

## Electrochemical Biosensor for Detection of Bioagents

Eva Švábenská<sup>1, 2</sup>, David Kovář<sup>1</sup>, Vít Krajíček<sup>1</sup>, Jan Příbyl<sup>1</sup> and Petr Skládal<sup>1, \*</sup>

<sup>1</sup> National Centre for Biomolecular Research and CEITEC MU, Masaryk University, Kotlářská 2, 61137 Brno; <sup>2</sup> VOP-026 Šternberk, Division VTÚO Brno; Czech Republic

\*E-mail: [skladal@chemi.muni.cz](mailto:skladal@chemi.muni.cz)

Received: 2 June 2011 / Accepted: 11 October 2011 / Published: 1 December 2011

---

Simple and rapid detection and identification of dangerous bioagents is important for preventing illness or even death of people due to infectious diseases and bioterrorist threats. Electrochemical detection has a large potential for development of portable instrumentation suitable for analysis in field and especially combination with specificity of immunoassays seems promising. Initially, the construction of the immunosensing layer was realised and the effect of Au nanoparticles on the response of peroxidase was studied. An amperometric immunosensor was developed using screen-printed transducer, it employed specific capture of microbes in the sensing area by formation of an immunocomplex and its subsequent labelling using the tracer - antibody conjugated to peroxidase. The obtained sandwich electrochemical immunoassay was applied for the model microbial cells of *B. subtilis*. The total time of analysis was 20 min and the detection of cells below 10<sup>4</sup> CFU/ml was feasible. The presence of microbial cells at the sensing surface was confirmed using atomic force microscopy. The results appear promising for combination of the immunosensor with a cyclone sampler and analysis of *Bacillus*-based bioaerosols in field trials.

---

**Keywords:** amperometric sensor, screen-printed electrode, immunochemical detector, immunosensor

### 1. INTRODUCTION

With the increasing activity of terrorist organizations, the danger of using biological (BWA) and chemical warfare agents (CWA) become a real threat. At the same time, the increasing demands on the detection of relevant pathogens and toxic substances using a portable, fast and simple instrumentation is in focus. Several examples of suitable and robust devices based on the bioanalytical detection principles are being used for the detection tasks in military operations, by civil rescue and security units and even become considered the homeland security. The detection of the biological attack is significantly more complicated compared to classic explosives or chemical weapons, as the biological agents are extremely variable and similar to natural harmless microorganisms [1].

Furthermore, the initial symptoms after infection from BWAs are difficult to distinguish from symptoms of infections associated with rather benign or common biological organisms. In the last two decades, the expansion of biosensor technologies for detection and identification of chemical and biological agents started. Several types of biosensors working on electrochemical, optical, magnetic, piezoelectric and thermometric principle are available [2,3,4].

Recently, nanomaterials and nanoparticles come into the focus of scientists as advantageous tools for preparation of electrochemical biosensing layers with potentially enhanced performance [5,6]. In this contribution, the effect of nanoparticles on the response of various types of peroxidase-based enzyme electrodes was investigated in order to improve communication between the enzyme and the gold electrode. Peroxidase (HRP) was chosen as it is widely used as a label in electrochemical assays. The gold nanoparticles usually enhance the electron transfer between electrode and peroxidase [7]. Nanoparticles were embedded in polymer structures formed using chitosan [8], sol-gel layers [9] and layer-by-layer assemblies [10]. The well defined system was obtained using covalently immobilised monolayer of suitably functionalised self-assembled monolayers based on cystamine and hexanedithiol [11]. Peroxidase may even become placed inside gold nanospheres [12]. In the case of immunosensors, the further role of nanoparticles is also to increase surface area thus providing higher loadings of the capture antibodies [13]. Using peroxidase as a label, the electrochemical immunosensor for the detection of *Bacillus subtilis* var. niger (= *B. atrophaeus*) was developed; most detection systems focused on bioaerosols are commonly tested using this gram-positive bacterium [14]. It is a safe model variant representing highly pathogenic strain *B. anthracis* [15]. *B. atrophaeus* is allowed to be spread in closed chambers and even in field trial facilities for testing purposes [16]. As our research of electrochemical immunosensors was focused on the gram-negative bacterium *Francisella tularensis*, which must not be spread in the air neither in closed nor open environments, the relatively safe variant *Escherichia coli* DH5 $\alpha$  was chosen as model microbe suitable for spreading in a closed air chamber [17,18]. However, we realised that *Bacillus subtilis* strains are most widely used and allowed for dissemination in open air at field trials; therefore, the existing electrochemical immunosensor-based assay of microbes becomes extended to *B. subtilis* in this contribution.

## 2. EXPERIMENTAL

### 2.1. Reagents

Glutaraldehyde (GA, 25% aq. solution), cystamine, bovine serum albumin (BSA) and gold nanoparticles (5 and 20 nm) were obtained from Sigma. Potassium ferricyanide, potassium iodide and hydrogen peroxide were obtained from Penta. Phosphate buffer (50 mM sodium phosphate, pH 7.0), phosphate buffered saline (PBS, phosphate buffer and 150 mM sodium chloride), acetate buffer (50 mM sodium acetate, pH 4.0 with 150 mM NaCl) were used for experiments. The mouse polyclonal antibody (Ab) specific against *Bacillus subtilis* was kindly provided by Dr. M. Pohanka, Central Military Institute of Health, Těchonín, 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).

## 2.2. Microorganisms

*B. atrophaeus* [19,20] previously named as *B. subtilis*, var *niger* or *B. globigii* was conveniently used as a substitute for *B. anthracis*. The microbes were cultivated using standard procedures in 8 g/l Nutrient Broth No. 4 (Fluka) supplemented with 10 mg/l MnSO<sub>4</sub>, 10 mg/l MgSO<sub>4</sub> and 10 mg/l CaCl<sub>2</sub>; the bacteria were collected after 5 days of aerobic growth at 30 °C, centrifuged for 20 min at 12000 r.p.m., and resuspended in 0.9% sodium chloride. The content of microbes was determined using the McFarland method (optical density at 600 nm [21]); the stock solution contained approximately 7x10<sup>10</sup> CFU/ml and was stored frozen. The working solutions were made fresh daily by diluting the stock solution in PBS. Content of endospores was determined using the counting chamber and the optical microscope Olympus BX-41, the endospores were highlighted by a simple staining by malachite green directly in suspension, as a reference method, the fluorescence detection with terbium ions was used [22].

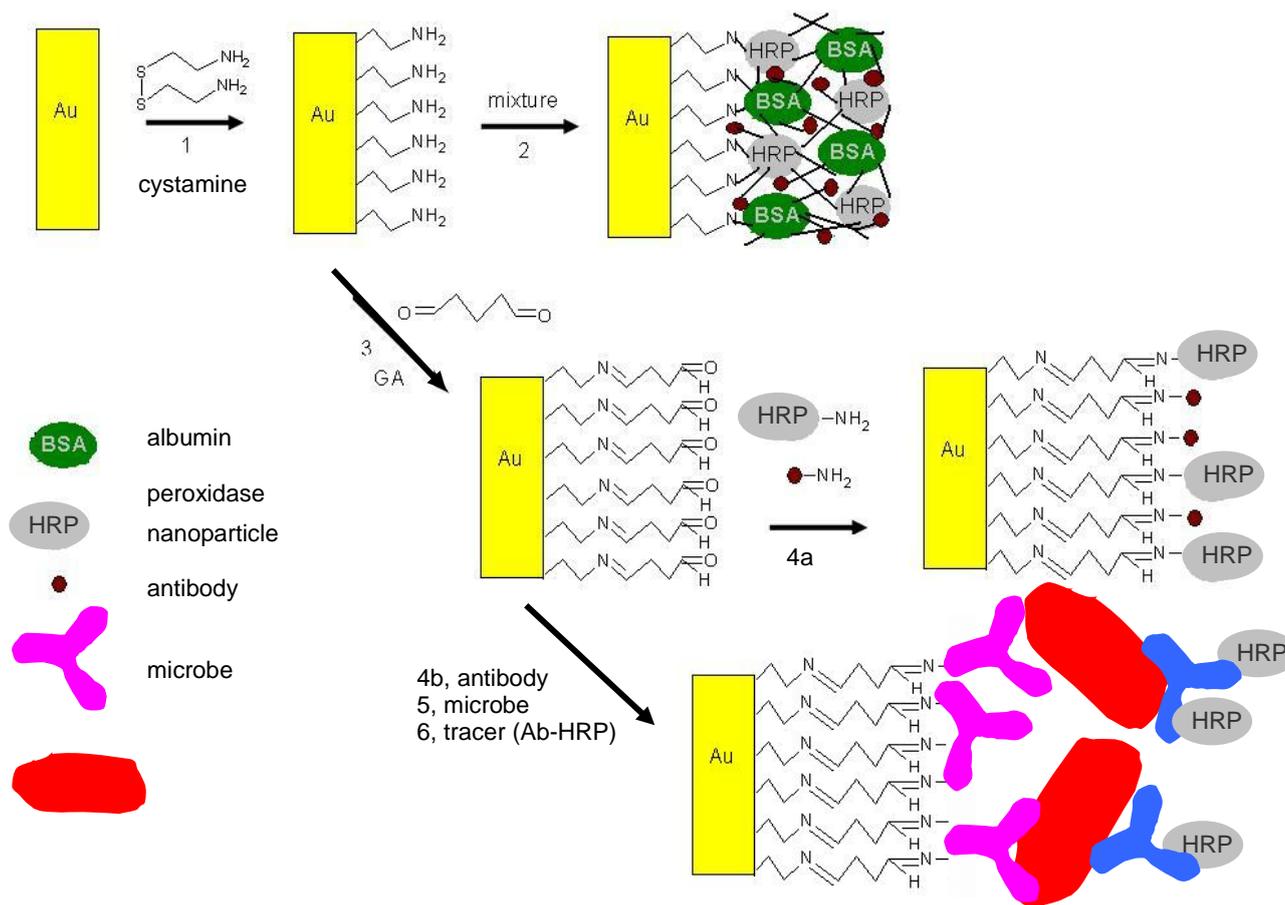
## 2.3. Preparation of biosensing layers

The exchangeable electrochemical strip with four working Au electrodes (1 mm diameter) was produced by screen-printing technology by BVT Technologies, Brno. The surface of metallic electrodes was always cleaned with acetone (30 min) and a self-assembled monolayer was formed during a 2-hour incubation with cystamine (10 mg/ml in water) (Fig. 1, step 1). Similarly, the Au nanoparticles (NP, 5 and 20 nm) were modified with the monolayer of cystamine; its excess was removed by dialysis overnight against surplus of phosphate buffer (10 mM, pH 7.0) using the cellulose membrane-based tubing (cut-off 10 kDa). Thus produced NP5-NH<sub>2</sub> and NP20-NH<sub>2</sub> obtained a free amino group.

Afterwards, different types of immobilisation of peroxidase were employed. Thick layers based on the mixtures of HRP, BSA as an inert protein and glutaraldehyde as a cross-linker were prepared; the mixtures contained 0.5 to 1 mg HRP, 0.5 mg BSA and 0.75 to 3 mg glutaraldehyde in 1 ml of phosphate buffer. When required, NP and NP-NH<sub>2</sub> were supplemented to the mixture, at the same level for all types of NP. Finally, 1 µl of the mixture was applied on the Au electrode and allowed to dry overnight in a wet atmosphere in the refrigerator (Fig. 1, step 2).

Alternatively, the amino groups from the monolayer of cystamine were activated for 2 hours with glutaraldehyde (3% in phosphate buffer, Fig. 1, step 3), washed and the monolayer of peroxidase was attached overnight by incubation with HRP (10 mg/ml in phosphate buffer) followed by washing with phosphate buffer and water; when required, the AuNP-NH<sub>2</sub> were added (Fig. 1, type 4a).

The immunosensing layer was prepared by covalently linking anti *Bacillus* Ab on cystamine activated using glutaraldehyde (Fig. 1, step 4b). The IgG fraction (1 mg/ml in phosphate buffer) was incubated overnight, after washing with phosphate and water, the resulting immunosensors were stored dried in the refrigerator. At the 4-channel strips, two electrodes were modified with the specific Ab and two were modified with the non-specific Ab AL-01 in order to obtain reference layers.



**Figure 1.** Schematic view of immobilisation of biolayers on electrodes. Deposition of the monolayer of cystamine (1) followed by formation of a glutaraldehyde-cross-linked thick layer consisting of peroxidase and albumin, supplemented with nanoparticles (2). Alternatively, activation with glutaraldehyde (3), washing and covalent binding of HRP was carried out (4a). The anti *Bacillus* antibody was covalently linked (4b, not shown). The sandwich immunocomplex was formed after capturing the *Bacillus* microbes (5) and finally labelled using the Ab-HRP conjugate as tracer (6).

#### 2.4. Characterisation of biosensing layers

Ferricyanide (5 mM) was used as a redox probe for measurement of permeability of biolayers. The cyclic voltammogram (CV) of the electrode was measured using the EmStat system (Palm Instruments) using Ag/AgCl reference electrode and platinum wire counter electrode. Measurements were carried out in PBS by scanning from -0.6 to 0.6 V at 0.05 V/s scan rate in non-stirred conditions. The change of the peak current obtained with 5 mM ferricyanide vs. signal in the background electrolyte was recorded (at -0.5 V).

The enzyme activity of peroxidase present at the electrode surface was determined using amperometric measurement at -0.05 V (vs. silver pseudoreference electrode) in the acetate buffer containing 0.5 mM hydrogen peroxide, the cathodic current after the addition of 1 mM iodide was followed in a flow-through mode. The 4-channel digital potentiostat ImmunoSMART system

(SMART, Brno) recorded the current from four working electrodes and provided also four miniperistaltic pumps for mixing of the working solutions. The own software LT\_ImmunoSMART provided automated timing of experiments based on scripts as well as graphic display and storage of measured values of current and temperature.

### 2.5. Electrochemical immunoassay

Prior to use, the measuring area of the immunosensor was incubated for 1 hour with 0.2% BSA in order to saturate any non-specific binding sites. The immunosensor was fixed in a flow-through cell containing also the embedded Ag pseudoreference electrode. The immunosensor was connected to ImmunoSMART, its four miniperistaltic pumps P1 to P4 were delivering the working solutions. P1 pumped either sample containing microbes or PBS with 1 mg/ml BSA, which served as a washing solution for the immunochemical steps. The sample was aspirated from a microtube for 10 min thus realising incubation of microbes with the capture antibody immobilised on the gold electrode in a flow conditions. Afterwards, P2 injected the tracer (25 µg/ml in PBS with 1 mg/ml BSA) for 5 min and the cell was briefly washed with PBS/BSA from P1. P3 and P4 mixed the substrates for HRP, both pumps contained acetate buffer plus either 8 mM H<sub>2</sub>O<sub>2</sub> (P3) or 8 mM potassium iodide (P4). P3 was working continuously, while P4 added a 5 min zone of iodide when the response was measured. The flow rates were 15 µl/min. A new immunosensor was always used for each immunochemical assay.

### 2.6. Visualisation of microbes using AFM

The gold surface (4 x 4 mm, SPI Supplies, West Chester, USA) was covalently modified with the capture antibody as described above for the electrochemical immunosensor (end of part 2.3.). The surface was incubated with 1 mg/ml BSA, washed and then incubated with the cells of *Bacillus atrophaeus* (~7x10<sup>7</sup> CFU/ml in PBS) for 1 hour with shaking, and washed again with PBS and water. Surface-bound cells were imaged using the atomic force microscope Ntegra Vita/Solaris (NT-MDT, Zelenograd, Russia). The scanned area (135 x 135 and 10 x 10 µm) was evaluated in the software Nova, the visual evaluation and the grain analysis were used to count the amount of bound cells. As a blank, the surface without incubation with cells was imaged.

## 3. RESULTS AND DISCUSSION

### 3.1. Electrochemical characterisation of biolayers

The appropriate design of the biosensing layer is a key element for its optimal performance. For electrochemical immunosensors, the excellent specificity of antibody as a recognition element becomes combined with an enzyme label suitable for electrochemical detection [23]. For the particular detection of microbes, the sandwich immunoassay format is conveniently adopted, the primary antibody is immobilised on the electrode surface and the specifically captured target cells further bind

the secondary antibody conjugated to enzyme label; horse radish peroxidase belongs to the most used labels [24] and it was also chosen here. Furthermore, the electrochemical immunosensors are typically combined with nanoparticles which should provide improved flow of electrons and facilitate the immobilisation of antibodies. The effect of gold nanoparticles of two sizes was studied here in combination with the gold-based screen-printed electrodes.

The biosensing layers with peroxidase were constructed as a thick reticulum consisting of HRP, nanoparticles and BSA cross-linked using glutaraldehyde (Fig. 1, part 2); this design is typical for enzyme electrodes measuring analytes as enzyme substrates, where it operates under diffusional control and part of the enzyme activity is latent. It is usually providing the highest responses and the effect of nanoparticles inside the biolayer was considered. The other type – a monolayer of HRP mixed with nanoparticles (Fig. 1, part 4a) provides only limited amount of the immobilised enzyme and is typical for applications where the amount of enzyme activity is limited (kinetic control of the response, measurement of inhibitors).

Finally, the typical immunosensor combines a (mono)layer of the capture antibody, layer of the bound microbes and the outer layer of antibody-enzyme conjugate as the tracer (Fig. 1, part 6). These biolayers were characterised by two parameters; permeability was described as a current obtained in CV with ferricyanide as the common redox probe, and the activity of peroxidase was determined from amperometric response for hydrogen peroxide and iodide in a flow-through mode, the cathodic reduction of the enzymatically formed iodine was measured. The results of measurements are summarised in Table 1.

Permeability of the thick HRP layers is not significantly modulated by the proportional amount of the bifunctional crosslinker – glutaraldehyde (Table 1, biosensors 1 and 2), the pores of the enzyme reticulum are not blocking access of ferricyanide to the electrode. However, the higher level of GA resulted in significant (~30%) reduction of the peroxidase response and for this reason the content of GA was further kept at 0.75 mg/ml in the immobilisation mixtures.

The inclusion of Au nanoparticles inside the biolayers was realised in two ways; the bare unmodified NPs (size 5 and 20 nm, biosensors no. 3 and 4) were embedded purely mechanically by entrapment inside the enzyme reticulum, the cysteine-modified NPs (NP5-NH<sub>2</sub> and NP20-NH<sub>2</sub>, biosensors no. 5 and 6) were in addition participating in the reticulation reaction with GA becoming chemically linked to the structure.

Overall, the addition of NPs did not result in significant effects on either permeability or peroxidase signals of the produced biosensors. Generally, the cystamine-modified NPs reduced the HRP signals to 57% and 74% for 5 and 20 nm sized NPs-NH<sub>2</sub>, respectively, the NP5-NH<sub>2</sub> caused also significant reduction of the permeability of the layer. The bare NPs had lesser effect on the HRP response, negligible change and decrease to 81% was obtained for 5 and 20 nm NPs, respectively. In conclusion, the cross-linked layers contain a surplus of HRP and thus its signal cannot be improved any more, the addition of nanoparticles is not beneficial but on the contrary it seems to slightly limit diffusion of substrates inside the biolayer.

**Table 1.** Comparison of performance of electrodes modified with peroxidase using a cross-linked layer (top part), monolayer (middle) and sandwich immunolayer (bottom). The effect of the addition of gold nanoparticles was investigated, too. The errors of measurements were typically 30% for  $\Delta i_{\text{ferri}}$  and 20% for  $\Delta i_{\text{HRP}}$ .

No.	Biosensor type, Au-Cystamine-X, X =	Permeability $\Delta i_{\text{ferri}}$ ( $\mu\text{A}$ )	Peroxidase $\Delta i_{\text{HRP}}$ (nA)
1	[GA, HRP, BSA] (cross-linked low 0.75)	470	1090
2	[GA, HRP, BSA] (cross-linked medium 1.5)	730	702
3	[GA, HRP, BSA, NP5] (cross-linked low)	498	1020
4	[GA, HRP, BSA, NP20] (cross-linked low)	750	875
5	[GA,HRP,BSA, NP5-NH <sub>2</sub> ] (cross-linked low)	290	619
6	[GA,HRP,BSA, NP20-NH <sub>2</sub> ] (cross-linked low)	670	811
7	GA-HRP (monolayer)	1360	27
8	GA-[HRP, NP5-NH <sub>2</sub> ] (monolayer)	1230	51
9	GA-[HRP, NP20-NH <sub>2</sub> ] (monolayer)	960	42
10	GA-Ab-microbe-[Ab-HRP]	350	34
11	GA-[Ab, NP5-NH <sub>2</sub> ]-microbe-[Ab-HRP]	240	26
12	GA-[Ab, NP20-NH <sub>2</sub> ]-microbe-[Ab-HRP]	290	29

In the case of the monolayer-based enzyme biosensors (nos. 7, 8 and 9), the role of NPs was rather beneficial and enhancement of response was to 190% and 156% for 5 and 20 nm sized cystamine-modified NPs, respectively; the non-modified NPs cannot be covalently immobilised in the monolayer and the simple adsorptive immobilisation was not used here due to limited robustness. Anyway, the permeabilities of the monolayers were always much higher compared to the cross-linked layers, however, the HRP signals were 20- to 40-times lower due to the limited content of HRP in the single monolayer. The addition of nanoparticles resulted in a significant increase of signal; in case of rather low amount of enzyme, the other factors as nanoparticles increasing effective size of the working electrodes were able to improve the response.

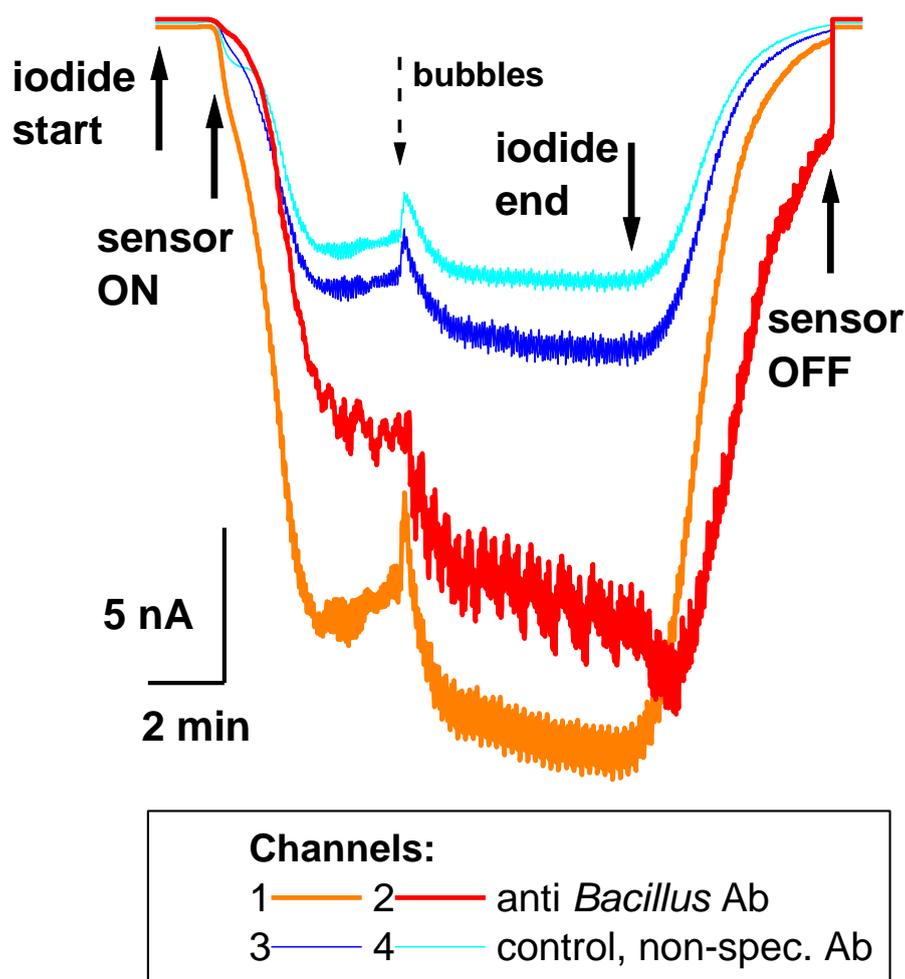
The immunocapture layers based on a monolayer of anti *Bacillus* antibody covalently linked to the gold (biosensor no. 10) were similarly as above prepared also mixed with NP5-NH<sub>2</sub> and NP20-NH<sub>2</sub> (nos. 11 and 12). However, in both cases the nanoparticles blocked the surface resulting in a slightly decreased permeability.

To evaluate the HRP response, the capture antibody was saturated with microbes (1 hour incubation,  $\sim 7 \times 10^7$  CFU/ml), washed, and HRP was linked to the sandwich through the Ab-HRP tracer interacting now with the surface of the specifically bound microbes.

The resulting signals were for all three biosensors at the level of a monolayer of covalently linked HRP (e.g. biosensor no. 7). As the presence of NPs did not improve the response, NPs were omitted in the construction of the immunosensor and the version no. 10 was finally chosen for immunoassay of *Bacillus*.

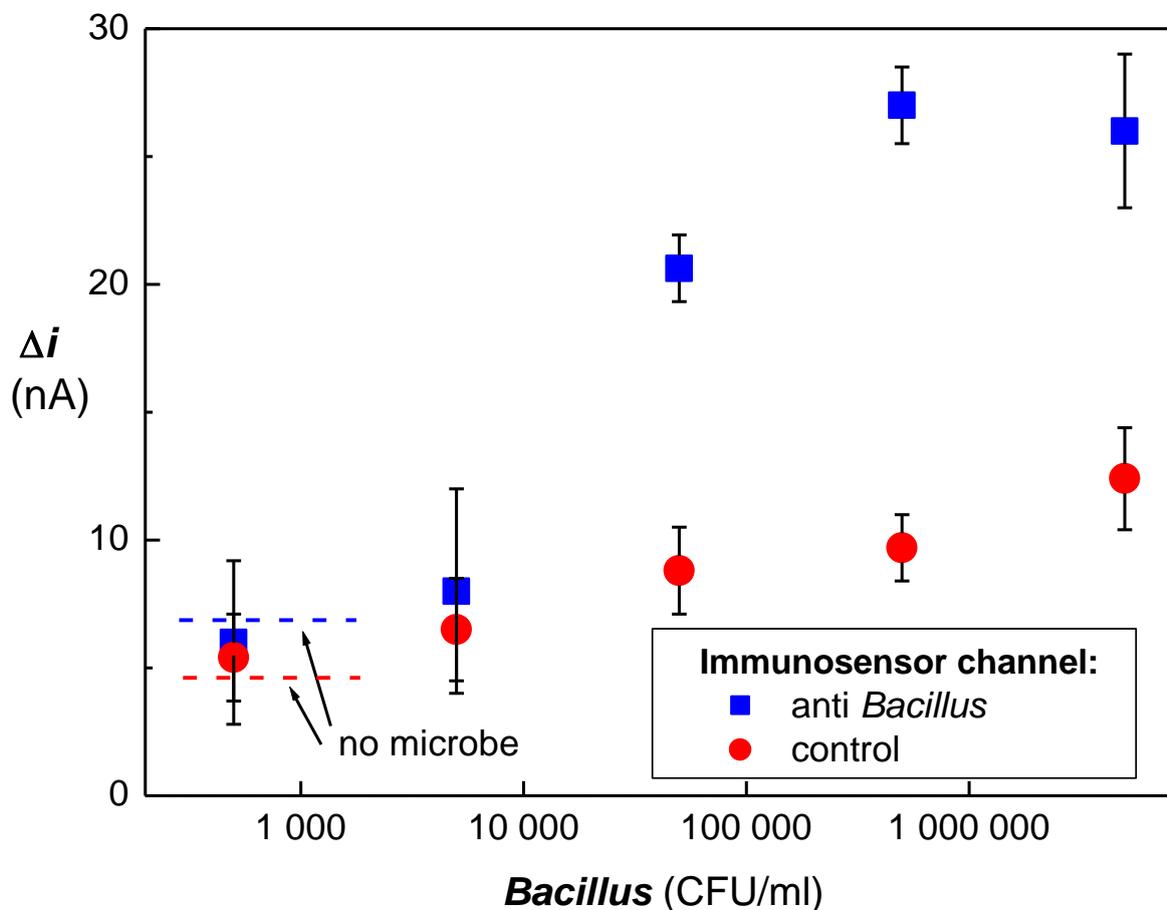
3.2. Performance of the electrochemical immunosensor

The electrochemical immunosensor employed the sandwich assay format schematically shown in Fig. 1 part 6. The antibody layer was immobilised on the gold electrode surface, the target microbes captured during incubation with sample (10 min) were subsequently labelled with the peroxidase-antibody tracer during the second incubation (5 min). The assay was heterogeneous, as washing steps were included after incubation steps. Finally, the surface bound peroxidase activity was determined electrochemically, the enzymatically-produced iodine was measured at a negative potential. The concept was quite similar as used previously for detection of *Francisella tularensis* [3,8]. A typical response of the immunosensor is shown in Fig. 2 as traces of current in time for the eventual amperometric measurement of HRP bound in the sandwich immunocomplex.



**Figure 2.** The response of the electrochemical immunosensor to *Bacillus subtilis*, the signal traces from the specific (orange, red) and reference (blue, cyan) channels are shown. The zone of sample with  $\sim 7 \times 10^5$  CFU/ml flowed for 10 min, after washing, the zone of tracer (Ab-HRP) followed for 5 min and washing zone again. The picture shows the response of thus bound HRP to the mixture of hydrogen peroxide and iodide (both 8 mM) as enzyme substrates.

This approach was used to generate a calibration curve for a wider range of amounts of microbes in the solution; the resulting dependences for specific and non-specific (reference) sensing channels are shown in Fig. 3.



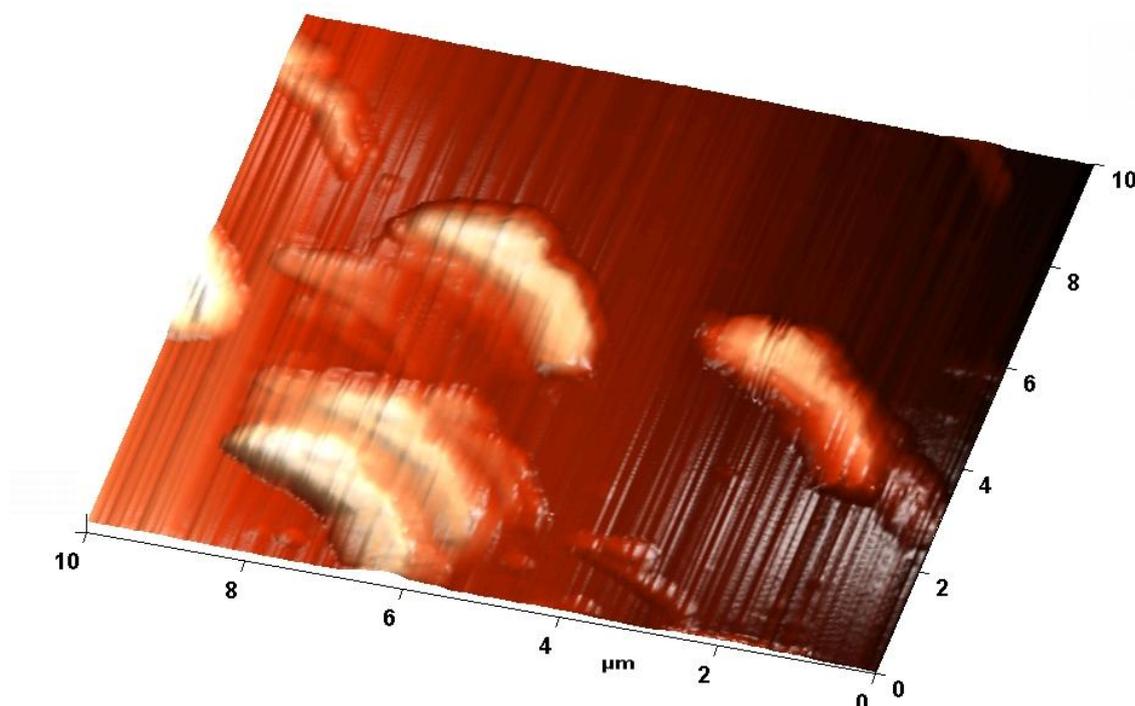
**Figure 3.** Response of the immunosensor to the cells of *Bacillus subtilis*, for procedure see Fig. 2. The specific (blue) and non-specific (red) sensing layers were used; the dashed lines indicate response in the absence of sample with microbes (i.e. signals due to the non-specific binding of the Ab-HRP tracer). Mean values from 2 independent immunosensors (i.e. 4 values) are shown, the bars indicate standard errors.

The useful working range of the immunosensor was from  $10^4$  to  $10^6$  CFU/ml; lower contents of microbes cannot be distinguished reliably from the background signal measured in the reference channels; this was mainly due to the binding of microbes also to the non-specific antibody in the reference channels. The non-specific binding (and the associated increase of the baseline) is common problem for immunoassays and in this case it will be addressed surely in future. On the other hand, the higher contents of microbes resulted in a slight decrease of the response due to the oversaturation or blocking of the sensing layer, thus products of the reaction of HRP have limited access to the electrode.

The achieved limit of detection seems sufficient for future tests, if one considers the intended use of this immunosensor together with a cyclone sampler – the device collecting bioaerosols from the air to the capture solution, a significant enrichment can be achieved using prolonged collection times.

### 3.1. Visualisation of microbes using AFM

To evaluate binding of microbes on the immunosensing surfaces independently, the technique of atomic force microscopy was adopted. The support for AFM imaging was made in the same way as for the immunosensor. After incubation with microbial cells ( $7 \times 10^5$  CFU/ml), the surface was carefully washed and allowed to dry. The captured cells were visualised using AFM scanning over  $10 \times 10 \mu\text{m}$  areas, the typical AFM image is shown in Fig. 4, where individual cells as well as clusters of several cells are clearly visible, the size of the shapes corresponds with the expected size of *Bacillus*, i.e. 3-6  $\mu\text{m}$  long rods [5].



**Figure 4.** Surface of the gold with covalently linked anti *Bacillus* antibody after incubation with the cells of *Bacillus subtilis*, washing and drying, imaged using the atomic force microscopy (system Ntegra, semicontact mode, probe MF001). The vertical extent of the (pseudo) 3D topographic AFM image was 450 nm.

Furthermore, the absolute numbers of surface-bound microbes (larger scans  $135 \times 135 \mu\text{m}$ ) were determined by both manual and software-based (grain analysis) methods; for the immunosensitive surface, the amounts were 53 and 60, respectively; only 5 / 4 “shapes” were identified on the reference surfaces incubated in the absence of microbial cells. Thus, around 3300

cells per 1 mm<sup>2</sup> were captured at the surface. For a comparison, during the performance of the immunosensor, the surface was exposed for 10 min to the flow of sample at ~15 µl/min, and this corresponded to 7x10<sup>5</sup> CFU/ml x 0.15 ml ~ 10<sup>5</sup> cells passed through the flow cell with the immunosensor.

The AFM system appears as a very useful tool for independent validation of the presence of microbial cells at the sensing surface; it would allow correlation of the measured responses with absolute cell counts. Furthermore, this technique is currently employed for improvement of specificity of the performance of the immunosensor when different methods of surface modification are compared.

#### 4. CONCLUSIONS

Electrochemical detection of biological agents has a large potential for rapid and specific response to target species of microorganisms. Initially, the construction of the immunosensing layer was realised and the effect of Au nanoparticles on the response of peroxidase was studied. However, the final immunosensor was developed using only antibody covalently linked to the sensing surface. The portable amperometric detector ImmunoSMART developed in our laboratory was successfully combined with the exchangeable electrochemical immunosensor and used for the sandwich electrochemical immunoassay of model microbial cells of *B. subtilis*. The total time of analysis was around 20 min and the detection of cell levels below 10<sup>4</sup> CFU/ml seems feasible. The presence of microbial cells at the sensing surface was confirmed using atomic force microscopy. The results appear promising for combination of the immunosensor with cyclone sampler and further tests on *Bacillus*-based bioaerosol both in chamber and in field trials.

#### ACKNOWLEDGEMENT

The work has been supported by the Ministry of Defence of Czech Republic (projects no. OVVTUO2008001 and OSVTUO2006003) and by CEITEC - Central European Institute of Technology (CZ.1.05/1.1.00/02.0068) from European Regional Development Fund.

#### References

1. J.J. Gooding, *Anal. Chim. Acta*, 559 (2006), 137.
2. F.S. Ligler, K.E. Sapsford, J.P. Golden, L.C. Shriver-Lake, C.R. Taitt, M.A. Dyer, S. Barone and C.J. Myatt, *Anal. Sci.* 23 (2007) 5.
3. M. Pohanka, P. Skládal and O. Pavliš, *J. Immunoass. Immunochem.*, 29 (2008) 70.
4. P. Skládal, Y. Symerská, M. Pohanka, B. Šafář and A. Macela, In: *Defense against bioterror. NATO Security through science series B* (2005) Dordrecht, p. 221.
5. Y. Li, H. Schluesener, S. Xu, *Gold Bull.*, 43 (2010) 29-41.
6. A. Escosura-Muñiz, C. Paroloa and A. Merkoçi, *Materials Today*, 13 (2010) 24.
7. J.J. Feng, G. Zhao, J.J. Xu, H.Y. Chen, *Anal. Biochem.*, 342 (2005) 280-286.
8. C.L. Xiang, Y.J. Zou, L.X. Sun, F. Xu, *Anal. Lett.*, 41 (2008) 2224-2236.
9. R.P. Liang, H.D. Qiu, P.X. Cai, *Anal. Chim. Acta* 534 (2005) 223-229.

10. Y. Zhuo, R. Yuan, Y.Q. Chai, *Sens. Actuators B*, 114 (2006) 631-639.
11. Y.Y. Xu, B.A. Chao, S.F. Chen, S.H. Xia, *Anal. Chim. Acta*, 561 (2006) 48-54.
12. D.P. Tang, J.J. Ren, *Anal. Chem.* 80 (2008) 8064-8070.
13. G.K. Ahirwal, C.K. Mitra, *Biosens. Bioelectron.*, 25 (2010) 2016-2020.
14. B.J. Hindson, M.T. McBride, A.J. Makarewicz, B.D. Henderer, U.S. Setlur, S.M. Smith, D.M. Gutierrez, T.R. Metz, S.L. Nasarabadi, K.S. Venkateswaran, S.W. Farrow, B.W. Colston, J.M. Dzenitis, *Anal. Chem.*, 77 (2005) 284-289.
15. C.A. Rowe, L.M. Tender, M.J. Feldstein, J.P. Golden, S.B. Scruggs, B.D. MacCraith, J.J. Cras, F.S. Ligler, *Anal. Chem.*, 71 (1999) 3846-3852.
16. J.O. Jensen, J.M. Theriault, In: *Chemical and biological standoff detection, Proc. SPIE*, Vol. 5268, pp. 163-172.
17. P. Skládal, J. Příbyl and B. Šafář, In: *Commercial and Pre-Commercial Cell Detection Technologies for Defence against Bioterror, Technology, Market and Society, NATO ARW Ser.*, 39 (2008), Amsterdam, p. 21.
18. P. Skládal, M. Pohanka, E. Kupská and B. Šafář, In: *Biosensors*, (2010), In-Tech, Zagreb, p. 115.
19. J.H. Hageman, G.W. Shankweiler and P.R. Wall, *J. Bacteriol.* 160 (1984) 438.
20. L.K. Nakamura, *Int. J. Syst. Bacteriol.* 39(1989) 295.
21. J.T. Kerr, B. McHale, In: *Applications in General Microbiology, A Laboratory Manual*. Hunter Textbooks, Winston-Salem, NC, USA (2007).
22. T.Y. Pun, E.D. Lester, G. Bearman and A. Ponce, *Biotechnol. Bioeng.*, 98 (2007) 864.
23. P. Skládal, *Electroanalysis* 9 (1997) 737.
24. M. Pohanka and P. Skládal, *Electroanalysis* 19 (2007) 2507