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Immunoassay of interferon gamma by quartz crystal microbalance biosensor

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ABSTRACT

Interferon gamma (IFNγ) is a cytokine and an immunochemical marker that can be used for revealing of infectious diseases and especially for distinguishing of viral and some types of bacterial infections. Blood tests for IFNγ are typically based on immunoassays like Enzyme-Linked Immunosorbent Assay (ELISA). In this paper, a biosensor working on the principle of quartz crystal microbalance (QCM) was developed as an alternative to the standard analytical methods for IFNγ. The biosensor contained antibodies against IFNγ immobilized on QCM and also on gold nanoparticles. A sandwich containing QCM, gold nanoparticles and IFNγ was formed and formation of the sandwich caused decrease of oscillation frequency. The assay exerted limit of detection 5.7 pg/ml for a sample sized 50 μl and one measuring cycle was finished within 90 min. The assay by biosensor fully correlated to standard ELISA. In a conclusion, the biosensor appears to be a fully applicable analytical tool for a simple assay of IFNγ. Overall simplicity and no special requirement on staff and equipment are the major advantages of the here presented assay.

1. Introduction

Interferon gamma (IFNγ) is a cytokine playing an important role in the both innate and adaptive immunity and it is also a necessary part of protective mechanisms against infections by viruses and bacteria. It is produced by Tc lymphocytes and Natural killer cells and it is effecting on macrophages and lymphocytes, it initiates targeted migration of leukocytes and also stimulates inducible nitric oxide synthase expression and block replication of viruses [1–6]. Immunity response based on IFNγ is typically necessary for resolving of infectious diseases caused by viruses and intracellularly growing bacteria. IFNγ interacts with interferon gamma receptor which is a heterodimer of two chains located in cellular membranes with salient intra and extracellular parts [7,8].

Increase of IFNγ in blood, blood plasma or serum can point to various pathologies including infectious diseases like viral, some bacterial diseases (especially the intracellular parasites) and some types of cancer [9–14]. In the blood from healthy people, IFNγ concentration is quite low and it can be even hard to detect it by some methods. Normal physiological level of IFNγ in blood serum or blood plasma from healthy individuals is approximately in the range 7–124 pg/ml. In a work by Liu and coworkers, IFNγ level in blood serum of healthy people was 136 pg/ml and 172 pg/ml in the cerebrospinal fluid [15]. The authors compared the level with patients suffered from amyotrophic lateral sclerosis having IFNγ level 281 pg/ml in the serum and 349 pg/ml in the cerebrospinal fluid.

IFNγ level in biological fluids can be measured by various methods but the immunochemical one like Enzyme-Linked Immunosorbent Spot (ELISPOT) or Enzyme-Linked Immunosorbent Assay (ELISA) are probably the most common [16–19]. In this work, piezoelectric biosensor based on quartz crystal microbalance (QCM) sensor is developed as a platform enabling a fast, simple and economically affordable assay for IFNγ able to compete with the standard methods but to be more convenient for application in field or homecare conditions because of portability and minimal processing of a sample. The decision to construct the biosensor on QCM sensor platform was initiated by promising specifications of the platform like direct recording of affinity interactions on their surface, low price of sensors and instrumentation, and high sensitivity [20–23]. The fact that piezoelectric biosensors work as gravimetric analysis make them quite unique comparing to other types of biosensors. General availability of QCM suitable for mass production and easy organization of gravimetric analysis by recording of oscillation frequency was recognized as a promising factors in various scientific reports and summarized in review articles [24–29]. In order to construct a biosensor, a biorecognition part should be attached to the sensor platform. Covalent immobilization of an antibody on the gold surface by self-assembled monolayer technique was chosen as an applicable protocol to prepare biosensor [30–33].

2. Material and methods

2.1. Biosensor construction

Standard 10 MHz QCM sensors with overall diameter 20 mm, thickness 0.166 mm and circled 7 mm gold electrodes on the opposite

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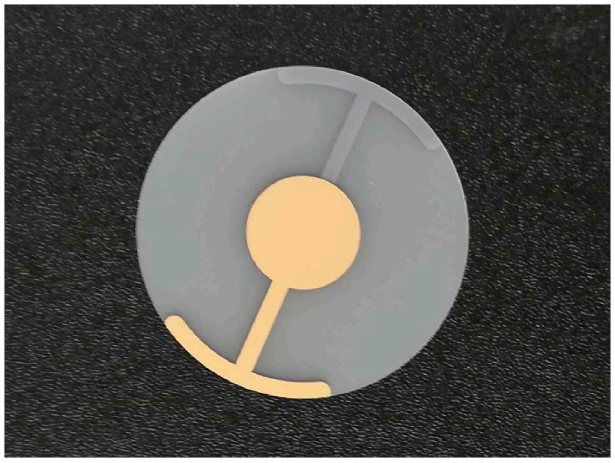

Fig. 1. QCM sensor with frequency of oscillation 10 MHz and gold electrodes on the opposite sides used for biosensor preparation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sides were bought from manufacturer Krystaly (Hradec Kralove, Czech Republic). Appearance of the used QCM sensor is perceptible from Fig. 1. The here described immobilization procedure is based on the previously published experiments [21,34]. The new sensors were placed into bath with 96% v/v ethanol for 1 h and then dried under laboratory conditions. The gold surface was activated by application of 50 mg/ml cysteamine in a total volume 50 μl per one electrode. The cysteamine solution was left to react with the surface for 5 h and washed by deionized water after this interval. After that, 5% w/w glutaraldehyde solved in water was given in an amount 50 μl per one electrode and was let to interact with the surface for another 5 h. The unreacted solution of glutaraldehyde was washed out by deionized water and let to dry. In the next step, monoclonal rat antibody against human IFNγ of IgG2a isotype produced in mouse (Sigma-Aldrich, St. Louis, MO, USA) was diluted in phosphate buffered saline pH 7.4 to a concentration 1 mg/ml and given on electrode in a volume 50 μl. The electrodes were rinsed by phosphate buffered saline pH 7.4 after 12 h of incubation. In the final step, gelatin 10 mg/ml in phosphate buffered saline pH 7.4 was spread over one electrode for another 12 h. The prepared biosensors were rinsed with phosphate buffered saline pH 7.4 with 0.1% w/w tween, then by phosphate buffered saline pH 7.4 and let to dry. Apart of drying, the aforementioned protocol was done in a wet box protecting from premature desiccation of reagents.

2.2. Particles modification

Spherical gold nanoparticles with size < 100 nm (Sigma-Aldrich) were used for the experiment purpose. 1 g of the particles was suspended in cysteamine 50 mg/ml with total volume 10 ml and let to incubate for 5 h. After that, the suspension was centrifuged at 9000 × *g* for 5 min, supernatant was removed by syringe with filter, the nanoparticles were resuspended in deionized water 10 ml, centrifuged again and then resuspended in 10 ml of 5% w/w glutaraldehyde for 5 h. Removing of glutaraldehyde and washing by deionized water followed. Centrifugation at 9000 × *g* for 5 min was placed between these steps. Finally, monoclonal mouse antibody against human IFNγ of IgG2a type produced in rat (Sigma-Aldrich) diluted in phosphate buffered saline pH 7.4 to a concentration 1 mg/ml was injected into the batch with nanoparticles in an amount 10 ml and let to incubate for 12 h. After incubation, the nanoparticles were isolated by centrifugation, washed by phosphate buffered saline pH 7.4 and bovine albumin 10 mg/ml in an amount 10 ml was given into the tube with nanoparticles and incubated for 12 h. In the final step, the particles were separated by centrifugation, washed by phosphate buffered saline pH 7.4 with 0.1% w/w tween, then by phosphate buffered saline pH 7.4 and finally resuspended and kept in phosphate buffered saline pH 7.4. The suspension was intensively shaken before further application. General principle of antibody immobilization on gold surface respective nanoparticle is depicted as Fig. 2.

2.3. Assay by biosensor

The oscillation frequency f_1 of each biosensor was measured in the beginning of assay. ICM Level Oscillator 10.000 MHz (ICM, Oklahoma City, OK, USA) and frequency counter UZ 2400 (Grundig, Nuremberg, Germany) served for the purpose. After that, a sample with volume 50 μl was spread over electrode and let to incubate for 30 min and after that 50 μl of modified particles suspension was added for another 30 min. Finally, the surface of biosensor was washed by deionized water, let to dry and oscillation frequency f_2 was measured. Difference $\Delta f = f_1 - f_2$ was calculated for each measurement. Limit of detection was calculated according the rule signal to noise ratio equal to three: S/N = 3. Principle of the IFNγ assay by biosensor can be learned from Fig. 3.

2.4. ELISA

Standard ELISA on 96 well microplate was performed as an assay for the purpose of biosensor validation. “Human IFN gamma ELISA Kit” by

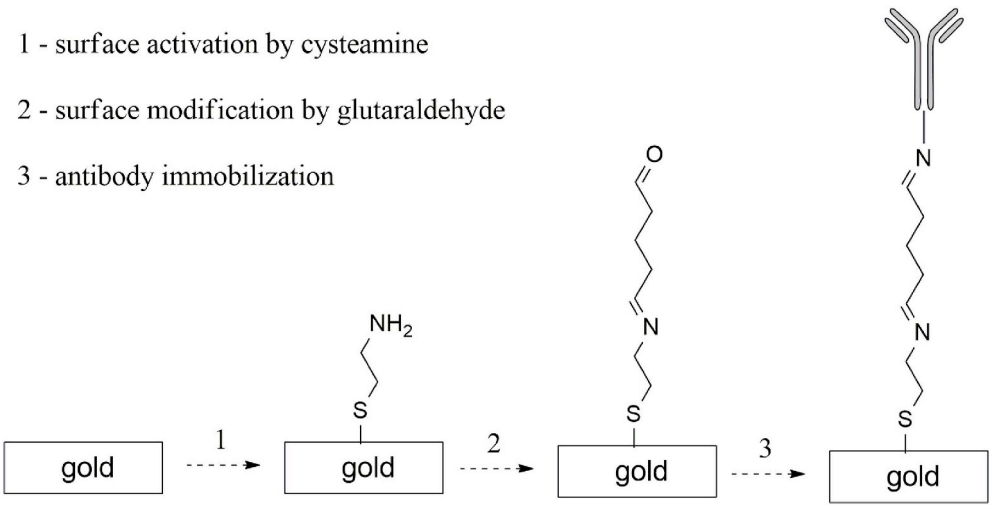

Fig. 2. General principle of antibody immobilization on gold electrode respective gold nanoparticles by cysteamine and glutaraldehyde.

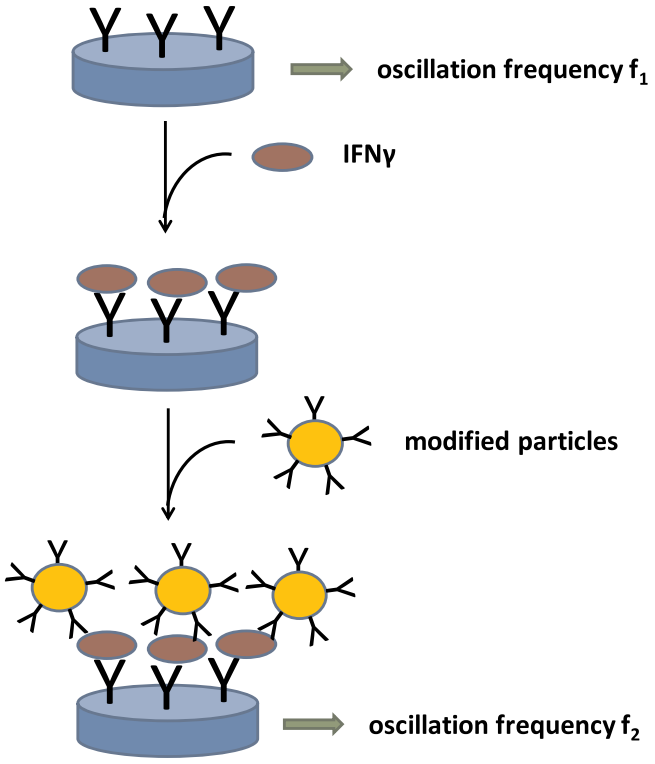

Fig. 3. Principle of IFNγ assay by biosensor using modified particles.

AbCam (Cambridge, United Kingdom) was chosen for the purpose. The kit was used in compliance with attached protocol. On assay consumed a sample sized 100 μl. The assays were made in five repeats.

3. Results and discussion

In the beginning of experiments, optimization of the immobilized antibody on gold nanoparticles and biosensor was performed in order to achieve the maximal signal with minimal costs per one constructed biosensors. The optimization for gold particles is depicted in Fig. 4. In a total five concentrations of antibody were tested: 0.04, 0.2, 1, 5 and 25 mg/ml. It is obvious that the concentrations of 0.04 and 0.2 mg/ml were too low to achieve maximal signal, increasing of concentration improved detected signal. No further improvement was achieved when concentration of antibody is higher than 1 mg/ml. The optimal concentration of antibody was considered to be just the 1 mg/ml. Similar optimization was performed for antibody immobilization on surface of QCM. In this experiment, no effect was observed which can be inferred that the lower concentration of antibody than 1 mg/ml would be enough. However, the concentration 1 mg/ml was kept for the next experiments in order to work with the same solution in immobilization of QCM and on gold nanoparticles. The volume applied on the electrode (50 μl) cannot be lowered because the smaller drop is too small to easily cover the whole electrode.

The biosensors were calibrated for standard solution of IFNγ. A solution of IFNγ in concentration 500 ng/ml was diluted by phosphate buffered saline in a two-fold dilution scale. Pure phosphate buffered saline pH 7.4 served for blank purpose. Calibration plot is depicted as Fig. 4. The calibration exerted coefficient of determination equal to 0.973 and the calculated limit of detection was equal to 5.7 pg/ml. As mentioned in the text before, healthy people have IFNγ in blood serum around 136 pg/ml [15]. Considering it, the assay by biosensor is suitable for testing of blood, blood plasma or serum samples with sufficient sensitivity. The biosensor can be also used for IFNγ assay in a label free mode, it means that the modified gold nanoparticles are not applied. Tough the assay in label free mode works well, avoiding of use of gold

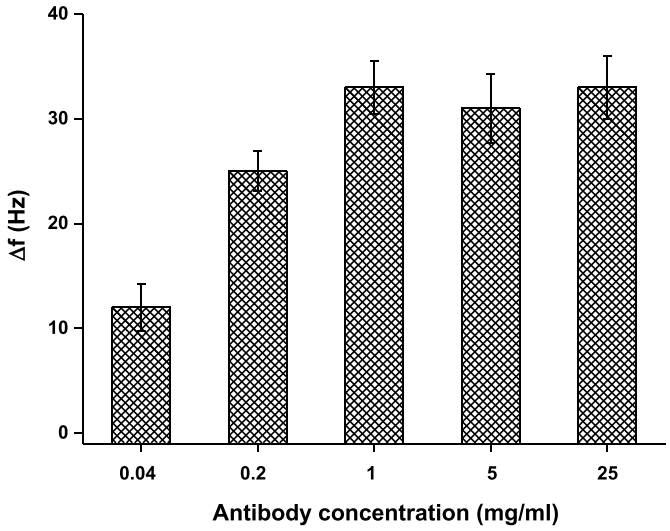

Fig. 4. Optimization of antibody concentration for preparation of modified gold particles. The biosensor and modified nanoparticles were performed for assay of a sample containing IFNγ in concentration 122 pg/ml.

nanoparticles causes significant worsening of limit of detection. The label free mode is simple in its principle and number of steps; on the other hand, it had limit of detection 263 μg/ml that is significantly worse than limit of detection of the aforementioned assay with the modified gold nanoparticles. Moreover, the limit of detection is not low enough to cover expected physiological concentrations of IFNγ. The biosensor assay with the use of modified gold nanoparticles is selected for the next experiments.

The biosensor was validated to the standard ELISA as a reference method. The same samples were used for assay by ELISA as for calibration purpose by biosensor. The results from validation are depicted as Fig. 5. The two assays appear to exert similar sensitivity to the tested samples but the biosensor had better limit of detection as the ELISA assay was able to detect IFNγ in a concentration from 28 pg/ml which was nearly five time worse than in the biosensor case. Coefficient of determination was 0.960 for regression of the two methods.

The biosensor was also tested for interference by various compounds and markers that can be presented in a tested blood, plasma or serum sample. Albumin in a concentration100 mg/ml, TNFα with concentration 0.1 mg/ml and murine IgM in concentration 100 mg/ml were assayed like a sample and the same was made with IFNγ in

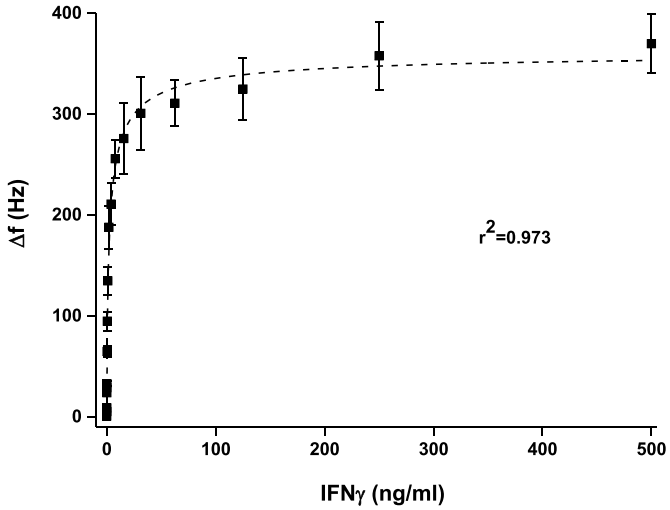

Fig. 5. Calibration plot for IFNγ when assayed by biosensor and modified particles. One sample was measured in five repeats.

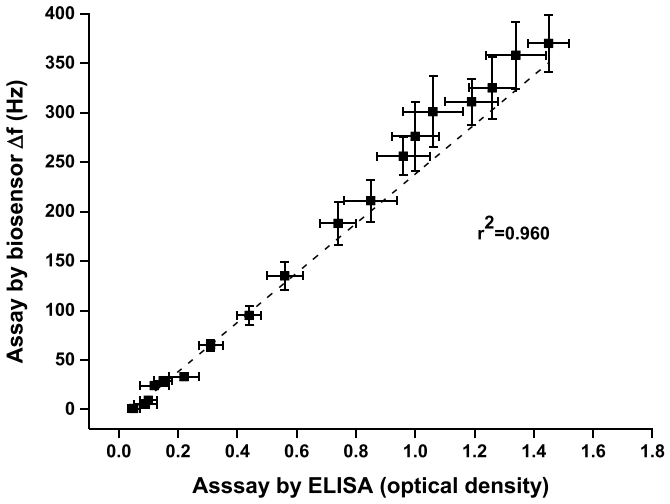

Fig. 6. Validation of the biosensor assay to ELISA. The same samples as used for calibration purpose were measured during the validation experiment. One sample was measured in five repeats.

concentration 122 pg/ml. Phosphate buffered saline pH 7.4 was also assayed for judgement whether solvent can cause an effect on the assay. The IFNγ represent a level that can be expected in a healthy man. Results from the experiment are shown in Fig. 6. The signal achieved by an assay of sample containing IFNγ in concentration 122 pg/ml was significantly differing (P = 0.01; ANOVA) to the other samples tested in this experiment. Assay of samples containing IgM, albumin and TNFα provided signals that were insignificant (P = 0.01 and 0.05; ANOVA) to assay of phosphate buffered saline pH 7.4. Considering the results, the assay can be enabled as selective one and the tested compounds does not represent a problem for the assay. Concentration of albumin, IgM and TNFα used here were highly above expected physiological concentrations in the human blood or plasma. Albumin level in blood plasma or serum of healthy people is between 35 and 55 mg/ml, IgM 0.5–2.5 mg/ml, TNFα has typical concentration around 10 pg/ml but it can exceed 100 pg/ml when inflammation is developed. The no sensitivity of the assay to the tested interfering compounds is an important parameter and resistance to interfering compounds is necessary for practical performance (see Fig. 7).

The assay was verified on real plasma samples achieved from human volunteers. In a total four plasma samples were tested five times by the both biosensor assay and five times by ELISA and concentration

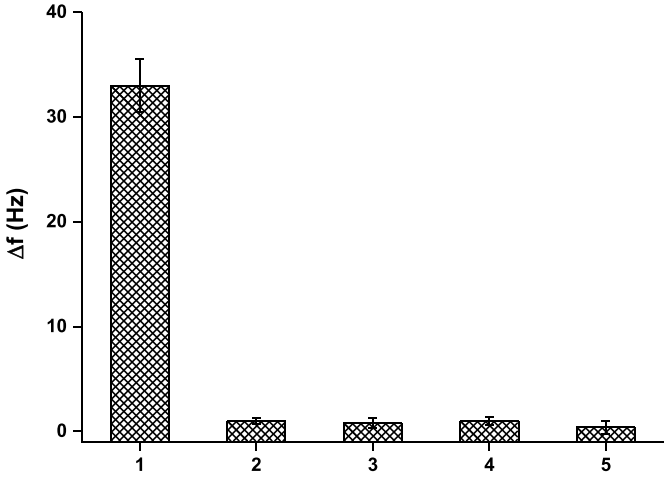

Fig. 7. Interference testing. Column 1) – IFNγ in concentration 122 pg/ml, 2) – albumin 100 mg/ml, 3) TNFα 0.1 mg/ml, 4) murine IgM 100 mg/ml, 5) phosphate buffered saline pH 7.4. One sample was measured in five repeats.

Table 1
Comparison of IFNγ assay in blood plasma samples by biosensor and ELISA.

Sample no.	IFNγ by biosensor (pg/ml)	IFNγ by ELISA (pg/ml)
1	102 ± 7	108 ± 5
2	135 ± 9	131 ± 10
3	92 ± 5	88 ± 8
4	158 ± 10	155 ± 12

of IFNγ was determined using the aforementioned calibration plots. The experimental data from the sample analysis are summarized in Table 1. The measured levels of IFNγ by ELISA and biosensor were mutually insignificant by ANOVA test on probability level P = 0.05. The comparison of the two assays can be concluded that the both methods provided very similar results and the assay by biosensor is fully comparable to the ELISA. The biosensor exerted also a good long-term stability. No significant difference for IFNγ assay was observed when compared new biosensors with the biosensors old three and six weeks.

When the biosensor assay presented here summarized, analytical parameters appears to be favorable to make the assay competitive to the standard immunoassays. Specifications of the biosensor are surveyed in Table 2. The here presented biosensor appears as a good alternative to the standard immunoassays. There are also known experiments devoted to the construction of biosensors to IFNγ in the recent literature and results from the already published papers can be compared to the experiment presented here. An electrochemical aptasensor with limit of detection 19 fg/ml [35], an electrochemical DNA biosensor with limit of detection 60 pmol/l [36] and a modified magnetic nanobeads with an aptamer and limit of detection 6 pg/ml [37] can be introduced as examples of the significant papers on the issue. The biosensor presented here had similar or slightly better limit of detection than the cited papers [36,37]. On the other hand, the aforementioned aptasensor seems to be more sensitive to IFNγ than the biosensor presented here [35]. Major advantage of the biosensor is overall simplicity and readability to production with keeping acceptable analytical parameters. The fact that piezoelectric microbalances can be applied with minimal analytical equipment, no elaborative step manufacturing process is necessary and overall time per assay is acceptable comparing to standard ELISA makes the assay promising for routine analysis. In a conclusion of comparison, the biosensor presented in this paper is fully competitive to the cited works.

4. Conclusions

The biosensor for assay of IFNγ was constructed as a tool suitable for small laboratories or home care conditions. On the other hand, it was proved to be a fully applicable alternative to standard immunoassays like ELISA hence use in routine laboratories is possible. The major advantage of the biosensor assay is overall simplicity, low cost per one assay, suitability for mass production of the biosensor devices, low volume of sample and no special requirements for laboratory staff.

Table 2
Survey of biosensor analytical specifications.

Specification	Value
limit of detection	5.7 pg/ml
sample volume	50 μl
coefficient of determination for calibration	0.973
coefficient of determination for validation to ELISA	0.960
interference by albumin 100 mg/ml, TNFα 0.1 mg/ml, murine IgM 100 mg/ml	not significant
time per one assay	60–90 min

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