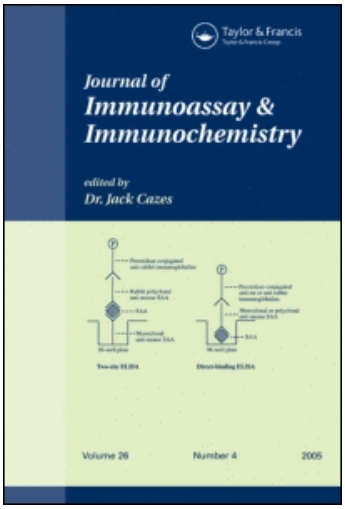

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Label-Free Piezoelectric Immunosensor for Rapid Assay of *Escherichia coli*

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Abstract: A piezoelectric sensor with immobilized polyclonal antibody was developed as a label-free assay for the model bacterium, *Escherichia coli*. The polyclonal antibody was prepared from mice BALB/c and covalently immobilized on the sensing gold electrode of the piezoelectric quartz crystal. The biosensor was able to detect *E. coli* in the range of 10^6–10^9 CFU/mL; signal of the negative control was not statistically relevant in the selected range. Samples could be analyzed in four minutes and one measuring cycle including regeneration was completed within ten minutes. Repeatability of the developed method is discussed; the signal obtained from three different biosensors was 12.9 ± 0.4 Hz for the sample containing 10^8 CFU/mL.

Keywords: Piezoelectric biosensor, Quartz crystal microbalance, Immunoassay, Immunosensor, *Escherichia coli*

INTRODUCTION

Timely detection and identification of microorganisms is often considered problematic, despite several existing methods developed in former times. The routine cultivation of suspect samples remains quite reliable, but often extremely time consuming. Immunological methods, such as ELISA (Enzyme-Linked Immunosorbent Assay) are frequently used in many clinical and microbiological laboratories. PCR (Polymerase Chain Reaction) became quite popular, especially in the research area; this method can

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provide unique information; on the other hand, it remains expensive and requires extraction of nucleic acid from complex matrices.[1]

Piezoelectric immunosensors are considered as promising alternatives for label-free detection of microorganisms. For piezoelectric biosensors construction, quartz crystal microbalances (QCM) with a basic frequency of 10 MHz are predominantly used. Physical characterization and description of quartz crystal piezoelectric behavior for solid state was done by Sauerbrey in the 1950s;[2] the relationship between the surface adsorbed mass and the accompanying frequency change was established.

Nearly 30 years later, the influence of liquids and their viscosities was examined and described.[3] A simple piezoelectric biosensor was published by Shons et al. in a model system consisting of immobilized bovine albumin and anti-albumin antibody.[4] Piezoelectric immunosensors are suitable for convenient sensing of several organic compounds. One of these arrangements was able to detect 0.03–100 μg/L of the herbicide, atrazine, in drinking water using sheep polyclonal antibody[5] and 34 ng/L cocaine was detected using anti-cocaine sheep polyclonal and mouse monoclonal antibody in a 15 min assay.[6] The applications of piezosensors in the food industry and in clinical diagnostics were discussed.[7] The immobilization procedure of anti-albumin antibody was optimized and albumin was determined as experimental analyte.[8] The antibody against IgM and single-stranded DNA aptamers as receptors immobilized on a quartz crystal were compared in a model system for IgM detection; aptamers proved to have similar specificity and sensitivity to antibody.[9] The 20 MHz piezoelectric sensor was employed for *Staphylococcal* enterotoxin B (SEB) detection using a competitive scheme resulting in the detection limit of 0.1 μg/mL.[10] A sensitive method for detection of the cholera toxin in sandwich format was introduced by Alfonta et al.[11] Monoclonal antibody specific against cholera toxin was immobilized onto the sensing area; the signal was amplified by using a secondary antibody and HRP-GM1-ganglioside-functionalized liposomes which mediated oxidation of 4-chloronaphthol in the presence of hydrogen peroxide. Thus, the obtained precipitate attached onto the gold electrode resulted in a significant decrease of the resonance frequency and a limit of detection equal to 0.1 pM was obtained.

Piezosensors were also often applied for sensing of microorganisms. *Escherichia coli* K12 contaminating drinking water was detected in the range 10^6–10^9 CFU/mL using antibody immobilized through protein A.[12] The *Vibrio cholerae* serotype O139 antigen equal to a concentration of 10^5 CFU/mL was detected in one hour.[13] The piezoelectric sensor with immobilized anti-*Chlamydia trachomatis* LPS antibody was able to detect 260 ng/mL of the LPS antigen from this microorganism.[14] A piezosensor monitored the agglutination reaction in a solution where *Staphylococcus epidermidis* and its specific antibody were present.[15] This assay took only 15 min. *Francisella tularensis* was detected as an immunoprecipitate with a limit of detection 5×10^6 cells/mL in a 35 min assay.[16] The interaction of

a gene probe with the *lacZ* gene was developed for *E. coli* detection.[17] DNA was purified from the lysate and amplified using PCR; the subsequent hybridization was carried out using the oligonucleotide probe immobilized on the sensing surface.

In this contribution, the piezoelectric immunosensor for direct, label-free, and rapid detection of bacteria represented by the model organism *E. coli* is proposed.

EXPERIMENTAL

Microorganism and Antibody Preparation

Escherichia coli (ATCC 9637) and *Bacillus subtilis* (ATCC 11774) were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Rep.) and cultured on the B10 agar at either 36°C (*E. coli*) or 33°C (*B. subtilis*). Cells were harvested after one day cultivation and suspended in either phosphate-buffered saline (PBS) or physiological solution. The obtained content of cells was estimated by using the standard cultivation test. Cell suspensions were kept at 4°C for max. one day or immediately used.

The polyclonal mouse serum was prepared from mice BALB/c (BioTest, Konárovice, Czech Rep.). Mice were inoculated by *E. coli* suspension equal to 10^6 CFU in 100 μL of the physiological solution. After 14 days from the beginning of immunization, mice were inoculated another dose of 10^7 CFU. After 28 days, inoculation continued with the dose of the *E. coli* antigen (100 μL, 5 mg/mL, prepared by repeated freezing/thawing cycles) and the Freund's complete adjuvant. After 36 days, the final dose followed with the same amount of antigen and Freund's incomplete adjuvant. Serum was collected after 40 days, centrifuged at 8,000 g for 15 min and frozen at −20°C if was not used immediately.

Antibody Immobilization

The piezoelectric crystal with smoothed surface and 5 mm gold electrodes on the opposite sides, with the basic frequency of 10 MHz, was obtained from International Crystal Manufacturing (ICM, Oklahoma City, OK, USA). The new crystal was washed by acetone and consequently modified by 25 μL of 20 mg/mL of water solution of cysteamine (Sigma, St. Louis, MO, USA) and activated by 3% glutardialdehyde (Sigma); each step lasted 2 hours. The immobilization procedure took place in a wet chamber. The obtained anti *E. coli* antibody was spread over the electrode area and incubated at 4°C overnight. The remaining reactive aldehyde groups were eliminated by incubation with bovine serum albumin (BSA, 10 mg/mL) for 2 hours.

Measuring Setup and Data Evaluation

The piezoelectric immunosensor was fixed in the flow-through cell (internal volume 10 μL) attached to the peristaltic pump (PCD 21M, Kouřil, Kyjov, Czech Republic) using silicone rubber tubes. A flow rate of 250 μL/min was chosen as optimal. The Lever Oscillator (ICM) driven by the crystal and the universal counter UZ 2400 (Grundig, Fuerth, Germany) measured, continuously, the resonant frequency. Signal was continuously recorded on a computer using the software, LabTools. The block schematic of this arrangement is presented in Fig. 1.

One measuring cycle consisted of the following steps: stabilization of the baseline signal with PBS as carrier buffer (1–2 min), 2 min flow of the sample zone, and stabilization of the resulting signal (typically 2–5 min). For regeneration of the biorecognition layer, 50 mM sodium hydroxide was used for 2 min.

The affinity interaction taking place on the sensing surface can be expressed as an association of antigen to the immobilized antibody followed by a spontaneous dissociation in buffer. The interaction was described by the kinetic equation.[18]

$$f = \frac{k_a c f_{max}}{k_a c + k_d}(1 - e^{-(k_a c + k_d)t}) + f_0 = f_{eq}(1 - e^{(-k_{obs}t)}) + f_0 \qquad (1)$$

where the actual frequency f (Hz), measured in time t with c meaning concentration of the free affinity partner (antigen) are variables, the initial frequency

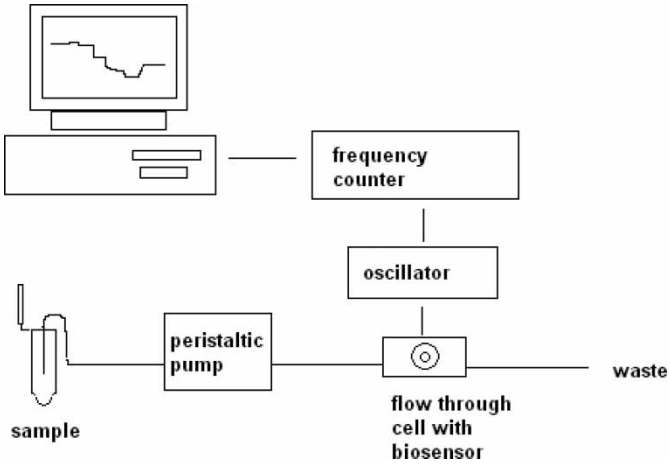

Figure 1. Block diagram of the piezoelectric immunosensor system. The signal collecting part is expressed by PC, frequency counter, and oscillator connected with the sensor. The flow through part consists of the microtube with sample, peristaltic pump, and flow through cell containing the sensor sandwiched between two soft o-rings. Only one side of the piezoelectric sensor is contacting the solution.

f_0, binding capacity f_{max}, and frequency at equilibrium f_{eq} represent sensing parameters. The kinetic parameters k_a and k_d are association and dissociation constants, respectively, and k_{obs} was a substitution used to simplify the non-linear fitting procedure. As in our experimental setup, the k_a value was difficult to obtain; concentration of the cellular antigen was not known, and the k_d value was obtained from the dissociation part of the assay and the measured frequency at the beginning of dissociation f_a:

$$f = f_a e^{-k_d t} \tag{2}$$

RESULTS AND DISCUSSION

The polyclonal antibody was successfully prepared; the highest titer (2-fold dilutions 1:10 to 1:10, 240) obtained by ELISA was 1:2, 560 for the whole immunoglobulin fraction, whereas 1:1, 280 for IgG and 1:320 for IgM subfractions using secondary HRP labeled antibodies (Serotec, Oxford, UK) specific against selected isotype. The protein content, which was determined using the Bradford total protein kit (Sigma, St. Louis, MO, USA), was 43.8 mg/mL. The polyclonal antibody was immobilized onto the sensor surface through its surface amino groups. The success of the immobilization procedure was confirmed by monitoring the resonant frequency after each immobilization step (Table 1). The introduced reactive aldehyde groups on the sensing surface were completely saturated with proteins from the polyclonal antiserum and the additional saturation with BSA was only a preventive step, as is obvious from Table 1.

The prepared immunosensor was tested for the measurement of the viable *E. coli* cells; the viable cells of *B. subtilis* served as a negative control, and both bacteria were suspended in PBS. The background signal was typically less than 1 Hz, which was determined in a zone of the carrier buffer (PBS)

Table 1. Summary of absolute frequencies obtained after each of the immobilization steps. Frequencies were measured in dry state. Standard deviations (SD) are expressed from three individual sensors. The corresponding mass changes were obtained using the Sauerbrey equation

Immobilization step	$f \pm$ SD (Hz)	$\Delta m \pm$ SD (ng)
Clean sensor	9996689 ± 2	0
Activation by cysteamine	9996656 ± 2	28.6 ± 1.7
Modification by glutaraldehyde	9996640 ± 2	13.9 ± 1.7
Antibody binding	9996309 ± 8	26.9 ± 6.9
BSA saturation	9996310 ± 11	0.9 ± 9.6

instead of the sample. No hysteresis or other fluctuation influence on the signal was observed after sampling. The signals obtained with the samples of *E. coli* and *B. subtilis*, both at 10^5 CFU/mL, were overlaid and there was no statistical difference between them (not shown). A sharp increase of signal was observed for the contents increased to 10^6 CFU/ml in the case of *E. coli*; the negative control realized by *B. subtilis* provided only a minimal change of signal compared to the previous lower amount. Thus, the limit of detection (based on the $S/N = 3$ ratio) was near 10^6 CFU/mL and distinguishing of *E. coli* and *B. subtilis* bacteria was statistically relevant at this level (t-test, probability > 0.99). The flow rate for all measurements was adjusted up 250 μL/mL; consequently, the total amount of cells was $5 \cdot 10^5$ CFU at the limit of detection. The slope obtained for the *E. coli* calibration curve was 4.24 Hz per decade, and the curve was relatively smooth ($R^2 = 0.98$) within the working range 10^6–10^9 CFU/mL. The maximal tested concentration was 10^9 CFU/mL, due to the observed instability of suspensions with higher bacterial contents. The highest obtained signal for *E. coli* at 10^9 CFU/mL was slightly less than 20 Hz. The dissociation rate constant k_d was $0.00034 \pm 0.00002\ s^{-1}$, indicating good stability of the immunocomplex. The immunosensor exhibited only minimal response to *B. subtilis*; the changes of signal in dependence upon the amounts of bacteria were within 1.5 Hz. The real binding curves, presented in Fig. 2, served for construction of the calibration curve (Fig. 3).

The long term stability of the immunosensor was verified by the repeated injection of sample containing 10^6 CFU/mL *E. coli* (Fig. 4). After each measurement, the regeneration step consisted of a 2 min zone

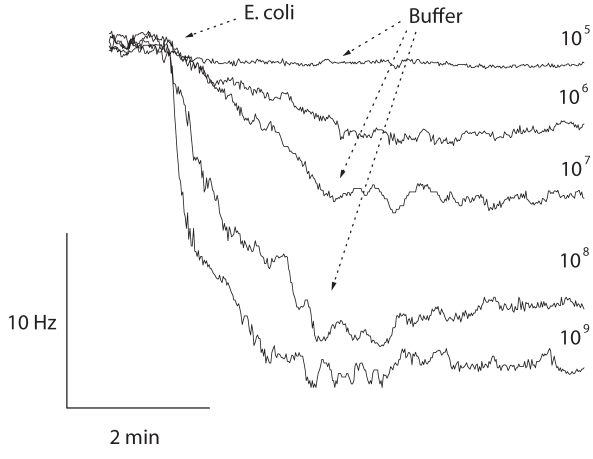

Figure 2. The acquired response curves (resonant frequency vs. time), recorded for samples containing different *E. coli* levels, measured by the piezoelectric immunosensor with immobilized polyclonal antibody specific against *E. coli*. The arrows indicate addition of the *E. coli* sample and/or carrier buffer flow.

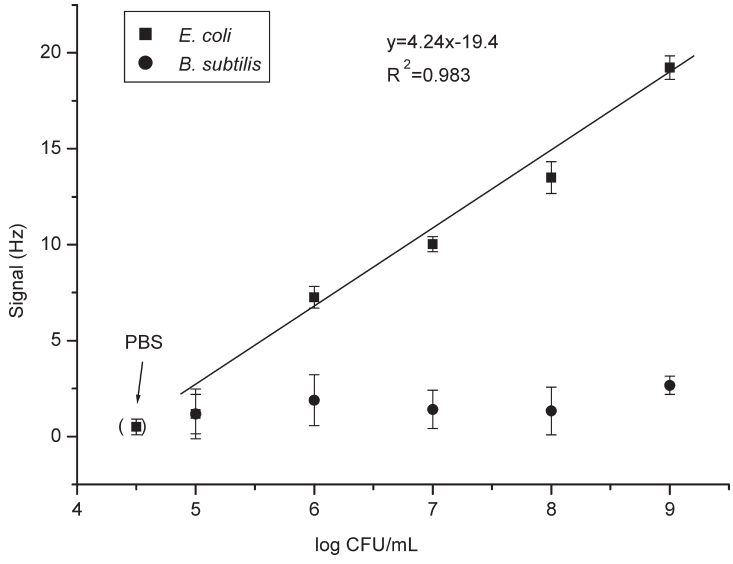

Figure 3. The calibration plot (signal in Hz vs. log of cell content) represents dependences obtained with *E. coli* (■) and negative control *B. subtilis* (●), respectively. The point in brackets represents the background signal obtained with PBS. The error bars represent estimated standard deviations ($n = 3$).

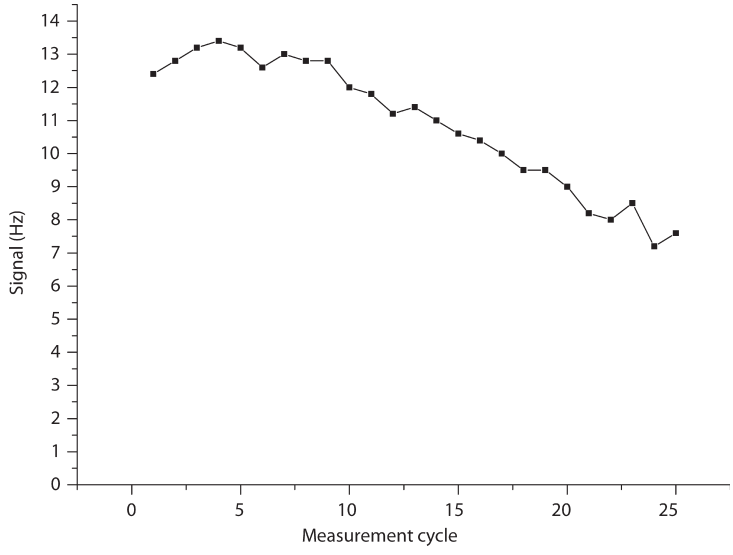

Figure 4. Long term stability of the immunosensor. Responses from the sequence of measurements of the same sample *E. coli* at 10^8 CFU/mL.

of 50 mM sodium hydroxide. The signals obtained in the initial twelve measuring cycles exhibited a total decrease of the signal below 10% (approx. 0.1 Hz/cycle) of the initial response. Furthermore, the decrease of signal became accelerated and approximately 0.5 Hz of the binding capacity was lost in each 13–25th cycle. No relevant fluctuations of response were noticed during inter-day/intra-day measurements. The sensor was kept mounted in the flow through cell filled with sterile PBS in the refrigerator when not used.

To evaluate the reproducibility of individual immunosensors, the signals received from three different sensors were compared. The immunosensors were prepared, consequently, from the same stock of antibody and each one was allowed to detect sample, including *E. coli* 10^8 CFU/mL three times in the same day. Three immunosensors provided signals 13.5 ± 0.8 Hz; 12.5 ± 0.8 Hz; and 12.7 ± 0.5 Hz, respectively. The average signal was 12.9 ± 0.4 Hz. The standard deviation for measurements with different immunosensors was overlayed with the normal variance. The method seems to be robust and its repeatability supports the simple production of immunosensors.

The immunosensor described here was able to detect sample, including *E. coli*, in six minutes, without any labeling or any other steps, with a limit of detection near 10^6 CFU/mL. Several developed immunosensors have been able to detect lower amount of bacterial cells; however, they have not allowed label-free detection and the time needed for one analysis was incomparably higher. Quite good results were obtained by DeMarco et al. using the evanescent wave biosensor.[19] This sandwich format assay detected 5.2×10^2 to 9.0×10^3 *E. coli* cells in 25 min. In other work,[20] a PCR assay was described as a reference method able to detect 10^3 CFU/mL after a 10 hour enrichment. The potentiometric biosensor developed for *E. coli* detection[21] reached a limit of detection 10 CFU/mL in 1.5 hour. As evident from these examples, our own immunosensor exhibited a higher limit of detection, but its main advantage is the very short time needed for the assay, as well as simplicity of this method. The label-free format, combined with a proposed flow-through arrangement can be simply applied for continuous monitoring such as fecal contamination of ground water where *E. coli* is a typical indicator,[21] and for very rapid analysis. When the continuous input of the testing liquid is applied, a substantially lower limit of detection can be reached proportional to the sample contact time. The presented immunosensor needs no reagents and no special steps for the analysis; consequently, the final price of such application becomes minimal in comparison with methods where, for example, expensive secondary labeled antibodies for sandwich formatting are needed. Especially for continuous analysis, the low cost per assay is the most important parameter. Finally, the proposed method can be easily adapted for other bacterial species when the corresponding antibody is applied.

CONCLUSIONS

An immunosensor for rapid detection of the model microorganism was constructed and evaluated in this work. Combination of the piezoelectric device allowing label free detection with a minimal amount of the immobilized polyclonal antibody can be considered as a low cost approach, considering both the consumption of reagents and the simple arrangement. This method is quite fast in comparison with other standard procedures for bacterial detection. The time required for one measuring cycle is under ten minutes, including regeneration; the results from the assay are, in fact, known after four minutes. The long term stability of the immunosensor was proven. No re-calibration is necessary for up to twelve assays. No complicated manipulation with samples is necessary; handling of potentially harmful samples is minimized due to the flow through measurement. The developed assay protocol can be easily applied for detection of pathogenic microorganisms.

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