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Piezoelectric and surface plasmon resonance biosensors for *Bacillus atrophaeus* spores

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Cheap, rapid and user friendly tools for detection of microorganisms were intensively discussed during last decades. The use of immunospecific biosensors seems advantageous for samples originating from clinical, environmental and agricultural area, food processing and military. The piezoelectric (quartz crystal microbalance, QCM) and optical (surface plasmon resonance, SPR) biosensors represented by QCM Analyzer and Biacore 3000 were compared for future development of real-time and label-free microbial biosensors. The spores of *Bacillus atrophaeus* were used as a non-pathogenic surrogate for *B. anthracis*. Seven commercially available antibodies were immobilised on the sensor surfaces to select the most promising one. Various immobilisation techniques were used. The total time of analysis did not exceed 30 min and the estimated limits of detection (LOD) were 10^6 CFU mL^{-1} and 10^5 CFU mL^{-1} in case of QCM and SPR, respectively.

Keywords: *Bacillus atrophaeus* spore; Biosensor; Immunosensor; Quartz Crystal Microbalance; Surface Plasmon Resonance

1. INTRODUCTION

Detection of microorganisms using conventional microbiological methods is time consuming, laborious and demanding on human operators. In most cases, the samples must be transported into specialised laboratory and therefore the analysis time is prolonged. In the last two decades, numerous trends to develop quick, cheap, and user friendly analysis of microorganisms appeared. The immunospecific biosensors seem to satisfy all these requirements. A certain advantage is portability of such devices and possibility of near real-time detection, which is important not only for military purposes. Biosensors consist of a sensitive biological element which is in close contact or tightly linked to a suit-

able physicochemical transducer. The biological part provides either biocatalytical or bioaffinity recognition. Most bioaffinity sensors employ antibodies as their biological element.

Piezoelectric biosensors are based on the piezoelectric effect – alternating current applied on the crystal stimulates high-frequency vibrations; for biosensing, the thickness shear vibrations represented by the quartz crystal microbalance systems are mostly used thanks to robust performance and simple construction of the driving electronics [1]. Optical biosensors are based on the measurements of light intensity or changes of advanced optical properties of biomaterials. In recent years, the intrinsic sensing represented by surface plasmon resonance has been receiving increasing attention [2].

The first QCM immunosensor for bacterial pathogens was pioneered by Muramatsu who detected *Candida albicans* [3]. Since 1986, a number of QCM immunosensors for microbes followed, including detection of *B. cereus* [4], *B. subtilis* [5], *E. coli* O157:H7 [6], *Listeria monocytogenes* [7], *Salmonella* Typhimurium [8,9] and *Vibrio cholerae* O139 [10]. Hao [11] detected 10^3 spores per millilitre of *B. anthracis* using oriented immobilisation of monoclonal antibodies via protein A on the monolayer-activated QCM in less than 30 min. LOD for most of the reported sensors was from 10^5 to 10^7 CFU mL^{-1}.

Similarly, SPR based immunosensors were reported for detection of *C. jejuni* [12], *E. coli* 0157:H7 [12-15], *Legionella pneumophilla* [16], *Listeria monocytogenes* [12,17-20], *Salmonela* Typhimurium [12,21], *S. paratyphi* [22], *S. enteritidis* [19,20], *Yersinia enterocolitica* [23] and *Vibrio cholerae* O1 [24]. A higher sensitivity of the SPR sensors allowed LODs in the range from 10^2 to 10^6 CFU mL^{-1} and times of analysis usually bellow 2 hours. Portable SPR devices were proposed, too [25]. Different ways of antibody immobilisation have been examined. Baccar [26] used the SPR biosensor for detection of *E. coli* K12 with limit of detection at 10^3 CFU mL^{-1}. The antibodies were immobilised using SAM monolayer. The antibody immobilisation through self assembled protein G was developed by Oh [22].

This work is focused on interactions of antibodies [27] and *Bacillus atrophaeus*. The commercially available antibodies against *Bacillus atrophaeus* spores were screened for potential use in biosensor development. The non-pathogenic *B. atrophaeus* is being used as a safe surrogate of *B. anthracis* [28], the causative agent of anthrax. *B. atrophaeus* is a Gram-positive facultative anaerobic endospore-forming bacterium, formerly known as *B. subtilis* subsp. *niger* or *B. globigii* [29,30], and also under the abbreviation BG.

2. EXPERIMENTAL PART

2.1. Chemicals and Reagents

Calcium chloride was from Merck (Germany). Cysteamine (Cys), ethanolamine, ferrous chloride, glutaraldehyde (GA), Staphylococcal protein A (SpA) and *O*-(3-carboxypropyl)-*O′*-[2-(3-mercaptopropionylamino)ethyl]-polyethylene glycol (PEG-SH(3k)) were acquired from Sigma-Aldrich (USA). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Biacore AB (Sweden). Hydrochloric acid, hydrogen peroxide, magnesium

sulphate, potassium chloride, and sodium hydroxide were obtained from Penta (Czech Republic). Manganese chloride, manganese sulphate, sodium acetate, disodium ethylenediaminetetraacetate and glycine were from Lachema (Czech Republic). Nutrient broth Nr. 4 for bacterial cultivation was from Fluka (Switzerland).

Phosphate buffer (PB, 50 mM sodium phosphate, pH 7.6) and phosphate buffered saline (PBS, PB and 150 mM sodium chloride) were used for QCM experiments. HBS-P buffer from Biacore was used for SPR measurements.

2.2. Antibodies

Seven different antibodies (Ab) were tested. Anti-*Bacillus* spores Ab (18-902-172157) and rabbit anti-*Bacillus* spores Ab (18-511-245214; Ab214) were provided by GenWay (USA). Anti-*Bacillus* spores Ab (LS-C103151; Ab151) and anti-*Bacillus* spores Ab-peroxidase (LS-C103411; Ab411) were from LSBio (USA). Goat anti-*B. globigii* IgG (TC-7014-001), mouse monoclonal Ab against *B. globigii* (TC-7025-001) and rabbit anti-*B. globigii* IgG (TC-7008-001) were from Tetracore (USA). Horseradish peroxidase (HRP) modification of antibodies does not play an important role in detection methods used in this work, but screening HRP modified antibodies provides valuable information useful for development of amperometric biosensors [31].

The immobilisation of antibodies on sensing surfaces was achieved using several approaches including random covalent coupling using glutaraldehyde, oriented capture through proteins A, G and direct immobilisation of fragmented antibodies using released native thiol groups adsorbed on gold. The partially reduced IgG (rIgG) from Ab214 was prepared using the following procedure [32]: 10 μL of Ab214 (4.5 mg mL^{-1}) was mixed with 1 μL of cysteamine (60 mg mL^{-1} in PBS-EDTA buffer containing PBS and 10 mM Na_2EDTA; pH 7,2). Cysteamine is a mild reducing agent and reduces the disulfide bond in the hinge region. The reaction mixture was gently stirred for 90 minutes at 37 °C. The mixture was diluted in 189 μL of PBS-EDTA buffer. The fragments were dialysed (two buffer washing steps) using centrifugal filter unit (Microcon YM-3, 3000 MWCO, Millipore Corp., USA) to remove the excess of cysteamine. The rIgG fragments were finally concentrated to 1 mg mL^{-1}.

2.3. Microorganisms

Bacillus atrophaeus (ATCC 9372) was obtained from the Czech Collection of Microorganisms. All procedures were done aseptically under the laminar flow hoods. Working media were autoclaved at 125 °C for 25 min. The culture method was adopted with minor modifications to the standard procedure [33]. The stock culture (200 μL) was inoculated in 25 mL of culture medium [34] consisting of 8 g of Nutrient broth Nr. 4 and 10 mg L^{-1} of manganese sulphate, pH was adjusted to 7.0 by 1 M sodium hydroxide. The inoculated medium was gently stirred (350 rpm) and incubated at 30±1 °C over night. Sporulation medium was prepared by dissolving 8 g L^{-1} of Nutrient Broth Nr. 4, 50 mM magnesium sulphate, and 1.5 M potassium chloride, pH value was adjusted to 7.6. 1 mL of the sterile solution containing 1 M calcium chloride, 1 mM ferrous chloride and 10 mM manganese chloride was added to

1 L of the medium prior use. The sporulation was initiated by inoculation of fresh bacterial culture in 250 mL of the completed medium. The flask was capped with a cellulose wadding stopper to allow air flow. The sporulation is strictly aerobic process and the oxygen consumption is higher than during growth. The sporulation media were incubated at 30±1 °C for 21 days under continuous stirring in order to provide for microbes sufficient time to exhaust nutrients and thus initiate the sporulation process.

After completed sporulation, the obtained suspension was divided into 50 mL Falcon tubes and centrifuged at 5,000 RCF (rel. centrifugal force) and 10 °C using Rotina 380 R (Hettich, Germany) for 15 min. The supernatant was discarded and the pellet was resuspended in 50 mL of cold sterile deionised water and mixed for 5 min. These two steps were repeated three times to remove the sporulation medium. After the final centrifugation, the suspension of spores was stirred for 24 hours at 4 °C. Three cycles of centrifugation (as above) were done to remove cell debris. Finally, the pellet of spores was suspended in 20 mM calcium chloride (pH 9.0) and stored at 4 °C in dark. Solutions for analysis were made fresh before every use by dilution of the stock solution in the corresponding working buffer. The spores were counted using a Bürker haemocytometer and phase contrast microscopy using Olympus BX41 (Olympus, Japan). Concentration of spores was expressed as colony forming units per millilitre (CFU mL^{-1}).

The process was optimised to achieve high yield of BG spores. Originally, the spores were harvested 10 days after inoculation (spores 1); the longer incubation significantly increased the yield. Furthermore, the standard spore suspension must be free of living cell or cell debris. Several purification procedures were reviewed [35]; the simplest one employed repeated washing and centrifugation of spore suspension in water. This method is effective but time-consuming, the alternative centrifugation in urografin gradient was modified according to Chen [36] who used sucrose gradient to separation of *Pasteuria penetrans* endospores. This procedure was adopted here and optimised for production of BG spores 2. The achieved 99 % purity was verified microscopically using phase contrast and dark field imaging.

2.4. Preparation of biosensing layers for QCM

Quartz crystals (10 MHz, AT-cut, gold electrodes) were purchased from ICM (USA). The resonance frequency was measured after each modification step. Prior to use, the gold electrodes were completely soaked in a freshly prepared piranha solution for 15 min and twice thoroughly washed with deionised water. The self-assembled monolayer was formed by aqueous cysteamine solution (20 mg mL^{-1}, 10 µL per electrode) immediately after cleaning. The crystals were incubated in the dark for 2 hours at room temperature, washed with deionised water and allowed to dry. Electrode surfaces were activated by 5 % GA in PBS for 1 hour at room temperature, washed and incubated with either SpA (1 mg mL^{-1}) or directly with Ab (0.2 mg mL^{-1}) for 20 hours at 4 °C (QCM@Ab). The monolayer of SpA binds antibodies specifically through their Fc fragment [7], this was achieved during incubation with Ab for 20 hours at 4 °C (QCM@SpA@Ab). The remaining aldehyde groups were deactivated with 50 mM ethanolamine for 15 min. After the last washing step, the crystals were either used or

stored in dry conditions at 4 °C. Alternatively, the crystals with the SpA monolayer were modified with Ab in a flow-through mode.

The partially reduced Ab fragments rIgG were immobilised using the following procedure with slight modification [37] to prevent conformation changes of rIgG; 10 μL of rIgG (100 μg mL^{-1}) and PEG-SH(3k) (150 μg mL^{-1}) were co-immobilised on the piranha treated QCM sensor surface for 2 hours at room temperature. The surface was thoroughly washed with water, allowed to dry and stored at 4 °C.

2.5. Piezoelectric immunoassay

The oscillation frequency was measured using the QCM Analyzer (Keva, Czech Republic). Data acquisition was performed using our own software LabTools. The immunosensor was placed in the flow-through cell and affinity interactions were followed in real-time [38]. The PB and PBS buffers were used as the carrier with flow rate of 17 μL min^{-1} (Minipuls MP3, Gilson, France). The baseline signal of the carrier buffer was stabilised for 5 min. The samples containing BG spores were flown over the sensor for either 5 or 10 min. Spontaneous dissociation of immunocomplexes was allowed for 10 min. Complete dissociation was done using regeneration solutions: 20 mM glycine pH 2.0, 50 mM sodium hydroxide and 50 % ethanol for 5, 5 and 0.5 min, respectively.

2.6. Preparation of biosensing layers for SPR

Sensor chips bare Au and CM5 (supplied by Biacore) were used for self-assembled monolayers (SAM) and covalent immobilisation, respectively. Both immobilisation methods were performed in a flow-through mode at 5 μL min^{-1}. For the CM5 chip, the sensing surface with covalently bound carboxymethylated dextran was activated using the mixture of EDC (400 mM) and NHS (100 mM), washed and antibody (~50 μg mL^{-1}) dissolved in acetate buffer (pH 5.0) was covalently attached. The channel Fc1 on the chips was used as a reference, the further three channels contained different immobilised antibodies. Finally, free reactive groups were deactivated with ethanolamine (1 M, pH 8.5) for 10 min.

The immobilisation using SAM was the same as for QCM (see above). Initially, the Au sensor surface was degreased using SDS (0.5 %, 10 min). Next, cysteamine (2 mg mL^{-1}), glutaraldehyde (1 %) and SpA (100 μg mL^{-1}) were subsequently covalently attached. The free aldehyde groups were deactivated using ethanolamine as in the case of the CM5 chip.

2.7. SPR immunoassay

Biacore 3000 was used for SPR measurements. HBP-P buffer and flow rate 5 μL min^{-1} were chosen. Samples (50 μL) containing either BG spores or cells suspended in the running buffer were injected into the flow-through system and the association and dissociation phases of the immunoaffinity interaction were measured. Complete dissociation was done using a short pulse of 50 mM hydro-

chloric acid [39]. The preliminary values of LOD for both QCM and SPR-based direct immunoassays were obtained using the ratio S/N = 3.

The visualisation of the bound spores was performed with the atomic force microscope Ntegra Vita with the HA_NC probes in non-contact mode; scans were processed in the Nova software (NT-MDT, Russia).

3. RESULTS AND DISCUSSION

3.1. Performance of the piezoelectric immunosensor

Interactions of seven antibodies with *B. atrophaeus* spores were studied using various immobilisation strategies. Under similar conditions, i.e. when being immobilised through the protein A layer, the bound amounts corresponding to signal changes of 48, 85 and 45 Hz were obtained for Ab151, Ab411 and Ab157, respectively. The aim was to immobilise as high amounts of antibody as possible, though the available quantity of antibodies was limiting. Only three of the screened Ab (Ab151, Ab411, Ab214) immobilised during step-by-step mode exhibited suitable affinity to spores and the sensors immobilised with these antibodies provided expected response. It should be noted that Ab411 corresponds to the HRP-labelled Ab151. Thus, it seems reasonable to suppose that affinities of these Ab are almost the same. The response of QCM@SpA@Ab151 was slightly more significant than QCM@Ab151. The LOD for both versions was about 10^6 CFU mL^{-1}. The typical real-time frequency shifts for standard spore suspensions are shown in Fig. 1, the regeneration phases are not shown.

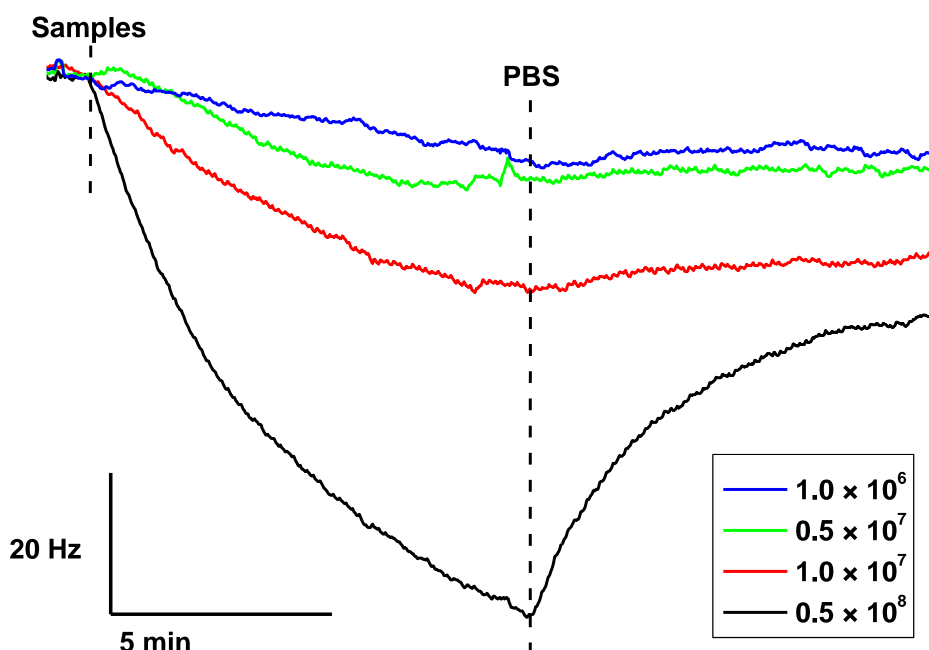

Figure 1. The interactions (QCM, resonant frequency shifts vs. time) between Ab151 (immobilised through protein A) and standard suspensions of BG spore. The association phase (10 min) and spontaneous dissociation of the formed immunocomplex are shown. Spore concentrations are expressed as CFU mL^{-1}.

The antibody Ab214 exhibits similar affinity as Ab151, it provided LOD equal to 2×10^6 CFU mL^{-1}. The association phase was 10 min long and 20 mM glycine was used for regeneration. Typical response is shown in Fig. 2, unfortunately, the regeneration of the sensing surface was not complete using common acid solutions of glycine. Alternatively, 50% ethanol was tested for regeneration, but no significant improvement was obtained. Other regeneration options should be studied to achieve a higher number of measuring cycles for the same immunosensor.

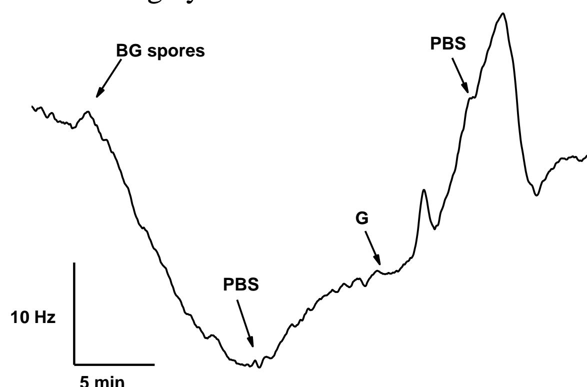

Figure 2. Typical response of the QCM sensor with immobilised protein A and antibody Ab214. The BG spores (10^7 CFU mL^{-1}) were flown-through for 10 min. Arrows indicate the beginings of individual zones of carrier buffer, spores and glycine solution. As it can be seen, the regeneration was not complete.

The obtained results stimulated testing of other immobilisation formats. The reduced antibodies were co-immobilised on QCM sensor together with a PEG layer and the response was measured immediately and a week later (Fig 3). The sensor lost the activity and affinity: the dissociation of immunocomplex was now partially influenced by PBS. A disadvantage of this immobilisation strategy was the lower stability of Ab. However, the frequency shift was 13 Hz for spore concentration of 10^6 CFU mL^{-1} at the beginning.

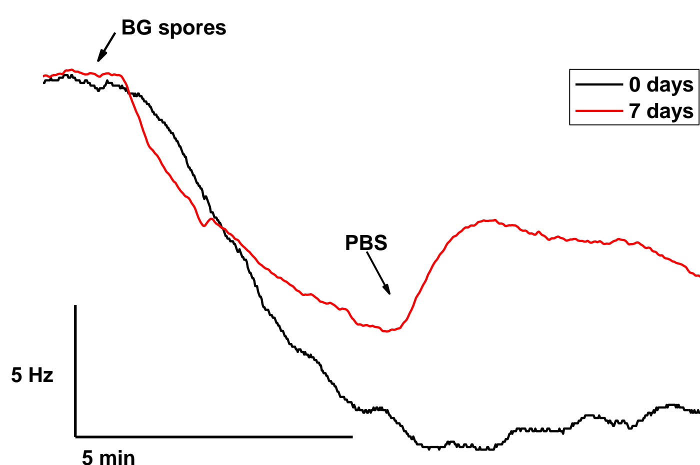

Figure 3. Association phase of BG spores (10^6 CFU mL^{-1}) with partially reduced antibodies immobilised directly on the QCM sensor surface. The black curve represents the first measurement immediately after immobilisation (zero time). The red curve shows the response of the crystal seven days after immobilisation.

Using the flow-through bound antibody, only Ab151 and Ab411 out of the seven tested antibodies were able to bind the spores of the chosen *Bacillus* strain. To improve sensitivity, the sandwich assay was used. In Fig. 4, the surface-bound Ab411 captured *B. atrophaeus* spores and the second layer of Ab411 formed the sandwich immunocomplex. Evidently, the rather low signal due to the direct binding of the microbial spores was nicely enhanced by the secondary Ab. Results with Ab immobilised in flow and using static incubation were quite similar. The former option provided lesser durability of Ab, but it can be exchanged more easily.

When comparing the obtained data with the literature, it was found that Alava [40] immobilised Ab similarly using the EDC/NHS activated surface and detected spores of *B. atrophaeus* with LOD 1.4×10^6 spores per 1 mL, but the frequency shift was only 0.5 Hz. Here, higher responses were achieved; the frequency shifts of 6 Hz and 11 Hz for 10^6 spores per 1 mL were obtained in case of Ab151 and reduced Ab214, respectively.

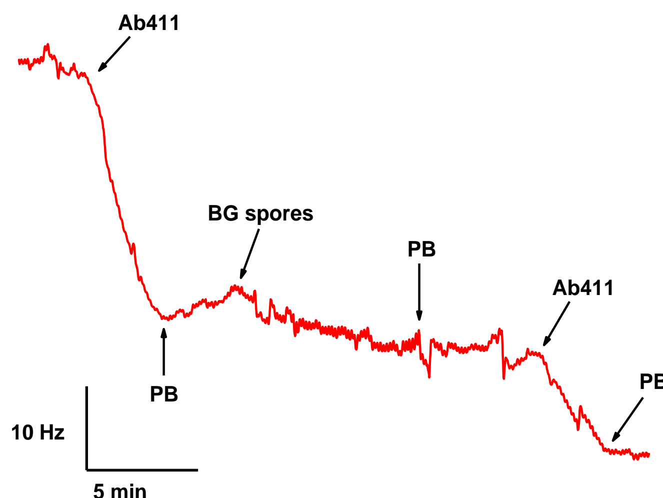

Figure 4. Interactions of *B. atrophaeus* spores (10^7 CFU mL^{-1}) with the immobilised antibody Ab411 in sandwich assay measured using QCM; antibody was bound through protein A in the flow-through mode. The change of resonant frequency Δf in time is shown.

3.2. Performance of the SPR immunosensor

For a comparison, several experiments were carried out using Biacore 3000; this SPR biosensor is generally expected to be more sensitive than the piezoelectric detector. Initially, antibodies were covalently immobilised to the sensor chips CM5 using the standard EDC and NHS reagents as recommended by the user manual. Similarly as for QCM, the attempt was to immobilise quite high levels of antibodies, the obtained signal changes were 2.4, 14 and 4.7 kRU for covalently bound antibodies Ab151, Ab411 and Ab157, respectively. Afterwards, interactions with *B. atrophaeus* were studied. The binding of *B. atrophaeus* spores and cells with Ab151 can be seen in Fig. 5. Spores showed higher change of response than cells, which agrees with assumed specifity of antibodies against bacterial spores. In addition, stability of the immunocomplex containing spores was slightly better. Anyway, the levels of signal around 10 RU were quite low considering that microbes are large objects. High in-

creases and drops of signal (sharp peaks) were caused by shifts of microfluidic channels – one containing spores while buffer was still in the second one.

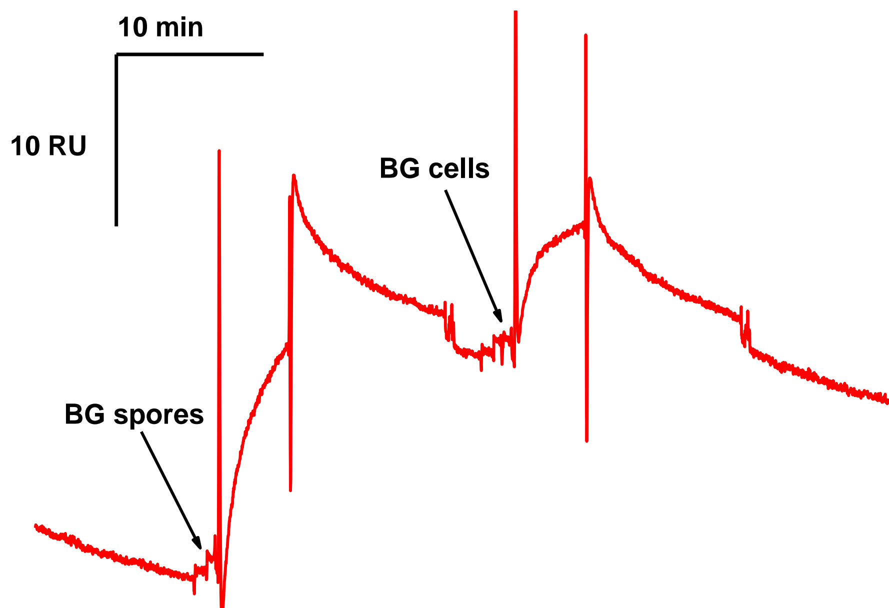

Figure 5. Comparison of binding interactions of BG spores and cells of (both 10^7 CFU mL^{-1}) with the covalently immobilised antibody Ab151 measured by SPR. Differential change of relative response in resonant units (RU) is shown.

Sandwich assay of *B. atrophaeus* spores can be seen in Fig. 6. Ab411 was covalently immobilised on the chip, *B. atrophaeus* spores were captured and then two different antibodies (Ab151 and Ab411) were allowed to form the sandwich complex.

Cultivation and purification of spores was optimised. It can be seen in Fig. 6 that the optimised procedure (spores 2) provided better results than the commonly recommended procedure (spores 1) with shorter sporulation. Antibodies Ab151 and Ab411 formed sandwich complex, however, its time stability was very limited. Sensitivity increased with antibody Ab151. Curiously, the signal dropped with Ab411. The most probable reason is that free antibody has higher affinity to *B. atrophaeus* spores than immobilised one and it was able to remove the spores out of the sensing surface.

Antibodies were immobilised also via the self-assembled monolayers using sensor chip Au and SpA. Fig. 7 shows binding of antibodies Ab151 and Ab411 to SpA and interactions of these antibodies with *B. atrophaeus* spores. Ab411 showed a higher temporary signal than Ab151, but practically the same amounts of both antibodies were bound on the sensor surface. Spores of *B. atrophaeus* have higher affinity to antibody Ab411. Measurements using the Au chip showed higher noise than in the case of the CM5 coated chip, but sensitivity of both methods was comparable.

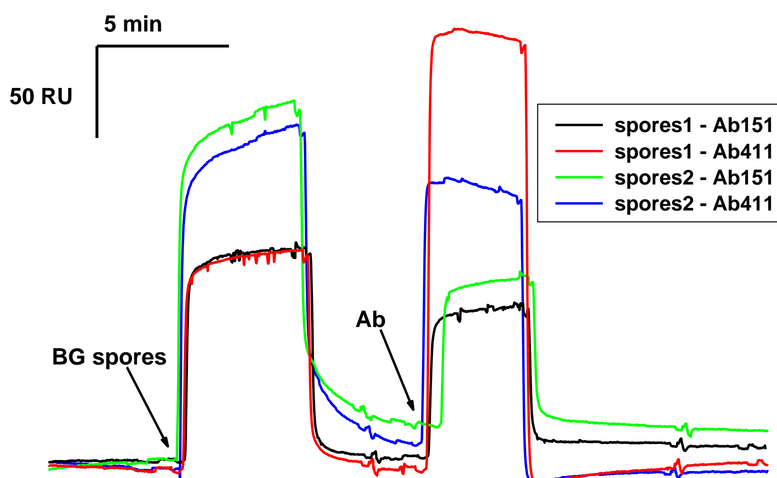

Figure 6. Binding interactions (SPR sensor) of BG spores (10^8 CFU mL^{-1}) with the covalently immobilised capture antibody Ab411, the other secondary antibodies followed to complete the sandwich assay (Ab151 or Ab411). Change of differential response in resonant units (RU) is shown.

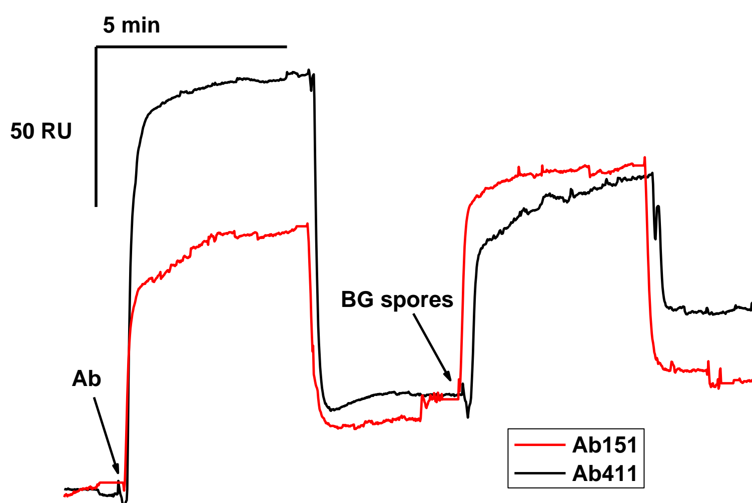

Figure 7. Binding interactions of BG spores 2 (10^8 CFU mL^{-1}) with antibodies Ab151 and Ab411 immobilised using the self-assembled monolayers in flow-through mode measured by the SPR sensor. Change of response in resonant units (RU) is shown.

The SPR experiments with Biacore 3000 confirmed interaction of *B. atrophaeus* spores only with antibodies Ab151 and Ab411. Detection limits of SPR measurements were 10^5 CFU mL^{-1}. Wang reported *B. anthracis* SPR sensor with LOD 10^4 spores per mL and detection time of 40 min [41]. However, Wang used monoclonal antibodies which were highly specific. Surprisingly, the results obtained with Biacore 3000 seemed not significantly better compared to the piezoelectric sensor; however, the automated operation of this system was a convenient advantage.

To confirm presence of spores at the sensing surface, the Au chip used in Fig. 7 was once again allowed to interact with the spores 2 for 5 min in the Biacore system, but very short (1 min) washing with buffer was realised and the chip was immediately removed from the system, disassembled and the

SPR active part was scanned using the atomic force microscope; the magnitude type of image providing higher contrast of the horizontal objects is shown in Fig. 8. The immunochemically bound spores correspond to the circular objects with approximately 1 µm diameter; the vertical dimension evaluated from the topography scan (not shown) was around 0.2 µm. The thin (left) and thick (right) vertical lines correspond to the boundaries of the sensing channel; these artefacts resulted from the contact zones between the chip and the microfluidic module of Biacore. The much smaller dots are most probably crystals of the HBS-P buffer, as the washing with water was not used in order to preserve the bound spores.

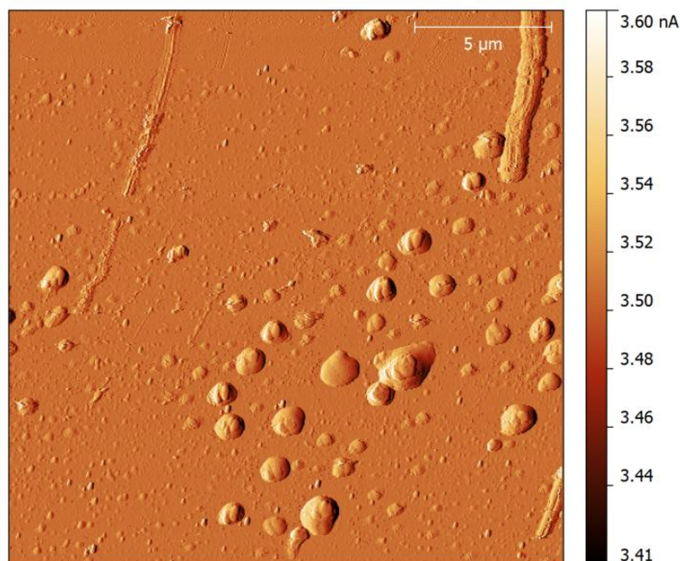

Figure 8. Atomic force microscopy image of the Au-based SPR chip modified with anti BG spores antibodies after interaction with the BG spores. The 20×20 µm scan presents the relative magnitude signal from the AFM system Ntegra Vita, non-contact mode of scanning.

4. CONCLUSIONS

The performance of the SPR and piezoelectric biosensors was compared for immunoassays of spores of the model microbial agent *Bacillus atrophaeus*. Seven commercial antibodies were characterised against the *B. atrophaeus* ATCC 9372 strain, but specific interaction was observed only with Ab151 (LS-C103151), Ab411 (HRP labelled, LS-C103411) and Ab214 (18-511-245214). The extent of interactions was significantly influenced by the method of bacterial cultivation and purification, a longer sporulation period provided spores of better quality. At the level of immunosensors, the response was also affected by the adopted immobilisation strategy. The partially reduced antibodies have the potential to lower the limit of detection as these thiol-containing fragments are directly bound on the gold surface. Generally, the SPR and piezoelectric biosensors provided similar results and rather limited sensitivities; the limits of detection were 10^5 and 10^6 CFU mL^{-1} for both systems, respectively. Sandwich assay was a good choice to somehow improve sensitivity. The results indicate that new im-

mobilisation strategies study must be further addressed and stability of immobilised antibodies has to be improved.

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