DETECTION OF ANTIBODIES AGAINST *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* USING SURFACE PLASMON RESONANCE

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ABSTRACT. Conventional methods of detecting *Corynebacterium pseudotuberculosis*, a bacterium that causes caseous lymphadenitis (CLA) in sheeps and goats focused on several serodiagnostic tests such as ELISA, Western blotting and various inhibition and precipitation techniques. This paper described a Surface Plasmon Resonance (SPR) protocol for the direct detection of polyclonal antibodies against *Corynebacterium pseudotuberculosis* with immobilisation of the antigen on unmodified transducer surface. The lower limit of detection was determined to be 2 μg mL^{-1} of immobilised antigen (Ag). Sufficient binding interaction was monitored on unmodified transducer; and saturation of the binding interaction was observed at 80 μg mL^{-1} of interacted antibody.

Keywords: Corynebacterium pseudotuberculosis, SPR, immobilisation, binding interaction

INTRODUCTION

Over the last decade, SPR biosensor has emerged as a form of tagless sensing device for the detection of analytes in liquid media. Small analytes such as atrazine [Brohnstein *et al*., 1997] which is relevant to environmental protection, morphine in medicine [Miura *et al*., 1997], methamphetamine [Sakai *et al*., 1999] and sulfamethazine [Li *et al*., 2010] in food safety have been detected using SPR. High molecular weight analytes such as viruses [Rich and Myszka, 2003 and Boltovets *et al*., 2004] and whole cell bacteria [Shankaran and Miura, 2007a; Mader *et al*., 2004 and Oh *et al*., 2005] have also been detected using this technique.

SPR response is directly related to changes in the mass of an analyte or ligand on a gold-coated glass disk surface of an SPR transducer. The gold-coated disk, with the coated side in contact with a dielectric media (such as a biological solution) is placed onto a hemi-cylinder glass prism separated by a layer of oil having the same refractive index as the glass disk and hemi-

cylinder prism. Free fluctuating electrons in the gold surface (known as Surface Plasmon) are excited when light (photons) hit the gold at a certain angle of incidence. In most SPR techniques, excitation of the Surface Plasmon by the photons followed the Kretchmann configuration (Kretchmann, 1971). The excited Surface Plasmon is referred to as Surface Plasmon Resonance; and under this condition the light waves are coupled to the oscillations of Surface Plasmon at the gold/solution interface producing a minimum of the reflected light. A detector placed at the path of the reflected light does not detect any light at Surface Plasmon Resonance, thus creating a dip when the light intensity is plotted against the angle of incidence (Shankaran and Miura, 2007a). Surface plasmons created through interaction of light with the gold atoms of the transducer surface changes with the concentration of ligands immobiliSed on it, allowing sensitive measurement of surface changes caused by adsorption and/or biomolecular interactions of immobiliSed ligands and its analyte in solution.

Compared to most immunoassay techniques, SPR immunosensors requires shorter detection time; and can be very highly specific and sensitive with greater simplicity (Shankaran *et al.*, 2007b). Furthermore, non-specific binding with unoccupied sensor surface can be minimised by saturating the surface with the ligand.

SPR sampling can be done in two configurations i.e. the flow cell and the cuvette system. Flow cell SPR introduces liquid samples into the sample compartment continuously following a programmed flow rate. In the cuvette-based SPR, working volumes of samples could be as small as 10 µL and is advantageous when limited amount of sample is available.

Caseous lymphadenitis (CLA), a disease caused by the *Corynebacterium pseudotuberculosis* is prevalent in small ruminant farm animals such as goats and sheeps (Fontaine and Baird, 2008). Diagnosis of CLA from infection by the bacterium has been focused on conventional serological-based techniques such as haemolysis inhibition test and haemaglutination (Kuria *et al.*, 2001), microaglutination (Menzies, 1989) and Western blotting (Paule *et al.*, 2003). Various ELISA techniques have also been reported in the assay of this potent pathogen. The first ELISA technique to identify *C. pseudotuberculosis* used an indirect double antibody sandwich (ELISA A) (Ter Laak *et al.*, 1992); consequently followed by ELISA B, C and D techniques (Dercksen *et al.*, 2000) which are modified double antibody sandwiched ELISA developed as strategies to improve the sensitivity of ELISA A. More recently, an anti-IgG ELISA (Binns, 2007) was developed and introduced in the United Kingdom as an alternative to the more expensive Dutch technique (Derchsen *et al.*, 2000 and Chirino-Zarraga *et al.*)

No SPR technique on the assay of this bacterium has been reported; until recently a flow-through SPR was used by a group

of researchers from Ireland [Stapleton *et al*., 2009] to detect this bacterium. We presented here the protocol for a direct single-step SPR analysis of antibody-antigen interactions of *Corynebacterium pseudotuberculosis* and its polyclonal antibodies using cuvette-based SPR configuration.

EXPERIMENTAL

Reagents and materials

All chemicals and solvents were of analytical grade, purchased from Aldrich and used without further purification. Water used for the preparation of buffers, antibodies and antigen solutions is of ultrapure grade purified through a Milli-Q ion exchange system (Millipore, Resistivity = 18 μS).

Phosphate buffered saline (PBS) (10 mmol L^{-1}) was prepared at pH = 7.4 with 140 mmol L^{-1} NaCl.

The gold-coated SPR sensor disks employed in this study were purchased from Eco Chemie, Netherland. They are of diameter 25.0 mm and can allow 8 sets of individual experiments to be carried out on it sequentially over a working area of 2 mm each (Figure 1).

Sample collection and preparation of antigen

The *C. pseudotuberculosis* strain was a local clinical isolate obtained from the abscess of infected goat. For antigen preparation *C. pseudotuberculosis* was initially cultivated on blood agar and a single colony of the bacteria was inoculated into 50 ml of Brain Heart Infusion, Oxoid, England (BHI) broth, incubated at 37°C with agitation for 24 hrs. 5 mL of the overnight culture were then transferred into 100 ml BHI broth and incubated at 37°C with agitation for a further 24 hr. period. The bacterial cell was harvested by centrifugation (8000 rpm for 10 min) and then washed twice with cooled PBS buffer solution. The pellet was then resuspended in 50 ml of PBS buffer and pulse-sonicated to disrupt the bacteria cell wall. The antigen protein concentration was quantified using Bradford, Coomassie Blue Kit (Bio Rad Protein Assay, U.S).

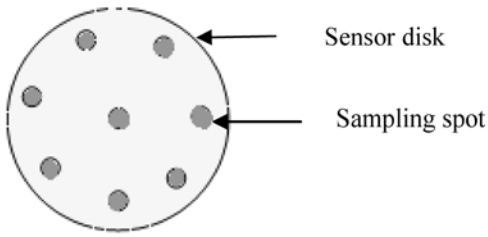

Figure 1. Gold-coated SPR sensor disk showing 8 different sampling spots on the disk which allow 8 individual sets of experiments to be carried out.

Prepared antigen was stored in 1.5 ml aliquots at -20°C before use.

The positive control serum serving as the reference standard was obtained from an infected goat with clinical symptoms of CLA (abscess on the right parotid lymph node). The diagnosis was confirmed by positive pus culture. A negative control serum was from a 2 year-old goat from a farm with negative history of CLA infection.

Development of Anti-CLA goat IgG polyclonal molecules

Purified polyclonal anti-CLA goat IgG was developed in goat using the whole cell bacteria protein of *C. pseudotuberculosis*. The antibody was purified from the serum and other unbound proteins using column chromatography Hi-Trap G HP (Pharmacia). The purified antibody was then desalted on a Sephadex G-25 column and lyophilized at a concentration of 1 mg/ vial.

Development of SPR assay

Equipment

The SPR assay was performed and optimized on an Autolab SPRINGLE system (Echo Chemie B.V., Utrecht, The Netherlands) operating on Microsoft Windows XP. Data are collected and analyzed using the Autolab SPRINGLE software version 4.2.

Modification of sensor surface

Modified gold-coated glass disks were produced by immersion of the disks in 100 mM solution of 11-mercaptoundecanoic acid (MUA) overnight. Activation of the carboxyl groups were done using 10 mM N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) and 10 mM N-hydroxysuccinimide (NHS).

Regeneration of sensor surface

All used sensor disks were regenerated by immersing in absolute ethanol overnight followed by immersion in 1.0 M HCl for 30 minutes. They were then rinsed copiously with ultra-pure water before drying with a stream of N_2 gas.

Overall SPR protocol

Instrument calibration after gold disk and cuvette installation; and baseline adjustment was performed according to manufacturer's suggestion provided in the SPR user manual. Washings of gold disk were performed after baseline adjustment at the start of the measurement; and after each ligand immobilization and association processes. A total of 130 µL of phosphate buffer pH 7.4 were introduced during the washing cycles. In every step within the SPR protocol, mixing of samples during measurement is efficiently carried out by a programmed intermittent introduction of air flow into the sample using a piston pump.

Saturation of antigens on sensor surface

Partial or incomplete occupation of ligands on transducer surface provides rooms on the sensor surface for non specific binding to occur. Typical procedure to overcome this problem is by blocking the exposed non reacted surface active sites with neutralizing compounds such as ethanolamine.

Alternatively, saturation of the sensor surface with ligand molecules eliminates the use of chemicals and unnecessary steps. Sequential addition of 5 µg/mL of antigen solutions were added to the unmodified transducer surface to determine the minimum concentration of antigen solutions required for saturation of the transducer surface.

For comparison, mercaptoundecanoic acid (MUA)-modified sensor surface was also used to immobilize antigen molecules. Modification of the sensor surface with MUA self-assembled monolayers was carried out as discussed in section 2.4.2.

Binding interactions of antigen-antibody

For the binding interactions between CLA antigens and its antibody of various concentrations, the unmodified sensor surface was initially saturated with antigen molecules by static mixing of 50 µg/mL[#] of antigen solutions over the sensor surface for a period of at least 16 minutes. After this period, the sensor surface was washed with PBS buffer and drained before introducing the antibody solutions. Binding interactions between the antigen and antibody of various concentrations were allowed to proceed for a minimum period of 16 minutes. 7 similar measurements on the same disk could be carried out by changing the position of the sampling spot (Figure 1). A 'one-stop' regeneration procedure of the sensor surface was performed after all the 8 sampling spots have been utilized; thus reducing further the analysis time.

[Note: [#]From this point onwards, the term **saturated antigen** will be used to denote antigen solutions of concentration 50 µg/mL introduced to the sensor surface]

RESULTS AND DISCUSSION

Saturation of ligands on sensor surface

Figure 2 shows the increase in the SPR response upon sequential addition of 5 µg/mL of antigen onto the unmodified sensor surface. The signal response reduces upon saturation of the surface. Antigen solutions of total concentration of 25µg/mL immobilized onto the sensor surface showed the highest SPR response indicating surface saturation. Further addition of antigen solutions do not significantly increases the SPR response. For the purpose of saturating the sensor surface with antigen molecules, antigen solutions of concentration 50 µg/mL were used in all antigen immobilization steps.

Immobilisation of antigen molecules onto sensor surface modified with MUA self-assembled monolayers is shown in Figure 3. The signal obtained was lower

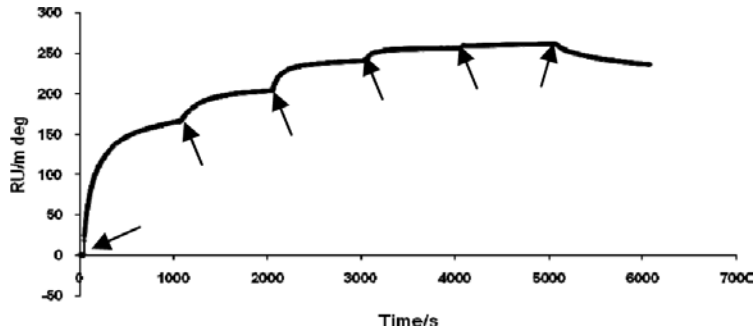

Figure 2. Sequential addition of 5 µg/mL of antigen onto unmodified sensor surface. Arrows show the point at each addition of antigen

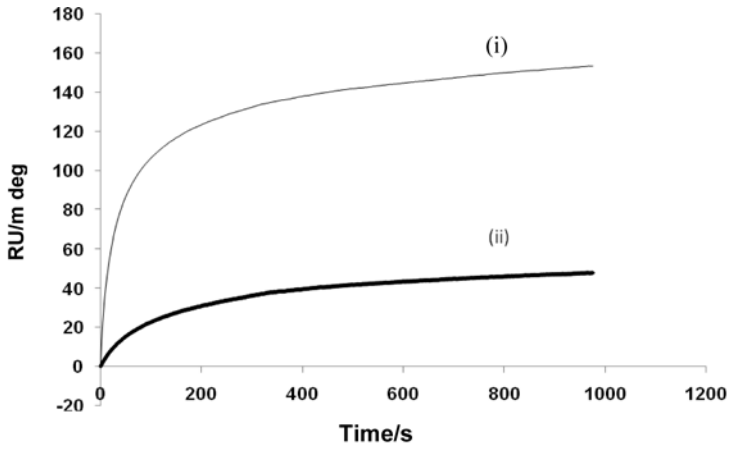

Figure 3. Immobilization of 50 µg/mL of antigen solutions on (i) unmodified sensor surface (bare gold) and (ii) sensor surface modified with MUA self-assembled monolayers

than on unmodified surface; following which consecutive experiments were done on unmodified sensor surface. This proved to be an advantage as it eliminates the step for modification of the sensor surface; thus reducing further the analysis time and problems of irreproducibility.

Binding interactions of antigen-antibody

A typical sensor gram showing the binding interaction between the saturated antigen and the CLA antibody is as shown in Figure 4. A positive response of 150 RU was observed indicating good association between the CLA antigen and its antibody. Subsequent washing with PBS buffer

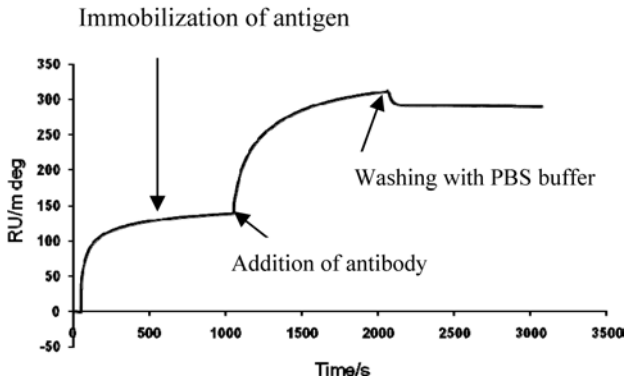

Figure 4. Interaction of antibody with immobilized antigen followed by washing of the sensor surface with PBS buffer

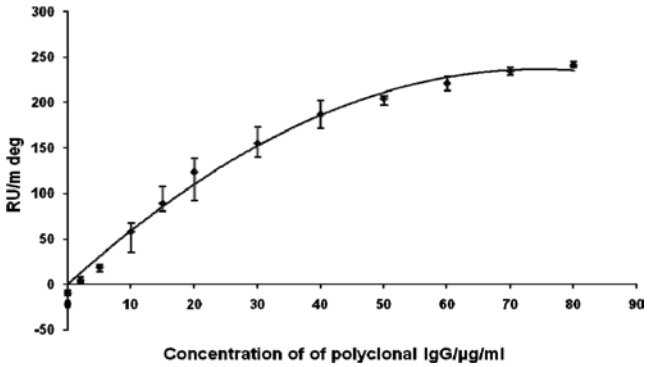

Figure 5. Dependence of signal response (RU) on concentration of polyclonal antibodies binded to saturated antigen molecules immobilized on unmodified sensor surface. Solid line shows fitting of experimental data to sigmoidal function. Error bars were generated from 6 samples for any particular antibody concentration

showed a small decrease in the RU response indicating irreversible binding interaction between the CLA antigen and its antibody.

Antibodies of increasing concentrations showed increased binding with the saturated antigen (Figure 5); and the binding tend to saturation for the addition of antibody solutions of concentration greater than 80µg/mL. The lower limit of detection was found to be 2 µg/mL of antibody molecules.

Non specific binding and Specificity of CLA antigen for other antibodies

Specificity of CLA antigen for other antibodies was tested by interacting anti-BSA and anti-*Brucella abortus* with the saturated antigens. Figure 6 showed the results from interaction of these antibodies with saturated CLA antigens. Both anti-BSA and anti-*Brucella abortus* showed positive interactions with the saturated

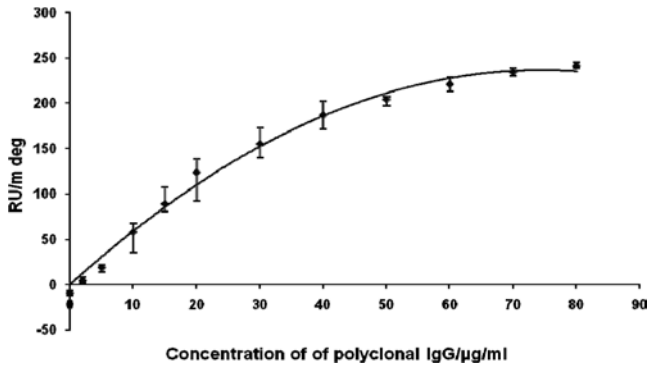

Figure 6. Interactions of 100 µg/mL of (i) anti-BSA, (ii) anti-Brucella abortus and (iii) CLA antibody with saturated CLA antigens immobilized on an unmodified sensor surface.

CLA antigens with a signal response of 50 and 120 RU respectively. However, interaction between CLA antibodies with the saturated CLA antigens produced a signal response of 310 RU. Interaction of anti-BSA with the saturated antigens immobilized on the sensor surface could be attributed to non specific binding. However, significant signal response observed in the interaction between anti-*Brucella abortus* and the saturated CLA antigens indicated that interactions of CLA antigens with CLA antibody are not highly specific. Anti-*Brucella abortus* is an antibody developed from bovines and cattles that are infected by the bacterial pathogen *Brucella abortus*.

CONCLUSION

The first SPR-based assay reported recently by a group of researchers from Ireland [Stapleton *et al.*, 2009] for the diagnosis of CLA in sheep employed a SPR system to detect antibodies specific to phospholipase D exotoxin of the bacterium *C. pseudotuberculosis* in sheep sera. The SPR assay was based on a flow cell whereby analyte solutions were continuously fed across the sensor surface at a specific flow rate. Blocking of unreacted active groups with blocking agents was routinely carried out as in a typical SPR assay. Regeneration of the sensor chips were performed after each analysis using appropriate regeneration solutions. In this study, the use of a cuvette-based SPR simplifies the immunoassay process with minimum use of the expensive antibody/antigen solutions. Blocking of uncovered sensor surface or unreacted reactive groups (for that matter) was avoided by saturation of the sensor surface with ligand molecules. Moreover, the one-stop regeneration process practiced in this assay further reduced the analysis time and eliminates unnecessary steps.

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