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Piezoelectric Biosensor for the Determination of Tumor Necrosis Factor Alpha

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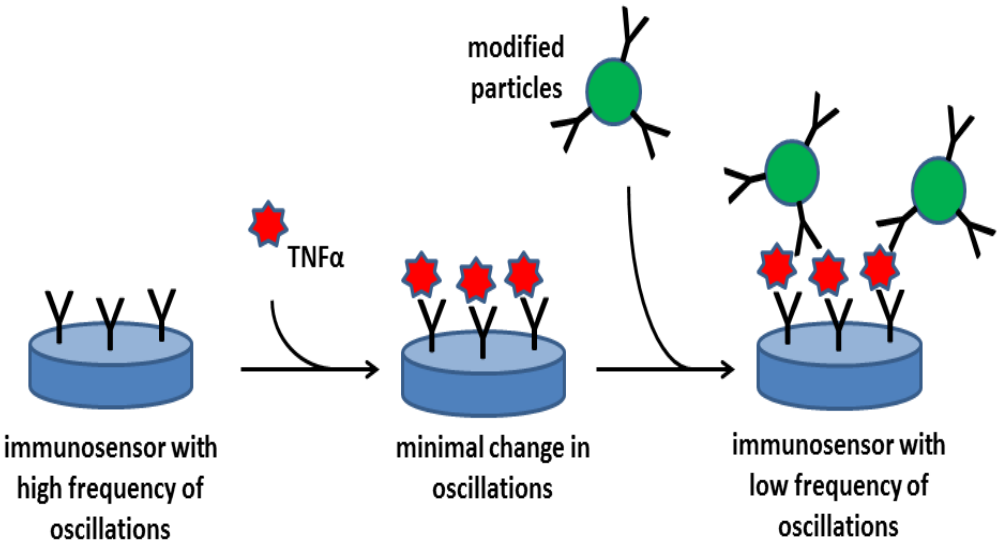
Abstract

Tumor Necrosis Factor Alpha (TNFα) is an important marker of inflammatory processes in human body. In the current healthcare, determination of TNFα blood or plasma level is done by Enzyme Linked Immuno-Sorbent Assay (ELISA) as a primary choice method. Piezoelectric immunosensors are analytical platform recording affinity interactions on their surface. It is inferred that the immunosensors would be a functional alternative to the ELISA. In this study, antibody against TNFα was immobilized on Quartz Crystal Microbalance (QCM) sensor and the same was made on magnetic particles. Human TNFα was measured in a way of interaction with QCM surface and then the particles were applied. The assay exerted sufficient limit of detection equal to 1.62 pg/ml and it also fully correlated with standard ELISA tests. No interference by interleukin 6 or albumin was observed. Long term stability of the immunosensors lasting for at least three months was found. The immunosensors appears to be readily for practical performance and it would be an alternative to the standard ELISA especially when diagnoses made in field, homecare conditions or conditions of small hospitals as an emergency test.

Graphical abstract



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

1. INTRODUCTION

Tumor Necrosis Factor Alpha (TNFα) named also as cachectin is a cytokine released mainly by macrophages and it is one of the key proteins initiating inflammation and innate immunity [1, 2]. Because of the crucial function in the immunity and releasing of TNFα into blood, the cytokine is an ideal marker for various pathological states. The level of TNFα can arise because of many pathological processes. Cardiovascular diseases, inflammatory bowel disease [3, 4], bacterial diseases and sepsis [5-7], diabetes mellitus where it even can considered as a causative cofactor for type 2 of diabetes mellitus [8, 9] and some types of poisoning [10-12] can be exampled as pathogeneses where TNFα plays a significant role.

Assay of TNFα is a common tool in the both human and veterinary medicine and it serves for the determination of inflammation and a positive prove of increased TNFα level can be attributed to one of the aforementioned pathological processes. When surveyed the current methods for TNFα determination, Enzyme Linked Immuno-Sorbent Assay (ELISA) should be mentioned as a method of the first choice. It is a standard method available in the most of immunochemical laboratories and when searched various studies on inflammation, ELISA is used there [13-15]. Despite of its reliability and good availability, ELISA has some drawbacks as well. The assay is also time consuming and it take some hours or even a day to reach the results. Fluorescence immunoassays, radio-immunoassays and chromatography techniques are other methods that can be chosen for assay of TNFα in biological samples as well.

The current paper is devoted to the construction of a piezoelectric biosensor for a fast-hand-held assay of TNFα in blood plasma samples. The piezoelectric platform appears to be suitable for this

purpose as it can directly follow affinity interactions on electrode surface [16-18]. It is expected that the platform can make assay simpler comparing to ELISA but analytical parameters typical for ELISA can be kept. For the assay purposes, the use of magnetic particles was chosen because theoretical sensitivity of QCM does not allow direct measurement of TNFα typical concentration because absolute mass in a biological sample is too low to be recorded by QCM. The here chosen principle provides significant advantage in this regard.

2. MATERIALS AND METHODS

2.1Sensor and antibodies immobilization

Quartz Crystal Microbalance (QCM) sensor was purchased from company Krystaly (Hradec Kralove, Czech Republic). The QCMs had basic frequency 10 MHz, the quartz disc had diameter 19 mm and wide 166 μm. Gold electrodes had diameter 7 mm and they were placed on the opposite sites of the quartz disc. Chromium interface was between the gold and quartz surface.

Prior to immobilization, the QCM sensors were washed by immersing into deionized water and then into ethanol (96 % v/v), each step lasted 30 minutes. After the washing, electrode was covered with cysteamine (analytical purity, Sigma-Aldrich; St. Louis, MO, USA) 50 mg/ml in a total volume 50 μl and let incubate in a dark and wet chamber under laboratory temperature and pressure for 5 hours. Unreacted cysteamine was washed out by a stream of deionized water and then dried. The self-assembled monolayer was finished by adding of glutaraldehyde which was injected on electrode in an amount 50 μl and concentration 5 % (w/w) and kept again under laboratory temperature and pressure for another 5 hours. In the next step, polyclonal antibody specific to human TNFα and produced in rabbit (Sigma-Aldrich) was diluted with recommendation of manufacturer 1:2,000 by phosphate buffered saline (PBS) pH 7.4 and spread over previously modified electrodes in an amount 50 μl and kept in the dark and wet chamber for 5 hours. In the final step, surface of electrode was blocked by adding of bovine serum albumin (1 mg/ml). Dark and wet chamber and incubation period 5 hours were chosen as well. Before storage in a refrigerator, freshly prepared immunosensors were washed by a stream of PBS.

2.2. Plasma samples

Blood samples were kindly provided by Faculty hospital in Hradec Kralove. In a total three samples were received (two male and one female origin; average age 34±4 years). The samples were collected to tubes treated with lithium heparin (Dialab, Prague, Czech Republic) and centrifugated at 1,000 ×g for five minutes. After the centrifugation, plasma was collected and stored at -80 °C until use.

2.3. Magnetic particles modification

Iron oxide based magnetic particles with size 1.0 μm and surface modified by carboxylate (Sigma-Aldrich) conjugated with the antibodies against TNFα which were described in previous

protocol. The magnetic particles (batch 5 ml, solid content of particles 5 %) were washed by PBS and separated by an external magnet for three times. After the final separation, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Litolab; Chudobin; Czech Republic) was solved in PBS up to concentration 20 µg/µl and total volume 2 ml was injected over the magnetic particles. The mixture was gently shaken and incubated under laboratory conditions for 20 minutes. After that, the particles were twice washed by PBS and isolated by the external magnet. In the next step, 4 ml of the antibody in PBS was given to the tube with magnetic particles, gently shaken for the whole time and let incubate for another 2 hours. Finally, the particles were two times washed by PBS and under holding by the external magnet. The washed and modified particles were re-suspended in 2 ml of PBS and stored in a fridge until use.

2.4. Piezoelectric assay

The constructed immunosensor worked on a principle of direct capturing of TNFα on immobilized antibodies. Iron oxide particles were the final layer. The mass bound on the sensor causes alteration in frequency of QCM oscillation [16]. Principle of assay can be learned from figure 1. The immunosensors having immobilized antibody against TNFα on QCM platform were fixed into holder and plug into ICM Level Oscillator 10.000 MHz (ICM; Oklahoma City, OK, USA) and measurement was performed using frequency counter UZ 2400 (Grundig; Nuremberg; Germany). Equilibrium frequency of oscillation was measured for each immunosensor. After measurement of the immunosensors before performance with samples, the immunosensors were covered with 50 µl of human TNFα (Sigma-Aldrich) solution in PBS in concentrations 0, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 or 4096 pg/ml or plasma sample. Solution of human serum albumin (100 mg/ml) and interleukin-6 (10 ng/ml) in PBS were chosen for interference testing. The potential interferents were purchased from Sigma-Aldrich. The drop of sample was incubated on surface of the immunosensor for two hours. After the two hours, 50 µl of modified magnetic particles was injected on the surface and it was rinsed by PBS with 0.1 % (w/w) Tween 20 after another two hours of incubation. After drying under laboratory conditions, frequency of oscillation was measured again and difference between frequencies before and after sample application (Δf) was calculated.

2.5. Enzyme-Linked ImmunoSorbent Assay test

Enzyme-Linked ImmunoSorbent Assay (ELISA) served as a reference method for the determination of TNFα. Standard 96 well microplates (flat bottom, MaxiSorp type, Nunc, Roskilde, Denmark) and ELISA reader Sunrise (Salzburg, Austria) were used throughout assay. The assay was made in compliance with following protocol.

- 50 µl of tested sample (blank or human TNFα solution or plasma sample) and 150 µl of phosphate buffered saline pH 7.4 were given per one well of microplate and they were mixed and incubated at laboratory temperature.
- After washing by phosphate buffered saline, the wells were blocked with 100 µl of 0.1 % (w/v) gelatin for one hour.
- 100 µl of polyclonal antibody specific to human TNFα (Sigma-Aldrich) diluted 1:2,000 was injected per well and incubated for four hours at 37 °C and wells were then washed by PBS.
- After that, 100 µl of secondary antibody specific to the primary from rabbit one and labeled with horse radish peroxidase (Sigma-Aldrich) was applied for another four hours at 37 °C and wells are washed with phosphate buffered saline with 0.1 % (w/w) Tween 20.
- A fresh solution of 0.5 mg/ml o-phenylenediamine and 5 mmol/l H_2O_2 was added for 1 min and reaction was stopped with 100 µl of 2 mol/l H_2SO_4 per one well.
- Optical density was measured at 450 nm.
- Wells with captured albumin (20 µl; 5 mg/µl) instead of the tested sample were used for purpose of negative control testing.

2.6. Statistics

Each measurement was repeated five times and the both average value and standard deviation were calculated. Calibration curves were fitted using Origin 8 (OriginLab Corporation, Northampton, MA, USA) software. Limit of detection was calculated as Signal to noise equal to three (S/N = 3) was used for limit of detection calculation. Comparison of human plasma samples assayed by immunosensor and ELISA was statistically tested using ANOVA (calculated in Origin 8 software) and probability level 0.05.

3. RESULTS AND DISCUSSION

The constructed QCM immunosensors were tested for standard solution of human TNFα. The calibration range was chosen in compliance with possible concentration of TNFα in human samples where the typical TNFα level in serum of health individuals 4 – 17 pg/ml is expected [19]. Increase of TNFα level can be mild in autoimmune diseases like psoriasis, where TNFα can variate from 15 to 36 pg/ml [19]. Severe infectious diseases cause more dramatic increase of TNFα comparing to the non-fatal one. Level of TNFα above 600 pg/ml is typical for septic patients [20]. In other sources, level up to 2.29 ng/ml for patients with survived sepsis and up to 3.45 ng/ml for the perished septic patients can be reached but these are rare values [21]. Considering the expected blood levels, calibration range 0 – 4 ng/ml can be considered as adequate. In order to test long term stability of immunosensors, concentration of TNFα equal to 512 pg/ml was chosen as an expected amount in samples of patients suffered from systematic inflammation.

Calibration curve for human TNFα is depicted in figure 2. Limit of detection was calculated to be equal 1.62 pg/ml and coefficient of determination was found to be 0.977. The limit of detection is low enough to cover standard concentration of TNFα in serum or plasma of health people. The curve

becomes flat when concentration of TNFα reaches approximate concentration 1 ng/ml. Though the concentrations of TNFα above 1 ng/ml are not fully covered by the immunosensor, they can be easily specified by dilution of collected sample. When considered diagnostic use of the immunosensor, distinguishing between normal level and common inflammations (level up to approximately 600 pg/ml) is fully covered. Distinguishing between high levels of TNFα does not further specify the type of inflammation. The reached limit of detection is quite low when considered theoretical sensitivity calculated from Sauerbrey equation for 10 MHz QCM [16, 22]. Microgram of a tightly attached mass can cause theoretically change of oscillations 230 Hz. The low detections limits presented here were reached due to application of the magnetic particles covered with antibody against TNFα. Without the particles, changes in oscillations were too low to be recorded even for the upper concentrations of the tested analyte. The use of magnetic particles for the determination of TNFα was tested in the past and some interesting adaptations were developed. Voltammetric assays of TNFα based on magnetic particles [23-26] can be exampled.

Tests on interference did not prove a significant affinity of either human serum albumin (100 mg/ml) or interleukin-6 (10 ng/ml) to sensitive layer on the immunosensor. While human serum albumin exerted signal 2.1±0.5 Hz, interleukin-6 caused decrease of oscillation equal to 0.7±0.3 Hz only. Concentration of albumin used here is approximately two times higher than the expected maximal physiological concentration is and concentration of interleukin-6 was also higher than the expected even in health and inflammation suffered patients [27].

The data achieved from immunosensors were validated to ELISA. Results from ELISA calibration are depicted as figure 3 and validation is given in figure 4. Limit of detection for ELISA method was found to be quite close to the immunosensor: 3.23 pg/ml. The both methods provided very similar calibration curve and the both calibration curves were well validated one to the other with coefficient of determination $r^2 = 0.951$. The fact that the curves correlated is not surprising because the same antibody was purposely performed in the both assays. The validation can be concluded that the immunosensor provides reliable results like ELISA but the assay based on immunosensor is simpler and it can be easily adapted on measurement in field conditions and use in homecare can be also performed. The two methods were also validated using real samples and the mentioned calibration plots. Each assay was made in pentaplicate. The test did not prove significant differences between the two methods. For the first sample, average TNFα level was equal to 4.1±0.3 pg/ml by immunosensor and 4.3±0.4 pg/ml by ELISA. The second sample had TNFα level 8.9±0.7 pg/ml by immunosensor and 8.7±0.9 pg/ml by ELISA. The last sample had TNFα level 9.1±0.8 pg/ml by immunosensor and 9.4±0.6 pg/ml by ELISA. The findings can be interpreted as a good correlation between the two methods. No significant difference when one sample assayed is a promising result in this sense.

Long term stability of the immunosensor was tested for 90 days. The resulting experimental data are presented as figure 5. The immunosensors and a stock of magnetic particles were prepared and five of them were tested immediately, the remaining immunosensors and stock of particles were kept in refrigerator. The assay was repeated by five of the stock each three days. No significant decrease of sensitivity to human TNFα was observed during the experiment and the immunosensors exerted full functionality for the whole 90 days period. The finding is promising because stability is a necessary condition for practical application. Further improvement can be made by preparation of special packages protecting the immunosensors but this type of experiment is out of this study scope.

4. CONCLUSION

Piezoelectric immunosensors appear as a promising platform for a simple determination of various biological markers and can be used even for a low amount of analyte when suitable tracer is employed. Though the immunosensors did not have significantly better analytical properties, they are readily applicable for a field or homecare use with no need of special training or education and no necessity of harmful reagents. Estimated price of one assay by the immunosensor is comparable with one assay (one well of a 96-plate kit) performed by a commercial ELISA tests.

ACKNOWLEDGEMENTS

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A competing interests statement

The author has nothing to declare.

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Figure 1. Principle of the constructed immunosensor based assay.

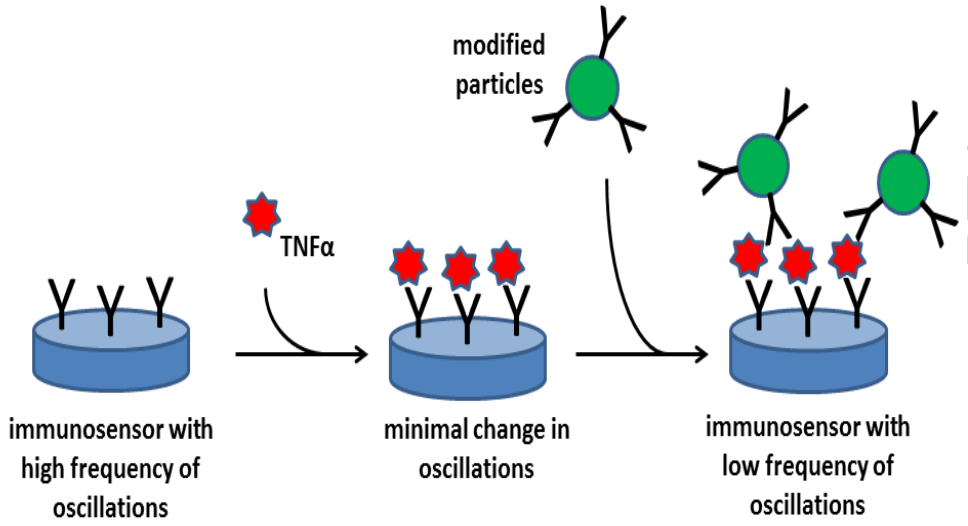


Figure 2. Calibration curve for human TNFα using QCM based piezoelectric immunosensor. Error bars indicate standard deviation for n = 5.

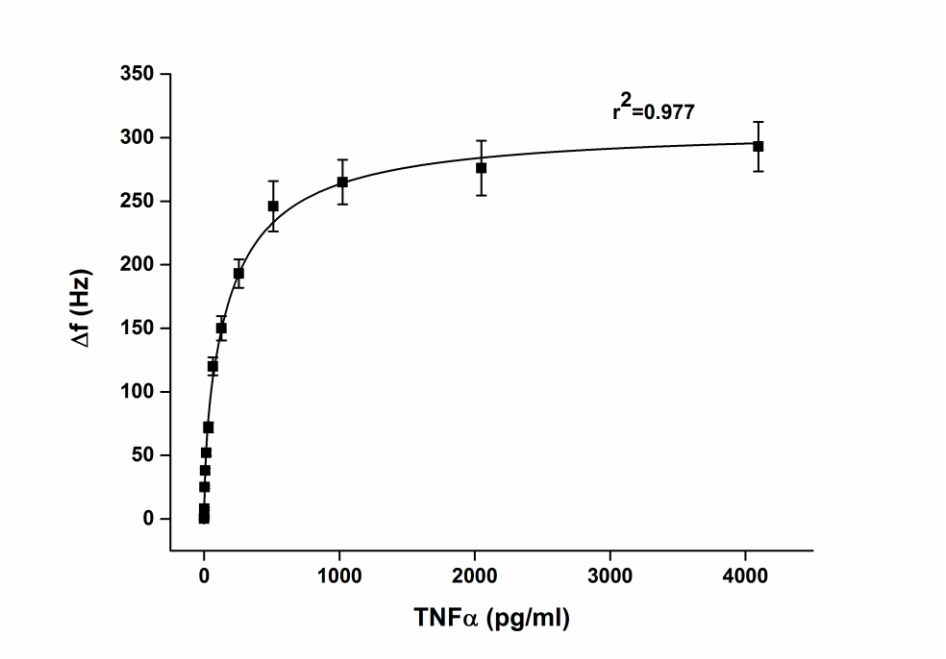


Figure 3. Calibration curve for human TNFα using ELISA method. Error bars indicate standard deviation for n = 5.

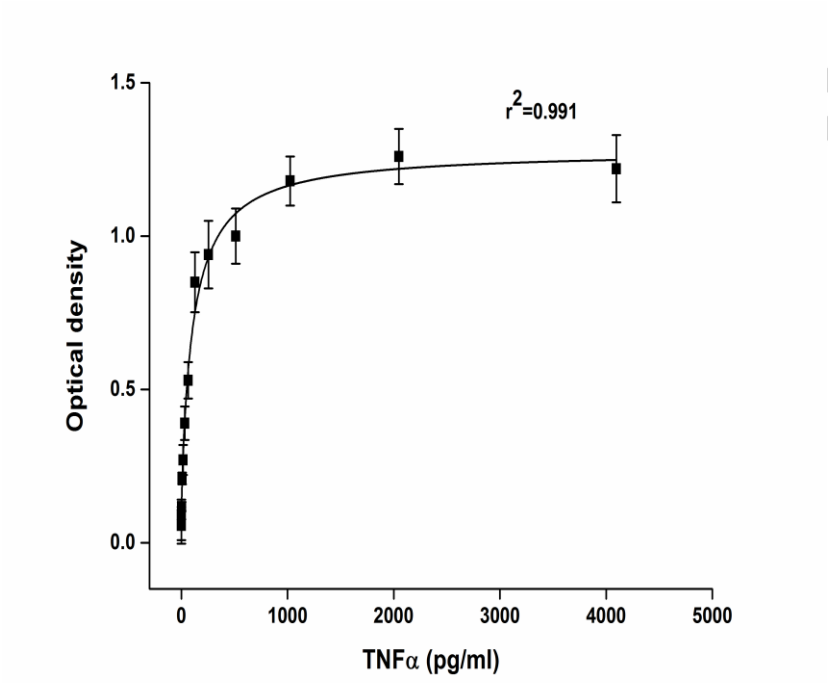


Figure 4. Validation of immunosensor providing Δf (Hz) to ELISA method providing optical density as an outputting value. Error bars indicate standard deviation for n = 5.

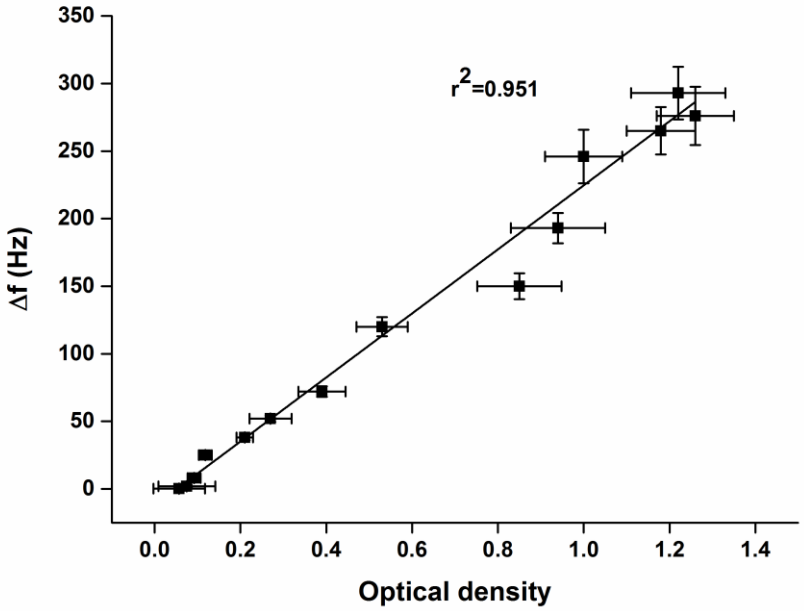
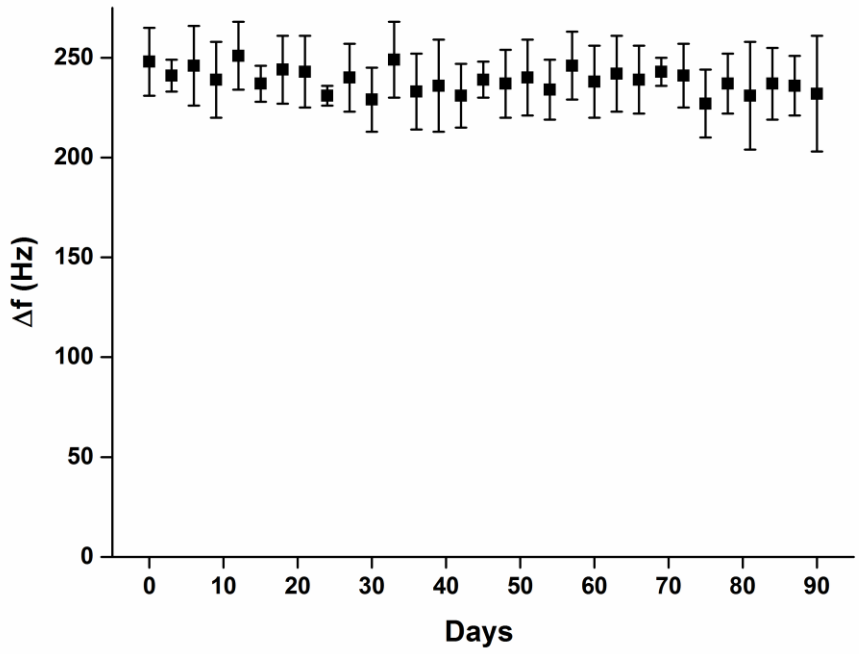


Figure 5. Long term stability of immunosensors presented as a repeated assay of human TNFα 512 pg/ml. Error bars indicate standard deviation for n = 5.



Highlights

- A piezoelectric immunosensor for simple assay of tumor necrosis factor α was constructed.
- The assay used modified magnetic particles in order to reach picograms of analyte.
- The assay had comparable results to ELISA with significantly simpler procedure.
- The immunosensors exerted long term stability.
- The assay covers full spectrum of TNFα concentration in expected samples.