Indian J Microbiol (Jan–Mar 2013) 53(1):48–55
DOI 10.1007/s12088-012-0334-3

ORIGINAL ARTICLE

Surface Plasmon Resonance Biosensor for Detection of *Bacillus anthracis*, the Causative Agent of Anthrax from Soil Samples Targeting Protective Antigen

N. Ghosh · G. Gupta · M. Boopathi · V. Pal · A. K. Singh · N. Gopalan · A. K. Goel

Received: 17 September 2012 / Accepted: 31 October 2012 / Published online: 10 November 2012

Abstract *Bacillus anthracis*, the causative agent of anthrax is one of the most important biological warfare agents. In this study, surface plasmon resonance (SPR) technology was used for indirect detection of *B. anthracis* by detecting protective antigen (PA), a common toxin produced by all live *B. anthracis* bacteria. For development of biosensor, a monoclonal antibody raised against *B. anthracis* PA was immobilized on carboxymethyldextran modified gold chip and its interaction with PA was characterized in situ by SPR and electrochemical impedance spectroscopy. By using kinetic evaluation software, K_D (equilibrium constant) and B_{max} (maximum binding capacity of analyte) were found to be 20 fM and 18.74, respectively. The change in Gibb's free energy ($\Delta G = -78.04$ kJ/mol) confirmed the spontaneous interaction between antigen and antibody. The assay could detect 12 fM purified PA. When anthrax spores spiked soil samples were enriched, PA produced in the sample containing even a single spore of *B. anthracis* could be detected by SPR. PA being produced only by the vegetative cells of *B. anthracis*, confirms indirectly the presence of *B. anthracis* in the samples. The proposed method can be a very useful tool for screening and confirmation of anthrax suspected environmental samples during a bio-warfare like situation.

Keywords Surface plasmon resonance · Biosensor · *Bacillus anthracis* · Protective antigen · Monoclonal antibody

N. Ghosh · G. Gupta · M. Boopathi · V. Pal · A. K. Singh · N. Gopalan · A. K. Goel (✉)
Biotechnology Division, Defence Research and Development Establishment, Jhansi Road, Gwalior 474 002, India
e-mail: akgoel73@yahoo.co.uk

Introduction

Bacillus anthracis, the main causative agent of anthrax is a Gram positive, aerobic, rod shaped, non-motile, spore forming bacterium, and has gained the status of the most potent biological warfare agent. *B. anthracis* produces spores which are highly resistant to environmental stress such as heat, drought, radiations, chemical assault and even the vacuum of outer space [1]. The virulence of *B. anthracis* is attributed to two major factors, i.e. a tripartite toxin consisting of protective antigen (PA), lethal factor (LF) and edema factor (EF), and the poly-γ-D-glutamic acid capsule [2]. The spores can drift gently in the wind and on finding their appropriate host (an animal or human) they change to the rod-like form and begin to multiply rapidly. Virulent *B. anthracis* vegetative cells form capsules of poly-D-glutamic acid which impedes the host immune system and inhibits macrophages from engulfing and destroying the bacteria [3].

A reliable identification of *B. anthracis* is difficult due to the monomorphic nature of the *Bacillus cereus* group, which comprises *B. cereus*, *B. anthracis*, *Bacillus thuringiensis*, and *Bacillus mycoides* [4]. There are several methods available for serodiagnosis of anthrax infection [5, 6]. However, rapid, sensitive and specific detection of *B. anthracis* is a challenging task. Sample processing for isolation of bacterium is risky due to its highly virulent and pathogenic traits [7]. Thus, during anthrax outbreaks, a rapid and highly sensitive detection method is required which could detect *B. anthracis* in environmental as well as clinical samples without isolation of the bacteria. Presently, several methods have been developed for detection of *B. anthracis* and many more are still in development phase [8, 9]. Recently, several sensor platforms like evanescent wave fiber-optic biosensors [10], electrochemiluminescence

sensor [11], quartz crystal microbalance [12] and surface plasmon resonance [13] have been devised for the detection of *B. anthracis*. However, many of these methods are time consuming, less sensitive and do not differentiate between live and dead spores/bacteria.

Here, we report a sensitive and specific method for the detection of PA using SPR technology. PA is produced by vegetative cells of all *B. anthracis* strains only as 83 kDa protein. Thus, the presence of PA in enriched soil samples indirectly confirms the presence of live *B. anthracis*. SPR is a dynamic method of real time detection in biological world which could detect and characterize the antigen antibody interaction in the absence of chemical labeling and with minimum sample preparation [14, 15]. In present study, the assay was used for detection and confirmation of anthrax spores in the soil samples spiked with a known number of *B. anthracis* spores by detecting PA by SPR.

Materials and Methods

Materials

N-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), phosphate buffered saline (PBS), 1 M ethanolamine and hydrochloric acid (HCl) were procured from Fluka. Citric acid, sodium hydroxide, dipotassium hydrogen phosphate and potassium dihydrogen phosphate were supplied by Sigma-Aldrich. All chemicals and reagents used were of analytical grade and purification was performed wherever necessary before use. A 0.05 M phosphate buffer (pH 6.0) was used as coupling buffer in the experiments and dilution of antibody was also carried out using this buffer.

Instrumentation

The studies of antigen–antibody interactions were conducted using a two channel cuvette based electrochemical SPR system (Autolab ESPRIT, Ecochemie B.V., The Netherlands). The outcome of the SPR measurement was automatically monitored using a software and data acquisition using the SPR software version 4.3.1 and all kinetic data were obtained using the SPR kinetic evaluation software version 5.1 (Ecochemie B.V.). Carboxymethyldextran (20 nm thickness) modified gold chip for SPR measurements was purchased from XanTec Bioanalytics (Germany). The pH of the buffers used was measured with a EUTECH instrument pH meter (pH 1,500, Singapore). The temperature of cuvette was controlled by a water bath (Julabo HE-4, Germany).

Preparation of Antigen

The antigen, *B. anthracis* recombinant PA (rPA, 83 kDa) used for evaluation procedure in this study was prepared and purified as described earlier [16].

Production of Monoclonal Antibodies

A mouse monoclonal antibody designated 3E5B8 was raised against 83 kDa rPA. Preparations containing 50 μg of rPA were injected subcutaneously into 8-week-old BALB/c mice. The immunization was repeated three times at 2 weeks intervals before boosting with 25 μg rPA per mouse. The spleen cells were removed 3 days later and fused with myeloma cells, according to the standard procedure [17]. The hybridomas were cloned by limit dilution, screened using enzyme linked immunosorbent assay (ELISA). The MAbs were purified by protein A column (Montage PROSEP-A Antibody Purification, Millipore), and analysed by SDS-PAGE to determine the purity.

ELISA

For determination of titre of antibodies, MaxiSorp Flat Bottom 96-well microtiter plates (Nalge Nunc International, Denmark) were coated with 100 μL per well of carbonate–bicarbonate buffer (pH 9.6) containing 2 μg/mL of rPA and incubated overnight at 4 °C. The antigen coated plates were washed three times with wash buffer (PBS containing 0.1 % Tween 20) using ELx 508_{MS} microplate washer (BioTek Instruments Inc., USA). The wells were blocked with 300 μL of blocking buffer (5 % skimmed milk in PBS) for 2 h at 37 °C. After washing, a concentration series of purified MAbs were added in 100 μL aliquots to individual wells and incubated for 60 min at 37 °C, and the plates were then washed three times with wash buffer. Horseradish peroxidase-conjugated goat anti-mouse antibodies (Sigma-Aldrich, USA) diluted in PBS containing 5 % skim milk and 0.5 % Tween 20 (100 μL/well) were added at a dilution of 1/1,000 and incubated for 60 min to detect the bound anti-PA-IgG. Plates were again washed three times with wash buffer and detected colorimetrically by using 100 μL per well of ortho-phenyl diamine/H_2O_2 substrate (Sigma-Aldrich, USA). Color development was stopped after 20 min by adding 50 μL of 2.5 N H_2SO_4 solution in each well and the plate was read at 492 nm using a ELISA plate reader (BioTek Instruments Inc., USA).

Specificity of Monoclonal Antibodies

Different *Bacillus* species including *B. anthracis* Sterne, *B. cereus*, *B. licheniformis*, *B. mycoides*, *B. pumilus*,

B. subtilis and *B. thuringiensis* were grown for 8 h in BHI broth. The culture broth was centrifuged and the supernatant of individual bacterial species was run on SDS-PAGE followed by Western blotting with MAb.

Preparation of Spores

Bacillus anthracis Sterne ($pXO1^+$ $pXO2^-$), *B. cereus*, *B. licheniformis*, *B. mycoides*, *B. subtilis* and *B. thuringiensis* were used in this study. Bacteria were grown on brain heart infusion (BHI) agar plates and incubated overnight at 37 °C. Subsequently, culture plates were transferred at room temperature for 6–7 days. Spores were harvested in normal saline from BHI plates, aseptically. The suspension was heated at 65 °C in water bath to inactivate the vegetative cells, if any. The concentration of colony forming units/mL in suspension was determined by plate counting method and Neubauer counting chamber. The final concentration of 1×10^9 spores/mL was prepared and stored at −80 °C for further use.

Immobilization of Anti-PA Monoclonal Antibody on the Carboxymethyldextran Modified Gold SPR Sensor Chip

Prior to start of the experiment, the milli degree change in resonance angle was recorded as the baseline and stabilized by passing 50 µL of 0.05M phosphate buffer (pH 8.0) over carboxymethyldextran modified gold chip at an interval of 120 for 600 s. The carboxymethyldextran modified gold chip was then activated by injecting a 50 µL of freshly prepared 1:1 mixture of EDC (400 mM) and NHS (100 mM) over the chip surface for 900 s in order to get more amine reactive NHS esters followed by immediate injection of 50 µL of α-PA monoclonal antibodies (0.34 mg/mL in 0.05 M phosphate buffer) in channel 2 and allowed to interact for 1,800 s to get an effective immobilization of α-PA monoclonal antibody over the activated dextran modified surface. The immobilized surface was blocked for 600 s with 1M ethanolamine (pH 8.5) to deactivate non-reacted NHS ester in order to avoid non specific binding of antigen over the immobilized surface during sensing process. For negative control measurement, the modified gold surface was activated with EDC/NHS and then blocked with ethanolamine in channel 1 as mentioned above and was used as the blank control surface.

Optimization of Experimental Conditions

In order to find out the optimum temperature for the interaction of antigen and antibody, temperature variation study was carried out in the range from 10 to 40 °C with an increment of 3 °C.

Antigen–Antibody Interaction and Characterization

For affinity measurements and detection limit of PA, varying concentrations of PA were prepared in phosphate buffer saline and injected into both channels from the 384 well micro titer plate and then association was performed for 500 s and dissociation for 400 s. Subsequently, 10 mM HCl was used to achieve regeneration of the sensor surface after each interaction for 120 s, and PA was recovered in order to bring the signal to baseline level so as to start a new cycle. The above procedure was performed with solutions containing 12, 120 fM, 1.2 and 12 pM concentrations of PA. A solution of phosphate buffer saline (pH 7.4) was used throughout the experiment as the running buffer.

Study of Kinetic Parameters

Kinetic parameters of affinity interactions were characterized on the basis of K_D values (equilibrium constant) and B_{max} (maximum binding capacity of analyte) using kinetic evaluation software version 5.1. The thermodynamic parameter was calculated using Van't Hoff equation [18].

Detection of *B. anthracis* from Soil Samples

Sterilized and unsterilized soils samples were artificially spiked with different number of spores of *B. anthracis* Sterne, *B. cereus*, *B. mycoides*, *B. licheniformis* and *B. thuringiensis.* One gram of spiked soil was suspended in 5 mL of BHI broth and incubated at 37 °C for 6–8 h under shaking conditions. After incubation, 1 mL of sample from upper layer of broth was centrifuged at 12,000 rpm for 10 min at room temperature. Supernatant was passed through 0.22 µm syringe filters and 1:10 dilution of supernatant in PBS was used directly for detection of PA. For negative control, 1 g of un-spiked soil was used in place of spiked soil. The concentration of spores used was 10^0–10^5 spores/g in both sterilized and unsterilized soil.

Results and Discussion

Generation of Monoclonal Antibodies

The purified monoclonal antibody (MAb) 3E5B8 raised against rPA in this study exhibited a very good reactivity (a titre of 1:512,000) with the PA in ELISA (Fig. 1a). The antibodies were highly specific to PA produced by *B. anthracis* as no cross reactivity was observed with the toxin produced by any of the other homologous bacterial species (Fig. 1b).

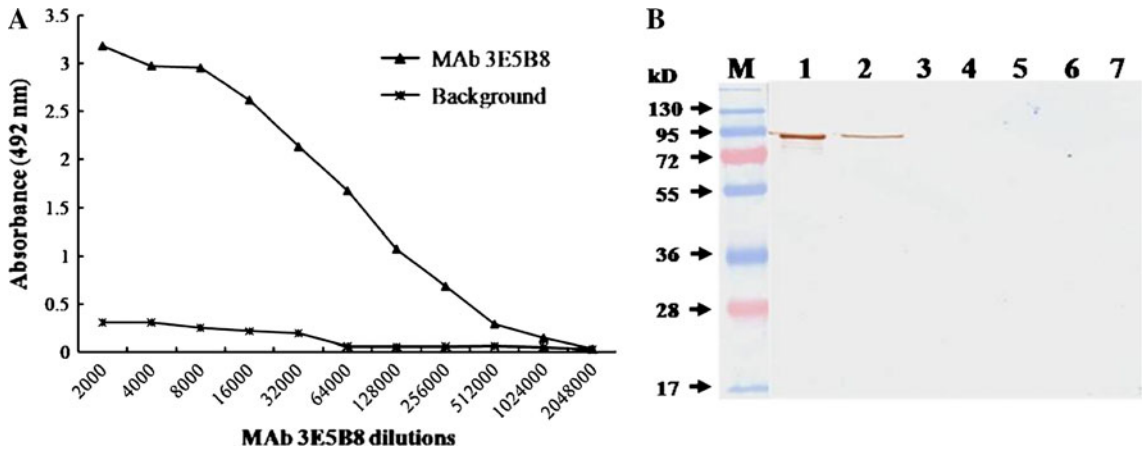

Fig. 1 Reactivity of purified monoclonal antibodies (3E5B8) with PA by plate ELISA (**a**) and with PA and other toxins produced by *Bacillus* species by immunoblot analysis (**b**). *Lane 1* purified recombinant PA, *lane 2 B. anthracis* Sterne, *lane 3 B. cereus*, *lane 4 B. subtilis*, *lane 5 B. thuringiensis*, *lane 6 B. mycoides*, *lane 7 B. pumilus*, *lane M* prestained markers (Fermentas)

Immobilization of Anti-PA Monoclonal Antibody on Carboxymethyldextran Modified Gold Chip for SPR Sensor

Immobilization of antibody on modified gold SPR sensor chip was done in nine steps as described in Fig. 2. In the first step, the base line was stabilized for 120 s in order to get a stable baseline signal. In the second step, the carboxyl groups on the dextran layer was activated with EDC–NHS (1:1 mixture) for 900 s for the generation of highly reactive *O*-acylisourea intermediates in order to make amide bonds between the carboxylic acid groups of modified sensor chip and the amino groups of anti-PA monoclonal antibody. The activation of the sensor chip resulted with 548 mdeg angle change in SPR. In the third step, washing was done with PBS, which brought the angle change nearly to the baseline value [19]. After washing, in the fourth step, 50 μL of anti-PA monoclonal antibody was injected on the sensor surface and allowed to interact for 1,800 s which resulted in angle shift of 102.67 mdeg. Subsequently, to remove unbound antibodies, a second washing was performed with PBS in the fifth step and during this process, the SPR angle decreased by 1.5 mdeg. To prevent non-specific binding of antigen on the sensor surface with un-reacted NHS group, blocking was performed for 600 s with 1M ethanolamine (pH 8.5) in the sixth step, which resulted in increase in SPR angle by 659.5 mdeg. In the seventh step, washing was done with PBS for 30 s followed by regeneration for 120 s in the eighth step. Finally, in the ninth step, baseline was brought back to initial value in 60 s. A net angle change of

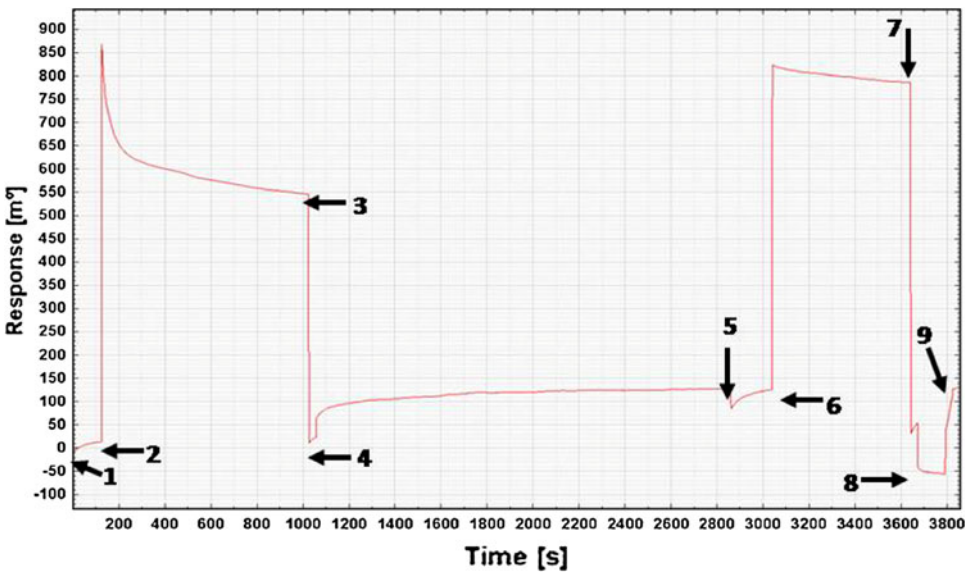

Fig. 2 Sensogram showing immobilization of anti-PA monoclonal antibody on modified gold chip. *1* Baseline, *2* EDC–NHS activation, *3* washing, *4* ligand coupling, *5* washing, *6* deactivation, *7* washing, *8* regeneration, *9* back to baseline

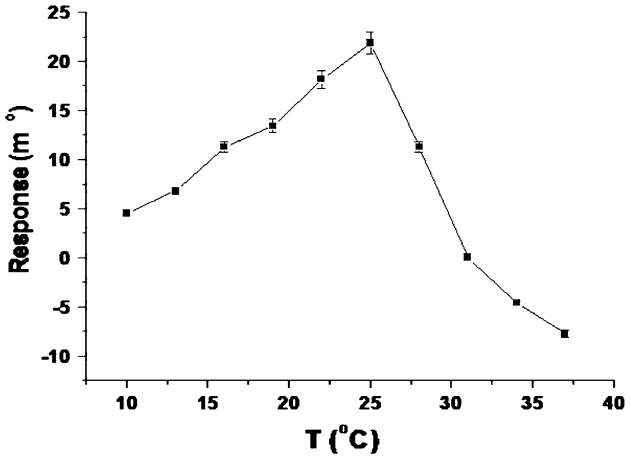

Fig. 3 Graph showing SPR angle shift with temperature

131.6 mdeg (Fig. 2) was observed which ascribed the attachment of 1.10 ng/mm^2 [120 mdeg = 1 ng/mm^2] of antibody on the sensor surface [20].

Effect of Temperature on Interaction of Antigen with Anti-PA Monoclonal Antibody

A varying range of temperature was used in this study. An increase in SPR angle was observed upon increasing the temperature from 10 to 25 °C and it decreased afterwards (Fig. 3). The maximum angle change in SPR was observed at 25 °C. This observation may be due to temperature dependent structural changes and electrostatic interactions that occurred on the sensor chip during the antigen antibody interaction as reported earlier [21]. Hence, the optimum temperature for the interaction of anti-PA monoclonal antibody and PA was found to be 25 °C.

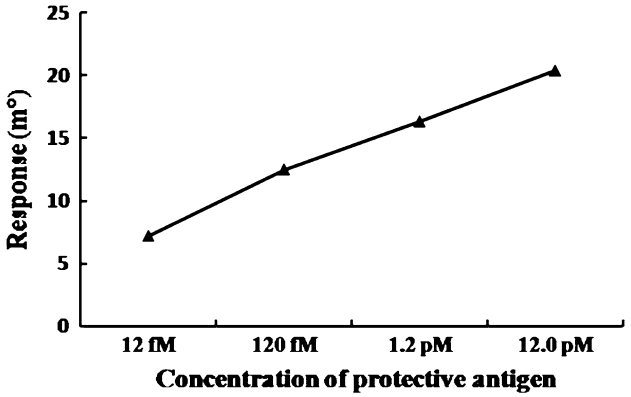

Fig. 5 Calibration plot of SPR signal v/s various concentrations of PA **a** 12 fM; **b** 120 fM; **c** 1.2 pM; **d** 12 pM

Detection of Anthrax with Anti-PA Antibody Immobilized Sensor Chip of SPR

The sensogram for the antigen antibody interaction for various concentrations of PA is shown in Fig. 4. The response increased in proportion with the concentrations of PA. A calibration curve was plotted using SPR signals from Fig. 4 (net angle change i.e. working channel minus reference channel) to find out the detection limit of sensor (Fig. 5). In purified condition, the sensor could detect the PA up to a concentration as low as 12 fM.

In spiked soil samples, SPR could detect PA produced even by a single spore/g in both, sterilized and un-sterilized

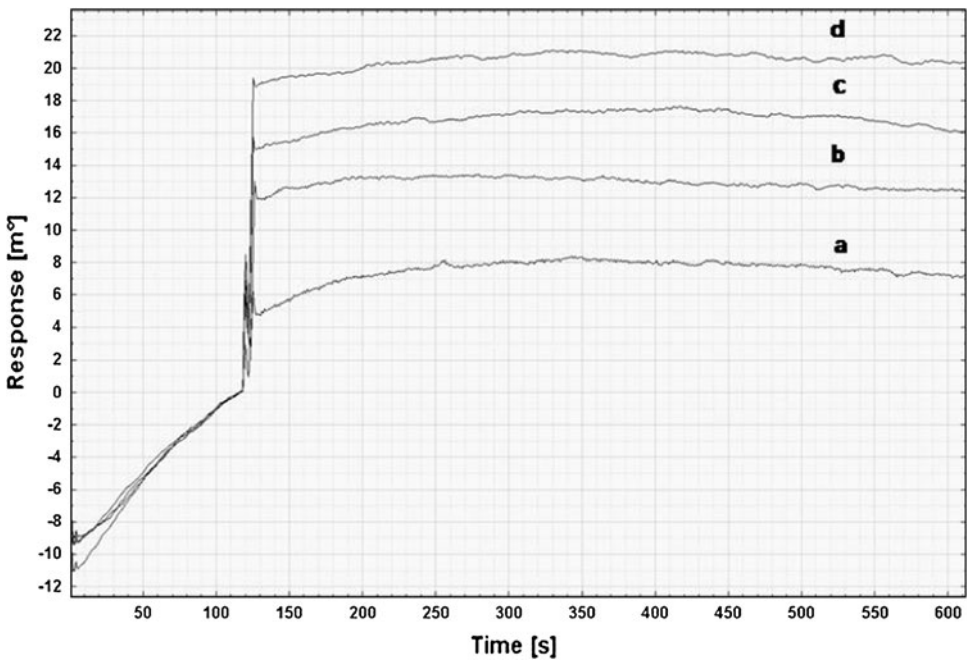

Fig. 4 Sensogram for antigen antibody interaction for various concentrations of PA *a* 12 fM; *b* 120 fM; *c* 1.2 pM; *d* 12 pM

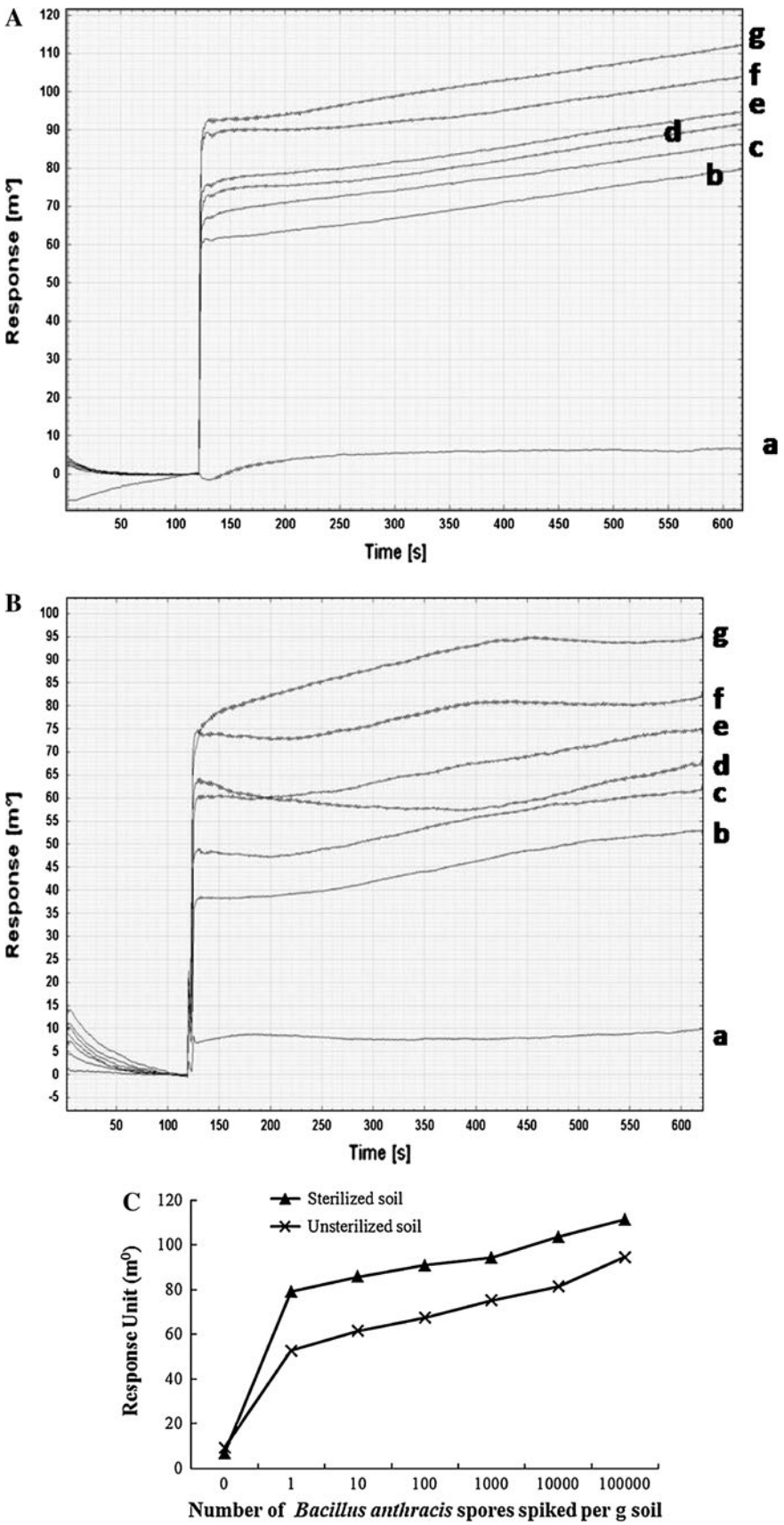

Fig. 6 **a** Sensogram showing responses in sterile soil. (*a*) Unspiked soil; (*b*) 10^0 spore/g soil; (*c*) 10^1 spores/g; (*d*) 10^2 spores/g; (*e*) 10^3 spores/g; (*f*) 10^4 spores/g; (*g*) 10^5 spores/g. **b** Sensogram showing response in unsterile soil. (*a*) Unspiked soil; (*b*) 10^0 spore/g soil; (*c*) 10^1 spores/g; (*d*) 10^2 spores/g; (*e*) 10^3 spores/g; (*f*) 10^4 spores/g; (*g*) 10^5 spores/g. **c** Detection of *B. anthracis* spores in sterilized and unsterilized soil samples by SPR

soil samples on enrichment (Fig. 6a, b). A separate graph (6c) was plotted using SPR signals from Fig. 6a, b to show the detection sensitivity of *B. anthracis* spores in sterilized and unsterilized soil samples. Thus, sufficient amount of PA is produced in the enriched samples after 6–8 h incubation for detection by SPR biosensor. The other advantage of this method is that it detects only live spores as dead or non-viable spores will not convert into vegetative cells and no PA will be produced. The method is much better to traditional methods like ELISA. Generally, the main drawback of ELISA is sensitivity and non-specific binding with antibodies when the unsterile soil is used as sample (Data not shown). The non-specific binding may be due to several other bacteria present in the soil, which produce various exoproteins and results in the nonspecific binding with antibodies. Immunoblot analysis confirmed that monoclonal antibodies used in this study were highly specific to PA, an anthrax specific toxin and no reactivity was observed with culture supernatant/toxins produced by other *Bacillus* species (Fig. 1b). The present SPR methodology is totally based on monoclonal antibody and also provides information about affinity of interaction along with kinetic information, it could easily discriminate between specific and nonspecific binding. Hence, the nonspecific binding is almost negligible in SPR based detection (Fig. 6a, b). Other homologous bacteria like *B. cereus*, *B. mycoides*, *B. licheniformis* and *B. thuringiensis* either in ELISA or in didn't give any signal indicating the specificity of the test. Moreover, in ELISA different types of antibodies are required for the detection including a labeled antibody, which make it very time consuming protocol for detection. Thus, SPR technology does not involve any labeling and is found to be better diagnostic system of *B. anthracis* for the detection from environmental samples in short time with very high specificity.

Kinetic Parameters of Antigen Antibody Interaction Used for the Detection of *B. anthracis*

The kinetics of the affinity binding between PA and anti-PA monoclonal antibody was analysed using the kinetic evaluation software as mentioned in materials and methods. The B_{max}, K_D and ΔG were found to be 18.74 mdeg, 20 fM and −78.04 kJ/mol, respectively. The low K_D value indicates the high binding affinity of antibody with antigen and negative value of Gibb's free energy shows the spontaneity of reaction.

On enrichment, spores enter the vegetative growth phase and begin to produce toxins. The anthrax toxins are secreted as three distinct proteins, namely PA, LF and EF and their activities have been well described [22, 23]. The PA component is produced as an 83-kDa protein and is responsible for the attachment of LF and EF to the cells surface receptor and their internalization within the cytosol.

Surface plasmon resonance based detection of PA produced by *B. anthracis* spores from soil samples is a unique method, where the anthrax spores can be detected in soil samples without isolation of spores/bacteria. Isolation of *B. anthracis* from soil sample is a very tedious procedure. In the present method, the samples can be autoclaved or disinfected after collection of supernatant for PA estimation. It is well known that soil is the reservoir of millions of bacteria which generally interferes with the growth of spores and other spores present in the soil produces multiple proteins which shows nonspecific binding in traditional immunological methods. SPR based detection rules out all the drawbacks present in the traditional methods of detection and fastens the detection procedure with simplicity, sensitivity and specificity. Another advantage of this SPR based method is that it could calculate the kinetic parameters of interaction by which we can calculate the affinity of binding between antigen and antibody. Higher the binding affinity more will be the specificity. Thus, SPR based detection of *B. anthracis* from environmental samples is a very useful tool for screening and confirmation of anthrax suspected environmental samples during a biowarfare like situation.

Acknowledgments Authors are thankful to Director, Defence Research & Development Establishment (DRDE), Gwalior for providing necessary facilities and funds for the work.

References

1. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64(3):548–572
2. Collier RJ, Young JAT (2003) Anthrax toxin. Annu Rev Cell Dev Biol 19:45–70
3. Ezzell JW, Welkos SL (1999) The capsule of *Bacillus anthracis*, a review. J Appl Microbiol 87(2):250
4. Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolsto AB (2000) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol 66(6):2627–2630
5. Ghosh N, Goel AK (2012) Anti-protective antigen IgG enzyme-linked immunosorbent assay for diagnosis of cutaneous anthrax in India. Clin Vaccine Immunol 19(8):1238–1242. doi:10.1128/CVI.00154-12
6. Quinn CP, Semenova VA, Elie CM, Romero-Steiner S, Greene C, Li H, Stamey K, Steward-Clark E, Schmidt DS, Mothershed E, Pruckler J, Schwartz S, Benson RF, Helsel LO, Holder PF, Johnson SE, Kellum M, Messmer T, Thacker WL, Besser L, Plikaytis BD, Taylor TH Jr, Freeman AE, Wallace KJ, Dull P, Sejvar J, Bruce E, Moreno R, Schuchat A, Lingappa JR, Martin SK, Walls J, Bronsdon M, Carlone GM, Bajani-Ari M, Ashford DA, Stephens DS, Perkins BA (2002) Specific, sensitive, and quantitative enzyme-linked immunosorbent assay for human

immunoglobulin G antibodies to anthrax toxin protective antigen. Emerg Infect Dis 8(10):1103–1110
7. Maraldo D, Rijal K, Campbell G, Mutharasan R (2007) Method for label-free detection of femtogram quantities of biologics in flowing liquid samples. Anal Chem 79(7):2762–2770. doi:10.1021/ac0621726
8. Rao SS, Mohan KV, Atreya CD (2010) Detection technologies for *Bacillus anthracis*: prospects and challenges. J Microbiol Methods 82(1):1–10
9. Goel AK, Kamboj DV, Singh L (2005) Enrichment and rapid detection of *Bacillus anthracis* spores by direct immunofluorescence assay. Indian J Microbiol 45(4):269–272
10. Tims TB, Lim DV (2004) Rapid detection of *Bacillus anthracis* spores directly from powders with an evanescent wave fiber-optic biosensor. J Microbiol Methods 59(1):127–130. doi:10.1016/j.mimet.2004.02.016
11. Bruno JG, Kiel JL (1999) In vitro selection of DNA aptamers to anthrax spores with electrochemiluminescence detection. Biosens Bioelectron 14(5):457–464
12. Vaughan RD, Carter RM, O'Sullivan CK, Guilbault GG (2003) A quartz crystal microbalance (QCM) sensor for the detection of *Bacillus cereus*. Anal Lett 36(4):731–747. doi:10.1081/al-120018796
13. Wang DB, Bi LJ, Zhang ZP, Chen YY, Yang RF, Wei HP, Zhou YF, Zhang XE (2009) Label-free detection of *B. anthracis* spores using a surface plasmon resonance biosensor. Analyst 134(4):738–742. doi:10.1039/b813038h
14. Bouffartigues E, Leh H, Anger-Leroy M, Rimsky S, Buckle M (2007) Rapid coupling of surface plasmon resonance (SPR and SPRi) and ProteinChip based mass spectrometry for the identification of proteins in nucleoprotein interactions. Nucleic Acids Res 35(6):e39. doi:10.1093/nar/gkm030
15. Buckle M, Williams RM, Negroni M, Buc H (1996) Real time measurements of elongation by a reverse transcriptase using surface plasmon resonance. Proc Natl Acad Sci USA 93(2):889–894
16. Shrivastva A, Tripathi NK, Sathyaseelan K, Jana AM, Rao PVL (2008) Production of *Bacillus anthracis* recombinant protective antigen in *Escherichia coli*. Adv Biotech 26:26–29
17. Kohler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517):495–497
18. Glasstone SD (1947) Thermodynamics for chemists. Van Nostrand Company, New York
19. Gupta G, Singh PK, Boopathi M, Kamboj DV, Singh B, Vijayaraghavan R (2010) Surface plasmon resonance detection of biological warfare agent Staphylococcal enterotoxin B using high affinity monoclonal antibody. Thin Solid Films 519(3):1171–1177. doi:10.1016/j.tsf.2010.08.064
20. Stenberg E, Persson B, Roos H, Urbaniczky C (1991) Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. J Colloid Interface Sci 143(2):513–526
21. Paynter S, Russell DA (2002) Surface plasmon resonance measurement of pH-induced responses of immobilized biomolecules: conformational change or electrostatic interaction effects? Anal Biochem 309(1):85–95
22. Mock M, Mignot T (2003) Anthrax toxins and the host: a story of intimacy. Cell Microbiol 5(1):15–23
23. Turk BE (2007) Manipulation of host signalling pathways by anthrax toxins. Biochem J 402(3):405–417. doi:10.1042/BJ20061891