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Detection of protective antigen, an anthrax specific toxin in human serum by using surface plasmon resonance

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ABSTRACT

In this study, surface plasmon resonance (SPR) technology was used for the sensitive detection of protective antigen (PA), an anthrax specific toxin in spiked human serum samples. A monoclonal antibody raised against *Bacillus anthracis* PA was immobilized on carboxymethyldextran-modified gold chip, and its interaction with PA was characterized *in situ* by SPR. 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 m°, 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 1 pg/mL purified PA. In PA-spiked human serum samples, 10 pg/mL of PA could be detected. Presence of PA in blood samples serves as an important early diagnostic marker for *B. anthracis* infections. Thus, SPR test can be a sensitive assay for detection of anthrax at early stages of infection.

1. Introduction

Bacillus anthracis is the causative agent of anthrax, a zoonotic disease of domestic live stock and wildlife. Naturally occurring anthrax is transmitted to humans through direct contact with contaminated animals or through consumption of infected animal products or anthrax spores (Iacono-Connors et al., 1994; Walsh et al., 2007). The virulence of *B. anthracis* is attributed to 2 major factors, i.e., a tripartite toxin and the poly-γ-D-glutamic acid capsule (Collier and Young, 2003). Virulent *B. anthracis* vegetative cells form capsules of poly-D-glutamic acid, which impede the host immune system and inhibit macrophages from engulfing and destroying the bacteria (Ezzell and Welkos, 1999). The anthrax toxins are secreted as 3 distinct proteins, namely, protective antigen (PA), lethal factor (LF), and edema factor (EF), and their activities have been well described (Mock and Mignot, 2003; Turk, 2007). PA combines with EF and LF to form the binary toxins edema toxin (ETx) and lethal toxin (LTx), respectively (Singh et al., 1999). ETx elevates intracellular cyclic-adenosine monophosphate levels, whereas LTx inactivates members of the mitogen-activated protein kinase family resulting in an imbalance in the production or release of a range of cytokines that may contribute to the pathogenesis of anthrax (Duesbery et al., 1998; Moayeri et al., 2003). Anthrax toxin is the main cause of host system failure and death (Turk, 2007). Hence, sensitive and rapid assays for detection of anthrax toxins are required.

Incidences of anthrax can be diagnosed by culture isolation of *B. anthracis* from the patients (Moayeri et al., 2003) or by detection of *B. anthracis* antigens or nucleic acid (Jernigan et al., 2002; Sacchi et al., 2002), if antibiotic treatment has not been initiated. Various assays like immunodiffusion, indirect micro-hemagglutination, and different enzyme-linked immunosorbent assays (ELISAs) with varying degrees of specificity and sensitivity have been developed for detection of α-PA or α-LF antibodies in anthrax infections (Ghosh and Goel, 2012; Ghosh et al., 2013b; Ghosh et al., 2013c; Iacono-Connors et al., 1994; Sirisanthana et al., 1988). However, due to acute and often fatal nature of untreated inhalation or systemic anthrax, the measurement of an immune response has not been a prominent feature of diagnosis. Moreover, during any outbreak or biological warfare–like situation, several methods like culture isolation, PCR, and biopsy may not be applicable. Under such circumstances, serological testing involving the direct detection of PA toxin in serum sample may be the confirmatory diagnostic tool. Presence of PA in the serum is a reliable marker for the detection, identification, and assessing the severity of the anthrax infection in human as well as animals (Kobiler et al., 2006). Moreover, PA plays a central role in the pathogenesis of anthrax, and there is a direct correlation between bacteremia and the PA concentration at the time of death (Fish and Lincoln, 1968). Therefore, sensitive and rapid assays for the detection of *B. anthracis* toxin are urgently needed to facilitate an early-stage diagnosis for successful treatment post exposure. The currently available ELISA for the detection of anthrax PA is able to achieve sensitivity levels of only up to 1 ng/mL (Mabry et al., 2006). In another study, europium nanoparticle-based immunoassay (ENIA) could detect anthrax PA in the range of 0.01 to 100 ng/mL (Tang et al., 2009). Hence, a highly sensitive assay is required which could detect anthrax toxin in human

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sera just post exposure when the concentration of circulating PA in blood is even in pg/mL.

Surface plasmon resonance (SPR) biosensors represent an emerging technology for a label-free, real-time, specific, rapid, and cost-effective diagnostic tool for bacteria that cause major public concern for food safety, bioterrorism, and nosocomial infections (Homola, 2008; Tawil et al., 2012). SPR is a dynamic method of detection in biological world, which could detect and characterize the antigen antibody interaction in the absence of chemical labeling and with minimum sample preparation (Bouffartigues et al., 2007; Dudak and Boyaci, 2009). It is based on optical phenomenon which measures refractive index changes produced by binding of molecule in the mobile phase to its biospecific ligand immobilized on the sensor (solid) surface. Analysis in real time provides the affinity of interactions or kinetic information of interactions, which can discriminate between the specific and nonspecific interactions. The presence of an analyte in the solution and specific binding to its ligands immobilized on the gold surface of the sensor chip results in a change in the refractive index of light reflected from the gold chip surface. Association and dissociation of the analyte are measured by detecting changes in the angle of incident light at which SPR occurs and is reported in millidegree in the sensorgram (Dudak and Boyaci, 2009; Homola, 2003; McDonnell, 2001; Rich and Myszka, 2000). Over the last years, extensive research efforts have been made to implement the SPR biosensors for rapid detection of bacterial pathogens (Ghosh et al., 2013a; Gupta et al., 2010; Taylor et al., 2006; Wang et al., 2012).

In this study, we report a sensitive and specific method for the detection of PA in the human serum samples using SPR technology. The proposed assay can be very useful for diagnosis of *B. anthracis* infections in clinical samples during an outbreak or biological warfare–like situation.

2. Materials and methods

2.1. Materials

N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), phosphate-buffered saline (PBS), 1M 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 mol/L phosphate buffer (pH 6.0) was used as coupling buffer in the experiments, and dilution of antibody was also carried out using this buffer.

2.2. Instrumentation

The studies of antigen-antibody interactions were conducted using a 2-channel cuvette-based electrochemical SPR system (Autolab ESPRIT; Ecochemie B.V., Utrecht, 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 (Duesseldorf, Germany). The pH of the buffers used was measured with a EUTECH instrument pH meter (pH-1500; Eutech Instruments Pvt Ltd, Ayer Rajah Crescent, Singapore). The temperature of cuvette was controlled by a water bath (Julabo HE-4, JULABO Labortechnik GmbH, Seelbach, Germany).

2.3. 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 (Shrivastva et al., 2008).

2.4. 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 3 times at 2-week intervals before boosting with 25-μg rPA per mouse. The spleen cells were removed 3 d later and fused with myeloma cells, according to the standard procedure (Kohler and Milstein, 1975). The hybridomas were cloned by limit dilution, screened using ELISA. The MAbs were purified by protein A column (Montage PROSEP-A Antibody Purification; Millipore, MD Millipore Corporation, Billerica, MA, USA) and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis to determine the purity.

2.5. ELISA

For determination of titre of antibodies, maxisorp flat bottom 96-well microtiter plates (Nalge Nunc International, Roskilde, 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 3 times with wash buffer (PBS containing 0.1% Tween 20) using ELx 508_{MS} microplate washer (BioTek Instruments, Inc, Winooski, VT, 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 3 times with wash buffer. Horseradish peroxidase–conjugated goat anti-mouse antibodies (Sigma Aldrich, St Louis, MO, USA) diluted in PBS containing 5% skim milk and 0.5% Tween 20 (100 μL/well) were added at a dilution of 1:1000 and incubated for 60 min to detect the bound α-PA IgG. Plates were again washed 3 times with wash buffer and detected colorimetrically by using 100 μL/well of ortho-phenyl diamine/H_2O_2 substrate (Sigma Aldrich). 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 an ELISA plate reader (BioTek Instruments, Inc).

2.6. Immobilization of α-PA monoclonal antibody on the carboxymethyldextran-modified gold SPR sensor chip

Prior to start of the experiment, the millidegree change (m°) in resonance angle was recorded as the baseline and stabilized by passing 50 μL of 0.05 mol/L phosphate buffer (pH 8.0) over carboxymethyldextran-modified gold chip at an interval of 120 s 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 mmol/L) and NHS (100 mmol/L) 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 mol/L phosphate buffer) in channel 2 and allowed to interact for 1800 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 nonreacted NHS ester in order to avoid nonspecific 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.

2.7. 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 38 °C with an increment of 3 °C.