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Piezoelectric Immunosensor for the Direct and Rapid Detection of *Francisella tularensis*

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ABSTRACT. A novel immunosensing device based on a piezoelectric sensor for direct detection of the biological warfare agent *Francisella tularensis* was developed. This sensor includes mouse polyclonal antibody immobilized in a layer of protein A covalently linked to the gold electrode of the sensor. The immunosensor is able to detect *F. tularensis* with the limit of detection 10^5 CFU/mL with a typical measuring cycle >5 min. The sensor was successfully evaluated for rapid detection of *F. tularensis* spikes in drinking water and milk; no deterioration of sensitivity in comparison with buffer solutions was observed. The proposed concept of a rapid measurement of microbial agents seems to be promising for evaluation of samples after short pre-cultivation enrichment.

Abbreviations

BTA	biothreat alert	PBS	phosphate-buffered saline
BWA	biological warfare agent	PCR	polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay	PZ	piezoelectric
LD_{50}	lethal dose	QCM	quartz crystal microbalance
LOD	limit of detection	RAPID	ruggedized advanced pathogen identification device
LPS	lipopolysaccharide		

The well known pathogenic bacterium *Francisella tularensis* is the causative agent of the tularemia infection. Although the mortality of tularemia disease is ≈1.4 % for the most pathogenic *F. tularensis* biovar *tularensis* occurring in North America (Dennis *et al.* 2001), due to the small number of cells able to initiate the infection and infection propagation through rabbits and rodents into human population, the infection is enrolled relatively high in the lists of potential BWA. *F. tularensis* belongs to Gram-negative bacteria with an amphipatic LPS structures incorporated into its outer membrane which plays an important role in the activation of the immune response (Weintraub 2003) and immunoassays frequently employ antibodies specifically targeted against LPS.

Several methods have been used for detection of *F. tularensis* and the associated infection. Tularemic infection can be monitored with the help of PCR (Emanuel *et al.* 2003) due to the nucleic acid released from *F. tularensis*. The portable real-time PCR device RAPID (*Idaho Technology*) claimed detection of 5 organisms within 1 h (McAvin *et al.* 2004), similarly as the LightCycler PCR from *Roche* (Fujita *et al.* 2006). An independent study comparing the previous two systems as well as the *Cepheid* Smart Cycler confirmed the claimed parameters (Christensen *et al.* 2006). The PCR procedure might be followed by a multiplexed fiber optic sensor assay (Song *et al.* 2006). The approaches focusing on the identification of *F. tularensis* subspecies were also reviewed (Johansson *et al.* 2004).

Whole cells of *F. tularensis* can be detected by cultivation with subsequent identification using ELISA (Grunow *et al.* 2000) or the commercial device BTA (*Alexeter Technologies*). The cultivation-based confirmation seems to be the most sensitive; however, 3 d are typically required (McAvin *et al.* 2004). The *japonica* biovar of subsp. *holartica* was selectively recognized by a dsDNA-based aptamer in a solid-phase assay (Vivekanda and Kiel 2006). Similar approach, the surface-immobilized molecular beacon, was tested for the detection of *F. tularensis* using its 16S rRNA specific sequence (Ramachadran *et al.* 2004).

Immunosensing-based approaches combine specific antibodies with different types of transducers, including the bidiffractive grating sensor (O'Brien *et al.* 2000). PZ biosensor based on QCM seems to be prospective for determination of viruses, bacterial and other cells, proteins, nucleic acids and some small

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molecules, *e.g.*, drugs, hormones or pesticides; moreover, affinity interactions of proteins (antibody and antigenes, receptors and ligands), nucleic acids (hybridizations, intercalation of metal complexes), lipids and saccharide-based layers can be effectively studied (Skládal 2003). PZ immunosensor for detection of the precipitate-like immunocomplex consisting of specific immunoglobulin M and *F. tularensis* was presented by Pohanka and Skládal (2005). The above type of biosensor is convenient for direct, label-free detection of pathogenic microorganisms; *Escherichia coli* (Plomer *et al.* 1992), *Vibrio cholerae* (Carter *et al.* 1995), *Listeria monocytogenes* (Jacobs *et al.* 1995) and *Salmonella* sp. (Wong *et al.* 2002) represent bacteria detected successfully using PZ immunosensors. Alternatively to the direct detection of *F. tularensis*, the rapid identification of the onset of tularemia is of a high importance (Splettstoesser *et al.* 2005). The delayed detection of the infection origin may result in the exposure of hospital stuff in patient care units and laboratory facilities requiring extensive prophylactic treatment (Shapiro and Schwartz 2002). Biothreat surveillance was critically reviewed by Cirino *et al.* (2004) from the technological point of view.

Here we present a PZ biosensor with immobilized polyclonal antibody as a low-cost device for direct (label-free) detection of *F. tularensis* as a model biological warfare agent.

MATERIALS AND METHODS

Microorganisms. As the local legislative regulations do not allow working with the fully pathogenic *F. tularensis*, the vaccination strain *F. tularensis* LVS (ATCC 29684), exhibiting pathogenicity to rodents was used; it was cultured on the McLeod agar supplemented with bovine hemoglobin and Iso VitaleX (*Becton-Dickinson*, USA). *F. tularensis* was harvested after a 1-d cultivation at 36.5 °C and suspended in PBS. The cultivation test provided data on the real concentration of bacteria in suspension. For negative control experiments, *Escherichia coli* (ATCC 9637) and *Bacillus subtilis* (ATCC 11774) were purchased and the suspension was made in the same manner as in case of *F. tularensis*. Live cells were used for detection purposes. The attenuated culture was obtained by adding phenol (final concentration 0.5 %) into the bacterial suspension.

Polyclonal antibody preparation. BALB/c female mice (*Anlab*, Czechia) were subcutaneously inoculated with LD_{50} of viable *F. tularensis* suspension (10^5 cells). Mice were bled under anesthesia by a cut of the ascellary artery 21 d after inoculation. The collected serum was stored for 1 h at 4 °C and centrifuged (8000 ***g***, 15 min). The prepared antibodies were kept at 4 °C for an intraday use or stored frozen. The indirect ELISA procedure (surface-bound antigen, anti-mouse antibody conjugated to peroxidase as a tracer) was employed to determine the titer of antibody; its value was >320.

PZ immunosensors. Quartz crystal resonators with the basic frequency 10 MHz and with two gold electrodes on the opposite sides were from *ICM* (USA). The crystal was washed by dipping in acetone and in distilled water. To form a self-assembled monolayer, 25 μL of 20 mg/mL aqueous cysteamine solution was spread over the gold electrodes for 2 h at 25 ºC. After washing with water, the free amino groups were activated by glutaraldehyde (25 μL of 3 % aqueous solution) for 2 h, washed and protein A was added (25 μL of 3 mg/mL; *Sigma*, USA) and incubated overnight. Finally, the murine serum (25 μL) containing polyclonal antibody specific against *F. tularensis* was spread over the electrode area and incubated at 4 °C overnight. The nonspecific binding sites were saturated with bovine serum albumin (25 μL of 10 mg/mL) for 2 h.

Measuring setup. The sensor was placed in a flow-through cell with the internal volume of 10 μL and connected with silicon rubber tubes (internal diameter 0.1 mm) to the peristaltic pump (PCD 21M, 250 μL/min; *Kouřil*, Czechia). The outlet tube from the system was dipped into a disinfectant solution. Bacterial samples and the carrier (PBS) were introduced from disposable plastic tubes. The PZ sensor was connected to the Lever Oscillator (*ICM*) and the counter UZ 2400 (*Grundig*, Germany) connected to PC measured the frequency. Our own software LabTools controlled data collection (1 s sampling interval, 0.1 Hz resolution). The measuring cycle consisted of the following steps:

– 2 min flow of PBS to stabilize the baseline signal;
– 2 min flow of sample;
– 2–5 min flow of PBS to obtain final stable signal.

The response was evaluated as a decrease of frequency between baseline and the final signal.

RESULTS AND DISCUSSION

Rapid immunoassay considerations. The main aim was focused on speeding up the measuring cycle of immunosensors. The rapid response of immunodevices is of a great importance for alarm tools protecting

facilities against potential bioterrorist attacks. Here, the vaccination strain of *F. tularensis* was chosen as a model microbial agent. For a rapid assay, the direct format is undoubtedly most promising; in conjunction with the label-free transducer, one does not need to perform further steps required in common sandwich assays. Also the pre-incubation time typical for competitive assays is not required. Our previous attempt at developing the direct PZ immunosensor (Pohanka and Skládal 2005) failed probably due to poor antibodies. For this reason, it was decided to develop a new mouse polyclonal antibody suitable for the direct assay.

The assay steps were adjusted to achieve a shortening of the whole procedure. The contact time with the sample was reduced to only 2 min; although this negatively influences the assay sensitivity, the length of assay was a priority here. The zone of sample should be "enclosed" with two zones of buffer, to remove temporary sample matrix effects (fluctuations in sample concentration and viscosity, and loosely binding materials). These "signal reading zones" were reduced to 2 min, which was sufficiently long to wash all sample components away from the measuring cell.

For direct affinity biosensors, after reading the signal the sensing surface is typically regenerated at the end of the assay, to re-establish again the full binding capacity. Reagents of extreme pH, high ionic strength, chaotropes and even proteinases are used; the regeneration might become long and the set-up needs to be washed clean of the regenerating reagents and baseline signal must be established. This process is not straightforward and extensive optimization is required. Here, the time-consuming regeneration step was completely omitted although the binding capacity of the sensing surface is known to be gradually reduced because of the negligible spontaneous dissociation of the immunocomplex. However, considering the planned type of assay – detection of bioagents – most samples are expected to be negative, do not reduce the binding capacity and do not require any regeneration.

Direct immunosensor performance. The PZ immunosensor was constructed using the previously optimized covalent immobilization of protein A and the following oriented immobilization of antibody. As the working pH remains constant during the whole assay, the stabilizing crosslinking of protein A and antibody using dimethyl pimelimidate was omitted. Typical responses of the immunosensor to the presence of the target *F. tularensis* cells as well as to the blank represented by *E. coli* are shown in Fig. 1. It is evident that the signal for *E. coli* was negligible; one can observe a minor peak of frequency decrease during the sample passing through the measuring cell, most probably due to mixing effects. The response to *F. tularensis* was quite significant even after only 2 min of contact, and the immunocomplex formed remained stable in the following buffer zone, providing a shifted baseline signal for the next measurement.

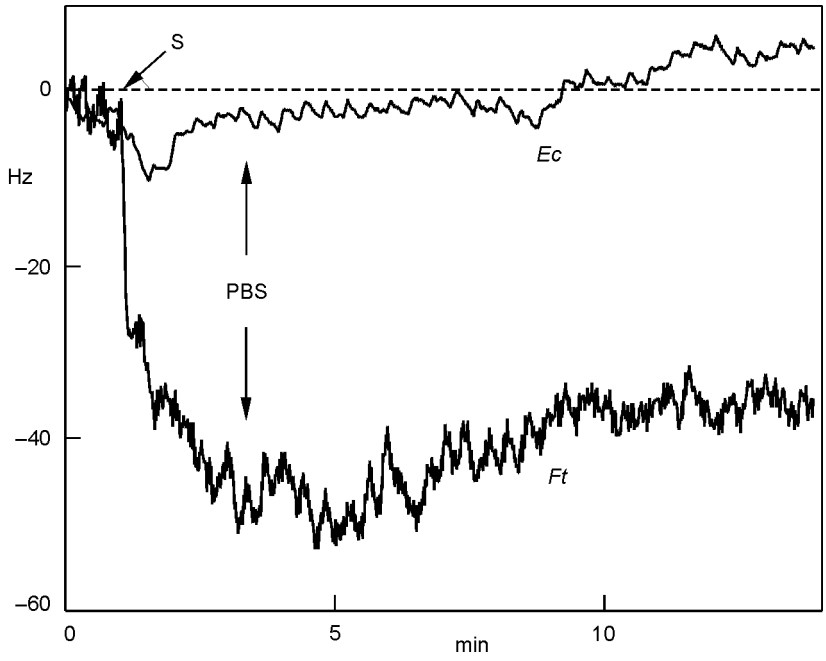

Fig. 1. The real time traces of the relative frequency signal (Hz) from the piezoelectric immunosensor recorded during the injection of *Francisella tularensis* (*Ft*) and *Escherichia coli* (*Ec*); concentration in both of 10^8 CFU/mL. The *vertical arrows* mark addition of sample (S; 2-min zones, corresponding to 0.5 mL) followed by the zone of buffer (PBS). The *dashed horizontal line* marks the baseline level.

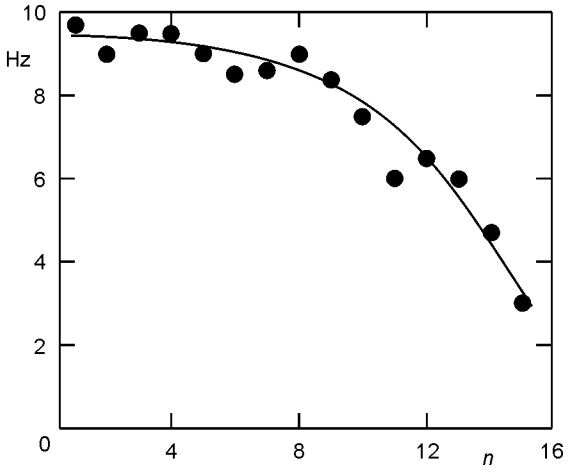

Fig. 2. The stability of the piezoelectric immunosensor with immobilized polyclonal antibody in repeated measuring cycles (*n*, number of measurement) without any regeneration; each point represents the response (Hz) obtained with 10^6 CFU/mL viable *F. tularensis* cells.

In fact, the regeneration of the immunosensor surface was considered and several reagents were tested, but this step remained unsuccessful in the case of a pH change (NaOH and formic acid up to 100 mmol/L) and Triton X-100 up to 5 %. Such reagents damaged not only the microbial cell–antibody immunocomplex but also the immobilized antibody–protein A, and even protein A alone became partially denatured. Therefore, the "regeneration free" format was adopted. As can be seen from the repeated measurements carried out with the same immunosensor (Fig. 2), one sensing layer was able to respond with a reasonably constant signal to 9 consecutive samples containing *F. tularensis* at 10^6 CFU/mL. Even though the binding capacity gradually decreased, a minimal loss of sensitivity occurred. For the additional measurement, the binding capacity became a limiting factor and the decrease of a signal was clearly evident. However, the detectable signal expressed as ratio of signal (S) and noise (N) was >3 even for the 14th measuring cycle. We supposed that this operational lifetime of the immunosensor sensor is quite sufficient and the regeneration attempts were therefore abandoned. The data (Fig. 2) were of course generated for all samples being positive; as mentioned *above*, this case is not typically expected for real situations.

Calibration of the immunosensor. Standard samples, *i.e.* live *F. tularensis* LVS suspended in PBS were used as a model biological agent. The calibration curve was constructed for the concentration of bacterial suspensions in the range of 10^4–10^9 CFU/mL (Fig. 3); the total amount of sample was 500 μL. The average N was 1.5 Hz (standard deviation of the mean signal during a 2-min interval), and the lowest concentration representing LOD expressed as S/N = 3 was thus 10^5 CFU/mL (4.8 Hz) for the viable cells. Additionally, a "safer" version of the microbe was also tested – attenuated cells in 0.5 % phenol. For such attenuated cells the immunosensor become significantly less sensitive and LOD shifted to 10^6 CFU/mL; in the presence of 0.5 % phenol the interaction of antibody with microbial cells was evidently weaker. The relative standard deviations (SD) for viable and attenuated cells (10^8 CFU/mL) were 5.6 and 10.6 %, respectively. The calibration curve was terminated at 10^9 CFU/mL; in this case, the viable cells provided S of 42 Hz, a value which was nearly twice higher than with the attenuated cells (25 Hz). The calibration curves for viable as well as attenuated cells are presented in Fig. 3.

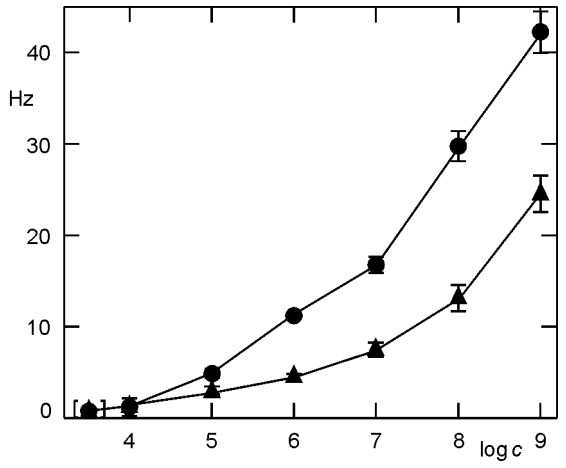

Fig. 3. Calibration curves for *F. tularensis* (*c*, cell concentration in CFU/mL; log *c*) obtained using the piezoelectric immunosensor (PZ response, Hz) with immobilized polyclonal antibody. The suspension of living viable cells in PBS was used (*circles*) and compared to the attenuated cells measured in PBS supplemented with 0.5 % phenol (*triangles*); the *points in brackets* represent blank (no bacteria); *error bars* indicate SD at *n* = 3.

Selectivity of the immunosensor. The suspensions of viable cells of *F. tularensis* at two levels (10^8 and 10^5 CFU/mL) were compared with suspensions of *E. coli* and *B. subtilis* at the same cell concentrations. The signal in *F. tularensis* assay for the higher cell concentration was 29.3 ± 1.7 Hz (*n* = 3), the negative

controls being under the threshold value (S/N = 3, *i.e.* 4.5 Hz) (Table I). The selectivity was examined close to the LOD level; the suspensions of *F. tularensis*, *E. coli* and *B. subtilis* of 10^5 CFU/mL were used. The average response for *F. tularensis* (4.8 ± 0.6 Hz) was clearly distinguished from signals obtained with *E. coli* and *B. subtilis* at the probability level >99 % (*t*-test). Thus, the obtained results were sufficient for distinguishing *F. tularensis* from other bacterial species.

Table I. Selectivity of the piezoelectric biosensor[a] for *F. tularensis* as compared with the negative controls (*E. coli* and *B. subtilis*)

CFU/mL	*F. tularensis*	*E. coli*	*B. subtilis*
10^5	4.8 ± 0.6	3.0 ± 0.4	1.4 ± 0.5
10^8	29.7 ± 1.7	2.3 ± 1.7	0.9 ± 0.7

[a]Responses (in Hz; mean ± SD for $n = 3$) for the target bacterium at concentrations of 10^5 and 10^8 CFU/mL.

Sample matrix effects. In the real situation of bioterrorist attacks, different conditions can be abused for the spreading of tularemia infection. For this reason, several sample matrices were tested including drinking water and milk as representatives of potential contaminated food. The bacterial suspension was prepared in the same manner as in the case of calibration curve preparation; PBS was replaced by drinking water from the common supply system and by packaged milk (fat content 1.5 %, UHT type). *F. tularensis* (viable) was added to a final concentration of 10^8 CFU/mL. The signals obtained by measuring such suspensions in PBS, PBS with 0.5 % phenol, drinking water and milk are presented in Table II. The immunosensor exhibited similar sensitivity for drinking water and PBS, the decreased response in the presence of phenol was expected. In case of the milk sample, the response was slightly higher; the milk composition probably promoted the immunointeraction and possibly also prolonged the contact time as the washing of the measuring cell took longer time (5 min).

Table II. Comparison of different conditions for detection of *F. tularensis* (10^8 CFU/mL) by the PZ biosensor with immobilized mouse polyclonal antibody

Sample matrix	LOD CFU/mL	Response[a] Hz
PBS	10^5	29.7 ± 1.7
PBS with 0.5 % phenol	10^6	13.2 ± 1.4
Tap water	10^5	29.1 ± 2.0
Milk (1.5 % fat content)	10^5	33.1 ± 2.7

[a]Mean ± SD for $n = 3$.

We demonstrate a promising approach to a rapid immunosensor for direct assay of the biowarfare agent *F. tularensis*. The reduced and simplified direct measuring format ensures a very fast assay; in fact, during repeated use, only 4 min are required for one analysis cycle, as the baseline zones between adjacent samples become shared. The issue of limited sensing capacity is less critical for real situations, when mostly negative samples are expected. The overall procedure becomes quite simple; no additional reagents are required as the PZ transducer measures formation of the immunocomplex directly. The fully flow-through arrangement requires minimal manipulation with samples and thus the risk of user contamination becomes reduced. The concept seems to be generally applicable for other microbial agents.

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