Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 255–259

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Surface plasmon resonance based immunosensor for serological diagnosis of dengue virus infection

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ARTICLE INFO

Article history:
Received 9 July 2009
Received in revised form
26 December 2009
Accepted 5 January 2010
Available online 11 January 2010

Keywords:
Surface plasmon resonance
Immunosensor
Self-assembled monolayer
Serological diagnosis
Dengue

ABSTRACT

Surface plasmon resonance (SPR) is a promising tool in sensor technology for biomedical applications. An SPR based immunosensor was established for label free and real time assay for the serological diagnosis of dengue virus infection employing the dengue virus antigen as the sensing element. The dengue virus antigen conjugated with bovine serum albumin is covalently immobilized on a gold sensor chip via activated self-assembled monolayer (SAM) of 11-mercaptoundecanoic acid, by amide coupling. Surface morphology of the biosensor was recorded using atomic force microscopy. Presence of dengue virus specific IgM antibodies in dengue positive sera was monitored by increase in resonance angle in direct immunoassay, whereas the principle of indirect competitive inhibition immunoassay was used to detect presence of dengue virus for early detection of the onset of dengue viral infection in clinical diagnostics. Results were compared with those obtained by MAC-ELISA. The regeneration was achieved by pepsin solution in glycin–HCl buffer (pH 2.2) and sensor surface displayed a high level of stability during repeated immunoreaction cycles. The proposed biosensor being simple, effective and based on utilization of natural antigen–antibody affinity, our study presents an encouraging scope for development of biosensors for diagnosis of dengue and dengue hemorrhagic fever (DHF) which continues to be a major health problem in the tropical and subtropical regions of world.

1. Introduction

Dengue virus, a mosquito-born flavivirus (family *Flaviviridae*), continues to be a major health problem in tropical and subtropical regions of the world affecting 50 million people every year [1]. The dengue virus causing dengue fever and dengue hemorrhagic fever (DHF) is transmitted to humans through mosquitoes of *Aedes* spp. involving a complex mechanism of transmission of viral pathogen across mosquito generations and retention of virus during inter epidemic periods of dengue in an endemic setting [2–4]. The enzyme linked immunosorbent assay (ELISA) [5,6] and immuno fluorescence assay (IFA) [7] are the gold standard serological tests for detection of specific antibody and the viral antigen, respectively. Recently molecular detection of dengue virus RNA employing reverse transcriptase PCR [8] and isothermal nucleic acid sequence-based amplification [9,10] has been reported.

The global resurgence of dengue is thought to be due to a failure to control the *Aedes* population, uncontrolled urbanization, climate change and increased air travel [11]. Currently, there is no effective vaccine or specific therapeutic agent to prevent or cure dengue and DHF, and therefore a timely diagnosis of dengue virus in vectors and human population employing real time biosensors is the demand of the day to prevent the spread of the disease. The surface plasmon resonance (SPR) based immunoassay, which combines the selectivity of molecular recognition of biomolecules and the high sensitivity of SPR signal transducer, has tremendous potential in biomedical field, viz. epitome mapping [12], bio-interaction studies [13], drug discovery [14], developing biomarker for Alzheimer's disease [15], acute myocardial infarctions [16] and for monitoring clinically significant analytes such as dopamine [16,17], cholesterol [19] and many more reviewed recently [20]. SPR offers the advantages of real time and label free analysis over conventional immunoassay techniques [21]. Briefly, SPR is an optoelectronic phenomenon where light incident on a metal surface at a given angle can excite a surface-bound electromagnetic wave, a surface plasmon, which propagates along the interface between the metal and the ambient medium. Associated with the surface plasmon is an evanescent field that probes local changes in the refractive index of the ambient medium that are induced, for example, by binding a

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doi:10.1016/j.jpba.2010.01.001

biomolecule (viz. antibody, receptor, enzymes, etc.) to the surface-bound ligand. A change in the refractive index will shift the angle of incidence at which SPR excitation occurs. This shift is tracked by monitoring the movement of the intensity minima of the reflected light as a function of time and the binding event is presented as a sensogram.

Herein we propose an SPR based immuno biosensor for single step, label free and real time assay for the serological diagnosis of dengue virus infection employing the dengue virus antigen as the sensing element.

2. Experimental

2.1. Surface plasmon resonance spectrometry

The SPR system was an Autolab Model SPRINGLE (Eco-Chemie, Utrecht, The Netherlands) equipped with an open cuvette system (20–100 μl of sample volume), optical functioning based on Kretchmann configuration and a flexible software package for controlling the functioning of the instrument. Gold coated BK7 type microscopic glass plates (Eco-Chemie, Utrecht, The Netherlands) were used as sensor chips. At each sensor chip, four sites could be made available to study molecular interaction by changing the position of sensor disc. All experiments were carried out at 25 °C. All injections were of 50 μl and after each injection the flow was switched back to carrier buffer, phosphate buffer saline automatically throughout the course of the experiment. A μ-Autolab potentiostat/galvanostat with GPES 4.9 software, Eco-Chemie, was used to record cyclic voltammogram in ESPR cuvette cell (150 μl), with an Ag/AgCl electrode (sat. KCl) as reference electrode and a platinum wire as counter electrode and gold film of sensor disc functioned as working electrode (diameter 5 mm). A scanning probe microscope, model NT-MDT Solver PRO (NT-MDT, Russia) with a silicon probe operated in tapping mode was used for topographic imaging of sensor surface.

2.2. Chemicals and reagents

Bovine serum albumin (BSA), 11-mercaptoundecanoic acid (11-MUA), N-hydroxysuccinimide (NHS), N,N′ dicyclohexylcarbodiimide (DCC), ethanolamine (EA) and pepsin were from Sigma–Aldrich, St. Louis, MO 63178, USA; and the remaining chemicals were of AR grade from E. Merck (India), Mumbai, India. Deionised water and HPLC grade solvents were used to prepare solutions. Phosphate buffer saline (PBS), pH 7.2 (0.01 M disodium hydrogen phosphate and 0.01 M potassium dihydrogen phosphate containing 0.8% NaCl and 0.02% KCl in deionised water) was used as carrier solution and flowed over sensor surface throughout the experiment. 1 mM 11-mercaptoundecanoic acid in ethanol was used for preparing self-assembled monolayer over the sensor chip. A stock solution of BSA (20 mg ml^{-1}) was prepared in 0.01 M sodium acetate buffer (pH 4.5). A 'dengue-IgM capture ELISA' kit was procured from National Institute of Virology, Pune, India. The strong positive control serum supplied therein was considered as a sample analyte containing dengue virus specific IgM antibodies (IgM Ab) and was diluted with PBS to get calibration standard for direct immunoassay for the presence of IgM Ab. The biotinylated flavivirus cross-reactive monoclonal antibody (den-MAb) supplied in the kit was used as high molecular weight (hmw) interactant in indirect competitive inhibition immunoassay procedure followed for detection of dengue virus antigen. The negative serum supplied in the kit was used as a control for the present study. Dengue antigen–BSA conjugate was freshly prepared prior to use by mixing the dengue antigen supplied in the kit with stock BSA solution in 40:60 (v/v) ratio containing 1 mg ml^{-1} DCC and NHS each; mixture was vortexed for 30 s and was then allowed to incubate for 15 min at room temperature.

2.3. Fabrication of biosensor and surface plasmon resonance assay

The SPR sensor chips, cleaned by ultrasonication in ethanol were left immersed overnight in 1 mM 11-MUA/ethanol, to allow self-assembled monolayer (SAM) formation. The SAM modified gold sensor disc was then attached to the prism of the SPR instrument using a matching liquid (refractive index = 1.515), a brief cleaning step of ~5 min was initiated by injecting a buffer of low salt concentration (0.01 M sodium acetate) to stabilize the self-assembled 11-mercaptoundecanoic acid monolayer. The next step was *in situ* activation of SAM, by introducing NHS–DCC reagent (1:1 aqueous solution of 100 mM NHS and 400 mM DCC) followed by immobilization of the dengue antigen–BSA conjugate. The optimum interaction for *in situ* activation of SAM and for immobilization of dengue antigen–BSA conjugate over activated SAM was found to be 5 and 10 min, respectively. The unbound reactive ester groups were deactivated by a brief flow of 1 M aqueous ethanolamine solution (pH to 8) to prevent non-specific binding.

Once the sensor surface was enriched with covalently anchored antigen–BSA, the analyte dengue virus specific IgM antibodies (IgM Ab) were injected over it. The immunoreaction between immobilized antigen and antibody (association phase) was studied for different periods. An optimum time period of ~15 min was found to be satisfactory to get a stable SPR signal for successful interaction and binding of reaction product on to the sensor surface. Every association phase was followed by a brief flow of carrier buffer for 2 min to wash out the unreacted/loosely bound species (dissociation phase). The effective rise at the end of dissociation phase with respect to resonance angle at initiation of interactive phase is correlated with the analyte concentration in direct immunoassay procedure. The regeneration step comprising a brief flow of 0.2 M pepsin solution (in glycine–HCl buffer, pH 2) was found appropriate to liberate the surface-bound analyte, leaving the sensor surface active for next injection.

3. Results and discussion

3.1. Fabrication of biosensor

The surface of a gold chip was functionalized for the specific binding of dengue antigen conjugated with BSA protein as sensing element since significance of biosensors for clinical diagnostics

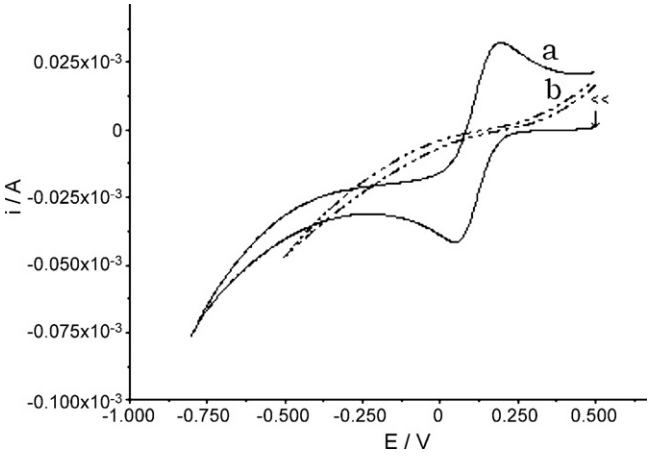
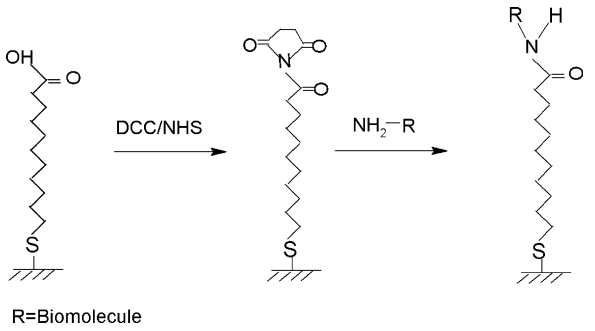


Fig. 1. Cyclic voltammogram of 1 mM $Fe(CN)_6^{3-}$ in PBS buffer (pH 7.2) as supporting electrolyte, on (a) bare gold film electrode and (b) self-assembled 11-MUA monolayer coated gold surface vs. Ag/AgCl electrode.



Scheme 1. Schematic representation of fabrication of dengue biosensor, R = dengue antigen–BSA conjugate.

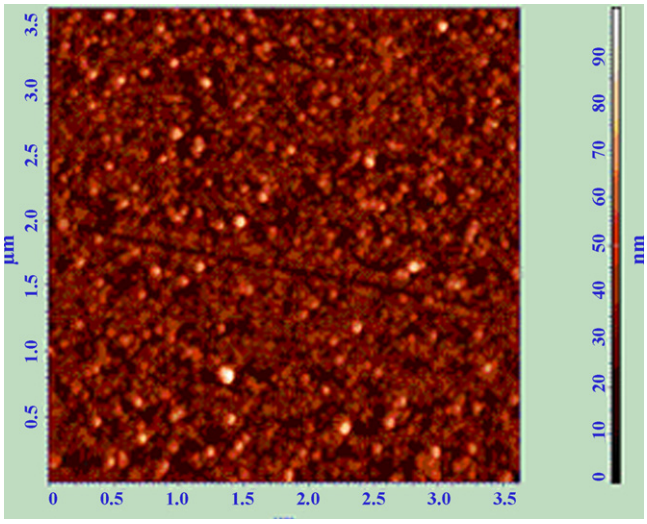


Fig. 2. AFM image of dengue antigen–BSA conjugate immobilized on to the DCC–NHS activated self-assembled 11-MUA monolayer coated gold chip.

lies in its specificity. To avoid non-specific adsorption/binding at the sensor and to provide covalent bonding sites to the ligand, a self-assembled monolayer of 11-mercaptoundecanoic acid (11-MUA) acted as matrix for immobilization the sensing element [19]. The strong affinity of gold for sulphur, results in a perfect orderly monolayer over the bare gold sensor chip. Cyclic voltammetry (CV) has been widely used to characterize the self-assembled monolayer on solid support surfaces [22]. For the present study, the CV

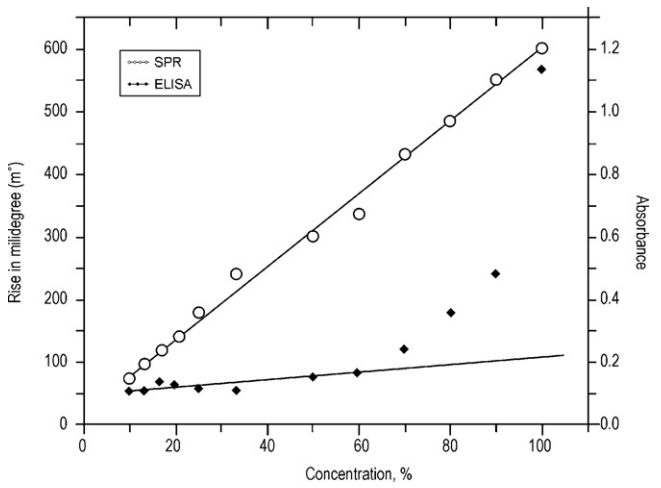


Fig. 4. SPR vs. ELISA response for detection of IgM Ab in positive control serum at different dilution.

response of a typical diffusion controlled, reversible $Fe(CN)_6^{3-/4-}$ redox probe on a bare gold surface (Fig. 1a) is totally suppressed on a self-assembled 11-MUA monolayer coated gold surface (Fig. 1b) indicating a successful formation of a stable SAM over the sensor surface. Ligands were immobilized via primary amine group following carbodiimide activation [23]. In SPR mode, a net rise of resonance angle (figure not shown) by 203 m° (milli degree) as compared to the bare gold chip signifies a good quality and stable matrix of SAM over the gold surface. The preliminary trials to immobilize dengue antigen on activated sensor showed poor binding, therefore an attempt was made to synthesize antigen–BSA conjugate to improve the stability. The immobilization of dengue antigen–BSA conjugate on to the DDC/NHS activated SAM of 11-MUA, through amine group of BSA and carboxyl group of activated SAM forms a stable amide bond ($-S(CH)_{10}CONHR$, where R = dengue antigen–BSA conjugate) for immobilization of sensing element on gold surface as shown schematically in Scheme 1. The surface morphology of dengue antigen–BSA conjugate immobilized SPR gold surface was studied by atomic force microscopy (AFM) and is shown in Fig. 2. The AFM image indicates a homogenous, compact and dense film of the conjugate. Thus, bovine serum albumin acts as a binding protein to the sensing antigen, which anchors very well to the sensor surface.

Fig. 3 shows the sensogram recorded for the immobilization of dengue antigen–BSA conjugate onto the active SAM immobilized

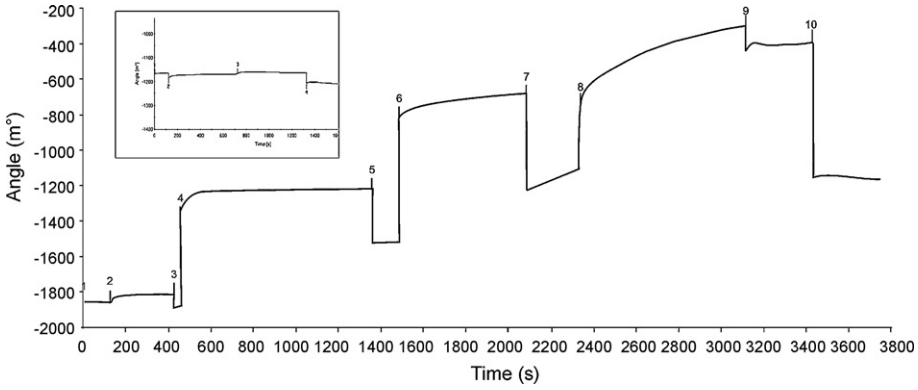


Fig. 3. Sensogram showing immobilization and immunoreaction of dengue antigen–BSA conjugate onto the activated 11-mercaptoundecanoic acid (MUA) modified gold surface [position 1, SAM coated gold chip; position 2, injection of 1:1 DCC + NHS; position 3, 5, 7, 9, PBS flow; position 4, injection of dengue antigen–BSA conjugate; position 6, injection of EA; position 8, injection of positive control serum containing IgM Ab at 50% dilution and position 10, injection of pepsin solution]. Inset: SPR response of negative serum [position 1, base line immobilization of dengue antigen–BSA conjugate; position 2, 3 injection of negative control serum at 50% dilution with PBS buffer and position 4, injection of pepsin solution].

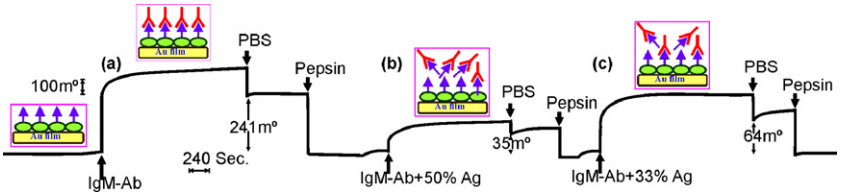


Fig. 5. SPR response for the immunoreaction of den-antigen–BSA conjugate with den-MAb in the absence and in the presence of dengue virus antigen (Ag).

gold surface, where position 1 represents the base line position of the SAM coated gold sensor chip. The SPR response of *in situ* activation of SAM by DCC–NHS mixture is seen at position 2 (Fig. 3), followed by a brief wash with PBS (position 3, Fig. 3) to remove unbound species from the sensor surface. The immobilization of dengue antigen–BSA conjugate onto the activated self-assembled 11-MUA layer immobilized gold surface is evidenced by a sharp rise in the resonance angle (position 4, Fig. 3) showing an appreciable rise in the refractive index of the sensor surface due to immobilization of conjugate. Desorption of the loosely bound conjugate with PBS washing (position 5, Fig. 3), is followed by blocking the unbound reactive esters by ethanolamine (position 6, Fig. 3) to avoid non-specific adsorption. A net rise of 400 m° indicates that the gold surface is covered with covalently bound dengue antigen–BSA conjugate and is stable on the self-assembled 11-MUA monolayer modified gold sensor surface. Considering change of 120 m° corresponds to 1 ng mm^{-2} protein binding in a standard SPR experiment, present biosensor chip may be considered to contain 28.3 ng of conjugate containing 11.5 ng of dengue antigen which is immobilized over an area 8.5 mm^2 being used by this SPR instrument for facilitating the bio-molecular interactions.

The SPR response at position 8 (Fig. 3) shows a steady interaction between antigen–BSA conjugate immobilized at sensor surface and the IgM Ab introduced over it and a net rise of 305 m° for positive control serum diluted to 50% with PBS buffer, demonstrating a very high sensitivity of proposed label free, real time immunoassay. The inset of Fig. 3 shows the SPR response of negative control serum, no appreciable rise in resonance angle was observed, signifying the specificity of the present biosensor.

3.2. ELISA vs. SPR for serological diagnosis of dengue virus specific IgM antibodies

The strong positive serum of varied dilutions with dilution buffer (PBS) supplied with Dengue MAC-ELISA kit was analysed by ELISA and SPR (*vide-supra*) for specific antigen–antibody interaction. An absorbance above 0.174 OD (2.1× negative OD) in ELISA assay and an SPR angle shift of at least 50 m° was considered positive for presence of dengue virus specific IgM antibodies in the serum. Fig. 4 shows the SPR response *vis-à-vis* MAC-ELISA response, which clearly signifies higher sensitivity of SPR as compared to ELISA. The linear regression equation for dengue virus specific IgM antibodies in the dilution range 10–100% of strong positive control serum was $y = 5.74 \pm 0.14x + 25.00 \pm 7.94$ ($n = 12$, $p < 0.0001$, $R = 0.9970$) for SPR direct immunoassay employing Au/11-MUA/DDC–NHS/den-antigen–BSA biosensor.

3.3. Serological diagnosis of dengue virus antigen by SPR

Detection of den-antigen was carried out using the principle of indirect competitive inhibition [18]. Here, equal volumes of dengue-MAb were incubated with different concentrations of den-antigen and introduced over a preset gold sensor chip immobilized with dengue antigen–BSA conjugate. It is expected that the interaction of dengue-MAb with immobilized den-antigen–BSA conjugate will be inhibited in the presence of free den-antigen in solution.

Fig. 5 shows the SPR response observed for the immunoreaction between dengue antigen–BSA conjugate and dengue-MAb in the absence, and in the presence of, dengue antigen. The dengue-MAb at 1:100 dilution with PBS and an interaction time of 15 min showed a good interaction with dengue antigen–PBS conjugate, as evidenced from a rise in resonance angle shift by 241 m° (curve a). For detection of den-antigen, introduction of dengue-MAb incubated with den-antigen at different dilutions revealed a decrease in resonance angle (Fig. 5, curves b and c) in proportion to the concentration of dengue antigen.

The conjugate immobilized assay format used in present study could be reused by regeneration of the conjugate surface by flowing an eluent (regeneration buffer) enabling multiple analysis. The conjugate surface remained stable and the immunoreaction with dengue-MAb did not vary significantly for nearly 30 cycles of measurements.

4. Conclusion

The proposed SPR based biosensor, Au/11-MUA/DDC–NHS/den-antigen–BSA is suitable for detection of dengue virus specific IgM antibodies in serum/plasma or CSF in a direct immunoassay as well as for detection of dengue virus antigen using indirect competitive inhibition immunoassay, employing den-Mab in human serum or mosquito head squash. The high sensitivity observed with present biosensing system is possibly due to the combined advantages of the highly specific immunoreaction, the sensitivity of SPR instrumentation, unique surface chemistry of immobilized dengue antigen–BSA conjugate and the indirect competitive inhibition immunoassay principle. The proposed method provides a new and promising route for a reliable, economical and label free real time technique with potential for fabrication of biosensors for detection of the onset of dengue in clinical diagnostics and surveillance of dengue virus in mosquito population.

Acknowledgments

Financial assistance from the Department of Biotechnology, Government of India, is gratefully acknowledged. We thank Dr. N. Kumar, Defense Laboratory for providing AFM facilities.

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