

# An attempt to develop surface plasmon resonance based immunosensor for Karnal bunt (*Tilletia indica*) diagnosis based on the experience of nano-gold based lateral flow immuno-dipstick test

Sadhna Singh <sup>a</sup>, Manoj Singh <sup>a</sup>, Ved Varun Agrawal <sup>b</sup>, Anil Kumar <sup>a,\*</sup>

<sup>a</sup> Department of Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities, G.B Pant University of Agri. & Tech., Pantnagar-263145, Uttarakhand, India

<sup>b</sup> National Physical Laboratory, Dr. K.S. Krishnan Marg, New Delhi-110012, India

## A B S T R A C T

Karnal bunt (KB) of wheat incited by *Tilletia indica* is an economically important quarantined fungal pathogen. Similarity in teliospore configuration makes it difficult to differentiate KB teliospores from the teliospores of other bunt fungi using conventional approaches. In order to determine the correct identity of KB teliospore— an infectious entity, it is essential to develop specific diagnostic probes and high quality of immunological reagents against infectious entities. The immuno-dipstick assay developed in our lab is quite sensitive to detect the antigens of even five teliospores. For on-site testing of KB, a nano-gold based lateral flow immuno-dipstick assay (LFID) was also developed in our lab using anti-teliospore antibodies. For development of LFID, colloidal gold was prepared and conjugated with anti-teliospore antibodies which were further characterized. However, species specific detection is yet to be achieved by generation of monoclonal antibodies against diagnostic antigens (34 and 66 kD teliospore's proteins). Based on the experience of nano-gold based LFID, we are proposing to develop surface plasmon resonance (SPR) based label-free detection system. The same may be employed not only for determination of the quality of immunological reagents in terms of sensitivity, specificity and precision but also development of suitable design of immunosensor for differential diagnosis of KB (*T. indica*). The approach involves the use of a mouse monoclonal antibody against diagnostic antigens and a suitable design of SPR sensor by the preparation of immuno-affinity layer over self assembled monolayer surfaces. The signal to noise ratio can further be enhanced using nano-gold particles. This will be first attempt for real-time monitoring of KB teliospores in wheat lots by SPR sensor and in a fully automated manner for establishing seed certification and plant quarantine standards.

## Keywords:

Karnal bunt  
Surface Plasmon Resonance  
T. indica  
Immunosensor  
Nano-gold

## 1. Introduction

Karnal bunt (KB) disease of wheat, caused by fungus *Tilletia indica* is an important disease and pathogen is placed as a quarantine pest. It is the target of strict quarantine regulations by most wheat growing countries and its presence raises trade barriers to wheat exports and now also categorized as one of the biological weapons [16]. Currently, KB disease is monitored by repeated visual examination of spores, which is time-consuming, expensive and requires skilled personnel. For the detection of KB or differentiating it from other pathogens several molecular detection systems such as conventional, biophysical technique, isozyme markers, immunological and DNA based techniques, which are laboratory-based label-dependent systems [4,9,15]. The immunological formats viz. Seed Immunoblot Binding Assay (SIBA), Indirect Immuno-fluorescent staining tests, Dyed Latex Bead

Agglutination Assay and Immuno-dipstick assay developed in our laboratory are quite promising for not only detection of KB infection in seeds but can also be used for the sensitive detection of seed borne inoculum present either in bunted seeds or as loose teliospores on the seed surface [8,10,11]. Nowadays label-dependent antibody-based dipstick methods and lateral flow devices are currently being used for fast on-site pathogen detection [3,5]. For specific detection of KB, characterization of diagnostic antigen(s) of teliospores is required for the development of monoclonal antibodies against it, which can be employed for development of single step diagnostic kit. However, development of label-free, fast and specific screening methods is, therefore, a priority. Antibody detection systems are more suited for future implementation in label-free immunosensors that could be mounted on platforms for 'on-site' continuous screening for various pathogens [2].

Such immunosensor exploits the highly specific and selective interaction between antigen and antibody for the detection of the target analyte of KB by the molecular recognition layer called immuno-affinity (IA) layer produced by immobilizing teliospore's antigens or anti-teliospore antibodies to the transducer surface on

\* Corresponding author. National Physical Laboratory, Dr. K.S. Krishnan Marg, New Delhi-110012, India.

E-mail address: [ak\\_gupta2k@rediffmail.com](mailto:ak_gupta2k@rediffmail.com) (A. Kumar).

which self assembled monolayers (SAMs) will be used as a linker layer. The surface plasmon resonance (SPR) detection principle is utilised in many optical biosensor systems for the study of 'real-time' molecular interactions [1,12,13,14]. The current efforts in our lab is centered around to develop label-free surface plasmon resonance (SPR) based immunosensor for detection of KB (Chart 1).

## 2. Generation of anti-teliospore antibodies

New Zealand White Rabbits were immunized by injection of suspension of teliospores of *T. indica* with adjuvant (Gupta et al., 1998). Anti-teliospore antibodies were isolated by purification of polyclonal antisera by affinity chromatography. Monoclonal antibodies are being generated against eluted specific proteins which are uniquely present in *T. indica*.

## 3. Characterization of diagnostic antigens

The antibodies developed were *Tilletia* specific (Gupta et al., 2009). In order to generate monospecific antibodies, unique, prominent immunoreactive proteins i.e. 34 and 66 kD of teliosporic protein of *T. indica* were identified using western blotting. These proteins were eluted from the preparative gel to obtain the protein in bulk (Fig. 1).

## 4. Preparation and characterization of nano-gold particle

Colloidal gold was prepared by slight modification of Frens method [6]. A nano-gold colloidal particle synthesized by addition of different concentrations of reducing agent was monitored by UV-vis spectroscopy between the wavelength range of 200 nm to 750 nm operated at 1 nm and it was found that the peak showed a maximum O.D. at 520 (Fig. 2b). Further nano-gold particle was characterized by transmission electron microscopy (Fig. 2a).

## 5. Conjugation of anti-teliospore antibodies with colloidal gold and its characterization

Nano-gold conjugate with anti-teliospore antibody was prepared under optimal condition, which was further characterized by UV-vis

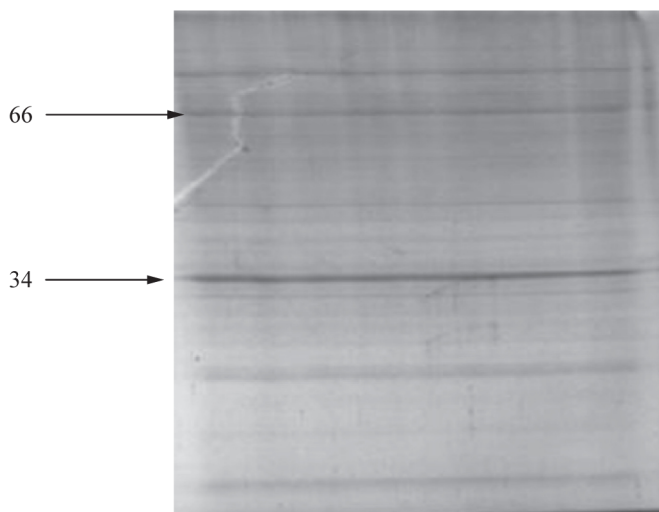


Fig. 1. Preparative gel for extraction of unique solubilized protein from teliospores.

spectra (Fig. 3a) and TEM. With the considerations of optimal conditions of antibody and colloidal gold conjugation, the pH of the as prepared colloidal gold solution was determined to be 8.5 and the minimal antibody concentration to stabilize colloidal gold was .02 mg/ml. In Fig. (3b), the blue curve is the spectra of colloidal gold solution, pink curve represents antibody colloidal gold conjugate immediately after addition of the antibody at pH 7.4 and after centrifugation and re-suspension of the conjugate in pH 7.4 buffer represented by yellow curve. A peak at  $\approx 520$  is due to the surface plasmon resonance of colloidal gold particles. After the addition of antibody, the surface plasmon band broadened and red shifted due to interaction of antibody with colloidal gold particles. After centrifugation and re-suspension of the conjugate, the intensity of the plasmon resonance band increased due to the enhanced colloidal gold concentration.

## 6. Development of lateral flow dipstick

Nano-gold labeled antibody conjugate was further used to develop lateral flow based immune-chromatographic assay for detection of KB. This immunochromatographic assay for diagnosis of KB depends on the transport of tag (colloidal gold-labeled anti-teliospore antibody probe to solubilize teliospore's protein) (Fig. 4).

## 7. Preparation of immuno-affinity (IA) layer

The IA layer is being prepared on the gold surface of the sensor chip at 37 °C. The IA layer is composed of the adsorption layer and linker layer (SAM). The adsorption layer specifically binds the target analytes and the linker layer connects the adsorption layer to the surface of the transducer.

## 8. Preparation of linker layer

The self assembled monolayers (SAMs) will be used as the linker layers. The SAM can be easily prepared by immersion of the substrate into a solution containing an appropriate amphiphile (Ulman et al., 1996). The self-assembled monolayer (SAM) will be prepared on the gold surface by incubating 5 mM 11-mercaptoundecanoic acid in ethanol for 2 h.

## 9. Coupling of antibody to self-assembled monolayer (SAM)

After the preparation of SAM (linker layer) on the gold surface, the protein (adsorption layer) will covalently coupled to the SAM by the well-

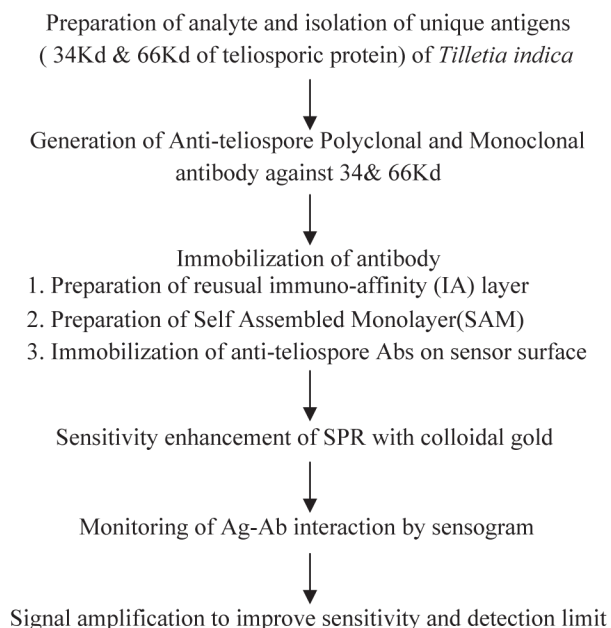
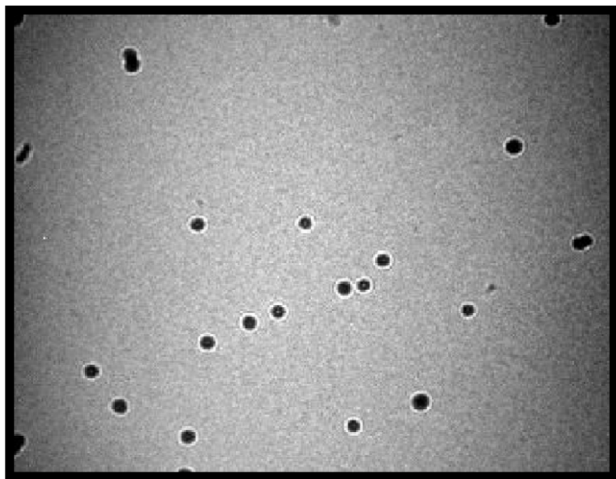
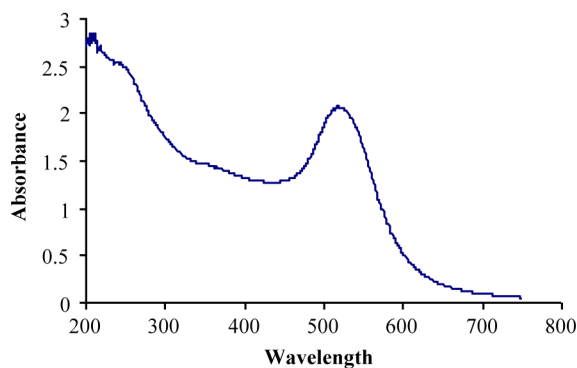


Chart 1. Attempts to develop SPR based immunosensor.

a



b



**Fig. 2.** (a) TEM image of colloidal gold particles ( $\times 80,000$ ) having maxima peak at 520. (b) Colloidal gold showing maxima peak at 520 nm.

known chemistry, which uses 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) as coupling reagents. In this reaction, the carboxylic groups of SAM are activated by a mixture of EDAC and NHS, and then amine groups of ligand are covalently bonded to the carboxylic groups of SAM. The remaining esters are deactivated by addition of ethanolamine (Johnsson et al., 1991). For the coupling of antibody, the sensor surface will be rinsed with ethanol and then with 10 mM PBS buffer (pH 4.5). The PBS buffer will be prepared by mixing 10 mM di-sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) with 0.15 M KCl and 10 mM sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ) with 0.15 M KCl. Antibody is coupled to SAM by using the coupling reagents of EDAC (2 mM) and NHS (5 mM) in 10 mM PBS (pH 4.5). The SAM is first treated with EDAC and NHS for 10 min and then the solution of antibody in 10 mM PBS (pH 4.5) is incubated. After immobilizing the recognition parts, the other reactive sites of SAM are blocked by 1 M ethanolamine (Naimushin et al., 2002; Kim et al., 2005; Lee et al., 2005).

## 10. Immobilization of antibody to the metallic surface of the transducer

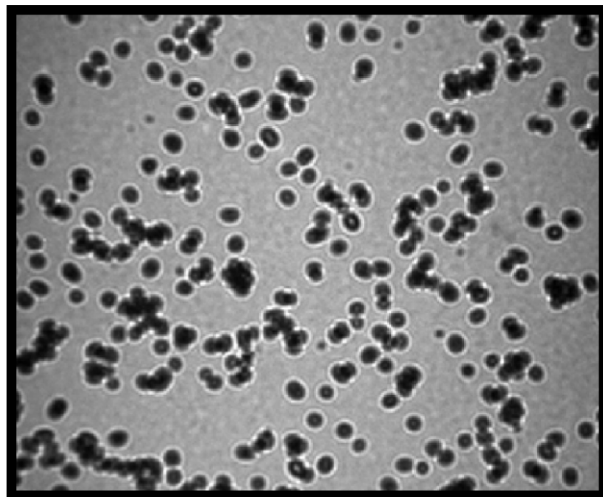
Various techniques can be used for the immobilization of antibodies (or antigens) to the metallic surface of the transducer. Such techniques can be classified into two categories: (1) physical adsorption to the metallic surface of the transducer, and (2) covalent coupling of antibodies (or antigens) to the transducer. Although the physical adsorption is simple to use, this technique is known to induce denaturation or conformational changes of the adsorption layer, poor

reproducibility, elution by the detergent of the washing solution and so on (Bae et al., 2005). Colloidal gold (Au) nanoparticles attached to thin film of an SPR biosensor exhibit more than ten-fold signal amplification.

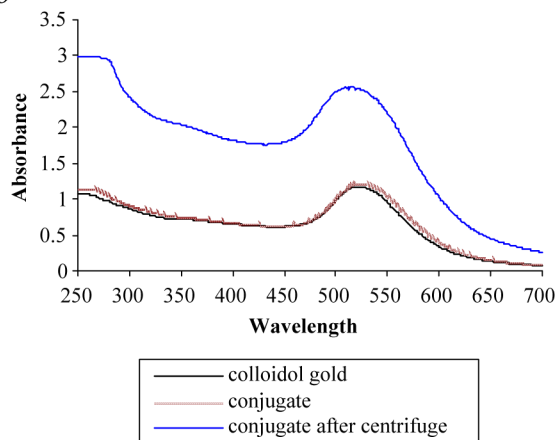
## 11. Coupling of antibody to self-assembled monolayer (SAM)

After the preparation of SAM (linker layer) on the gold surface, the protein (adsorption layer) will covalently coupled to the SAM by the well-known chemistry, which uses 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) as coupling reagents. In this reaction, the carboxylic groups of SAM are activated by a mixture of EDAC and NHS, and then amine groups of ligand are covalently bonded to the carboxylic groups of SAM. The remaining esters are deactivated by addition of ethanolamine (Johnsson et al., 1991). For the coupling of antibody, the sensor surface will be rinsed with ethanol and then with 10 mM PBS buffer (pH 4.5). The PBS buffer will be prepared by mixing 10 mM di-sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) with 0.15 M KCl and 10 mM sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ) with 0.15 M KCl. Antibody is coupled to SAM by using the coupling reagents of EDAC (2 mM) and NHS (5 mM) in 10 mM PBS (pH 4.5). The SAM is first treated with EDAC and NHS for 10 min and then the solution of antibody in 10 mM PBS (pH 4.5) is incubated. After immobilizing the recognition parts,

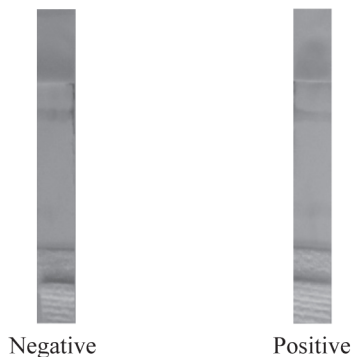
a



b



**Fig. 3.** (a) TEM image of antibody-gold particles ( $\times 80,000$ ). (b) UV-visible spectra of colloidal gold and antibody-gold conjugates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Lateral flow dipstick with positive and negative results.

the other reactive sites of SAM are blocked by 1 M ethanolamine (Naimushin et al., 2002; Kim et al., 2005; Lee et al., 2005).

## 12. Sensitivity enhancement of SPR with colloidal gold

The sensitivity of surface plasmon resonance biosensor is enhanced by the conversion of the real-time direct binding immunoassay into the sandwich immunoassay, in which colloidal gold particles coated with anti-teliospore antibody will be used. Our effort will be made to optimize and enhance the sensitivity of SPR using protein coated colloidal gold particles. Anti-teliospore antibody will be dissolved in 25 mM carbonate/bicarbonate buffer (pH 9.0) and then will be added to a colloidal gold solution with gentle stirring. The supernatant is removed by centrifugation at 15,000 rpm for 20 min. The precipitate is washed with PBS repeatedly, dissolved and diluted with PBS until O.D. reached 1.9 at 520 nm.

## 13. Monitoring of antigen–antibody interaction by sensorgram

As the analyte binds to the sensor surface, the refractive index and SPR angle change according to the increase of the mass at the sensor surface [17]. When the interaction between analyte and the immobilized receptor occurs at the sensor surface, a sensorgram can be obtained in real time by plotting the signal against time. The sensorgram will show the sequential procedures and responses for the immobilization of anti-teliospore Ab to carboxymethyl dextran of sensor surface.

## 14. Signal amplification to improve the sensitivity of detection limit

The mass change caused by the binding of analyte to the sensor surface affects the amount of plasmons to change the SPR angle, which is converted to the binding amount of analyte in the SPR-immuno-

sensor. The sensitivity of immunosensors can be improved by the mass label and the control of the IA layer (orientation and density). The first method (by using mass label) is to increase the signal by using label proteins such as secondary antibody [7], latex particle [18] and avidin-biotinylated liposome [19]. The second method is to control orientation, avidin, LB film, self-assembly and protein A will be used and the surface density and orientation of antibodies could be improved.

## 15. Conclusion

Based on the capabilities and leads available within our group, such as generation of anti-teliospore antibodies, identification of diagnostic antigens, standardization of components of LFID such as colloidal gold and its conjugation with anti-teliospore antibody, design of dipstick and lateral migration of antigen and antibodies led to provide the clues for the development of label-free immunosensor for detection of KB. Immuno-affinity sensor based on SPR will not only help in the characterization of immunological reagents used for development of diagnostic kit but also discriminating the identity of the teliospores of contaminating fungus with better signal to noise ratio in repetitive manner. Further, teliospores or its antigens can be examined with high sensitivity, more quickly, cheaper, in a fully automated manner and with a higher number of samples by developing such types of immunosensor. The developed LFID and portable immunosensor based on LED could be frequently employed in the seed certification and plant quarantine regulations for on-site testing and declaring the wheat lots KB free.

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