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The Determination of Human Albumin by a Quartz Crystal Microbalance Immunosensor

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Albumin is a protein serving as a standard biochemical marker which is assayed in the blood and blood plasma as a liver function test but its diagnostics importance is much wider. In the current time, albumin can be determined by standard immunochemical and spectral methods. In this work, immunosensor based on the piezoelectric10 MHz QCM was developed to determine albumin in levels corresponding with expected concentration in blood plasma. The immunosensor contained an antibody specific to albumin interdigitated to electrode surface through protein A. The immunosensor was performed for the determination of albumin and limit of detection 0.234 mg/ml was achieved. The limit of detection is lower than the expected plasma level of albumin (35 – 55 mg/ml). The immunosensor assay fully correlated with the standard ELISA and the immunosensor exerted good long-term stability for at least two months. The immunosensor based determination of albumin appears to be a promising tool for practical use in field or home care conditions. No elaborative manipulation with samples or necessity to use specific reagents is a significant advantage of the immunosensor based assay.

Keywords: affinity; albumin; antibody; biosensor; biorecognition; immunochemistry; label free assay; liver function test; piezoelectric; quartz crystal microbalance

1. INTRODUCTION

Albumin is a protein created in the liver and secerned into blood stream in a high amount. In the body, the albumin plays a significant role which includes keeping of colloid osmotic pressure (or oncotic pressure in some sources) of the blood circulatory system, transport disparate compounds including hormones, drugs and some metabolites, and it is also a significant pool of amino acids for the time of starvation [1-10]. There is also a specific task of albumin in the immunity but the role remains

poorly understood though some works revealed interesting links between the immunity and albumin [11-13].

Diagnosis of various diseases is the reason why albumin serves as a marker in clinical biochemistry. In the plasma of health individuals, albumin is presented in a level 35 – 55 g/l and it composes more than half of blood plasma proteins. Because albumin is produced by hepatocytes, its plasma level serves as one of the liver function test markers. Various liver malfunctions including toxic and pathogen damaging of the hepatocytes can be revealed by the determination of blood plasma albumin level.

The determination of albumin can be done by several ways but only some of them are cheap and reliable enough to be done in the current clinical laboratories. Colorimetric determination by application of bromocresol green or bromocresol purple is a typical protocol [14,15]. Immunoassays including Enzyme Linked Immunosorbent Assay (ELISA) are another option for the albumin determination [16,17]. Albumin can be also determined by chromatography which is more reliable but also more elaborative and expensive. Traces of albumin can be determined by the chromatography techniques, the chromatography is also suitable for the recognizing of adducts like albumin – drug, and albumin can even serve as a matrix for affinity interactions [18-21].

Piezoelectric biosensors are a group of analytical devices containing a piezoelectric crystal as a vital part necessary for the assay purposes. They also contain a part of biological origin which is necessary for the interaction with analyte. In the case when the biological origin part is an antibody, then the biosensor is called an immunosensor. The piezoelectric immunosensors are suitable for the determination of a wide number of analytes by an affinity interaction which results in the change of electrochemical properties, namely in decrease of oscillations frequency [22-24]. In this work, a piezoelectric platform was proposed as a sensor suitable for an antibody immobilization and direct determination of albumin. Simple design of the assay and analytical parameters demanded for a contemporary clinical method are expected. It is expected that the assay based on the immunosensors will be reliable and more available for general use than the standard methods.

2. MATERIALS AND METHODS

2.1. Quartz Crystal Microbalance (QCM) and immobilization of antibody on electrode surface

The used QCMs were purchased from Krystaly (Hradec Kralove, Czech Republic; http://www.krystaly.cz/en/). The QCM sensors have parameters as follow: basic frequency of oscillations 10 MHz; external diameter of quartz disc 19 mm, two gold electrodes on chromium core layer on the both opposite sides with a diameter 7 mm, thickness of the quartz disc 166 μm. Appearance of the QCM sensors used in the experiments can be learned from figure 1.

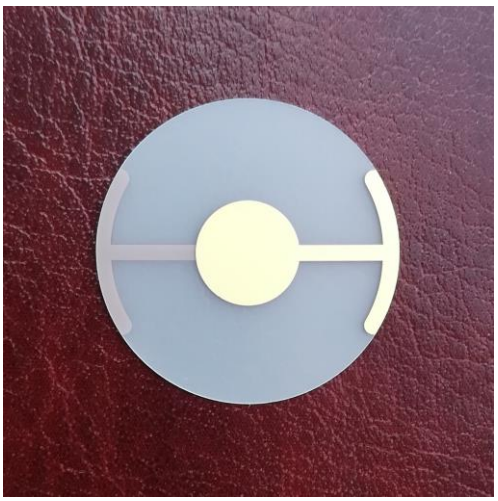

Figure 1. Appearance of the QCM used in the experiments.

Every new batch of QCM sensors was washed by immersion into 96 % v/v ethanol (Litolab, Chudobin, Czech Republic), left to dry and then washed in deionized water produced by a device Aqua Osmotic 02 (Tisnov, Czech Republic). After repeated drying, cysteamine 50 mg/ml was spread over each electrode in an amount 50 µl, placed into a wet chamber preventing from premature desiccation and left there for five hours. Surface of the sensors were rinsed by deionized water, dried and 5 % w/w glutaraldehyde (Sigma-Aldrich) was applied in an amount 50 µl per electrode. After another five hours in the wet chamber, the QCMs were washed and dried again and then protein A from *Staphylococcus aureus* (Sigma-Aldrich) was solved in phosphate buffered saline (PBS) with pH 7.4 and concentration 1 mg/ml. The protein A solution was incubated on the electrode surface for 12 hours. In the final step, monoclonal antibody against human albumin (Sigma-Aldrich) was interdigitated on the electrode surface. The antibody was IgG2a type, mouse type and provided as a conserved solution. It was diluted 1:5,000 by PBS in compliance with the manufacturer instructions. The antibody solution (50 µl of the solution per electrode) was incubated with the QCM in the wet chamber for 12 hours. Then the solution was sucked out and gelatin (Litolab) 10 mg/ml in an amount 50 µl was given per electrode and the electrodes were kept in the wet chamber for further 12 hours. After that, the electrodes were subsequently rinsed with PBS with 0.1 % (w/w) Tween 20 and by deionized water. After drying under laboratory conditions, the constructed immunosensors were stored at 4 °C until use in the experiments described in the following text.

2.2. The determination of albumin by QCM immunosensors

Principle of the assay as well as the immunosensor construction can be learned from figure 2. As seen in the figure, the immunosensor worked on a simple, label free principle where no specific reagent was necessary in the assay. The immunosensor was plugged into ICM Lever Oscillator 10.000 MHz (ICM; Oklahoma City, OK, USA). Oscillations were recorded by frequency counter UZ 2400 (Grundig; Nuremberg; Germany). Frequencies of the immunosensors were recorded before a sample application. In the next step, 50 µl of a sample was applied on the sensor surface and left to incubate

for a half of an hour. After the incubation, the immunosensors were washed by PBS with 0.1 % w/w Tween 20 and dried under laboratory conditions. Finally, frequency of oscillations was measured when the immunosensors were dry. Change in the frequency of oscillations Δf was calculated from the frequency before and after the sample application.

Human serum albumin 96 % lyophilized powder (Sigma-Aldrich) served as an analyte. The albumin was solved in PBS and concentrations 0.0488, 0.0975, 0.159, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 mg/ml were used for calibration purposes. The calibration range covered expected physiological concentrations in human blood plasma (35 – 55 mg/ml). Pure PBS served for blank purposes. Fibrinogen from human plasma, immunoglobulin G from human serum and human hemoglobin were bought from Sigma Aldrich and served as examples of typical plasma proteins for testing of interferences.

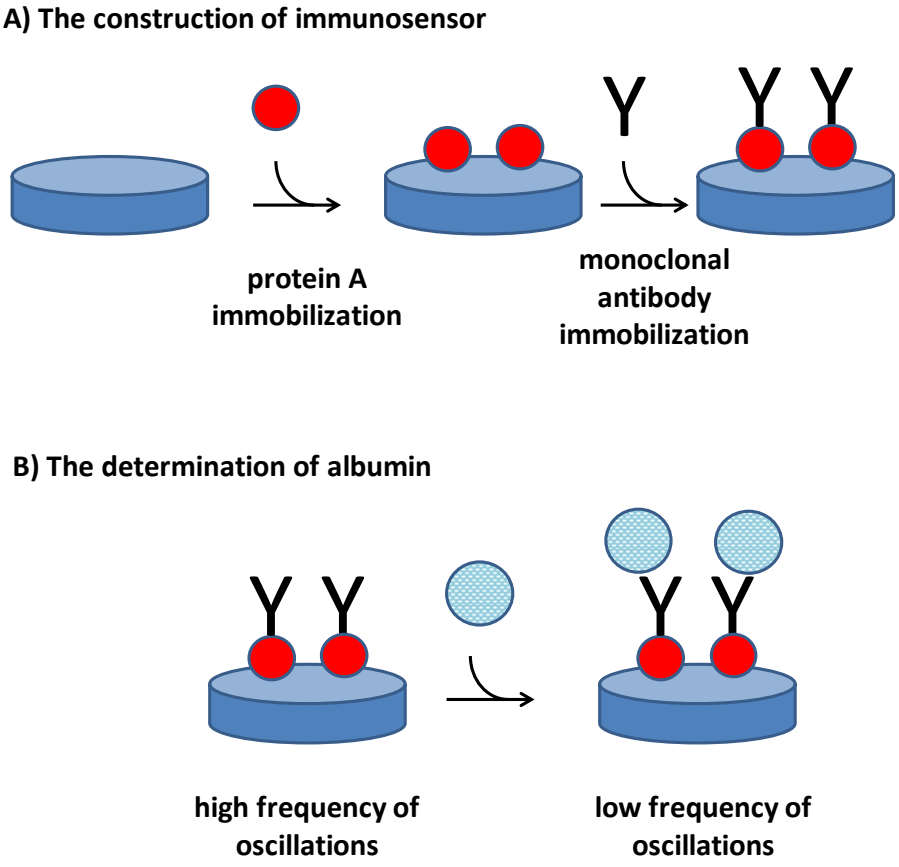

Figure 2. Schematic presentation of the immunosensor construction (step A) and the determination of albumin (step B) as an analyte (blue ball).

2.3 Measurement of albumin concentration by ELISA as a standard method

In the beginning, 96 well flat bottom microplates (MaxiSorp, Nunc, Roskilde, Denmark) were covered with tested sample. In a total 50 µl of a sample was poured with 150 µl of PBS in one well and left to incubate in the wet chamber for 12 hours. After the interval, the solution was sucked out and

100 µl of 0.1 % w/v gelatin was injected per one well and let interact for one hour. In the next step, 50 µl of the monoclonal antibody against albumin solution (the same as described in the immunosensor construction) was poured in one well with 150 µl of PBS and incubated for four hours. The solution was sucked out and a solution of horse radish peroxidase labelled antibody against mouse IgG (source Sigma-Aldrich) was applied in an amount 200 µl and incubated for another four hours. After sucking the solution out, the wells were washed by PBS with 0.1 % w/w Tween 20. Finally, a mixture containing final concentration of o-phenylenediamine 0.5 mg/ml and hydrogen peroxide 5 mmol/l was injected in an amount 100 µl and the reaction was stopped aby adding of 2 mol/l sulfuric acid (100 µl) after one minute. Coloration was measured as a value of optical density measured at 450 nm by an ELISA reader Sunrise (Salzburg, Austria).

2.4. Statistics

The both assays, ELISA and assay by immunosensor, were performed in pentaplicates. It means that every sample was measured in five repeating. Mean and standard deviation of the pentaplicate was calculated for each sample measurement. Limit of detection was reckoned using the rule that it corresponds with the point in the calibration where signal is three times higher than noise which was represented by the deviation of blank assay. The rule of the limit of detection characterisation is also known as S/N=3. Statistical significance was calculated by analysis of variance (ANOVA) on the probability levels 0.05 and 0.01. The experimental and statistical testing data were calculated in Origin 8 (OriginLab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSION

The immunosensor was prepared just the way how described in the experimental part. When the immunosensor constructed, frequency of oscillations was recorded for every step of immobilization. Average oscillations of the new 10 MHz QCM sensors did not drop when the surface of electrodes was activated by cysteamine and modified by glutaraldehyde. It can be explained by the fact that molecular weight of the both molecules is low and total mass bound on the electrode surface is under the method limits. The immobilization of protein A was the next step. The immobilization caused decrease of frequency equal to 635±52 Hz. Immobilization of antibodies against albumin caused further drop of the oscillations: 497±43 Hz.

The assay of albumin followed and calibration curve was constructed. The resulting calibration is depicted as figure 3. The immunosensor was applicable for the whole calibration range 0.0488 – 100 mg/ml but surface of the immunosensor respectively free antibodies immobilized on the surface appeared to be saturated by albumin in the concentrations concentration exceeding 100 mg/ml and distinguishing of concentrations in the concentration range 100 – 400 mg/ml is limited. The low sensitivity in the region 100 – 400 mg/ml is not however crucial because physiological concentration of albumin in plasma is expected in the range 35 – 55 mg/ml and it is even lower under pathological conditions. In the physiological range and under it, the assay exerted full sensitivity and applicability is expected. The assay had also promising correlation between change of oscillation frequencies and

albumin concentration which is apparent from coefficient of determination r^2 = 0.998. Limit of detection for the assay was equal to 0.234 mg/ml which is deeply under physiological range of expected albumin concentration in the blood plasma. Fibrinogen from human plasma, immunoglobulin G and human hemoglobin were tested as possible interferents because these compounds are typical proteins presented in plasma samples. Expected physiological concentration for free hemoglobin in blood is approximately 120 – 170 mg/ml, plasmatic level is lower and it depends on proper isolation of plasma where hemoglobin can occur when erythrocytes become disrupted. Fibrinogen is a protein with expected plasma level in a range 1.5 – 4.0 mg/ml. Immunoglobulin G from human serum served as another protein with possible interaction with the immunosensor. Expected serum level for immunoglobulin G is 0.7 – 4 mg/ml. All the proteins were applied in the concentration 200 mg/ml that is highly above their physiological range. The resulting frequencies Δf were compared to the control assay when buffer was applied only and ANOVA was calculated. There were no significant signals represented by the Δf value. It can be noted that the assay appears to be specific to the albumin and typical plasma proteins are not interferents.

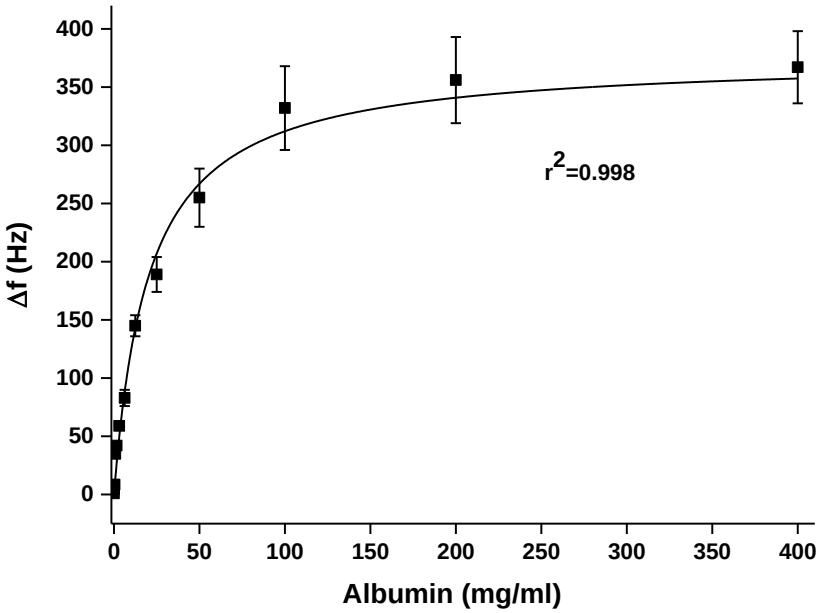

Figure 3. Calibration curve for albumin by a QCM immunosensor containing interdigitated antibody against albumin. Error bars indicate standard deviation for n = 5.

The samples used for the immunosensor calibration were also analyzed by ELISA method. Results from this experiment are depicted as figure 4. As seen from the correlation, quite high coefficient of determination (r^2=0.959) was achieved which confirms plausibility of the immunosensors based assay. It is obvious that the both methods were fully applicable for the determination of albumin and the immunosensor presented here had comparable sensitivity to the analyte like the standard ELISA. It should be, however, written, that while the ELISA is a standard laboratory method having limitation in size of the device and elaborateness of the assay. It can be

performed for various analytes with good results [25-27], but time necessary per one assay knowledge demanded from a worker doing the methods are limiting to use it outside standard laboratories. The immunosensor is suitable for a label free analysis and no elaborative manipulation with samples is necessary hence even uneducated people can perform the assay in home or field conditions.

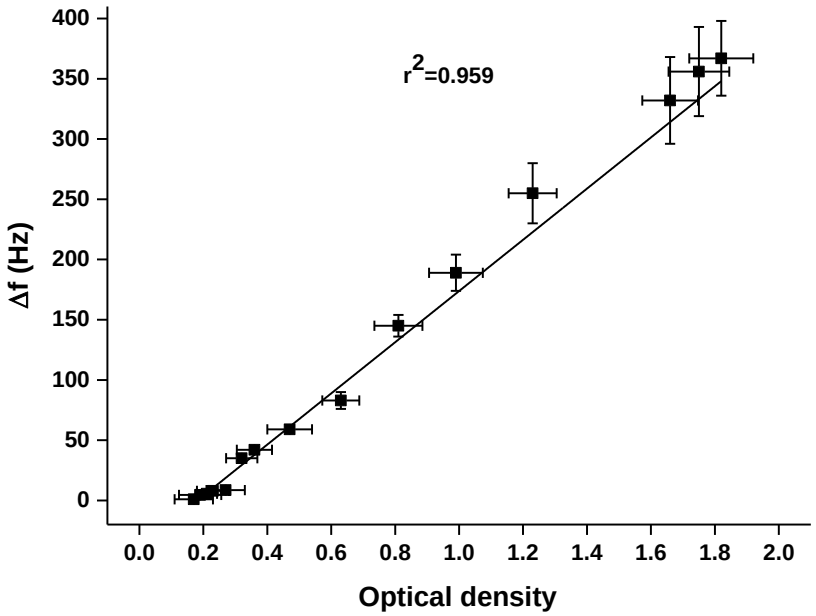

Figure 4. Validation of immunosensor to the ELISA as a standard method. Optical density of the ELISA and Δf of the immunosensors are given in the graph. Concentration range of albumin 0.0488, 0.0975, 0.159, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 mg/ml was tested for the validation purpose. Error bars indicate standard deviation for n = 5.

The immunosensors were tested for long term stability as the last parameter that should be known before a sensor is considered for implementation into praxis. In this experiment, period 60 days was chosen for the immunosensors characterization. The resulting plot of the long-term stability is depicted as figure 5. The immunosensors were stored in laboratory conditions in a closed box and no specific protection or maintenance of the immunosensors was done. The long-term stability test has not revealed decrease of sensitivity because of storage. After the tested interval, 95.6 % of the initial signal was reached (drop of sensitivity 4.4 %). This fact is another promising parameter. Instability in storage would be a problem in practical use hence the exerted stability is an important condition. The stability can be further improved for a storage in intervals above months or years by specific packaging of the immunosensors. Relevant papers in this field can be used for the purpose [28,29]. Survey of achieved analytical parameters is depicted in table 1.

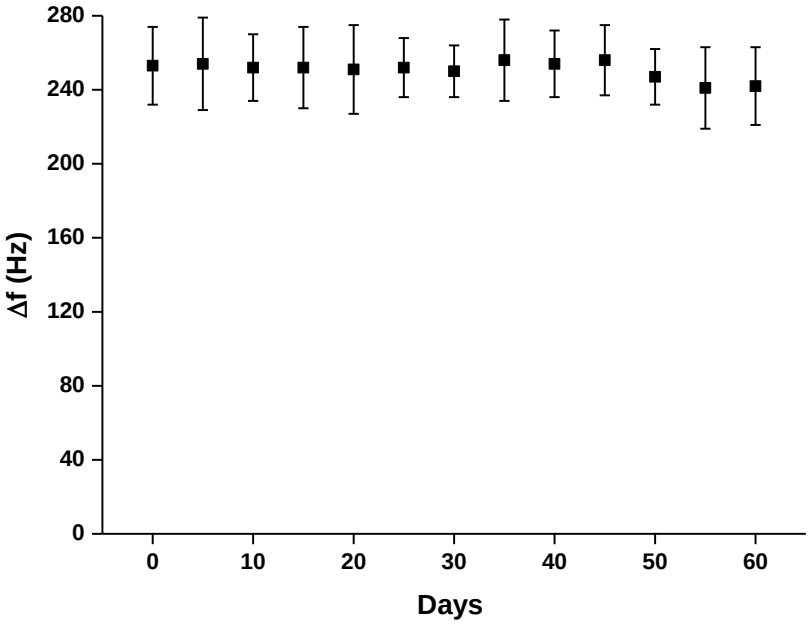

Figure 5. Use of immunosensors for the determination of albumin (50 mg/ml). The albumin was assayed in intervals 5 days for 60 days. Error bars indicate standard deviation for n = 5.

Table 1. Survey of achieved analytical parameters.

Limit of detection	0.234 mg/ml
Drop of sensitivity after 60 days	4.4 %
Coefficient od determination / correlation coefficient to standard ELISA method	r^2=0.959 / r=0.979
Interference by fibrinogen from human plasma, immunoglobulin G from human serum and human hemoglobin in physiological concentrations	not significant by ANOVA on probability level 0.05

The assay of albumin by piezoelectric immunosensor can be also compared with the experiments known from literature. Lim and Ahmed developed an immunosensor for the detection of porcine serum albumin as a marker for meat adulteration [30]. The researchers detected porcine serum albumin in a label free electrochemical assay based on screen printed electrodes and they reached very low limit of detection 0.5 pg/ml and liner range up to 500 pg/ml. The assay is more sensitive that the assay presented in our study. On the other hand, the cited work is focused to prove traces of albumin, clinical samples have high level of albumin so there is no necessity to have so sensitive immunosensor which is more expensive for production. There are also biosensors for the determination of specific forms of albumin. Glycated albumin as a marker of diabetes mellitus can be mentioned as the most relevant one. Some studies for this purpose were done [31,32]. Demands on such devices are stricter because there must not interfere the unmodified version of albumin. The immunosensor introduced in this paper is a device readily for practical performance with no necessity to apply expensive

technology. No similar study on a piezoelectric immunosensor for direct, label free, determination of albumin in biological samples have been found.

4. CONCLUSION

In a conclusion, piezoelectric immunosensor for the determination of albumin was constructed. The immunosensor proved promising analytical parameters and it is fully comparable to standard ELISA method. The assay has a significant advantage to the standard method which subsists in the overall simplicity, no necessity to apply specific reagent or pretreat biological sample. The immunosensor appears to be suitable for practical performance.

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