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Surface plasmon resonance characterization of monoclonal and polyclonal antibodies of malaria for biosensor applications

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ABSTRACT

Surface plasmon resonance (SPR) screening of monoclonal and polyclonal antibodies of *Plasmodium falciparum* (Moab*Pf* and Poab*Pf*) for recombinant Histidine rich protein-II antigen (Ag) of P*f* (rHRP-II Ag) was conducted in a real-time and label-free manner to select an appropriate antibody (Ab) for biosensor applications. In this study 4-mercaptobenzoic acid (4-MBA) modified gold SPR chip was used for immobilizing the Ag and then Ab was interacted. SEM image showed modification of SPR chip with 4-MBA and EDAX confirmed the presence of 4-MBA on the SPR chip. Equilibrium constant (K_D) and maximum binding capacity of analyte (B_{max}) values for the interaction of Moab*Pf* or Poab*Pf* with the immobilized rHRP-II Ag were calculated and found to be 0.517 nM and 48.61m° for Moab*Pf* and 2.288 nM and 46.80m° for Poab*Pf*, respectively. In addition, thermodynamic parameters such as ΔG, ΔH and ΔS were determined for the interaction between rHRP-II Ag and Moab*Pf* or Poab*Pf* and the values revealed that the interaction is spontaneous, exothermic and driven by entropy. The kinetics and thermodymanic results of this study revealed that the interaction between Moab*Pf* and rHRP-II Ag is more effective than that of Poab*Pf* due to the fact that Moab*Pf* was derived from a single epitope (single clone) whereas the Poab*Pf* was from the mixture of a number of epitopes (polyclones). Finally, SPR methodology was developed for the sensing of malarial antibodies. The limit of detection was found to be 5.6 pg with Moab*Pf* which was found to be the best in our study.

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1. Introduction

Malaria is an infectious disease and this is due to the parasite *Plasmodium falciparum* (*Pf*). The world malaria fact sheet report (World Health Organization [WHO] report) of 2011 reveals data received from 104 malaria-endemic nations and territories for the year 2011. The data from 99 of these countries indicates about the on-going nature of malaria transmission. Moreover, the data indicates about the prevention of 1.1 million malaria deaths between the years 2000 and 2010 due to prior interventions. The world malaria report 2012 released by WHO (2012) indicates about an estimated number of 247 million human malarial infections (98% in Africa with 70% being in the age of 5 years or younger). In addition, in the year 2010 the WHO estimate reveals about 219 million cases of malaria and also about 0.66 million deaths. Africa is the most affected continent: about 90% of all malaria deaths occur there. Malaria is more prevalent in sub-Saharan Africa when compared to other regions of the world; in most African countries, more than 75% of cases were due to *Pf*, whereas in other countries the malaria transmission is predominantly due to less virulent plasmodial species. Almost every malarial death is caused by *Pf* (WHO world malaria report 2012 and www.unicef.org).

Pf is one of the species of *Plasmodium* and it is a protozoan parasite which is responsible for malaria in humans. *Pf* is transmitted by the female Anopheles mosquitos. Malaria induced by *Pf* (also called malignant or falciparum malaria) is the most dangerous form of malaria with the highest rates of complications and mortality (Rich et al., 2009; Perkins et al., 2011). More than 120 species of the parasite genus *Plasmodium* are available; however, only four of them infect humans to cause malaria (*P. falciparum, P. vivax, P. ovale and P. malariae*) and among these only *Pf* can cause severe (life-threatening) malaria when compared to the other three species (Coppel et al., 1986). *Pf* entry to the human blood cell leads to the change of shape of red blood cell within 48 h of asexual blood stage cycle; the mature forms change the surface properties of infected red blood cells, and make them stick on blood vessels. This induces the obstruction of microcirculation and results in dysfunction of multiple organs, including the brain in cerebral malaria (Perlmann and Troye-Blomberg, 2000;

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Dondorp et al., 2004). Histidine-rich protein-II (HRP-II) is a naturally occurring histidine and alanine rich protein and is localized in several cell compartments, including the cytoplasm of *Pf*, and it is closely associated with the development and proliferation of the parasite and therefore is perfectly suited to reflect growth inhibition as a measure of drug susceptibility (Harald et al., 2002). Five malaria proteins (HRP1, HRP2, EMP1, EMP2, and EMP3) have been identified on the surface or in association with the cytoskeleton of erythrocytes infected with *Pf* (Rock et al., 1987). HRP-II was identified in every *Pf* parasites regardless of knob phenotype, and was recovered from culture supernatants as a secreted water-soluble protein (Panton et al., 1989). HRP-II may also participate in parasite mature stages evasion of the immune system and their subsequent destruction in the spleen (Rock et al., 1987). HRP-II is produced and secreted by the parasite during its growth and development (Howard et al., 1986). There is evidence for an intracellular route of transport for the malarial protein from the parasite through several membranes and the host cell cytoplasm (Howard et al., 1986; Magowan, 2000).

In view of the above, timely detection of antibodies of HRP-II Ag to know about the malarial outbreak is very vital to save the life of humans for taking appropriate medical measures. Many methods are currently available in the literature for the diagnosis of malaria by detection of HRP-II Ag with dipstick antigen-capture assay or enzyme-linked immunosorbent assay (ELISA) (Beadle et al., 1994; Gaye et al., 1998). Moreover a number of methods are also developed for the detection of *Pf*, including various biological assays like immune fluorescence microscopy (IFA) (Matile and Pink, 1990), fluorescence microscopy (Lenz et al., 2011), western blot analysis (Parra et al., 1991), ELISA (Wirtz et al., 1989), competitive ELISA (Bualombai et al., 1990), dot blot analysis (Lee et al., 2006), and also a method based on isothermal titration calorimetry (ITC) (Pierce et al., 1999), polymerase chain reaction (PCR) (Patsoula et al., 2003; Pieroni et al., 1998) and surface plasmon resonance (Helg et al., 2003) using merozoite surface protein-1. Though many methods are reported for malaria detection, still methods development and improvisation are vital to avoid labeling which is required with enzyme substrate, much volume of samples requirement, time consuming nature and also non-availability of kinetic and thermodynamic data for most of the malaria antigen (Ag)–antibody (Ab) interaction to know about the nature of reaction. In view of the above, there is a need for the development of immunosensors which are highly specific for Ag–Ab interaction for identification and quantification of specific analytes. SPR immunosensors have recently attracted a lot of attention due to their high sensitivity, real time and label-free monitoring capability of biological interactions (Toyama et al., 1998).

In SPR the interaction of a biomolecule immobilized on the SPR chip surface with its counterpart in solution is monitored without any labeling of the biomolecules by using the interfacial refractive index changes associated with the affinity binding interactions (Myszka, 1999). The important parameters that can be obtained with the help of SPR include protein binding (Ahmad et al., 2003), association/dissociation kinetics (Nordin et al., 2005), and affinity constants (Babol et al., 2005) and these contributed a role of SPR in large application areas such as molecular engineering (Calender, 2006), food analysis (Sternesjo et al., 1995), clinical diagnosis (Inamori et al., 2005), proteomics (Natsume et al., 2002), environmental monitoring (Dillon et al., 2003), bacteriology (Mader et al., 2004), virology (Athmaram et al., 2014), cell biology (Quinn et al., 2000), drug discovery (Cimitan et al., 2005) and warfare agent detection (Gupta et al., 2011a).

In line with our earlier studies on the development of SPR detection methodologies for biological warfare agents (BWAs) such as *Brucella abortus, Salmonella typhi* and *Staphylococcal enterotoxin B* (Gupta et al., 2011b, 2012, Singh et al., 2010), in the present work we employed SPR for the characterization of Moab*Pf* and Poab*Pf* and also for the direct detection of monoclonal and polyclonal antibodies (Moab*Pf* and Poab*Pf*) of recombinant histidine rich protein antigen (rHRP-II Ag) in buffer, using rHRP-II Ag immobilized on a 4-mercaptobenzoic acid (4-MBA) modified gold surface as (to our knowledge) no SPR based detection of rHRP-II Ag antibodies is available in the literature. The main advantage of using 4-MBA for modification of SPR chip is to get a lesser thickness and faster electron transfer on the chip when compared to conventionally used dextarn modified SPR chip as it is well known that SPR response depends on the thickness of the modification on the SPR chip. (Schasfoort and Tudos, 2008: Mendesa et al., 2004). Moreover, parameters affecting the response of SPR were optimized and finally affinity constant (K_D) and maximum binding capacity of analyte (B_{max}) were calculated; in addition, thermodynamic parameters such as change in Gibb's free energy (ΔG), change in enthalpy (ΔH) and change in entropy (ΔS) involved in the interaction between rHRP-II Moab or rHRP-II Poab with rHRP-II Ag of *Pf* were also deduced in this study.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), phosphate buffered saline (PBS), sodium acetate, ethanolamine and hydrochloric acid (HCl) were of Fluka grade and obtained from Sigma-Aldrich, Bangalore, Karnataka, India. Moreover, glacial acetic acid, glycine, sodium hydroxide (NaOH) and methanol (MeOH) were supplied by Sigma-Aldrich, Bangalore, Karnataka, India. 4-MBA Aldrich purchased from Sigma-Aldrich, Bangalore, Karnataka, India, was used for the modification of the SPR gold chip (Xantech Bioanalytics GmbH, Metrowingerplatz, Germany). Moab*Pf*, Poab*Pf* and rHRP-II Ag of *Pf* were developed in house by the trained biologists. All chemicals and reagents used in this work were of analytical grade and purification was done wherever necessary before use.

In order to perform SPR measurements, 4-MBA modified SPR gold disc was used. Different buffer solutions were used in this study depending on pH [acetate buffer (pH 4.0–5.5), PBS (pH 6.0–7.5) and glycine–NaOH buffer (pH 8.0–9.0)] for the optimization of pH. All solutions were prepared using water from a Milli-Q system (Millipore India, Bangalore, Karnataka, India) throughout the experiment.

2.2. Instruments

The biomolecular interactions were investigated using a two channels cuvette based electrochemical surface plasmon resonance system (Autolab ESPRIT, Ecochemie B.V., Utrecht, The Netherlands) and a diode laser was a source with a fixed wavelength of 670 nm in conjunction with a scanning mirror to modulate the plane polarized light beam on the SPR substrate. The outcome of the SPR measurement was monitored using a PC with data acquisition software version 4.3.1.

2.3. Preparation of 4-MBA modified gold chip

Bare gold SPR chip was modified with 0.01 M 4-MBA using a spin coater (Autolab Spin coater, Ecochemie B.V., Utrecht, The Netherlands). First 75 µl of 4-MBA was dispensed on bare gold chip at 100 rpm. After this, spin speed was increased up to 2500 rpm and kept for 5 min in order to spread the liquid on

the SPR gold chip. This 4-MBA modified SPR gold chip was utilized for further modifications and sensing studies. All kinetic data were obtained using kinetic evaluation software version 5.0 (Ecochemie B.V., Utrecht, The Netherlands). The pH of buffers was measured with a EUTECH instruments pH meter (pH 1500, Ayer Rajah Crescent, Singapore). All experiments were carried out at 25 °C unless otherwise specified and the temperature of cuvette was controlled by a Julabo HE-4 (Julabo Labortechnik GmbH, Seelbach, Germany) water bath.

2.4. Preparation of protein rHRP-II Pf by recombinant DNA technology

The Ag of *Pf* rHRP-II fusion protein was used for the sensing of the Abs of *Pf*. The procedure for the preparation of the Ag was adopted from an earlier report of our laboratory (Sharma et al., 2011a). Culture time is 12 h, temperature 37 °C and 120 revolvings per minute were used for harvesting the culture. Polymerase chain reaction (PCR) was used to amplify *Pf* rHRP-II gene sequence and then purified. The purified PCR product was cloned in pQE-30 UA cloning vector. Transformation and expression of the histidine fusion protein was conducted with *Escherichia coli* strains M15.

2.4.1. Cloning and expression of an rHRP-II gene fragment

The cloning and expression of rHRP-II gene fragment was conducted as reported earlier (Merwyn et al., 2011). DNA was extracted from cultured *Pf* by processing the infected RBCs with a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) as per the instructions from manufacturers. Primers were designed and a part of exon2 region of rHRP-II gene was amplified with the help of PCR. Subsequently, the resultant PCR fragment was cloned in pQE-UA cloning and expression vector (Qiagen, Hilden, Germany) in frame with the N-terminal histidine tag (His-tag), based on the manufacturer's instructions. The cloned gene was transferred into *E. coli* strain M15, and then the successful transformants were induced to express rHRP-II using 1 mM isopropyl-β-D-thio-galacto-pyranoside (IPTG). The cells harboring the recombinant protein were harvested and analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophorosis (SDS–PAGE; Fig. 1a) in order to confirm the protein expression.

2.4.2. Purification of recombinant protein

Purification of rHRP-II protein was carried out by affinity chromatography in the native conditions using commercially available Ni-NTA columns (Qiagen, Hilden, Germany) as per the manufacturer's instructions as reported earlier (Merwyn et al., 2011) and the SDS–PAGE for rHRP-II protein is shown in Fig. 1b. Briefly the harvested pellet was analyzed with the help of ultra-sound waves and the soluble recombinant protein was captured onto the Ni^{2+} ions in Ni–NTA resin. After a brief washing using 40 mM imidazole, the protein of interest was eluted with 350 mM imidazole.

2.5. Production of MoabPf and PoabPf against rHRP-II Ag of Pf

2.5.1. Immunization and generation of MoabPf

A set consisting of five female BALB/c mice, each weighing 20 g were individually immunized with 50 mg of purified rHRP-II emulsified in complete Freund's adjuvant through the subcutaneous route. Moreover, five booster doses with the same amount of immunogen emulsified with incomplete Freund's adjuvant were given at an interval of 7 days through the intramuscular route. A final intraperitoneal injection of rHRP-II (50 mg) was also given to mice 3 days before fusion (Merwyn et al., 2011).

2.5.2. Immunization and generation of PoabPf

White rabbits of The New Zealand origin were used for raising hyper-immune sera against rHRP-II Ag. The rabbits were first immunized by subcutaneous route with 100 μg of recombinant antigen and Freund's complete adjuvant (FIA) intramuscularly at 15 days interval for 45 days. The rabbits were bled from the heart and then the sera was subjected to separation and stored at −20 °C in a deep freezer (Sharma et al., 2011b).

2.5.3. Cell fusion and culture

Fusion experiments were performed by a modified procedure as reported earlier (Kohler and Milstein, 1975). Briefly, spleenocytes harvested from the immunized mice were fused with Sp2/0-Ag14 myeloma cells by using the fusing agent polyethylene glycol (PEG 2000). The fused cells were then resuspended in selective hypoxanthine–aminopterin–thymidine [HAT] medium containing Dulbecco's modified Eagle's medium consisting of 20% fetal calf serum and 1% of selective supplement HAT. The re-suspension was distributed in culture plates and incubated at 37 °C with 5% CO_2.

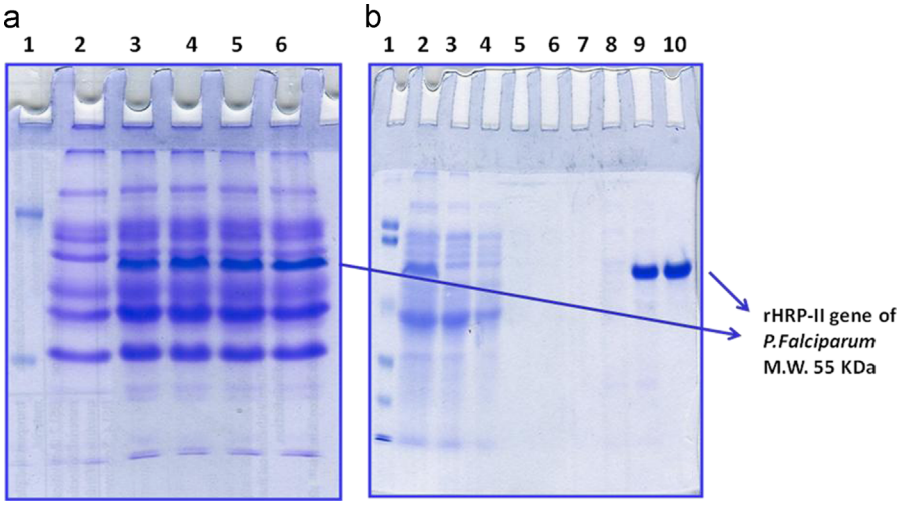

Fig. 1. (a) SDS–PAGE profiles showing expression of induced protein of HRP-II gene of *P. falciparum* (55 kDa) Lane 1 [MW marker], Lane 2 [Uninduced cells], Lane 3 [Clone 2 (1 h)], Lane 4 [Clone 2 (2 h)], Lane 5 [Clone 2 (3 h)], Lane 6 [Clone 2 (4 h)] and (b) SDS–PAGE profiles showing expression of purified HRP-II protein (55 kDa) Lane 1 [MW marker], Lane 2 [Cell lysate], Lane 3 [Flow through], Lane 4 [Wash 1], Lane 5 [Wash 2], Lane 6 [Eluant 1], Lane 7 [Eluant 2], Lane 8 [Eluant 3], Lane 9 [Eluant 4], and Lane 10 [Eluant 5].

After 4 days, half of the portion of media was replaced with fresh HAT medium and on day 7, HAT was fully replaced by hypoxanthine–thymidine medium [HT] (Merwyn et al., 2011).

2.6. Immobilization of rHRP-II Ag on 4-MBA modified gold SPR sensor chip

Prior to the immobilization of rHRP-II Ag on 4-MBA modified gold chip, 50 µl of PBS buffer (pH 7.5) was passed at every 120 s interval for 600 s in order to get a stable baseline in both the channels. A 4-MBA modified gold chip was chemically activated by the injection of 75 µl of a 1:1 mixture of 400 mM EDC and 100 mM NHS. Subsequently, 75 µl of rHRP-II Ag (1:100 dilution in 10 mM PBS) was injected in channel 1 for 1800 s so as to get an effective immobilization of rHRP-II Ag on the activated 4-MBA modified gold chip surface. After rHRP-II Ag immobilization, the remaining active sites were then blocked with 75 µl of 1000 mM ethanolamine. Afterwards, 10 mM HCl was injected to achieve regeneration of immobilized sensor surface. For negative control measurements, the modified gold surface was activated with EDC/NHS and then quenched with ethanolamine (channel 2) as mentioned above and was used as blank control surface. The rHRP-II Ag immobilized SPR sensor chip was interacted with different concentrations of Moab*Pf* or Poab*Pf* of rHRP-II in order to detect the rHRP-II antibodies and also deduce kinetic and thermodynamic parameters involved in the interaction.

2.7. Biosensing protocol

In order to conduct the binding studies, different dilutions of Moab*Pf* and Poab*Pf* were prepared in PBS. The entire SPR sensing methodology was executed by a sequence of automatic procedure (baseline, association, dissociation, regeneration and back to baseline steps). A sample solution containing a selected concentration of Moab*Pf* or Poab*Pf* in the PBS was injected in both channels from a 384 wells microtiter plate and then association was performed for 500 s and dissociation was performed for 400 s followed by the regeneration of the sensor surface by addition of 10 mM HCl for 120 s. PBS (pH 7.5) was used as the running buffer solution in the entire work. For SPR sensing, an aliquot of the analyte solution (75 µl) was injected and mixed at 16.7 µl/s and this protocol was adopted with different dilutions of Moab*Pf* 1:12,800, 1:6400, 1:3200, 1:1600 and 1:800 and also different dilutions of Poab*Pf* 1:3200, 1:1600, 1:800, 1:400, and 1:200.

2.8. Optimization of experimental parameters

2.8.1. Effect of temperature on interaction between MoabPf or PoabPf and rHRP-II Ag

It is well known that temperature is able to influence the sensitivity of SPR measurement. By conducting this temperature variation study one can get certain thermodynamic parameters like ΔG, ΔH and ΔS by using the data obtained in this study based on the ratio of kinetic association and dissociation rate constants (Lutz et al., 1997). To know the optimum temperature for binding of Moab*Pf* and Poab*Pf* with rHRP-II Ag, experiments were conducted by passing Moab*Pf* or Poab*Pf* with a dilution (minimum dilution where SPR response was observed) of 1:12,800 (Moab*Pf*) or 1:3200 (Poab*Pf*) on immobilized rHRP-II Ag by varying the temperature between 10 and 37 °C with a 3 °C increment as reported earlier (Gupta et al., 2010a).

2.8.2. Effect of pH on interaction between MoabPf or PoabPf and rHRP-II Ag

In order to know the effect of pH on the change in SPR angle, pH variation study was carried out using different buffers in the pH range from 4.0 to 9 with 0.5 increments. To maintain the pH, acetate buffer (pH 4.0–5.5), PBS buffer (pH 6.0–7.5), and glycine–NaOH buffer (pH 8.0–9.0) buffer were used for the interaction of Moab*Pf* or Poab*Pf* with immobilized rHRP-II Ag.

3. Result and discussion

3.1. Preparation of protein rHRP-II Pf by recombinant DNA technology and purification of rHRP-II Pf

The rHRP-II Ag having 55 kDa molecular weight was isolated (Fig. 1a) as discussed in the experimental part. The purity of the rHRP-II protein was confirmed by SDS–PAGE analysis for the presence of any contaminating proteins (Fig. 1b). Moreover, to confirm the functionality of rHRP-II *Pf*, this protein was checked using a commercially available MRDT system (at present known as Malaria, Binax, Scarborough, Maine) and it detected rHRP-II *Pf*.

3.2. Production of MoabPf and PoabPf against rHRP-II Ag of Pf

After immunization in mice and rabbit, antibodies were successfully collected and these are Moab*Pf* and Poab*Pf*, respectively. In order to confirm the functionality of Moab*Pf*, the western blotting technique was performed and we observed single band for rHRP-II Ag. Poab*Pf* was checked by using the indirect ELISA method against rHRP-II Ag.

3.3. SEM and EDAX characterization of bare and 4-MBA modified SPR chip

In order to know the modification of SPR gold chip with 4-MBA, SEM and EDAX experiments were conducted in order to know the morphology and elemental composition, respectively and the resultant SEM images and EDAX data are presented in Fig. 2. Fig. 2a is a SEM image for bare SPR gold chip where no modification was conducted and it is not possible to covalently bind the Ag with this chip. In order to covalently immobilize the Ag on the SPR gold chip, the chip was modified with 4-MBA in order to use the carboxyl group of 4-MBA to make covalent attachment with the amine group of Ag with the help of EDC/NHS. The SEM image of 4-MBA modified SPR gold chip is shown in Fig. 2b and this image shows some crystals due to the modification of 4-MBA; however, SEM image of bare gold SPR chip does not have this kind of crystals and this fact confirms the modification of SPR gold chip with 4-MBA. The SEM images of Fig. 2a and b are different from each other and this observation indicates about the modification of SPR gold chip. Fig. 2c is EDAX for bare gold SPR chip and Fig. 2d is EDAX for 4-MBA modified SPR gold chip and these figures show the elemental composition present on the SPR chips, Fig. 2d shows sulfur and Fig. 2c does not. The presence of sulfur element in Fig. 2d and absence of sulfur element in Fig. 2c confirms the modification of SPR chip with 4-MBA.

3.4. Immobilization of rHRP-II Ag on 4-MBA modified SPR sensor chip

The pictorial representation of the steps involved for the immobilization shown in Fig. 3a and b indicates the various steps involved in the immobilization of rHRP-II Ag of *Pf* on 4-MBA modified SPR gold chip and this process comprises nine steps. In the first step of Fig. 3b, stabilization of baseline was performed for 120 s. For chemical binding between the 4-MBA adsorbed on the gold surface and free amino-groups of rHRP-II Ag, in the second step, activation of carboxyl groups on 4-MBA modified chip was carried out for 900 s with EDC–NHS. In the third step, washing was conducted with PBS and the SPR angle shifted nearly to baseline

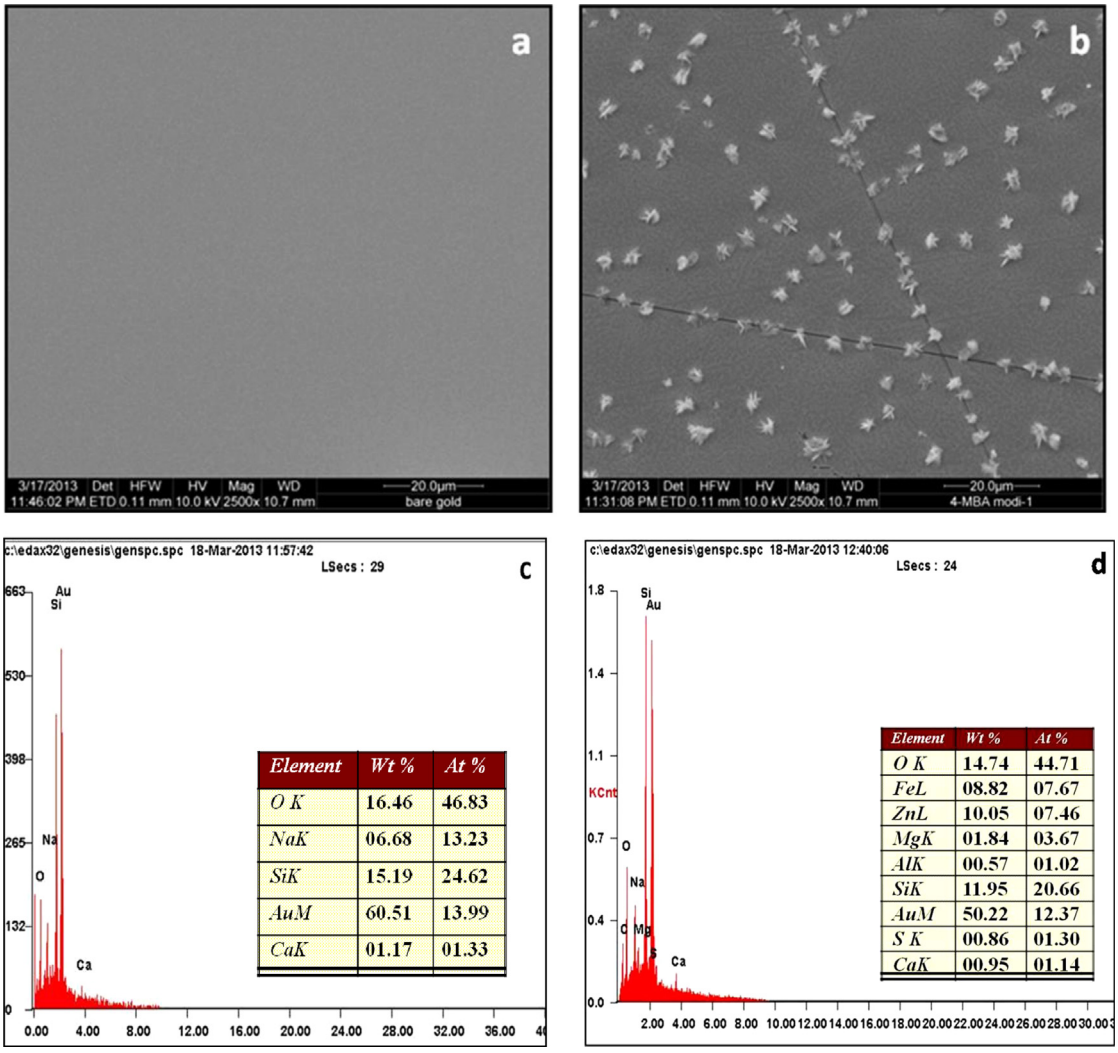

Fig. 2. (a) SEM image of bare gold SPR chip, (b) SEM image of 4-MBA modified gold SPR chip, (c) EDAX for bare gold SPR chip and (d) EDAX for 4-MBA modified gold SPR chip.

(Tsai and Li, 2009). In the fourth step, 0.04 mg/mL of rHRP-II Ag was injected on 4-MBA modified gold chip, allowed to interact for 1800 s and an increase in SPR angle is observed. In the fifth step, washing was performed and in the sixth step, to prevent non-specific binding and also for the blocking of un-reacted NHS-ester groups on 4-MBA chip, 1000 mM ethanolamine was used and allowed to react with sensor surface for 600 s. In the seventh step, washing was performed for 30 s as discussed in the experimental part. In the eighth step, regeneration was carried out for 120 s. At last in the ninth step, back to baseline process was conducted for 60 s. A net angle change of 113.27m° is observed and this indicates about the attachment of 0.94 ng/mm^2 of rHRP-II Ag on 4-MBA SPR chip (Stenberg et al.,1991). This rHRP-II Ag immobilized SPR chip was utilized for the interaction of Moab*Pf* of rHRP-II Ag. Similarly, for Poab*Pf* of rHRP-II Ag interaction, immobilization of the same rHRP-II Ag was performed on a different SPR gold chip modified with 4-MBA and interaction studies were carried out.

3.5. SPR characterization

The SPR angle for before and after the attachment of rHRP-II Ag on 4-MBA modified chip is shown in Fig. 4 and the SPR angle is found to shift significantly from −825m° (Fig. 4a is meant for 4-MBA modified SPR chip) to −511m° (Fig. 4b is meant for rHRP-II Ag attached 4-MBA modified SPR chip) due to the covalent attachment of rHRP-II Ag on 4-MBA modified gold chip (Rogers and Mulchandani, 1998; Lundstrom, 1994). Thus, the shift in SPR angle can be attributed to the formation of thin layer of rHRP-II Ag on 4-MBA modified SPR gold chip.

3.6. Interaction of rHRP-II MoabPf or PoabPf with the immobilized rHRP-II Ag of Pf on 4-MBA modified gold chip

The rHRP-II Ag immobilized SPR sensor chip was utilized for the sensing of different concentrations of Moab*Pf* or Poab*Pf* and the results are depicted as SPR sensorgram in Fig. 5A and B, respectively and these figures exhibit concentration dependent angle changes at various concentrations. The limit of detection (LOD) of the present method is found experimentally to be 5.6 pg for Moab*Pf* and this is the minimum concentration Moab*Pf* which shows the response during the interaction with its immobilized rHRP-II Ag on 4-MBA modified gold chip. Moreover, LOD for Poab*Pf* was found experimentally found to be 0.4 ng and this is the minimum concentration Poab*Pf* which shows the response during the interaction with its immobilized rHRP-II Ag on 4-MBA modified gold chip. The better LOD obtained with Moab*Pf* than that of Poab*Pf* is due to the sensitivity and specificity of Moab*Pf*; it will be always higher because they are derived from a single epitope (single clone), whereas the Poab*Pf* is from the mixture of a number of epitopes (polyclones). Cross reactivity of this Moab*Pf*

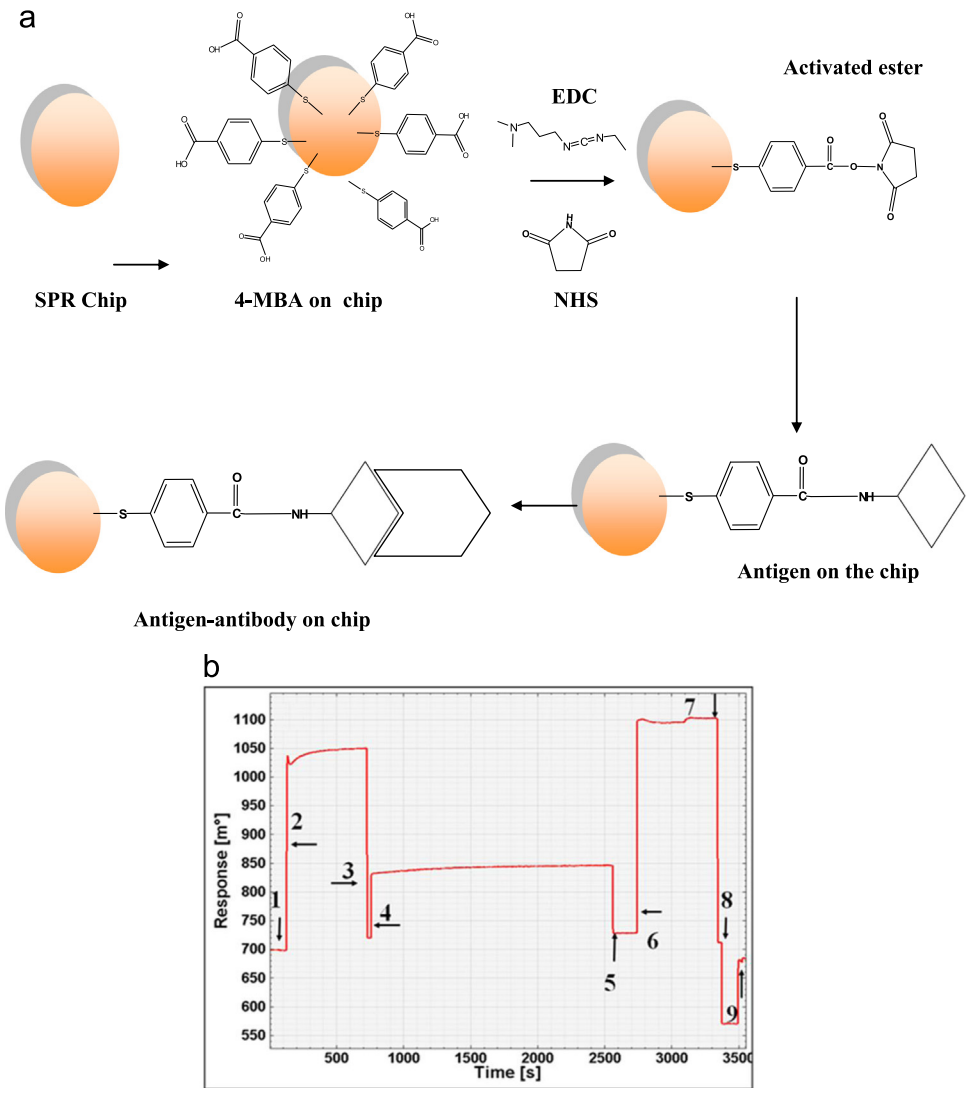

Fig. 3. (a) Pictorial representation of steps involved for the immobilization is indicated and (b) various steps involved in the immobilization of rHRP-II Ag of *Pf* on 4-MBA modified SPR gold chip. Sensorgram showing different steps [(1) Baseline, (2) EDC–NHS activation, (3) Washing, (4) rHRP-II Ag of Pf coupling, (5) Washing, (6) Deactivation, (7) Washing, (8) Regeneration and (9) Back to baseline].

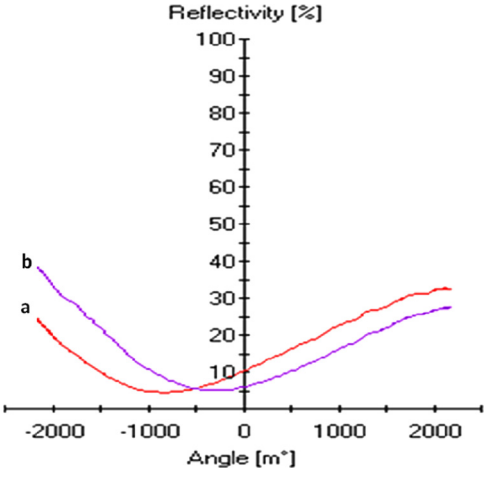

Fig. 4. SPR angle shift curve for (a) 4-MBA modified gold chip before and (b) after immobilization of rHRP-II Ag of *Pf*.

was also checked with other Moabs by using western blotting and the outcome results of Moab*Pf* do not have any cross reactivity with other Moabs; this is the main cause for the high response of Moab*Pf* with rHRP-II Ag when compared to the Poab*Pf*.

These LOD values are better than earlier reports based on amperometric immunosensor (Sharma et al., 2011a) and QCM (Sharma et al., 2011b) for the detection of rHRP-II *Pf*. Calibration plots using the SPR data of Fig. 5A and B are shown as Fig. 5C and D for Moab*Pf* and Poab*Pf*, respectively with the coefficient of variation (R) value for Moab*Pf* 0.99208 and for Poab*Pf* 0.99281 and standard deviation (SD) of 1.73152% and 1.88055% for Moab*Pf* and Poab*Pf*, respectively.

3.7. *Evaluation of kinetic parameters for the interaction of MoabPf or PoabPf with rHRP-II Ag of Pf*

The affinity interactions between immobilized Ag and Ab were characterized by the equilibrium constant (K_D). The data were fitted using a simple 1:1 interaction model (Liu et al., 2008), A+B=AB, where 'A' is the injected analyte, 'B' is the immobilized ligand and 'AB' is the analyte–ligand complex formed during the interaction process. In the SPR system, the signal R is proportional to the amount of [AB] and the R_{max} is proportional to the initial [B]. Hence, in this study kinetic parameters such as K_D and B_{max} value were calculated for the binding of Moab*Pf* or Poab*Pf* with

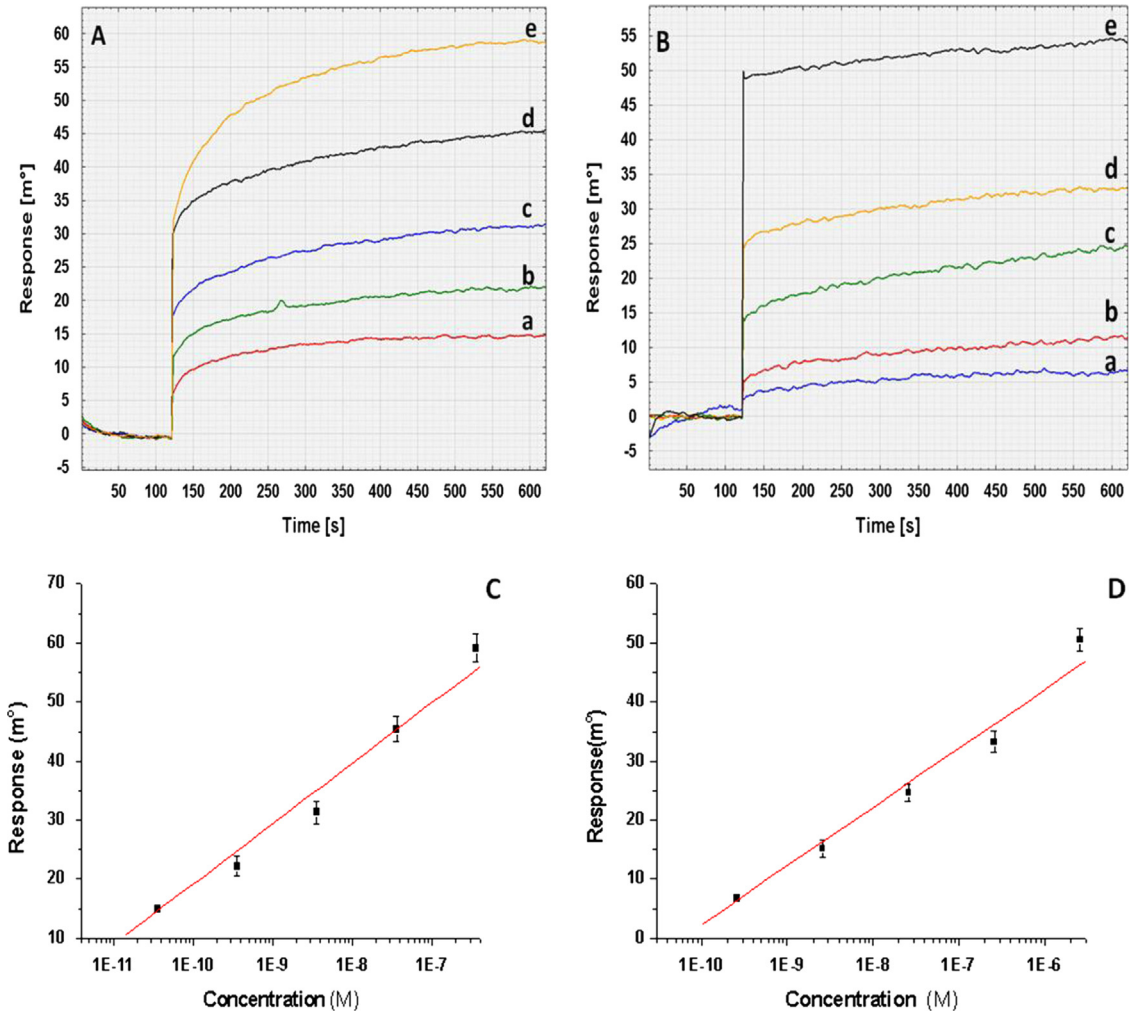

Fig. 5. SPR sensor response for the interaction of different dilutions of (A) Moab*Pf*: (a) 1:12,800, (b) 1:6400, (c) 1:3200, (d) 1:1600 and (e) 1:800 and (B) Poab*Pf* (a) 1:3200, (b) 1:1600, (c) 1:800, (d) 1:400, and (e) 1:200 with immobilized rHRP-II Ag of *Pf* and calibration graph for the response of angle changes against concentration of (C) Moab*Pf* and (D) Poab*Pf* with immobilized rHRP-II Ag of *Pf*. Temperature: 25 °C and pH 7.5.

immobilized rHRP-II Ag using the software and were found to be 0.517 nM (K_D) and 48.61m° (B_{max}) for Moab*Pf* and 2.288 nM (K_D) and 46.80m° (B_{max}) for Poab*Pf*, respectively and these low K_D values (if $K_D \leq 10$ nM) indicate the high affinity interactions of the Moab*Pf* or Poab*Pf* with the immobilized rHRP-II Ag (Wassaf et al., 2006).

3.8. Evaluation of thermodynamic parameters

The K_D and B_{max} values obtained from kinetic evaluation software for the binding of Moab*Pf* or Poab*Pf* with its immobilized Ag were further utilized for the calculation of thermodynamic parameters like ΔG, ΔH and ΔS involved in the binding of Moab*Pf* or Poab*Pf* with the immobilized rHRP-II Ag using Van't hoff equations (Savara et al., 2009; Glasstone, 1947):

$$\Delta G = -RT \ln K_A = \Delta H - T\Delta S \quad (1)$$

$$\Delta H = R\,T_2T_1 \ln K_2T_2 - T_1/K_1T_2 - T_1K_1 \quad (2)$$

$$K_A = 1/K_D \quad (3)$$

where R is the universal gas constant, K_A is the affinity constant, T is temperature and K_1, K_2 are affinity constants for association with T_1 and T_2 temperature, respectively. ΔG, ΔH and ΔS are, respectively, changes in Gibb's free energy, change in the enthalpy and change in the entropy due to the binding of Moab*Pf* and Poab*Pf* with immobilized Ag. The value of ΔG for binding of Moab*Pf* and Poab*Pf* with immobilized Ag was found to be −52.89 kJ/mol and −49.22 kJ/mol at 298 K, respectively. The negative values of ΔG indicate the spontaneous interaction of Moab*Pf* and Poab*Pf* with the immobilized Ag on 4-MBA modified SPR chip. The calculated value of ΔH using Van't Hoff Wizard in kinetic evaluation software was found to be −27.07 kcal/mol and −16.05 kcal/mol for Moab*Pf* and Poab*Pf*, respectively and this negative ΔH reveals the interaction of Moab*Pf* and Poab*Pf* with immobilized rHRP-II Ag as an exothermic process (Cabilio et al., 2000). The value of ΔS for binding of Moab*Pf* and Poab*Pf* with immobilized rHRP-II Ag was calculated using software and was found to be 28.79 and 37.44 cal/mol K respectively. The magnitudes of $T\,\Delta S$ values were found to be higher than the observed ΔH values, indicating that the net influence of enthalpy on Moab*Pf* and Poab*Pf* with immobilized Ag is minor and the apparent gain in entropy is actually the driving force for the interaction of Moab*Pf* and Poab*Pf* with immobilized Ag (Cabilio et al., 2000; Kamyshny et al., 2001). The positive values of entropy observed indicate that the interactions can be explained by the Langmuir replacement reaction that exhibits Langmuir type isotherm (Savara et al., 2009). The Langmuir replacement reaction shows that the observed positive entropy is because of desorption

of water molecules from either Ab or Ag or both. The importance of desorption of water molecules from protein surfaces during ligand–protein binding was reported earlier (Gregory, 1995).

3.9. Effect of temperature on interaction between MoabPf or PoabPf and rHRP-II Ag

Temperature variation study was performed here in order to know the effect of temperature on SPR response during the interaction of Moab*Pf* or Poab*Pf* with immobilized rHRP-II Ag. Upon increasing temperature from 10 to 25 °C, an increase in SPR angle is observed as shown in Supplementary Fig. 1S (Moab*Pf*) and Supplementary Fig. 2S (Poab*Pf*) and beyond 25 °C, SPR angle decreased (Gupta et al., 2010). Hence, 25 °C is used as optimum temperature for the interaction of Moab*Pf* or Poab*Pf* with its immobilized rHRP-II Ag.

3.10. Effect of pH on interaction between MoabPf or PoabPf and rHRP-II Ag

Supplementary Figs. 3S and 4S indicate the effect of pH on SPR angle change due to the interaction of Moab*Pf* and Poab*Pf*, respectively with immobilized rHRP-II Ag. It is observed from Supplementary Figs. 3S and 4S that the SPR angle is increased with increase in pH up to 7.5 and then decreased up to pH 9.0. This observation is probably due to the pH dependent structural changes and electrostatic interactions occurring on the SPR sensor disc between Ag and Ab as reported earlier (Paynter and Russell, 2002). The above observations suggest that in pH 7.5 PBS buffer, the interaction of Moab*Pf* or Poab*Pf* with immobilized rHRP-II Ag is more effective and thereby results in more angle change; hence, pH 7.5 was preferred in the studies here. Maximum SPR angle response was found at pH 7.5 for the minimum dilutions 1:12,800 (Moab*Pf*) or 1:3200 (Poab*Pf*) in the present investigation. This pH range was used for this experiment because below pH 4.0 and above pH 9.0 the protein loses its activity (Gupta et al., 2010b).

4. Conclusion

Moab*Pf* and Poab*Pf* of rHRP-II Ag were characterized in a label free and real time manner using a 4-MBA modified SPR gold chip. K_D and B_{max} values were calculated by using kinetic evaluation software, and found to be 0.517 nM and 48.61m° for Moab*Pf* and 2.288 nM and 46.80m° for Poab*Pf*, respectively with the immobilized rHRP-II Ag. The K_D values derived in this study indicate that the interaction of Moab*Pf* with rHRP-II Ag is more effective than that of the interaction between rHRP-II Ag and Poab*Pf*. Moreover, thermodynamic parameters such as ΔG, ΔH and ΔS were determined for rHRP-II Ag and Moab*Pf* and Poab*Pf* interactions and the values revealed that the interaction between rHRP-II Ag and Moab*Pf* or Poab*Pf* was spontaneous, exothermic and entropy driven. The Moab*Pf* will be used in our laboratory in future for the detection of malaria using the serum collected in local hospitals from the infected patients and this sensing methodology may also find application in the effective and timely screening of malaria affected patients in the pathology laboratories equipped with SPR system.

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Appendix A. Supporting information

Supplementary data associated with this paper can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.04.025.

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