic thin-film transistor [26], impedance measurement [27], lateral-flow chromatography [28] and colorimetry [29] can be introduced as other devices suitable for an assay outside equipped laboratories and these biosensors had limit of detection under the biosensor presented here. The biosensor described in this work is, however, a promising tool in the regard of practical applicability and simplicity for manufacturing and assay performance. A short survey of analytical parameters for the presented biosensor is provided in table 1.

Table 1. Survey of analytical parameters

Limit of detection for CRP	0.080 mg/l
Correlation between biosensor assay and ELISA (r^2)	$r^2=0.956$
Interference by albumin, IgM, TNF α	not significant

4. CONCLUSION

Simple tools for analytical measurement of pathological markers in body fluids are highly desired and they are highly actual for home care conditions, examination of markers in small labs and small hospitals and field hospitals organized by a military or international health institutions. The biosensor presented here fill this demand because it is suitable for an easy measurement of CRP in biological samples with no demand on specific manipulation or pretreatment. The assay is the both quite easy and cheap. In this experiment, silica nanoparticles used as a blocking reagent and contemporary as a material amplifying frequency change were used. The antibodies on electrode surface were properly oriented due to use of protein A. The whole assay device consisting from the biosensor, oscillation circuit and frequency counter is also low cost and portable. Despite the overall simplicity and low cost, the assay provides results similar to the standard laboratory ELISA.

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