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Electrochemical immunosensors for detection of microorganisms

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Electrochemical immunosensing devices for detection of microbial agents are briefly reviewed. The practical results describe amperometric immunosensors based on screen-printed electrodes as a general platform for sandwich-based assay of microbes. The examples are focused on the determination of *Francisella*, *Salmonella*, *Escherichia* and *Bacillus* species. The achieved analytical parameters seem promising for real applications. In particular, combination of the immunosensor with cyclone device allowed fully automated testing of the system for detection of bioaerosols of *E. coli* as a model agent; the preliminary results confirm that levels below 100 CFU/L in air can be detected within 20 min.

Keywords: Amperometric Sensor, Screen-Printed Electrode, Immunochemical Detector, Atomic Force Microscopy

1. INTRODUCTION

Detection and identification of microbial agents is a challenging task. Prevention or early treatment of infectious diseases and even death of people due to possible bioterrorist attacks became significant issues for the modern society. For analysis, suitable procedures (PCR, polymerase chain reaction, and ELISA, enzyme-linked immunosorbent assays) and devices (MS, mass spectrometry) are available; however, slow operation, complicated portability and high running costs are limiting widespread application. Assays of pathogens are further complicated by small differences between common and dangerous microbial species. As alternatives, numerous types of biosensors are considered as well suited for this purpose [1-3], interest is currently focused on food borne pathogens

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[4,5] and monitoring of water [6]. The focus on detection of bioagents originated from military, however, rescue and security services, protection of public buildings and homeland security are participating, too. The activity of terrorist organizations increases the danger of using biological warfare agents (BWA), too. Consequently, portable, rapid and simple instrumentation based on immunoanalytical principles became widely supported [7,8].

For microbial agents, immunochemical devices are preferred for the early response, good sensitivity and continuous monitoring capabilities. The detection is based on phenotype and no extraction of the genetic material from the bioagent is required. Here, the electrochemical immunosensors will be addressed as an approach combining sensitivity, simple construction and portability of the sensing part with excellent specificity of antibodies as affinity-based recognition elements. The principles of electrochemical immunosensors are well-known [9] and their potential for detection of pathogens was realized as well [10,11]. Recently, nanomaterials and nanoparticles come into the focus of scientists as advantageous tools for preparation of electrochemical biosensing layers with potentially enhanced performance [12].

The electrochemical measurement system is highly sensitive, quite cheap and already exists in portable formats. Progress in electronics allows miniaturizing the whole electrochemical system to a single-chip format; the embedded digitally controlled potentiostat LMP91000 (Texas Instruments) is programmed through serial interface (I2C) and consumes minimum power. Even the advanced pulsed, voltammetric and galvanostatic techniques are available in hand-held instruments from several companies: PalmSens and EmStat (Palm Instruments), μStat (DropSens), PG581 (Uniscan Instruments), 910 PSTAT mini (Metrohm), as well as other prototypes designed in laboratories.

As the measuring element, the screen-printed electrodes (SPE) are widely applied due to easy and reproducible fabrication at both laboratory and mass production scales [13]. The reasonable production costs allow the single use of the resulting immunosensors, thus, no complicated regeneration procedures are required. The suppliers of SPEs include companies as BVT Technologies, DropSens and Gwent Group; however, researchers can print the sensing patterns themselves using commercial inks and pastes or even using custom mixtures containing carbon nanotubes [14] and metal nanoparticles [15] for improved communication with biomolecules. Sensing systems are printed on plastics or alumina ceramics, the latter allowing use of high temperatures for manufacturing and providing electrodes quite similar to pure metals or sputtered metal layers. The SPE approach allows designing various shapes and arrangement of electrodes, production of several sensing channels is favorable for detection of several target species during one assay.

The measurements are commonly carried out in the amperometric mode where the indicating molecule (e.g. product of the enzyme label reaction) becomes converted. The working potential can also be pulsed in order to improve the signal / noise ratio (continuous pulses); alternatively, the accumulated product is converted after applying a single step of potential (chronoamperometry) to achieve higher response. More complex voltammetric techniques are particularly suitable when several different indicating molecules need to be detected in multiplexed assays. The impedimetric measurements either follow formation of affinity complexes on the surface of the electrode, when impedance of the sensing surface becomes significantly affected; the alternative voltage is applied and the phase and amplitude of the non-faradaic current is interpreted. Blocking of the electrode with

immunocomplexes can be probed using external redox label (ferri / ferrocyanide) and voltammetric measurement [9].

The immunosensor is obtained when antibody as the immunorecognition element becomes immobilized on the surface of the electrode. Antibodies used are immunoglobulin G (IgG) and rarely also IgM; the fragments made from native antibodies by chemical and proteinase-based cleavage (Fab) and recombinant forms (single chain variable fragments, scFv) might provide immunosensing surface with higher density of the binding sites. The molecule of IgG should be immobilized on the electrode, and the covalent linkage is the preferred option. The bare metal and carbon electrodes are able to adsorb proteins including antibodies, but the resulting layers are not robust enough as slow spontaneous release of proteins occurs. The noble metal electrodes (gold, platinum, including the screen printed versions) are first chemically activated using deposition of thiol-based self-assembled monolayers (SAM). The other end of the typically linear molecule of thiol brings a suitable group used for subsequent attachment of antibodies. Thiol modifiers providing amino, carboxy and hydroxyl groups are widely used; cysteamine, merkaptoundecanoic acid and merkaptodecanol representing these options, respectively. The appropriate conjugation reactions and reagents are available in the literature [16]. The advanced approaches include mixed SAMs, where active (e.g. biotin) and inactive (hydroxyl, polyethylenglycol, oligosaccharide) end-groups allow controlled density of the immobilized binding sites and limit the non-specific adsorption. Immunoglobulins become linked directly, the oriented linkage through proteins A and G is other option; this protein A – IgG affinity complex should be covalently cross-linked for enhanced stability if longer or repeated use of the immunosensor is expected.

The primary immobilized antibody captures the target microorganisms from the sample and after washing, the obtained surface-bound immunocomplex should be specifically labeled using the secondary antibody linked to enzyme label, this conjugate is known as tracer.

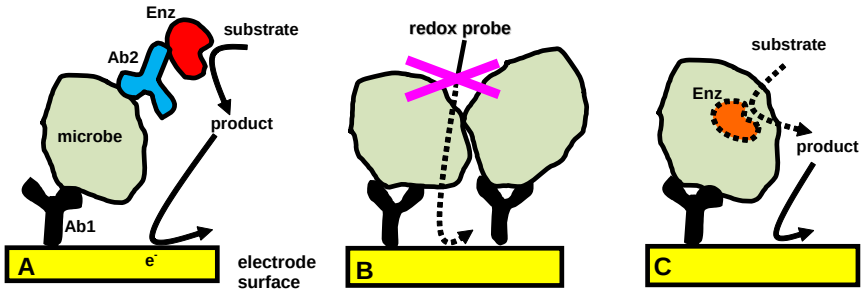

Figure 1. Examples of electrochemical immunosensor formats. (A) Sandwich assay, primary antibody Ab1 captures the target microbe. This is labeled using the secondary antibody-enzyme conjugate (Ab2-Enz), and the added enzyme substrate becomes converted to electrochemically active product measured at the electrode. (B) Direct assay, the captured microbe is blocking access of a suitable redox probe to the electrode. Alternatively, impedance of the electrode is measured directly. (C) Viable (living) cells are initially captured; afterwards, the chosen microbe-originated reporting enzyme is measured.

The final sandwich complex is again washed, substrate for the enzyme is added and the signal is recorded (Fig. 1A). The heterogeneous sensing format is widely used; the double antibody system

enhances specificity of the procedure and the enzyme label amplifies the useful signal which together with the electrochemical measurement provides high sensitivity and robustness [9].

Alternatively, the captured microbial species can be detected directly; typically, the formation of the immunocomplex on the surface of the electrode blocks access to the surface (Fig. 1B). This is measured either using cyclic voltammogram of a suitable redoxactive probe (e.g. ferri/ferrocyanide redox pair, signal decreases in case of positive response), or the increased resistance (generally, impedance) of the electrode is determined from electrochemical impedance spectroscopy (EIS). However, in this case, the non-specifically captured microbes might contribute to the measured signal, too.

Sometimes, not only the presence of microbes, but also viability of the captured cells should be determined. For this purpose, intracellular activity of a suitable reporting enzyme (e.g. β-galactosidase) is measured (Fig. 1C). The microbe is captured on the electrode, the enzyme activity is enhanced using suitable inductor and its activity is measured using suitable substrate providing electroactive product (e.g. phenyl-β-D-galactoside). The specific detection of target microbial pathogens is carried out in food, water and air samples. Many publications focused on the proof of concept or proposing novel detection schemes are using non-pathogenic strains of *Escherichia coli*. *Bacillus globigii* is typically used for detection of model bioaerosols in military testing facilities. The examples of electrochemical immunosensors for microbial cells are presented in Table 1.

Table 1. Selected electrochemical biosensors for assay of microbial pathogens.

Microbe	LOD (CFU mL^{-1})	Time	Sensing surface, principle	Ref.
Bacillus globigii spores	112	~10 min	Ag NP-Au/Ab, direct/blocked RP, DPV	[17]
Escherichia coli	$4 \cdot 10^4$	1.5 h	B-doped diamond, βGal assay, CV	[18]
	150	3 h	Pd-IrO_2, βGal assay, amper	[19]
	10	~1 h	Au/Ab, direct/imped.	[20]
	10^3	~1 h	Au/Ab, direct/blocked RP, imped	[21]
	10^4	~30 min	Pt,Pt-black, Ab, μchannel, imped	[22]
4 strains	$9 \cdot 10^5$	~30 min	C SPE array/lectins, direct/coulom	[23]
O55	50	~10 min	O_2 sensor, membrane/Ab, catalase, amper	[24]
O157	10^3	~10 min	porous Si/Ab, direct/imped	[25]
	$2 \cdot 10^6$ (20)	1 (7) h	magn. P/Ab, Au, βGal assay, amper	[26]
Salmonella Typhimurium	$5 \cdot 10^3$	2.5 h	C/tyrosinase, magn. P/Ab1, sandw/Ab2-ALP	[27]
	$5 \cdot 10^4$	2 h	C/Ab1, sandw/Ab2-ALP	[28]
	21	2.5 h	C SPE/CM-dextran-Ab1, sandw/Ab2-HRP	[29]
Salmonella enteritidis	10^3	5 min	Au IME/Ab, direct/gap imped	[30]
Streptococcus pneumoniae	$1.5 \cdot 10^4$	3.5 h	Au SPE/magn. P/Ab1, sandw/Ab2-HRP	[31]
E. coli, S. aureus, S, cholerasuis	(10^6)	3 h	C-Fc/GOD, glucose consumption, antibiotics	[32]
E. coli, S.aureus, M. phlei	$5 \cdot 10^3$	>1 h	Au-SPE/lectins, direct/imped + βGal/tyrosinase	[33]

Abbreviations: ALP, alkaline phosphatase; amper, amperometry, coulom, chronocoulometry; CV, cyclic voltammetry; DPV, differential pulse voltammetry; Fc, ferrocene; βGal, galactosidase; GOD, glucose oxidase; IME, interdigitated microelectrode; imped, impedance; magn. P, magnetic particles; NP, nanoparticles; RP, redox probe; sandw, sandwich assay

The variability of testing principles is really high considering both sensing surfaces and assay formats. The ELISA-inspired sandwich assay using capture antibody and the tracer seems most complicated as it requires several incubations and washing steps; measurements is typically a simple amperometry. On the other hand, this heterogeneous enzyme-label based format ensures high robustness and reliability and was successfully tested on real samples. Advantageously, the magnetic particles can be used for pre-concentration of the target and washing of sample matrix components, thus enhancing sensitivity and protecting the sensing surface from fouling with biomolecules [27].

Many attempts exist towards the simple direct pseudohomogeneous formats (e.g. Fig. 1B), which potentially simplify the working protocol. However, the label-free measurement of the captured microbes requires more sophisticated approaches in order to achieve the desired low limit of detection. In this way, the assay becomes less robust as the various amplification strategies and complex surfaces and procedures are more sensitive and become easily influenced by matrix of the real samples; model microbial samples are mostly used for evaluation of the performance and the reported surprisingly low detection limit are hardly accessible for complex real samples. Not only measurement, but also the method of sampling, storage and pre-incubation conditions should be considered [34]. Critical validation of results is mostly missing; for this purpose, the publications reporting on parallel measurement of samples using different approaches are highly valuable [35,36].

2. EXPERIMENTAL PART

2.1 Chemicals and material

Cystamine, glutaraldehyde (GA, 25% solution), bovine serum albumin (BSA) and horse radish peroxidase (HRP) were obtained from Sigma. Potassium iodide and hydrogen peroxide were from Penta. Phosphate buffer (50 mM sodium phosphate, pH 7.0), phosphate buffered saline (PBS, phosphate buffer and 150 mM NaCl), acetate buffer (50 mM sodium acetate, pH 4.0 with 150 mM NaCl) were used for experiments.

2.2 Antibodies (Ab) and immunoreagents

The mouse polyclonal antibodies anti *Bacillus subtilis* and anti *Escherichia coli* [37] were kindly provided by Miroslav Pohanka, Central Military Institute of Health, Těchonín, Czech Rep. The rabbit polyclonal anti *Francisella tularensis* Ab was obtained from Aleš Macela, University of Defence, Hradec Králové, Czech Rep. The anti *Salmonella* Typhimurium mixed polyclonal serum was provided by Ivan Rychlík,Veterinary Research Institute, Brno, Czech Rep. The mouse monoclonal Ab AL-01 (anti albumin, used for reference purposes) was provided by Exbio Praha (Prague, Czech Rep., 10 mg/mL IgG).

The polyclonal rabbit anti *E. coli* Ab 4329-4906 conjugated to horse radish peroxidase was supplied by AbD Serotec. Other tracers were prepared using the standard periodate-based conjugation protocol [16]; briefly, 4 mg of dialyzed periodate-oxidized HRP was mixed with antibody (an amount

corresponding to 10 mg of IgG) and incubated for 2 hours in 50 mM carbonate buffer pH 9.5. Next, 0.4 mg mL^{-1} sodium borohydride was added; mixture was incubated for 2 hours and dialyzed overnight in 100 mM borate buffer pH 7.4. Thus obtained conjugates were mixed with 60% glycerol (1:2) and stored frozen.

2.3 Microorganisms

B. atrophaeus (also known as *B. subtilis*, var *niger* and *B. globigii*) served as a substitute for *B. anthracis*. It was cultivated aerobically at 30 °C in 8 g L^{-1} Nutrient Broth no. 4 (Fluka) supplemented with $MnSO_4$, $MgSO_4$ and $CaCl_2$, each at 10 mg L^{-1}. Bacterial cells were collected after 5 days, centrifuged for 20 min at 12,000 r.p.m., and resuspended in 0.9% NaCl. Sporulation medium was prepared by dissolving 8 g L^{-1} of Nutrient Broth Nr. 4, 50 mM $MgSO_4$ and 1.5 M KCl, pH value was adjusted to 7.6. 1 mL of the sterile solution containing 1 M $CaCl_2$, 1 mM $FeCl_2$ and 10 mM $MnCl_2$ was added to 1 L of the medium prior use. The sporulation was initiated by inoculation of fresh bacterial cell culture in 250 mL of the completed medium and incubation proceeded aerobically for 21 days at 30 °C. The obtained suspension was centrifuged at 5,000 r.p.m. at 10 °C for 15 min. After washing with water for 24 hours, three cycles of centrifugation were carried out to remove debris and the pellet of spores was suspended in 20 mM $CaCl_2$ (pH 9.0) and stored in cold. To verify homogeneity of the produced cells and spores, AFM images of microbial suspensions deposited on a glass support were obtained (Fig. 2) and confirmed negligible contamination of spores with cells or in the opposite.

The *E. coli* DH5α strain was cultivated on the Luria Broth medium (Duchefa Biochemie, Netherlands), 30 g L^{-1} with added 5 g L^{-1} NaCl, cultivation was aerobic at 37 °C for 1 day, cells were centrifuged at 4,000 r.p.m. for 20 min and resuspended in phosphate buffer. The fresh cells of *Salmonella* Typhimurium were provided by I. Rychlík. The fresh cells of *Francisella tularensis* LVS (live vaccination strain) were provided by A. Macela; all experiments with *Francisella* cells were performed in specialized certified category 3 microbiological laboratories of the State Institute of Nuclear, Chemical and Biological Protection in Kamenná, Czech Rep. Concentrations of cells were determined spectrophotometrically using the McFarland method; the working solutions of microbes were made fresh daily by diluting the stock solution in PBS.

2.4 Immunosensors

SPE with four working Au electrodes (1 mm diameter, type AC8.W1) were supplied by BVT Technologies, Brno. After cleaning with acetone (30 min), the cystamine SAM was formed (2 hour incubation, cystamine 10 mg mL^{-1} in water). The washed surface was activated with GA (1 hour, 3% solution in phosphate), washed again and incubated with the appropriate antibody (typical concentration 200 μg/mL, overnight in refrigerator). Next day, the surface was washed with phosphate and water and stored dry in cold. Prior to use, the sensing part of SPE was incubated for 1 hour with 0.2% BSA in order to saturate any non-specific binding sites. The same procedure was also used for immobilization of antibodies on Au coated Si chips (4 x 4 mm, SPI Supplies, West Chester, USA) utilized for microscopic scanning.

2.5 Measuring procedure

The immunosensor was fixed in a flow-through cell (~10 μL) containing also pseudoreference silver electrode. The immunosensor was connected to ImmunoSMART (Smart Brno), its four miniperistaltic pumps delivered the solutions: P1 pumped either sample with microbes or PBS with 1 mg/mL BSA (washing solution for the immunochemical steps); P2 injected the tracer (dissolved in PBS with 1 mg mL^{-1} BSA); P3 and P4 mixed the substrates for HRP, both pumps contained acetate buffer plus either 4 mM H_2O_2 (P3) or 8 mM KI (P4). P3 was working continuously, while P4 added a few minutes long zone of iodide when the response was measured. The flow rates were 15 μL min^{-1} and the applied potential was –50 mV; this ensures reduction of iodine produced in the peroxidase enzyme reaction (Fig. 1A):

$$H_2O_2 + 2I^- \xrightarrow{\text{peroxidase}} I_2 + 2OH^-$$

$$I_2 \xrightarrow{\text{electrode, } -50\,\text{mV}} 2I^-$$

2.6 Experiments with bioaerosols

Bioaerosols were formed inside a metal chamber (62 m^3, FOI, Umea, Sweden) where the tested systems were placed. Using a nebulizer-based spreader system, the source suspension of microbes (*E. coli* DH5α, ~10^{10} CFU/mL) was disseminated inside the closed chamber and distributed with the help of rotating ventilators within few seconds. The timing of dissemination was controlled externally as well as ventilators and HEPA filters allowing decontamination of the chamber. All persons entering the decontaminated chamber were properly equipped with protective suit and wear the chemical protective mask according to the safety regulations.

Sampling of the air was done using the portable air sampler SASS 2300 (Research International, Seattle, USA). It is a wetted wall cyclone system, the aspirated air is contacting circulating phosphate buffer and the present microbes became captured in the solution (5 mL sample, 5 min sampling). The output solution from the sampler was aspirated to a holding coil using the peristaltic pump of the sampler and then pumped through the flow cell with immunosensor for 2 min using one of the peristaltic minipumps of ImmunoSMART. The subsequent operation was the same as for liquid samples (chapter 2.5). The program LT_ImmunoSMART synchronized the sampler and detector and controlled all timing, washing, incubation steps and data recording; the parameters were edited as a working script thus allowing simple programming of operation steps and reproducibility of measuring procedures. The reference measurements were performed using rotating agar plate slit samplers (BC-AM-11, Biotrace F. Baker), mass flow meter (EL-FLOW, Bronkhorst) and vacuum pump (17 L min^{-1}); the air flow passes through a narrow slit and contacts the rotating petri dish plate with solid agar cultivation medium, the captured microbes are cultivated overnight and the counted number of colonies converted to CFU per liter of air.

2.7 Atomic force microscopy

Surface-bound cells were imaged using the atomic force microscopes (AFM) Ntegra Vita/Solaris (NT-MDT, Zelenograd, Russia) and Bioscope Catalyst (Bruker, Mannheim, Germany) both with custom control and evaluation software packages. Scanning was performed in dry conditions. The diluted (~10^3 cells/mL) microbial suspensions were directly spread over the surface of glass or gold-coated Si chips, allowed to dry in the stream of air for 15 min and then immediately imaged (Fig. 2).

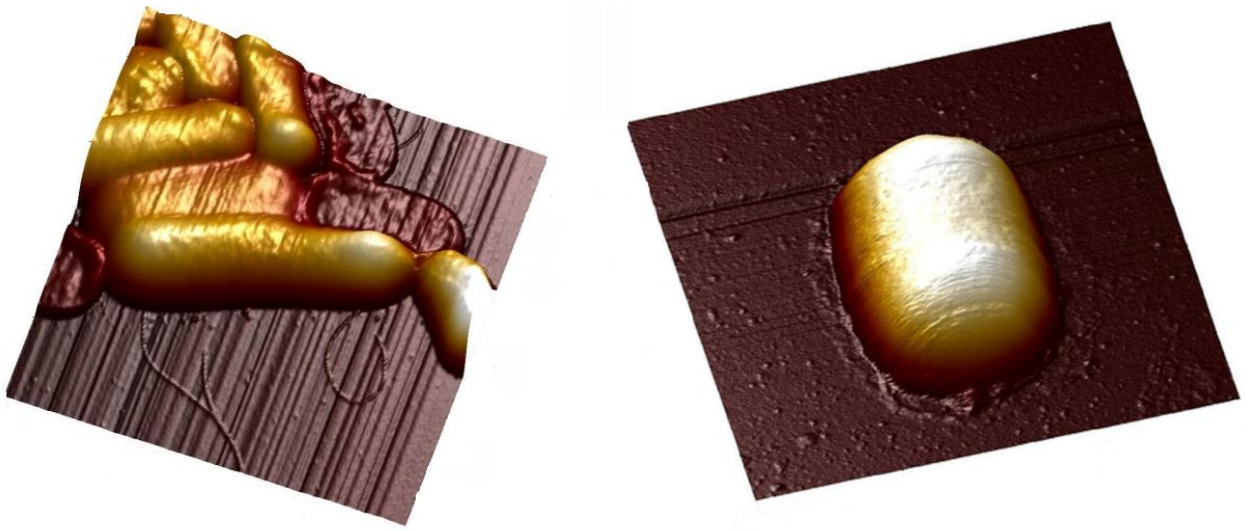

Figure 2. AFM images of *Bacillus atrophaeus* cells (left, 5x5 scan size) and a spore (right, 2.8x2.8 μm scan size); pseudo-3D visualization, vertical range 0.6 μm. The samples were deposited on glass support, allowed to dry and immediately imaged. Bioscope Catalyst, PeakForce mode.

3. RESULTS AND DISCUSSION

3.1. Amperometric immunosensors for microbes

The development of immunosensors for microbial agents was in our case stimulated by Czech Army, which decided to initiate research in the field of biosensors potentially useful for rapid detection of biological weapon agents. Our proposal to direct activities towards electrochemical immunosensors was accepted, and the screen printed sensors with gold electrodes were adopted as the transducer. The multichannel sensing format was chosen from the beginning in order to measure simultaneously in control reference zone non-specific interactions, and also to allow future screening of several microbes during one analysis.

The immunoassays were realized in the sandwich format (Fig. 1A) with capture antibody covalently linked to the cystamine SAM deposited on gold. The incubation with sample containing the target microbes was performed and finally the bound microbes were labeled with the secondary antibody linked to peroxidase (tracer). This heterogeneous format employs washing steps after each incubation in order to remove loosely bound non-target cells and tracers interacting non-specifically

with sensing and working surfaces, tubings etc. The heterogeneous formats are more complicated and time-consuming; however, the robustness of the assay based on two specific interactions seems to be an advantage. The prototype immunosensor detector ImmunoSMART was designed in order to provide multichannel amperometric measurements and automated flow operation [38].

The initial experiments focused on *Francisella tularensis*; this highly pathogenic microorganism belongs to the top list of biological weapon agents compiled by the Centers of Disease Control, and was chosen by army to work with 'real' model microbe. The examples of measuring traces for heat inactivated (for safety reasons) and fully viable cells are shown in Fig. 3.

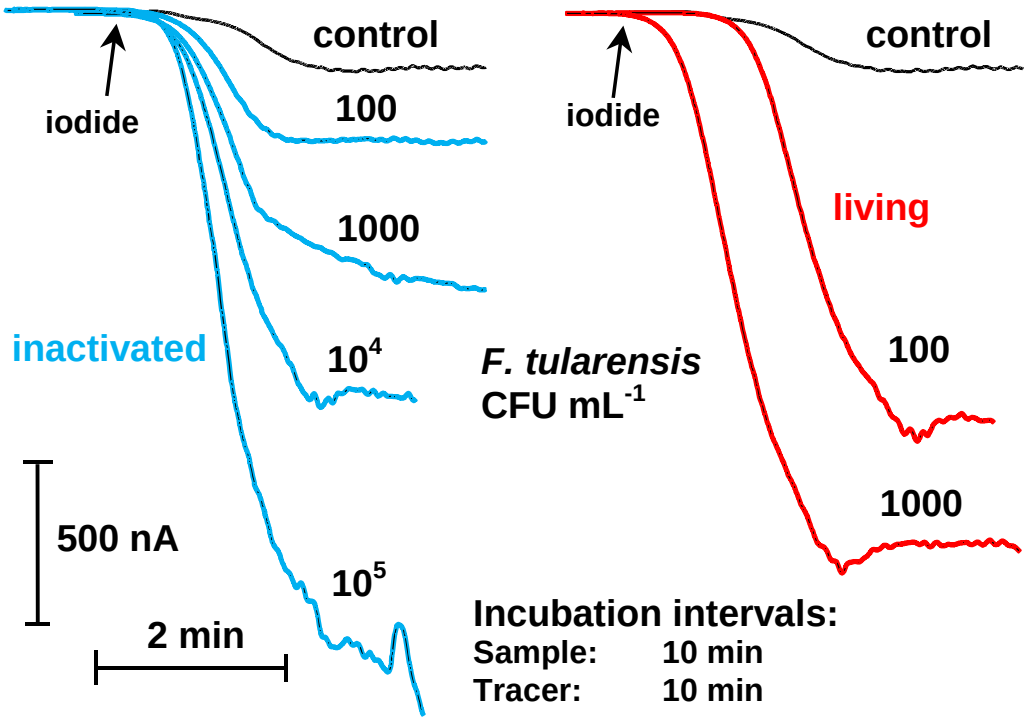

Figure 3. Signal traces from the immunosensor for *F. tularensis* LVS. Immunosensors with immobilized Ab after incubation (10 min in flow) with different samples of heat inactivated (left) and viable (right) cells were evaluated in the carrier acetate buffer containing 5 mM H_2O_2, response to the injection of 10 mM KI into the flowing medium is shown. The traces of current correspond to the amount of captured cells labeled with the peroxidase tracer. Control indicates samples without any cells.

Compared to standard ELISA procedures, which require more than hour to complete, the flow-through operation of the immunosensor allowed to shorten the incubation with sample and tracer significantly while providing comparable limit of detection. The sandwich assay format provided good sensitivity; a big difference existed between the control and the sample containing 100 CFU mL^{-1} of cells. This indicates that even less microbes can be detected. However, the inactivated cells (Fig. 3, left) exhibited significantly lower responses indicating that the surface antigen interacting with the used Ab was partially damaged. Even lower responses were obtained for killed microbes (Tab. 2).

Similar concept of the amperometric immunosensor was also extended for other microbial species. In Fig. 4, the calibration for *Salmonella* Typhimurium is shown together with microbes deposited on the gold surface.

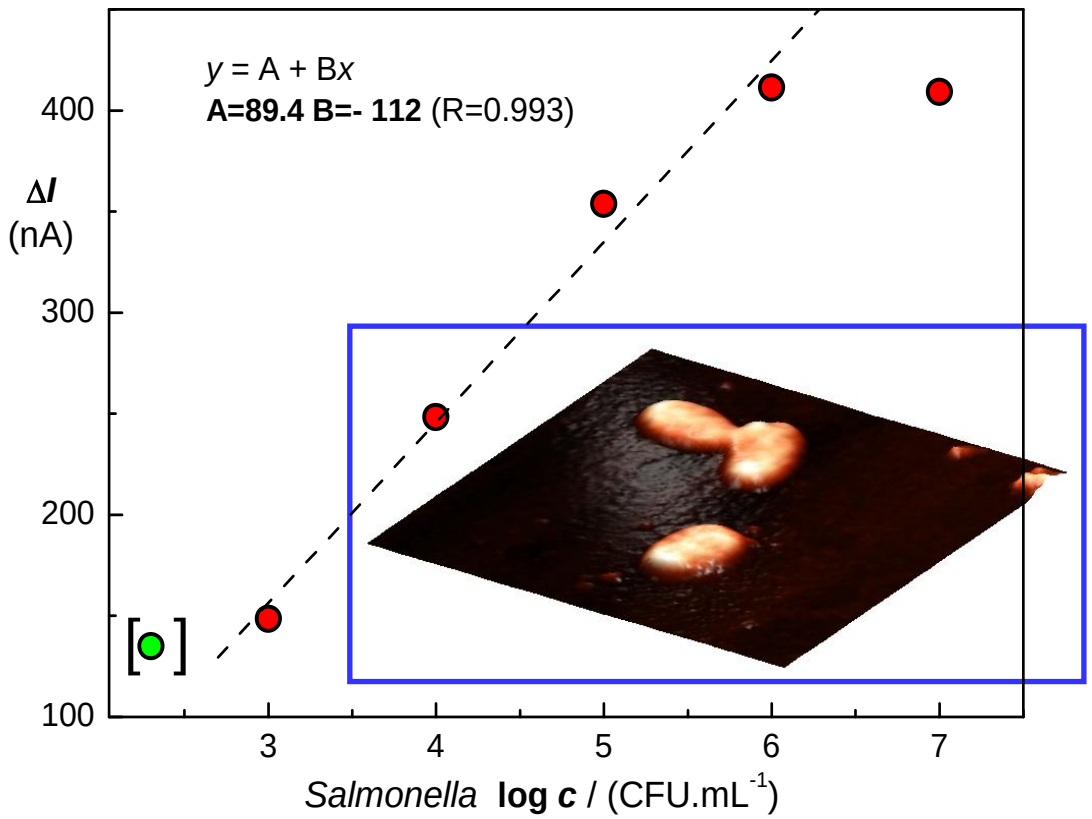

Figure 4. Calibration of the immunosensor for *Salmonella* Typhimurium. Incubation was 60 min with the sample and 15 min with the tracer. The point in brackets represents control response in the absence of microbes. The standard deviations of the points were below 20%. The insight graph presents AFM image of *Salmonella* cells, scan size 5x5 μm, vertical range 0.15 μm, Ntegra Vita, non-contact scan of cells bound on the gold substrate.

In the following period, the focus was on non-pathogenic microorganisms which are better suited for normal laboratory work and also for the planned testing of microbial bioaerosols required by the army partners. For this purpose, *Escherichia coli* DH5α [39] and *Bacillus atrophaeus* [40] were chosen. The comparison of immunosensors for microbes developed in our laboratory is summarized in Tab. 2.

Under similar arrangement of the assays (transducer, immobilization procedure, flow rate, level of tracer, etc.), the resulting analytical parameters are quite different for individual microbes. This indicates the significant role of the antibodies chosen for the immunosensor; especially the immobilized capture Ab plays the most significant role and its proper selection is the key issue for successful performance of the immunosensor. Furthermore, an interesting comparison appears also in the case of *Francisella*, where the state of the microbe is influencing the assay, too. Therefore, the

collection and sampling procedures should be carefully considered [34]. This might be quite important when microbial aerosols should be detected.

Table 2. Amperometric immunosensors for detection of microbes based on screen-printed sensors. Comparison of performance of the sandwich assays, peroxidase as a label and hydrogen peroxide / iodide as the substrate mixture. Measurements were performed in flow-through mode at room temperature.

Microbe	Range[a] (CFU mL^{-1})	Time[b] (min) [sample / tracer]	Saturation signal[c] (nA)
F. tularensis (killed)	$5 \cdot 10^7$ - $2 \cdot 10^9$	100 [60 / 30]	32 ± 3
(innactivated)	100 - 10^5	25 [10 / 10]	1200 ± 100
(viable)	<100 - 10^4	25 [10 / 10]	1300 ± 100
S. Typhimurium	10^3 - 10^6	80 [60 / 15]	270 ± 50
B. subtilis (cells)	$5 \cdot 10^3$ - 10^6	20 [10 / 5]	17 ± 2
E. coli DH5α	$7 \cdot 10^2$ - 10^6	30 [15 / 10]	380 ± 60

Notes: [a] from the LOD till the saturation of the response; [b] optimized incubation intervals; [c] difference of the response at saturation and the background signal with standard deviation.

3.2 Immnosensing of bioaerosols

In real military and civil monitoring situations, the dangerous microbes should be preferentially monitored in the air; the spreading in the form of bioaerosols is typically expected in the target areas as transportation terminals and public buildings. For testing purposes, the relevant military and civil protection institutes are organising field tests of equipment [38] where model simulants (e.g. spores of *B. atrophaeus*) are spread and monitored. However, the laboratory aerosol chambers allow achieving better control of the environment (temperature, humidity, air flow) and the disseminated microbes. A typical response from the immunosensor combined with the air sampling cyclone system is presented in Fig. 5.

The main graph presents the profile of *E. coli* cells in the air inside the chamber during the dissemination interval (red horizontal line) and afterwards. In fact, the stability of the aerosol was very limited; the cells were very quickly disappearing from the air due to decomposition under limited humidity (25%). An increase of humidity (35-37%) helped to improve this situation. The sampling was running for 5 min (green horizontal line) and thus obtained solution of microbes was transferred to the immunosensor system and analyzed. The inset graph present responses obtained for positive (*E. coli* disseminated) and negative control (no dissemination) experiments. The positive response corresponded to some 120 CFU L^{-1} in air, as determined from the reference slit sampler and cultivation.

The minor signal change obtained for the blank control measurements was probably due to the non-specific binding and interaction of other cells present permanently in the chamber as spores. As can be seen from the agar plates used for the monitoring of air during the experiment (Fig. 6), the cultivation revealed not only colonies of *E. coli* DH5α, but also *B. globigii* and *B. thuringiensis*, which

were used in previous tests (few month ago) and successfully 'survived' the subsequent chamber cleaning and disinfection procedures.

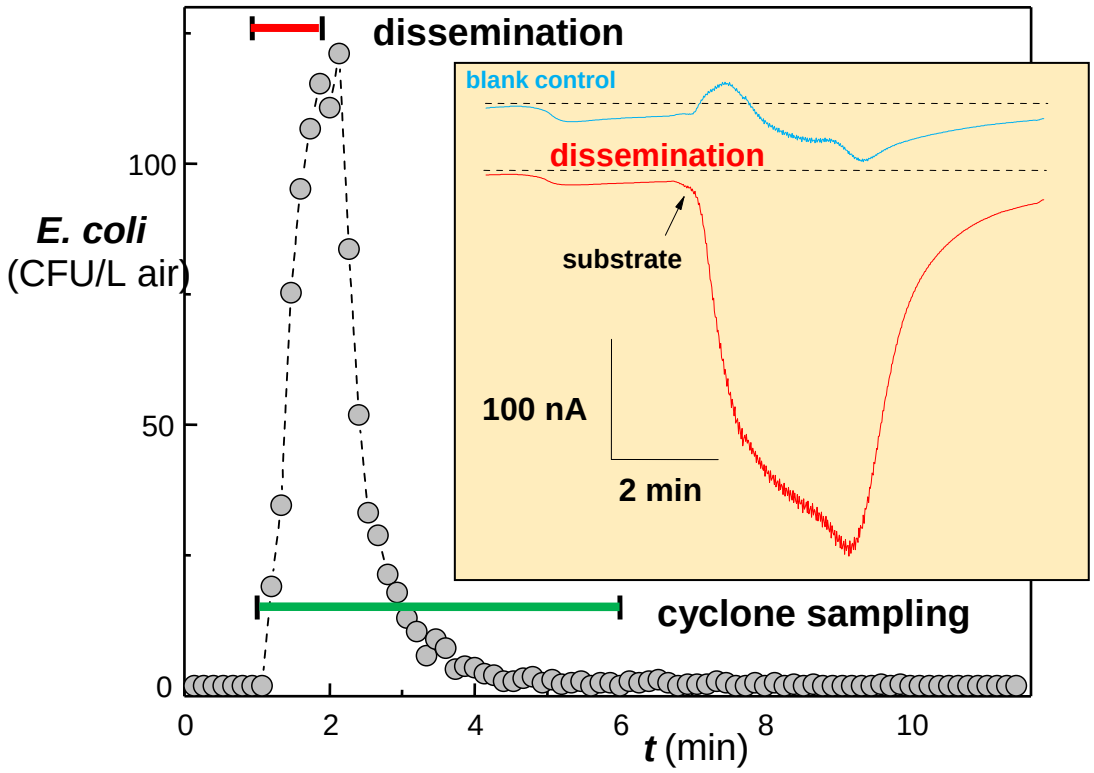

Figure 5. Monitoring of viable cells of *E. coli* disseminated inside aerosol chamber using the slit sampler (rotating agar plates, colonies counted after overnight cultivation). The intervals of dissemination and cyclone sampling are marked. The inset graph presents corresponding positive signal trace from the electrochemical immunosensor where the captured bioaerosol sample was transferred; for the blank control, no dissemination was realized.

Recently, a smaller version of the aerosol chamber was constructed in our laboratory. The whole aerosol chamber and linked systems function as a portable unit; the only requirements for operation are power and network connections. The chamber has small working volume (60x65x92 cm = 0.36 m^3) which is however sufficient for stable conditions in combination with the employed flow rates of the cyclone and the particle counter. The advantage is portability, economic performance and simple cleaning / disinfection of the inside space. The option to control internal humidity seems very attractive with respect to more realistic simulation of variable weather conditions. The tests will be focused on the spores of *B. atrophaeus*, in order to be compatible with field trials organised by other institutions.

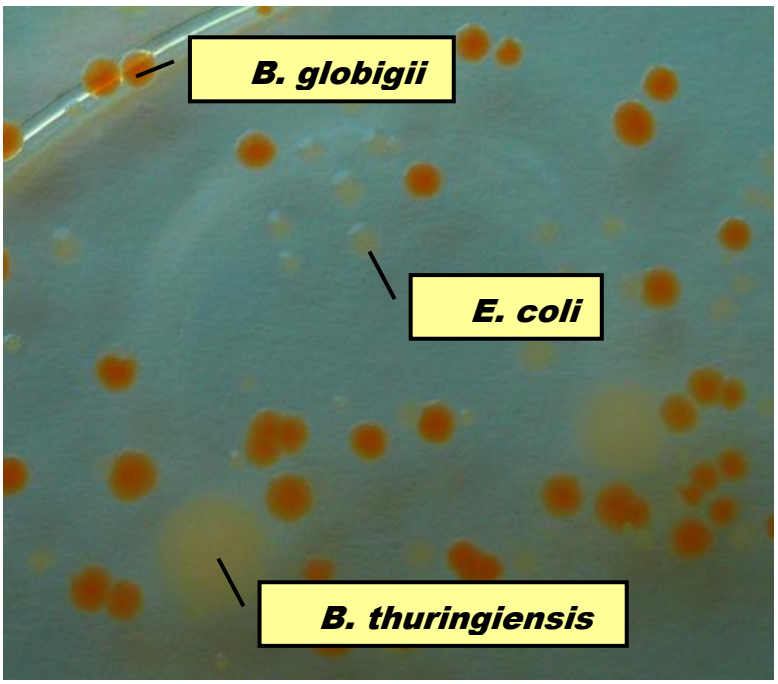

Figure 6. Image of the surface of agar plate after cultivation, the colonies of three microbial strains detected in the sampled bioaerosol in the chamber during dissemination of *E. coli* are shown.

4. CONCLUSIONS

The development of the electrochemical immunosensing devices for detection of microbial agents was briefly reviewed. At present, two types of assays are in focus. The heterogeneous sandwich immunoassay format seems to be robust and reliable due to two immunointeractions involved, sensitivity is provided by enzyme labels generating electrochemically measured products. As alternative, the direct assay formats employ different strategies how to evaluate capture of the target microbes without any label.

The practical results describe amperometric immunosensor based on screen-printed electrodes as a general platform for sandwich-based assays of microbes. The selected examples focused on the determination of *Francisella*, *Salmonella*, *Escherichia* and *Bacillus* species. The achieved analytical parameters seem promising for real applications. In particular, combination of the immunosensor with the cyclone air sampler allowed fully automated testing of the system for detection of bioaerosols of *E. coli* as a safe model; the preliminary results indicate that levels below 100 CFU L^{-1} in air are feasible within 20 min monitoring cycle interval.

At present, the user decides about the positive detection of the target bioagent; in future, this evaluation should be implemented in the control software. The combination of the analytical devices with chemometrics should provide really smart biosensors suitable for automated measurements and early detection of potentially danger microorganisms in the monitored area.

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