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Label-free detection of *B. anthracis* spores using a surface plasmon resonance biosensor

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This study demonstrates the first use of surface plasmon resonance (SPR) technology for the rapid, sensitive and label-free detection of whole *B. anthracis* spores. The approach involves the use of an SPR biosensor (Biacore® 3000), and a monoclonal antibody which was raised against the *B. anthracis* spore (mAb 8G3). By means of subtractive inhibition assays, whole *B. anthracis* spores with concentrations as low as 10^4 colony-forming units (CFU) ml^{-1} can be detected within 40 min, and other related *Bacillus* spores, even in high concentrations, can be differentiated from *B. anthracis* spores.

Introduction

Bacillus anthracis is a rod-shaped, Gram-positive and spore-forming bacterium. Fully virulent *B. anthracis*, carrying two plasmids (pXO1 and pXO2), is the causative agent of *anthrax* and can enter the host through open wounds, by ingestion or by inhalation.[1] In response to nutrient deprivation *B. anthracis* will produce spores that can withstand harsh environments, including extreme temperatures, radiation, chemical assault, time and even the vacuum of outer space.[2] In the Fall of 2001, *B. anthracis* spores, as an airborne pathogen, were intentionally disseminated through the American postal system, resulting in the death of people and societal disruption. Furthermore, *B. anthracis* is genetically very similar to other members of the *Bacillus* genus.[3] Therefore, the threat of *B. anthracis* spores as a biowarfare agent has created an urgent need to develop a convenient, sensitive, and specific method for the detection of *B. anthracis* spores in real time.

Currently, many methods have been proposed for the detection of *B. anthracis* spores, including bacteriological assays, immunological assays, PCR-based methods and other advanced approaches.[4] However, these techniques have significant limitations as they tend to be time consuming or low in sensitivity. Over the last decade, with the progress in the development of biosensors and their applications in biology, there have been several sensor platforms allowing the direct, rapid and real-time detection of *B. anthracis* spores. These detectors include various sensor devices such as evanescent wave fiber-optic biosensors,[5] electrochemiluminescence sensor,[6] quartz crystal microbalance (QCM)[7] and sensors based on a cantilever,[8] but as yet no SPR-based sensors.

SPR biosensors are well established platforms for the study of biomolecular interactions. They are highly sensitive, label free, convenient and work in real time. Biacore® and other commercial SPR sensors are available, and have been used for the detection of various analytes ranging from small molecules to intact cells.[5–12] The Biacore® sensor is a flow-through-based system. It measures an average SPR angle over an area of approximately 0.25 mm^2, and the penetration depth of the evanescent field arising under conditions of total internal reflection in an SPR system cannot exceed 300 nm.[11] Therefore, an SPR instrument has limited sensitivity for the direct capture of intact cells, *via* immobilized antibodies on the sensor chip, as large cells not only fail to evenly cover the area measured but also prevent effective penetration of the evanescent field. Meanwhile, the intact cells can block the microfluidic path by direct injection into microflow channels, resulting in damage of the instrument hardware. Therefore, a subtractive inhibition assay has been employed for the detection of low concentrations of intact cells using an SPR sensor.[11–14] This method is based on pre-incubation of cells and antibodies, followed by removal of cell-bound antibodies. The remaining free antibodies, related to the initial cell concentration, is then quantified by a Biacore® biosensor.

In this study, a monoclonal antibody, designated 8G3 and directed toward the *B. anthracis* spore surface antigen, was employed to detect intact *B. anthracis* spores in subtractive inhibition assays using a Biacore® 3000 biosensor. The results demonstrate a new biosensor alternative for sensitive and label-free detection of whole *B. anthracis* spores.

Results and discussion

In this study mAb 8G3, raised against *B. anthracis* spores, was examined for its reactivity using ELISA (Fig. 1). These results showed that the mAb, using a range of low concentrations, always produced obvious positive signals when reacting against *B. anthracis* spores (Fig. 1a). Furthermore, the mAb was also employed for the detection of *B. anthracis* spores with sandwich

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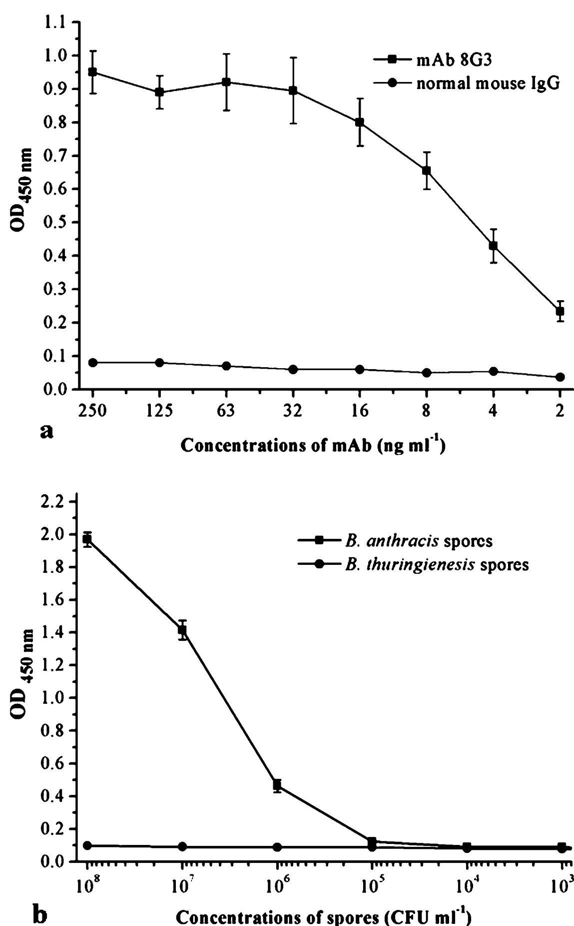

Fig. 1 Examination of mAb activity. Different concentrations of mAb 8G3 reactions with *B. anthracis* spores using indirect ELISA (a). mAb 8G3 used for the detection of *B. anthracis* spores using sandwich ELISA (b).

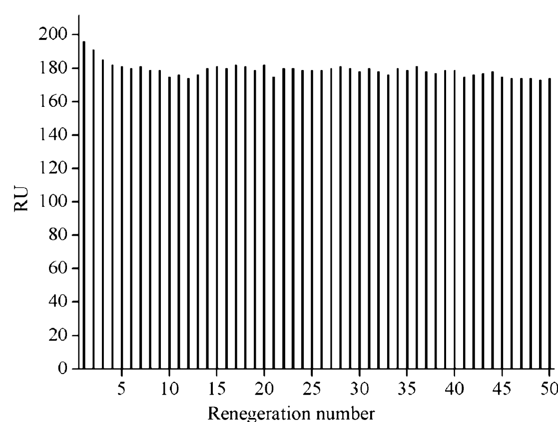

Fig. 2 Surface stability in regeneration cycles. Repeated 5 μg ml^{-1} mAb 8G3 injections (2 min) and surface regeneration using 30 μL 20 mM HCl at a flow rate of 10 μL min^{-1}. The stability of the sensor surface was confirmed with a decrease in surface activity of 11.7% after 50 regeneration cycles.

ELISA, and within 90 min, it could achieve the detection limit of 10^5–10^6 CFU ml^{-1} taking 0.2 as the cut-off value of optical density (Fig. 1b). Therefore, it was determined that the mAb 8G3 had high affinity with *B. anthracis* spores, and can be used for our future SPR assays.

In the SPR assays, approximately 7000 response units (RU) of goat anti-mouse immunoglobulin G (IgG) was immobilized onto the activated CM5 dextran surface, and injection of 30 μL 20 mM HCl was determined to be optimal for surface regeneration (data not shown). After each analysis, the chip would be regenerated, followed by another injection of 35 μL running buffer for the next analysis. To assess specificity of the sensor chip, the mAb 8G3 was simultaneously injected over the functionalized chip surface and blank dextran surface. No significant antibody binding was observed on the blank surface, compared with the chip surface immobilized by goat anti-mouse IgG (data not shown). The stability and activity of the chip surface were further investigated by repeating mAb 8G3 injections and regenerations. As shown in Fig. 2, the first binding cycle produced a 196 RU response signal, and a 174 RU binding response was measured after 50 injections. Although there was a decrease of 11.7% in surface activity, it was less than 20% which is the recommended cut-off value in repeated regeneration assays using Biacore® sensors.[15] In addition, approximately 7% loss of surface activity occurred in the initial 4 regeneration cycles, while the binding response decreased by no more than 5% during the later cycles. The results showed that some initial regeneration cycles were necessary to stabilize the activity of the sensor surface following immobilization. It was also concluded that the functionalized chip surface can be regenerated without significant loss of binding response, and thus can be used for the analysis of a larger number of samples.

The subtractive inhibition assays were used for the real-time detection of *B. anthracis* spores. The mAb 8G3 against *B. anthracis* spores were incubated with a serial concentration of spore solutions. Following sequential centrifugation, the supernatant containing the unbound antibodies was analysed using the Biacore® biosensor, and the binding response of each sample was observed (Fig. 3). A representative experimental result is shown by an overlay plot against the spore concentration (Fig. 3a), and the normalized binding values (R/R_0) from three separate assays were used to construct a calibration curve (Fig. 3b). The coefficients of variation (CV) of each point of the curve were found to be in the range 1.02–7.22%. From this calibration curve, there were apparent differences in binding response for each spore concentration, and the 10^4–10^8 CFU ml^{-1} spores can be detected using subtractive inhibition assays. In addition, *B. anthracis* spores of 10^3 CFU ml^{-1} produced a response similar to the blank sample (data not shown). Therefore, the detection limit was determined to be 10^4 CFU ml^{-1} spores, as the spores resulted in a significant inhibition based on 10% inhibitory concentration (IC_{10}) values.

The mAb 8G3 specificity to the *B. anthracis* spores was investigated with the SPR sensor in subtractive inhibition assays. A range of high concentrations of *Bacillus* spores (10^7 CFU ml^{-1}) were reacted with mAb 8G3 and analysed as above. In these assays, the *B. anthracis* spores gave a binding response of 55 RU, while the other types of spores, as well as the blank sample, all produced signals over 200 RU (Fig. 4), which indicated no significant amount of mAb was bound to these *Bacillus* spores except *B. anthracis*. So, the mAb was highly species-specific, and

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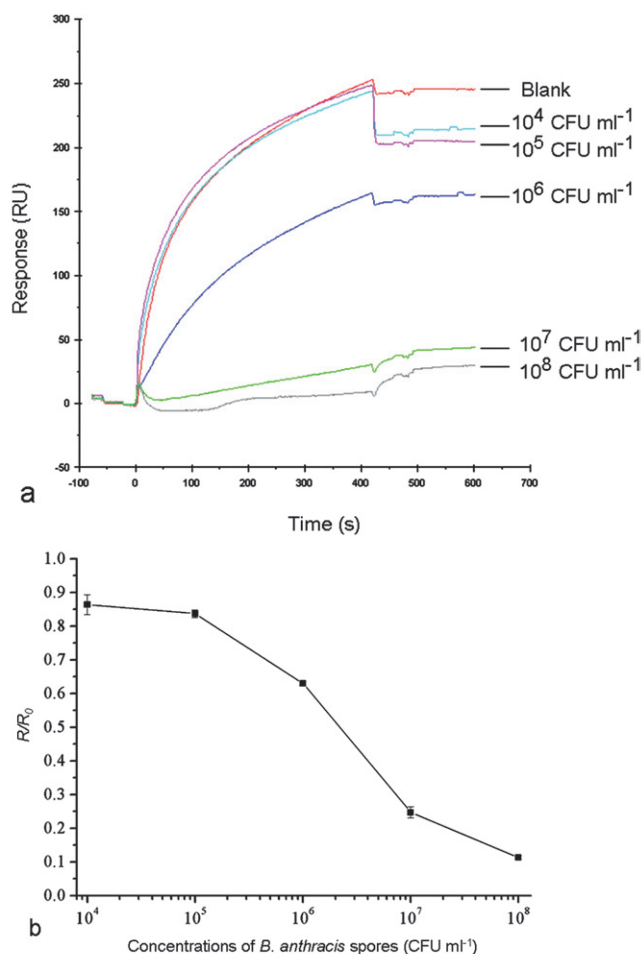

Fig. 3 Overlay plot and calibration curve. The overlay plot presents the decrease in binding of *B. anthracis* mAb 8G3 to the functionalized chip surface with increasing *B. anthracis* spore concentrations. Representative results from spore concentrations ranging from 10^4 to 10^8 CFU ml^{-1} are shown (a). The mean binding response at each spore concentration (R) was divided by the mean blank sample response (R_0) only consisting of mAb 8G3, yielding a normalized binding value (R/R_0). The calibration curve was constructed from the R/R_0-values and standard deviations from three repeated assays, and the detection limit of 10^4 CFU ml^{-1} was achieved based on the IC_{10} value (b).

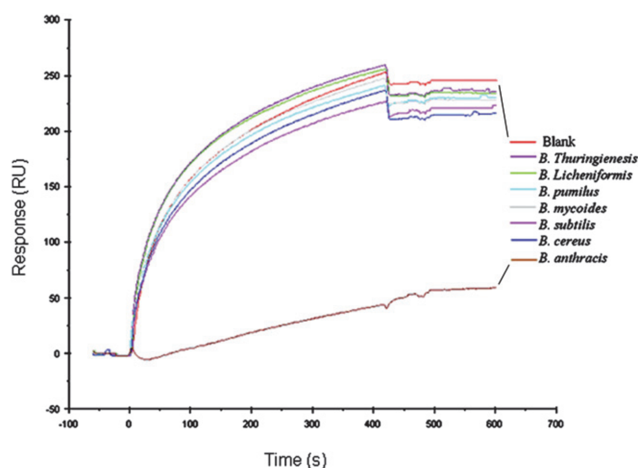

Fig. 4 Cross-reactivity studies. High concentrations of *Bacillus* spores (10^7 CFU ml^{-1}) were investigated for the cross-reactivity with mAb 8G3 in the subtractive inhibition assays. The *B. anthracis* spores gave a binding response of 55 RU, while the other types of spores and the blank sample produced signals of over 200 RU.

even the closest relatives of *B. anthracis*, *B. thuringienesis* and *B. cereus*, had no apparent cross-reaction with our mAb. It was concluded that the current approach was suitable for the specific detection of *B. anthracis* by immoblishing mAb 8G3 on the sensor chip to act as a binding site.

The results in this study demonstrated assays for the direct and label-free detection of *B. anthracis* spores using an SPR biosensor, and achieved a detection limit of 10^4 CFU ml^{-1} *B. anthracis* spores. Compared with ELISA, this method is relatively sensitive, because the *B. anthracis* spores could not be detected in our sandwich ELISA assays even at concentrations of 10^5 CFU ml^{-1} (Fig. 1b). Although many PCR-based methods have been developed for highly sensitive detection of *B. anthracis* spores and the reported detection limits for real-time PCR can be as low as 5–10 spores,[16] the current detection limit is enough to detect *B. anthracis* spores from terrorist activities, as the 50% lethal dose (LD_{50}) estimated for inhalation of anthrax in humans is approximately 3000–50 000 spores.[17] Furthermore, this approach based on an SPR biosensor is superior in terms of analysis time and other performances. Firstly, the whole assay was complete within 40 min, including sensor chip functionalization, sample incubation, centrifugation and measurement of binding response. To emphasize, the sensor only took 7 min to detect the binding signal (Fig. 3), and the next sample could be analysed following regeneration of about 2 min. Secondly, it eliminates the need for complicated protocols such as spore disruption, and no labeling reagents with fluorescence or radioactive tags were required for this technique, thereby realizing direct, convenient and label-free detection of whole *B. anthracis* spores. Lastly, the Biacore® 3000 sensor has many unique benefits because of an automatic and microfluidic system. There is little sample consumption and no washing steps are required to replace the sample with buffer. In addition, this instrument reduced hands-on time and increases sample through-put, as large numbers of samples could be included in the centrifugation step and analysed in unattended runs.[12]

SPR biosensors have already been applied for the detection of other microorganisms in subtractive inhibition assays. The reported detection limits were 10^5–2.2×10^6 CFU ml^{-1},[11–13] which is less sensitive than the current detection limit of 10^4 CFU ml^{-1} *B. anthracis* spores. This may be due to a higher affinity of the mAb used in our experiment or the large number of epitopes on the *B. anthracis* spores surfaces. Therefore, the performance of mAb undoubtedly played an important role in the detection of *B. anthracis* spores, and thus determined our test results. If only suitable biomolecular recognition elements such as antibodies, peptides and aptamers can be exploited, the method presented in this paper is capable of detecting relevant microorganisms.

Experimental

Spores preparation

B. anthracis A16 (pXO1$^+$, pXO2$^+$), *B. subtilis, B. cereus* CCTCCAB 93038$^+$, *B. thuringienesis* subsp. alesti, *B.*

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licheniformis, *B. mycoides* and *B. pumilus*, were used in this study. The spores were prepared by growing the bacteria at 37 °C on the sporulation medium (DSM) containing 6 g tryptone, 3 g yeast extract, 10 g NaCl, 1 g KCl, 0.25 g $Mg_2SO_4 \cdot 7H_2O$, 0.23 g $Ca(NO_3)_2$, 0.197 g $MnCl_2 \cdot 4H_2O$, 0.0002 g $FeSO_4$, and 15 g agar per liter.

mAb production

A mouse monoclonal antibody designated 8G3 was raised against intact *B. anthracis* A16 spores. Preparations containing 10^6 spores of *B. anthracis* strain A16 and inactivated by 1.5% formaldehyde were injected subcutaneously into six-week-old BALB/c mice. The immunization was repeated 3× at two week intervals before boosting by intraperitoneal injection. The spleen cells were removed 3 d later and fused with myeloma cells, according to the procedures of Kohler and Milstein. The hybridomas were cloned by limit dilution, screened using enzyme linked immunosorbent assay (ELISA). The mAbs were purified by caprylic acid–ammonium sulfate precipitation of ascites, and analysed by SDS-PAGE to determine the purity.

ELISA

For indirect ELISA assays, 96 well microtiter plates were coated with 100 μL per well of carbonate–bicarbonate (CB) buffer (pH 9.6) containing either 10^7–10^8 ml^{-1} spores and incubated overnight at 4 °C. The wells were blocked with 200 μL of blocking buffer (5% skimmed milk in PBS) for 2 h at 37 °C. A concentration series of purified mAb were added in 100 μL aliquots to individual wells and incubated for 30 min at 37 °C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (prepared in our laboratory) were added at a dilution of 1/900 and incubated for 20 min.

For sandwich ELISA, the microtiter plates were coated with purified mAb 8G3 (10 μg ml^{-1}) and blocked as described above. The plates were incubated with a range of concentrations of spores at 37 °C for 30 min and then reacted with 1 μg ml^{-1} rabbit polyclonal antibodies against *anthracis* at 37 °C for 20 min. HRP-conjugated goat anti-rabbit antibodies were added to the plate at a dilution of 1/10 000 (Boster, China) and reacted for 20 min at 37 °C.

For both indirect ELISA and sandwich ELISA, five washes with PBS containing 0.05% Tween 20 (PBST) were carried out between each step and all antibody dilutions were prepared in PBS, containing 1% skimmed milk. A tetramethylbenzidine substrate (100 μL per well) was added for approximately 5–10 min at 37 °C to start the reaction, and 50 μL of 2 mol L^{-1} H_2SO_4 was added to stop the reaction. Then the absorbance was measured at 450 nm, and each assay was performed in quadruplicate.

Antibody immobilization

Analysis were carried out on a Biacore® 3000 instrument (Uppsala, Sweden) using filtered and degassed PBS containing 0.005% Tween 20 as a running buffer. Sensor chip CM5 (Biacore®) dextran surfaces were explored throughout this study. The CM5 chip was first activated by mixing 50 μL of 100 mM *N*-hydroxysuccinimide (NHS) with 50 μL of 400 mM *N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and injecting 70 μL of the mixture over the CM5 dextran surface at a flow rate of 10 μL min^{-1}. A 100 μg ml^{-1} solution of commercial goat anti-mouse (Boster, China), diluted in 10 mM sodium acetate with varing PH (3.8–5.0), was injected over the acitvated dextran surface at 5 μL min^{-1} for 12 min. Unreacted sites were subsequently deactivated by injecting 1 M ethanolamine, pH 8.5 (Biacore®) at 10 μL min^{-1} for 7 min.

Surface regeneration

The surface was regenerated using 30 μL 20 mM HCl and 35 μL running buffer at a flow rate of 30 μL min^{-1}. The mAb 8G3 with 5 μg ml^{-1} diluted in the running buffer was passed over the regenerated chip surface for 2 min at 10 μL min^{-1} The binding signal, detected at 100 s after injection finished, was used to assess the stability of the immobilised anti-mouse antibody surface.

SPR assay design

Both mAb 8G3 and spores were diluted in the running buffer (PBS containing 0.005% Tween 20). Two hundred microlitres of mAb (10 μg ml^{-1}) was added to the equal volume of spores solution with a varying concentration, and each mixture was incubated for 30 min at 37 °C with gentle shaking (70–100 rpm). After incubation, free mAb were separated from the spore-bound antibodies by sequential centrifugation as reported previously.[11] The supernatant was carefully removed without touching the remaining spores, and was assayed by automated analysis using the Biacore® 3000 instrument. For each assay, 35 μL of supernatant were injected over the CM5 chip surface at 5 μL min^{-1}, and the resulting response unit change was measured at 100 s after the injection finished. Following each sample injection, the CM5 chip surface was regenerated as above. The mean values were from triplicate assays of each spore concentration (R), and a mean blank sample response (R_0) only consisted of mAb 8G3. The mean values were divided by the mean blank sample response, yielding a normalized binding value (R/R_0) which showed the degree of inhibition for each spore concentration. A calibration curve was constructed to show correlation between degree of inhibition and spore concentration, and the detection limit was calculated as the lowest spore concentration resulting in 10% inhibition (IC_{10}).[18]

Specificity of mAb

To evaluate the mAb cross-reaction with other related species, *B. subtilis*, *B. cereus*, *B. thuringienesis*, *B. licheniformis*, *B. mycoides* and *B. pumilus* were investigated in this study. The spores of each strain (200 μL, 10^7 CFU ml^{-1}) were incubated with equal volume of purified mAb 8G3 (200 μL, 10 μg ml^{-1}). The free antibodies removal and the sample analysis were performed as described above.

Conclusions

This study develops a method based on an SPR sensor for fast, sensitive and label-free detection of whole *B. anthracis* spores using high affinity mAb in subtractive inhibition assays. The detection limit of 10^4 CFU ml^{-1} is achieved within 40 min, and

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other related *Bacillus* spores can be easily differentiated from *B. anthracis* spores, even with high concentration, by this approach. Therefore, the method can be used as an alternative detection means in central laboratories and potentially applied in field conditions when a portable SPR machine becomes available.

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