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DEVELOPMENT OF A NANO BIOSENSOR FOR DETECTION OF BRACT MOSAIC VIRUS IN BANANA (*Musa* spp.)

by

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Development of a nano biosensor for detection of *Bract mosaic virus* in banana (*Musa* spp.)"** is a bonafide record of research done by me during the course of study and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Place- Vellanikkara
Date- 23-09-2016

CERTIFICATE

Certified that this thesis entitled **"Development of a nano biosensor for detection of *Bract mosaic virus* in banana (*Musa* spp.)"** is a record of research work done independently by Saurav Saha (2014-11-106) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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Abbreviations

APS	Ammonium per sulphate
β	Beta
AR	Aspect ratio
bp	Base pair
BBrMV	*Banana bract mosaic virus*
BSA	*Bovine serum albumin*
BSV	*Banana streak virus*
BBTV	*Banana bunchy top virus*
CPBMB	Centre for Plant Biotechnology and Molecular Biology
C-MET	Centre for Material and electronics
CMV	*Cucumber mosaic virus*
MPA	3-Mercaptopropinic acid
HAuCl4	Hydrogen tetra chloroauratehydrate
CCB	Coomassie Brilliant Blue
CTAB	CetylTrimethyl Ammonium Bromide
C-DNA	Complementary Deoxyribonucleic Acid
CDMV	*Cardamom mosaic virus*
CMV	*Cucumber mosaic virus*
DNA	Deoxyribonucleic Acid
DIC	Distributed Information Centre
DAC- ELISA	Direct antigen coating Enzyme linked immunosorbant assay
DIBA	Dot immuno binding assay
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immunosorbant Assay
FAO	Food and agricultural organization
GNRs	Gold nanorods

g	Gram
ha	Hectare
Ig	Immunoglobulins
Kb	Kilo basepairs
kDa	Kilo dalton
KP	Potassium phosphate
l	Liter
M	Molar
MUA	Mercaptoundecanoic acid
MPTMS	(3-mercaptopropyl) trimethoxysilane
mM	Mili molar
LSPR	Localized Plasmon Resonance
TEM	Transmission electron microscope
mg	Mili gram
ml	Mili liter
NHB	National Horticulture Board
NIR	Near infra red
NCS-TCP	National Certification System for Tissue Culture Raised Plants
PBS	Phosphate buffer saline
µg	Microgram
µl	Micro liter
ng	Nano gram
°C	Degree Celsius
OD	Optical Density
ODD	Ouchterlony Double Diffusion
%	Percentage
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration

pM	Pico molar
PAGE	Polyacylamide gel electrophoresis
PEG	Poly ethylene glycol
PBS	Phosphate buffer saline
pNPP	para-Nitrophenylphosphate
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SD	Standard deviation
TEMED	Tetramethylethylenediamine

Introduction

1. Introduction

Banana and its plantain (Musa *spp.*) are some of the traditional crop that has been domesticated by human and one of the valuable industrial fruit crop provide staple food and income for millions of small holding farmers across the world. Almost all widely cultivated varieties were parthenocarpic result from inter or intra specific crosses of seedy species, *M. balbisiana* (2n=2×BB) and *M. Acuminata* (2n=2×AA) (Ortiz, 2013). Human migration and trade especially in the Asia-pacific region plays an important role in the dissemination of cultivar to America and Africa. Currently over a 100 accessions with a wide range of morphological character and genome organization have been identified in the whole world-wide (Ortiz, 2013; Pollefeys *et al.*, 2004).

Botanically the fruit of banana called berry, which is produced from the several kinds of herbaceous flowering plant under the genus *Musa* and mainly cultivated in a humid region of the tropic and subtropics, but it can also acclimatize in a wide variety of climates (Heslop & Schwarzacher, 2007).

Presently banana is grown about 150 countries across the world and ranked fourth after the rice, wheat and maize in terms of total production. It was estimated that total production of banana in the worldwide is 139 million tons (FAO, 2014).In India banana mainly cultivated in Tamil Nadu, Maharashtra, Karnataka, Gujarat, Andhra Pradesh, Bihar, Assam and Madhya Pradesh with the total production of 297.24 Lakh Tones(NHB, 2014). The varieties mainly cultivated in India are Dwarf Cavendish, Poovan, Robusta, Rasthali, Grand naine, Red Banana and Nendran, *etc.*

Banana is a crop with dual propagation ability, sexual through seeds and asexual through suckers, seed propagation mainly followed in diploid wild species where normal meiosis and fertilization and seed formation take place but it is not a normal way of propagation of banana. Propagation by sucker is the only natural means of their multiplication. An artificial method of propagation includes macro

propagation and micro propagation is most commonly followed. For that farmer generally used young sucker from the old mother plant for the establishment of a new plant. This practice among the farmer community causes the major outbreak of disease of banana, mainly viral diseases.

Nearly about 20 viruses till now reported from five different families that are infected banana and its plantain like *Banana bunchy top virus* (BBTV, genus Babuvirus, family Nanoviridae) and *Banana bract mosaic virus* (BBrMV, genus Potyvirus, family Potyviridae) are the most economically important viruses. These viruses are readily transmitted from plant to plant during vegetative multiplication and therefore it poses a threat for banana cultivation. Besides banana bunchy top virus, bract mosaic disease caused by BBrMV is widely spread among the banana growing areas like Kerala, Tamil Nadu, Karnataka and Andhra Pradesh (Singh *et al.*, 1996) and causes subsequent yield reduction.

Till now no effective resistance has been developed in *Musa* against all of these viruses and once this virus infects the plant it is difficult to control these viruses. So the only control measure is production of planting material that are virus free or virus indexing of planting material before distributing to the farmers.

Production of planting material of vegetatively propagated crop through national certification system has been adopted in all over the countries in the world. In our country virus free tissue culture planting material provided the through National certification system for tissue cultured plants (NCS-TCP) where an individual, organization or company needs certification for production of healthy virus free plant. The most important element of certification scheme is sensitiveness, reliability and quick detection technique to check the sanitary status of planting materials. Currently, most of the laboratory practices depend upon ELISA for the detection of virus from planting materials, some other techniques like targeting nucleic acid through RT-PCR, molecular bacon hybridization etc. are commonly

followed. New technology such as next-generation sequencing may expect to further enhance the frequency of virus detection. Though real-time and ELISA are specific and sensitive approach it need tremendous and cumbersome sample preparation procedure and other techniques need to maintain highly sophisticated equipment are not realistic for quick detection of viruses. Therefore, in commercial standpoint of view need such type technique which is rapid and easy to detect the viruses.

Over the last decade, increasing effort has been put into for the development of novel analytic sensor. In present days biosensor based on the metallic nanoparticles are frequently used for the development of a noble analytic device, which can enable faster detection and reproducibility in much better way. The detection is mainly carried out by either through colorometric based or chip based nanobiosensor (Wang *et al.*, 2012a).Gold nanoparticles posses a distinct optical and physical property, which can make them excellent scaffold for biological and chemical sensing. First it can be easily synthesized in a simplest way and highly stable in any environment. Second they possess an electromagnetic property, third they are having high surface to volume ratio and biocompatible in nature and final most important property is optical property which can make them a unique novel metallic nanoparticles (Saha *et al.*, 2011).

In this background Centre for Plant Biotechnology and Molecular biology (CPBMB) took an initiative for the development of an antibody-based nanobiosensor for the quick detection of *Banana bract mosaic virus*, in banana with the objective of to develop an antibody based nanobiosensor for quick detection *of Bract mosaic virus* in banana

The study will enable rapid analysis of an immense number of samples for selection of disease free planting materials from mother plants and in the supply of virus free plants to farmers. The technology will be highly useful for virus indexing of tissue cultured planting material of banana and also for plant quarantine measures.

Review of literature

2. REVIEW OF LITERATURE

Banana (*Musa sp*) and its plantain is a major horticulture crop produced over a large region of the world. It is the foremost necessary fruit of Asia and pacific, which provides stable food and income over a large number of farmers. Banana also played an important role in the agricultural economies of the other countries of the world (Jones, 1995).

Banana bract mosaic disease(BBrMD) has been recently described as a viral disease of banana and incidence of disease reduces the yield from 5 to 36 per cent, more in cv. Nendran in Kerala (Kiranmai *et al.,* 2005).The infected plants produce very small bunches with curved brittle fruits. Very severely affected plants may fail to flower and may die by stunted growth and necrosis of the pseudo stem (Selvarajan and Jeyabaskaran, 2006). Serological technique (ELISA) based on antigen and antibody interaction and PCR method is most commonly followed for detection this virus (Sharman *et al.,* 2002).

In present day, biosensors depend on localized surface Plasmon resonance gains much more importance and show significant advances over the traditional detection techniques, including high sensitivity refractive index, fast sensor response, real-time detection, and a label-free detection of viruses. The objective of the present study was to develop an antibody based nanobiosensor for quick detection *of Bract mosaic virus* in banana. The relevant literature regarding various aspects of this study is reviewed in this chapter.

2.1. Viral diseases of banana

In recent year increasing the international movement of banana germplasm mostly in the developing countries in the form of micro propagated plants lead to spread of a new virus or strain in large number of areas. Banana is generally affected by five well known characterized viruses: *Banana bunchy top virus* (BBTV), *Banana*

bract mosaic virus (BBrMV), *banana steak mosaic virus* (BSV), *banana cucumber mosaic virus* (CMV), *Abca mosaic virus* (AMV).Other than those a new filamentous virus (die-back virus) has been reported in bananas from America and African countries which significantly contributed the yield reduction in of banana (Lockhart, 1995; Thomas *et al.*, 1997; Hughes *et al.*, 1998).

2.1.1. Banana bunchy top disease (BBTD)

Bunchy top disease in banana is one of the most economically important viral diseases of banana, which causes up to 100 percent yield reduction. The first incidence of this virus was first reported in Fiji in the year of 1989 where banana cultivation was completely destroyed (Magee, 1927; Simmonds, 1934; Taylor, 1969).

In the year of 1913the virus was introduced into a Sri lanka and later in southern India by 1940 and gradually it covered entire country by the end of 1970 (Wardlawa *et al.*, 1972).

The virus is mainly transmitted by aphid and characteristic symptoms are appearing 26 days after infection. BBTV is a single sanded circular DNA virus, designed as a DNA U3. R. M, S, N and C with aapprox size of one 1 KB (Harding *et al.*, 1991; Burns *et al.*, 1995; Vetten *et al.*, 2005).

It was observed that BBTV causes gradual reduction in hill banana cultivation from 18000 to 20000 ha (Kesavamoorthy, 1980; Selvarajan *et al.*, 2010; Elayabalan *et al.*, 2013)

Banerjee *et al.*(2014) identified a new isolate of BBTV in Meghalaya region of the country. The characteristic symptoms of the disease are dark green colour streaks in leaf, vein, petioles and midribs with a cluster of leaves looking like a bunchy appearance.

2.1.2. Banana bract mosaic virus (BBrMV)

Banana bract mosaic virus (BBrMV) locally known as *Kokkan* disease causes serious threat to banana production and it spreads at a high rate in almost all the banana varieties cultivated in Kerala, India. The disease was first reported from Philippines by Magnaye (1979) and occurrence of this virus spread frequently in few other countries like Asia, south pacific, including India, Srilanka, Vieatnam. In India it was first reported Chennai and Bangalore in 1992 (Jones, 1992) and Kerala by 1996 (Samraj *et al.*, 1966).

BBrMV mainly developed characteristic spindle shape, purple coloured streak on bract and pseudostem, even in leaf and cause minor to severe yield reduction in banana (Rodoni *et al.*, 1997). Cherian *et al.* (2002) observed that in banana due to BBrMV yield reduction in cv. Robusta (AAA) (70%), followed by cv. Nendran (AAB) (52%).

Naturally the main host of BBrMV is *Musa* spp. and is mostly grown in the Philippines (Sharman, Gambley, Oloteo, Abgona, & Thomas, 2000). The other natural host includes small cardamom in India (Siljo *et al.*, 2011) and ornamental ginger plants (*Alpinia purpurata*).Recently, the occurrence of this virus in a commercial farm was recorded in Ecuador (Quito-Avila *et al.*, 2013).

BBrMV primarily transmitted through the infected planting material and in a non persistence manner through several aphid species viz., *R.maidis, P. nigronervosa, A.gossypii* (Magnaye& Espino, 1990).BBrMV particles are filamentous and flexuous with a (+)ss RNA as its genome (Bateson & Dale, 1995). The genome contains 9710 nucleotides and a typical large ORF of 9378 nucleotides that code for a poly protein of 3125 amino acids, which consist of a 9 cleavage sites for protease that potentially yielding 10 matured functional proteins that have motifs conserved among homologous proteins of other potyviruses (Balasubramanian & Selvarajan, 2014).

2.1.3. Banana streak disease

Banana streak disease cause chlorotic streak on infected plant and is known to be most widely infected virus among the banana and its plantain. The virus belongs to the genus Badnavirus, family Caulimoviridae. The virus particles are in bacilliform shaped and its genome contain circular double stranded DNA with genome size approximately 7.2–7.8 kb long (Harper &Hull, 1998). The disease was first observed in Nieky Valley on the Ivory Coast in 1958 (Lockhart & Jones, 2000).

BSV infect different species of *Musa* and its plantain produces discontinuous yellow dots/streaks that turn necrotic on the leaves and also pseudo-stem splitting. It was estimated that yield loss ranges up to 90% on Poyo (AAA, Cavendish subgroup). BSV is not mechanically transmitted and spread by the mealy bug in semi persistent manner (Daniells *et al.*, 2001).

2.2. Quality analysis of viral protein by SDS-PAGE

Purified BBTV protein was analysed in SDS-PAGE and visualized by silver staining (Wedrychowski *et al.*, 1996).

BBTV protein were denatured in 2 percent SDS and five percent 2-mercaptoethanol and analysed in discontinuous SDS-PAGE. The separated protein was stained with silver nitrate (Blume *et al.*, 1996).

Wu and Su (1990) determined the relative molecular weight of BBTV coat protein by SDS-PAGE. The molecular mass was estimated by relative mobility in the gel.

Thomas *et al.*(1997) determined the relative molecular mass of BBrMV coat protein subunit by SDS-PAGE. The major protein band with estimated size 31, 37 and 38 kDa were obtained.

Estelito (1998) analysed protein derived from midribs, leaf shoot and rhizome of healthy and BBTV infected banana plants by SDS- PAGE, and found that infected tissue contain extra cellular protein in the midrib portion compared to the healthy one.

Oben *et al.* (2015) isolated the CMV coat protein and analysed in SDS-PAGE and found that special 29 kDa band was obtained in CMV infected sample compared and no bands were observed in healthy sample.

2.3. Methods of virus detection

It was well known that plant species which are propagated vegetatively may be infected by many plant viruses or virus like particle, which over a time induce severe detoriation in the health of a plant. Therefore, one of the most important ways to control these viruses is by the identification, development and propagation of virus-free (VF) clones. Therefore the identification of such clones requires reliable, quick detection and sensitive method in order to ensure disease free planting material. There are three main groups of diagnostic method, i.e., bioassays, serological, and molecular methods.

2.3.1. Bioassays

Biological testing means grafting of planting material into indicator plant and symptom that eventually developed in later stages is compared with positive or negative samples. Normally this method is time consuming and need a green house facility for keeping sample, but in some certification system it is still followed mainly in the case of grape vine certification system (Martelli, 1999). Biological indexing is normally applied in combination of serological or molecular technique.

2.3.2. Serological

Serological methods, which are mainly based on antigen and antibody reaction, are the most common method followed for checking sanitary status of the plant or animal. Even though many serological methods have been developed like DIBA, western blot. ELISA is the most common method i.e. followed and significantly affects the plant virus diagnostic by shortening the time needed to get results, simplifying detection and allowing the testing of large numbers of samples.

2.3.2. a. ELISA

Gonsalves *et al.* (1986) performed DAC-ELISA with partial purified antigen for detection of *cardamom mosaic virus* and they observed virus can detect at dilution range from 10^5-10^6.

Mariappan and Mathikumar (1992) found that direct antigen coating ELISA is a best method of detection BBTV pure antigen at even a 1: 10000 dilution.

On the basis of serological relationship Reddy *et al.*(1996)reveled that all antiserum of potyvirus could be detected BBrMV infection and it was more related *Potato virus Y.*

Choueri *et al.* (1996) mentioned that the reliability of ELISA is dependent on the concentration of antiserum that we used, mainly in case of woody perennial fruit crop where viruses affecting these crop plants are phloem-limited, appears in low concentrations. Therefore, it is necessary to collect plant sample from a part of a plant at the time of a year when infection is high.

Sing *et al.* (1996)detected BBrMV by DAS- ELISA using antiserum obtained from Philippines. Positive reaction obtained for all the infected samples.

Rodoni *et al.* (1999) compared ELISA,IC-PCR and reverse transcriptase PCR for the detection of banana *Bract mosaic virus* and the results confirmed ELISA and IC-PCR is most sensitive for virus detection.

Different types of ELISA (direct and indirect) using polyclonal and/or monoclonal antibodies have been developed and successfully applied to routine virus indexing of mostly vegetatively propagated agricultural crops (Cambra *et al.*, 2000a).

In recent times, monoclonal antibody have been successfully used in serological technique like tissue print- ELISA, which allows thousand plant sample analysis without plant extract preparation, by directly printing section of a plant material on nitrocellulose membrane (Cambra *et al.*, 2000b).

Dasanayaka and Cooper(2004) performed DAC-ELISA for the determination of antibody titer of BBrMV antiserum and identified best antiserum dilution is 1: 500 for the detection of a BBrMV.

Production of monoclonal antibodies against Citrus tristeza virus (CTV) solved the problems of specificity and increased sensitivity of ELISA tests (Boonham *et al.*, 2014).

Loconsole *et al.* (2014) used ELISA as a serological technique to detect Olive Quick Decline Syndrome (OQDS) caused by *Xylellafastidiosa.* Another serological method lateral flow dipstick immunoassay now a days are widely used, which are more sensitive than ELISA.

2.3.2. b. Dot blot analysis

Dot blots analysis a simple diagnostic assay where virus is detected on nitrocellulose membrane. The procedure is same as ELISA, but in this case crude sample doted on to a nitrocellulose membrane where in case of ELISA samples were coated on a solid support.

Are (1989) reported that among the serological method developed for detection of a sugarcane mosaic virus DIBA is best and provide quick detection of virus, within in three hour around 40-60 samples were analysed.

Noda *et al.*(1989) detect the presence of LYSB in a sample using a DIBA on a nitrocellulose membrane. The results found that use of Phosphate buffer saline pH-7.4 for extraction gave good reaction and use of PBS-T as a washing buffer removed green colour from a doted area without reducing the colour intensity of a positive reaction of an infected samples dilution up to 1: 1000.

Raizada *et al.* (1991) used ELISA, Immunosorbant electron microscope and DIBA for detection of a *Yellow Been Mosaic* in faba bean seed and they observed there is a clear correlation between the three techniques.

Selvarajan(1997) used DIBA for detection of BBrMV and result found that there are clear blue dots observed on nitrocellulose membrane in infected sample and no blue colour dots were observed in healthy samples.

Selvarajan(2000) compared the results between DAC-ELISA and DIBA and they observed that DIBA is best for detection of a BBTV compared to ELISA.

2.3.3. Molecular methods

Molecular methods of detection mainly focus on viral nucleic acid detection. Among the molecular methods of detection developed PCR based methods were widely followed. The primary benefits of the PCR based methods of detection are reliability and specificity, especially at low concentrations of virus load. The different variants of PCR have been developed and successfully used for virus detection in of a vegetatively propagated plant species for example, nested RT-PCR, immune capture RT-PCR, or multiplex PCR assays.

Thomas *et al.* (1997) amplify the specific coat protein gene of a *Bract mosaic virus* in banana by reverse transcription assay using Potyvirus specific degenerated primer.

Multiplex PCR is a modification of a normal PCR which are capable of amplifying the many genes simultaneously at a time and can be valuable for certification system by reducing the time, cost and labour needed for virus indexing Sharman *et al.*(2000) detected three banana virus by multiplex PCR.

Marie *et al.*(2008) developed reliable single step IC-RT-PCR for the detection of a BBrMV, which are able to detect this virus before the introduction of RT-PCR by introducing a specific set of oligos. These primers were tested for Indian and Philippines BBrMV- infected isolates.

Recently, quantitative PCR has been developed and widely used in many laboratories. This technique mainly based on the fluorescence signal generated during DNA amplification in a tube, gave higher specificity and had less contamination than the conventional PCR method. (Siljo and Bhat,2014) developed loop-mediated isothermal PCR assay, which was highly sensitive, and the detection limit was 100 times more efficient than the conventional RT- PCR.

Wu *et al.* (2016) developed loop mediated fluorescent based real-time assay for the detection of a Huanglongbing (HLB) a destructive disease of citrus which are caused by bacterium *Candidatus Liberibacter asiaticus* (Las)' in China and the sensitivity of this assay was found approximately 1 pg/ml template DNA.

2.4. Biosensor

Biosensor is the bio analytic device that has a molecular recognition entity which is associated with a physical transducer. The rapid, easy and quick detection of pathogen or molecule are the major problem associated with the biosensor. Recently, few diagnostic platforms are using dimension of a nanomaterial called nanobiosensor,

which can be detect and measure the bioanalyte with high sensitivity. In such type of biosensor nanoparticles are used as a transducer (Tothill, 2009).

The main function of biosensor is to detect the bioanalyte like antibodies, proteins, enzymes, immunological molecules, and so on. This is done by the bioanalyte called bioreceptor which has affinity to the target. Second component is the transducer, which converts the interaction of bioreceptor and bio analyte into electrical signal. The third component is the detector which amplifies the signal that is coming from the transducer, so the corresponding response can be readable and studied properly (Haun*et al.*, 2010).

Huang *et al.* (2011) developed biosensors based on grapheme molecule for the detection of bacteria, where grapheme molecules are immobilized with antibody of E-coli and significant changes were observed in the electrical conductance of graphene based device, while it comes into contact of *E-coli* at a concentration of 10 cfu/ml while no changes are observed in case of another bacterium with higher concentration.

Zeng *et al.* (2013) reported biosensor based on the SPR for the detection of a maize chlorotic mottle virus. Gold nanoparticales is modified with 11-Mercaptoundecanoic acid to form self-assemble monolayer and a layer of an anti-MCMV antibody is cross-linked with the SAM layer and the study found sensor was capable of detecting dynamic range of 1 to 1000 ppb antigen.

Izadib *et al.* (2016) developed an electrochemical DNA-based biosensor for the detection of *Bacillus cereu.* In this experiment gold nanoparticles were modified with PEG is self-assembled with single stranded DNA (sedan) of *rhea* gene which can covalently bind to the gold nanoparticle by a thiol group and detection was carried out by changes in the charge transfer resistance of a biosensor while hybridization with the complementary sedan.

2.5.Nanobiosensor: Variations and Types

The classification of nanobiosensor is very diverging. It is mainly based on the nature of Nanomaterials incorporated in the biosensing operation like nanotube, nano wire, and nanorod. More over the classification of biosensor as compared to the nanobiosensor is not much easier. In case of biosensor, it was classified as a electrochemical, colorimetric, optical, and acoustic type of sensor. Each of these categories includes diverse range of classes of sensor.

Spadavecchia *et al.* (2012) reported changes in the size, shape and as well as linear arrangement GNRs after bioconjugation to an antibody. These bioconjugate Goldnanorods are used in model Fourier Transform based immuno sensor (FT-SPR) for the detection of target and result show sensitivity of sensor increased by 8 fold.

Yang *et al.* (2013) developed labeled free electrochemical based biosensor based on the wheat-germ agglutin lectin as a molecular recognition element for determination and discrimination of an Alfa feroprotein (AFP). The EIS biosensor was fabricated by absorbing the lectin molecule on the surface of the carboxyl-functionalized single-wall carbon nanotubes (SWNTs) and results shows detection limit up to 1 to100 ng/L.

Nayak *et al.*(2015) reported light dependent fiber based biosensor based on the gold(Au)nanoparticle coated with graphene oxide sheet for the detection of sucrose. Au nanoparticle coated with the GO in a fixed ratio to prevent the aggregation of Au particle, which was broaden the absorbance spectra by more than 25 %. The Go encapsulated AuNPs immobilized on the core of the optical fiber was subsequently used for the sensing of sucrose.

2.6. LSPR based nanobiosensor

Localized surface plasmon resonance (LSPR) is an optical phenomena generated by light when it interacts with conductive nanoparticles (NPs) that are

smaller than the incident wavelength. As in surface plasmon resonance, the electric field of incident light can be deposited to collectively excite electrons of a conduction band, with the result being coherent localized plasmon oscillations with a resonant frequency that strongly depends on the composition, size, geometry, dielectric environment and separation distance of NPs. Due to its characteristic LSPR band, it show different optical property according to the size of the particle (Lu *et al.*, 2009).

Sharma *et al.* (2009) and Huang *et al.* (2009) demonstrated that metallic nanoparticles of different shape, size and metal show a different absorption property and thus it's may appear in a different colors. Such as example seen in the LSPR plasmon band of a spherical nanoparticle, where one absorption band can be seen in UV-Vis spectra (Fig 1). But in case of GNR two absorption bands are observed in its absorbance spectrum, one is longitudinal plasmon band (LPB) and another one transverse plasmon band (TPB),due to oscillation of electron in short and long axis of GNRs (Fig 2).

The transverse plasmon band of gold nanorods has been found not be sensitive to changes in the size of the gold nanorod, where as the longitudinal plasmon band very sensitive to change in the size of the particle, with increasing in the aspect ratio of GNRs red shift was observed in the longitudinal plasmon band (Lin *et al.*, 2011).

Hall *et al.* (2011) reported antibody-labeled gold nanoparticles increase LSPR peak shift 40, when it comes in contact with the analyte as compared to the native antibody concentration.

(Petryayeva and kortil 2011; Le *et al.*, 2012) reported that commonly novel metal like Ag and Au are used for production of the nanomaterial due to their LSPR phenomena in visible ranges. Despite the fact Ag display strongest and sharpest LSPR phenomena among all the metals, Au particle was more preferable due to its

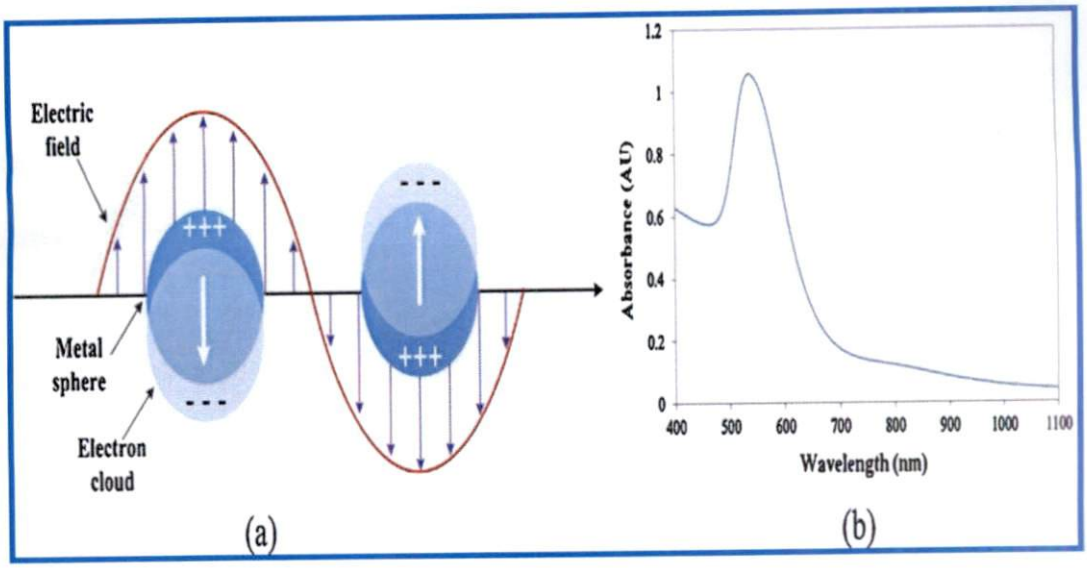


Fig 1. (a) Schematic presentation of LSPR excitation for GNSs. (b) A typical LSPR absorption band of GNSs. (Willets *et al.*, 2007)

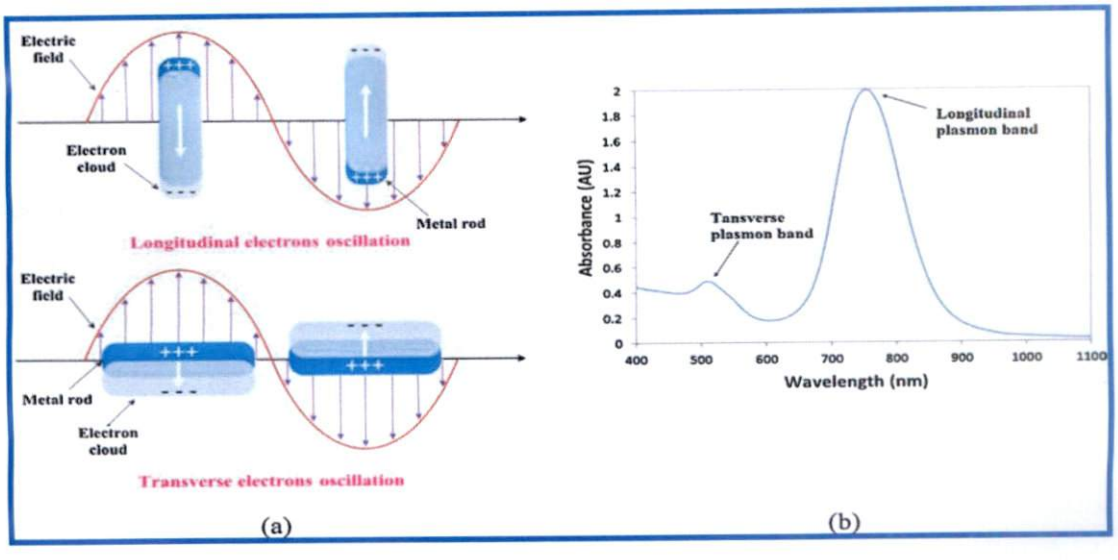


Fig 2. (a) LSPR excitation of GNRs (b) longitudinal and transverse Plasmon bands corresponding to the electron oscillation along the long axis of GNR. (Willets *et al.*, 2007)

inhabitant nature and biocompatibility and gold thiol association for binding of biomolecules.

2.7. Gold nanorod based LSPR biosensor

LSPR based biosensor work is based on the interaction between light and metallic nanoparticle .The sensor configuration for both GNS and GNR are almost similar, gold particle can be immobilized in the transparent substrate like glass substrate or just simply leaving the functional nanoparticle in suspended stage in a solution, detection of a target place. In general gold nanorod based LSPR biosensor can be classified into three categories chip-based, optical fiber-based and solution-phase-based LSPR biosensors (Table 1).

Guo and Chim (2012) demonstrated sensitive approach for the detection of a protein, based on the LSPR peak shift induce by aptamar- antigen- antibody complex. The applicability is demonstrated on detection of a alfa thromb protein, where receptor of a alfa thomb protein is immobilized on the gold nanorods, caused a measureable shift along with the antigen.

He *et al.* (2013) various gold nanorods with different aspect ratios were used to fabricate the optical sensor. Five kinds of gold nanorods with different aspect ratios were chosen to construct five throughputs of MLSPR. Various LSPR peaks imply that different acceptor–ligand pairs can be detected simultaneously in the wavelength range from 530 to 940 nm.

De *et al.* (2013) used Surface of the TGNPs is easy applied to the further modification. The subsequent bioconjugation of TGNP films with goat anti-mouse immunoglobulin G (anti-m-IgG) is successfully employed for the detection of mouse-immunoglobulin G (m-IgG) in a model based on the specific binding affinity between the antigen and antibody. The spectrophotometre sensor shows concentration-dependent binding for m-IgG

Table1: Characteristics of GNR-based LSPR sensors with different configurations.

Sensor configuration	Characteristics	LSPR sensing method	Detector	Ref.
Chip-based	GNRs immobilized on a silica substrate	Based on refractive index change at GNR surface	Bulk UV–Vis spectrophotometer	Willets *and Van (* 2007)
Optical fiber-based	GNRs immobilized on a decladedopticalfiber	Based on refractive index change at GNR surface	Mini spectrometer	Chen *et al.*, (2008)
Solution-phase-based	GNRs suspended in solution	Based on refractive index change at GNR surface; based on GNRs aggregation	Bulk UV–Vis spectrophotometer	Yu and Irudayaraj (2007)

2.7.1. Chip based LSPR biosensor

Most probably biosensor based on chip based configuration frequently used for making LSPR based sensor. This was fabricated by immobilization of gold nanorods on silica substrate such as glass slide, cover slip *etc* (Gole and Murphy, 2005). There are two main methods for gold nanorod immobilization. The first method is electrostatic force, where substrate is dipping into the poly electrolytic solution, which makes the substrate charge opposite to the gold nanorod. Then the charged substrate deep into the gold nanorod solution where, due to electrostatic interaction GNRs bind to the clean substrate. However this method suffers from poor

stability and uniformity (Marinakos *et al.*, 2007). A second method is SAM technique, where glass slide is first immobilized with the alkyl silane solution, such as MPTMS(3-mercaptopropyl trimethoxysilane) to form thiol terminated saline group or SAM layer on the glass substrate, subsequently the saline substrate is dip into the gold nanorods (GNRs) solution and due to covalent interaction gold particle bind to the glass substrate and further can be modified by with various receptors for target detection (Huang *et al.*, 2009).

Chemnasiria and Hernandez (2012) reported the gold nanorods mercury sensor. The immobilization of the gold nanorod on the glass substrate by covalent binding of the gold nanorod to the mercaptopropanoic acid modified glass slide. Then they demonstrated that mercury sensor, using immobilized gold nanorods were much more sensitive than the solution based sensor, which are capable to detect the mercury level at very low concentration. Fig 3 shows the schematic diagram of the gold nanorods immobilization.

Wang and Tang (2013) reported excessive CTAB can adversely affected assembly of GNRs to the substrate due to electrostatic shielding affect to stabilize the colloidal gold solution so the attraction of nanoparticles to the substrate become secondary .

Chena *et al.* (2014) developed effective strategies for immobilization of gold nanorod on the ITO glass slide without using any binding molecule, they directly binding the gold nanorod on the glass slide during gold nanorods preparation procedure or during growth of the gold nanorod. The connectivity of gold nanorod on the glass slide confirmed by SAM image and Goldnanorods (GNRs) occupy a dominant position on the glass slide. It shows both transverse and longitudinal peak.

Wang and Tang (2015) used gold nanorods-based biochip as a multiplex sample analysis. The aspect ratio (AR) dependent LSPR spectra phenomena provide the ideal multiplex mechanism. GNRs of selected sizes can be combined to ensure

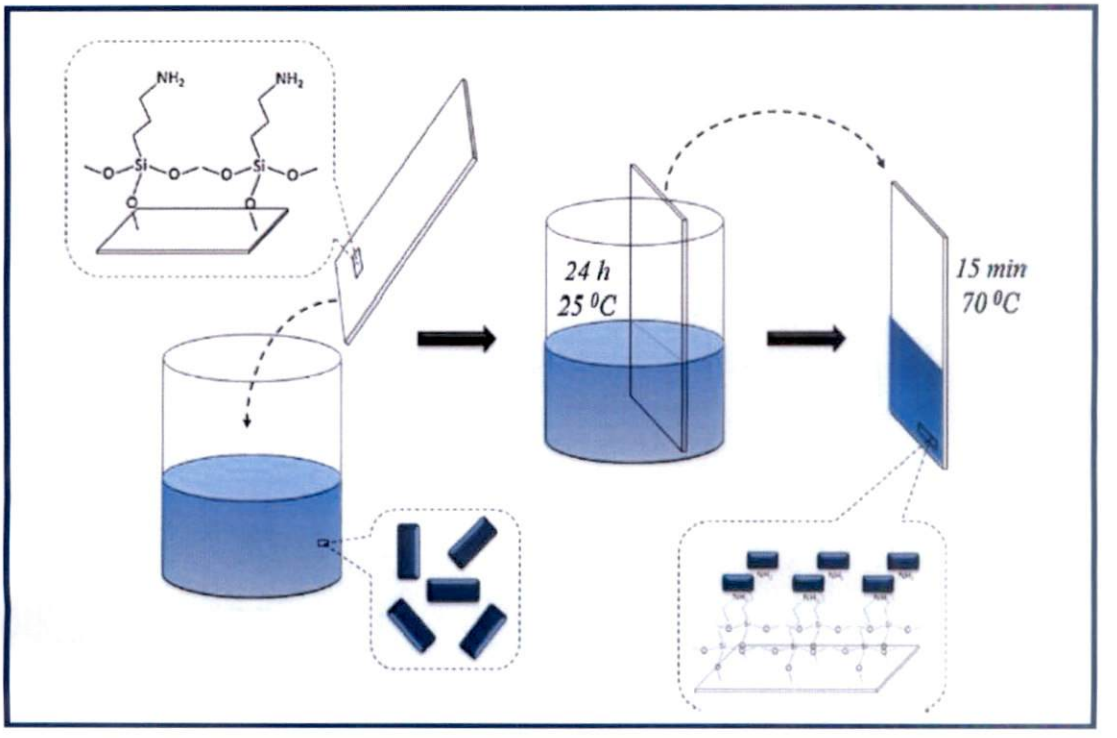


Fig 3. Schematic diagram of functionalize Silane coated glass with GNRs

distinct plasmon peaks in absorption spectrum. Monitoring the spectral shift at the dedicated peak allow the simultaneous detection of a multiple sample. They normally used gold nanorods of aspect ratio (AR) from 2.6 to 4.5.

2.7.2. Optical fiber-based LSPR sensors

Optical based LSPR sensor show many advantage over other, including small volume of sample needed, simplify optical deigned, capable to do remote sensing, resistance to electromagnetic interference. In 2003 Cheng and Chau first developed optical fiber based sensor based on gold nanoparticle. The sensor showed high sensitivity with local refractive index changing and capable to working as a biochemical sensor. Since, then there are so many LSPR based optical fiber sensor have been developed and used for various applications. In most of the cases gold nanoparticles are immobilized on the core of the optical fibre. (Mayer *et al.*, 2008).

Crosby *et al.* (2013) developed fiber optic based sensor using a gold nanorods as a sensing material for the detection of atmosphere elementary mercury. Mercury readily adsorbs on the nanoparticles causing a measurable shift in the longitudinal localized surface plasmon resonance. Depositing the nanorods on the surface of a bare, bent fiber optic cable provides a means to excite the resonance and determine the absorbance through the evanescent wave at the surface. The response of the system is linear with concentration, and they have been able to directly measure concentrations down to 1.0 g/ml.

Cao *et al.* (2013) developed gold nanorod (GNRs) based LSPR sensor. They immobilized the gold nanorods that are prepared by seed mediate method to the core of the optical fiber. In a test carried out for checking the reflective index of sensor. Result showed the reflective change of the gold nanorods is more sensitive than the gold nanospheres.

Lin *et al.*(2014) developed a fiber optic Plasmon resonance based immuno sensor for the labeled free detection of a orchid virus using a gold nanorod as a sensing material and they reported that GNRs create a window to solved the colour interference problem of sample matrix for direct detection of a target analyte, but in case of gold nano spheres, this work cannot be achieved because the signal of sample color absorption largely overlaps the signal of molecular recognition events in the visible spectrum, making the signal interpretation much more difficult.

2.7.3. Solution-phase-based LPSR sensor

Solution based LSPR based biosensor are those, which gold particle are suspended in solution rather than immobilized on the substrate (Potara *et al.*, 2011). Which allows surface modification of gold particles and as well the detection was carried out within in the solution itself. The fabrication of such type sensor often involves by gently mixing the nanoparticle with functional molecule and by multiple centrifugation process purifies the functional nanoparticle. The analyte detection was normally carried out by in a small cuvate by UV-Vis spectrophotometer.

Wang *et al.* (2012b) demonstrated binding of an analyte to a nanoprobe, where local reflactive index are change when target bind to a immuno capture probe. They also reported this nanobiosensor can be detecting wide range of analyte in Tris buffer ranging from 0.01 IU/mL to 1 IU/mL, which cannot be detected in ELISA.

If in case of gold nanorods were used for the development of LSPR based sensor, there are mainly two type assay are used for the detection one is based on the reflective index changing and another one based on the aggregation of the GNRs .

Wang *et al.*(2013) detect IgG of human through aggregation of gold nanorods based on the interaction driven by antigen-antibody. The surface of the CTAB capped Goldnanorods first modified into a negatively charge by the use of polyelectrolyte PSS. Such negatively charged GNRs then functionalized with the positively charged

antibody through electrostatic reaction. The aggregations of functionalized GNRs were observed after the addition of human IgG, which resulted in the red-shift of the longitudinal peak and subsequently broaden of peak.

Lesniewski *et al.* (2014) reported colorometric based nanobiosensor for T7 bacteriophage based on the gold nanoparticle modified with T7 bacteriophages antibody. The T7 virion causes immunological complex resulted in aggregation of a nanopartical, which can be visually identify by seeing the changes in colour from red to purple colour as well as with a UV-Vis spectrophotometer.

Moghadam *et al.*(2015) reported visual detection of lysozome through heat induced aggregation of gold nanorods. Exposure of nanoprobe to a nanomolar level of lysozyme leads to direct aggregation of nanostructure at optimum temperature, which was greatly improved by introduction of small time exposure of heat.

2.8. Kinetics of GNRs along with analyte

Soman and Giorgio (2009) studied kinetics of quantum dot-antibody conjugates with different concentration of analyte at very low concentration for finding the aggregation pattern nano assembly; they found that in time interval aggregation percent of nanoparticle increase and after a particular time the kinetics curves remain flat.

Weng *et al.*(2013) demonstrated kinetics of gold nanoparticles for detection of a Cu^{2+} ion, they observed that with time interval due to formation of new core assembly of nanostructure with gold nanoparticle and Cu^{2+} ion, the kinetics curve of a Cu^{2+} ion of a sample increased over a time and after some time curve relatively remain flat. In contrast of kinetics curve of a blank sample remain flat over a time.

Song *et al.*(2013) conducted study for obtaining the kinetic adsorption curves of rabbit anti-transferrin at different concentration of transferring and they observed

that LSPR peak shift at different concentrations changed and kinetics curve after some time remain flat.

2.9. Synthesis of GNRs

The successful development of LSPR based biosensor depends upon the accurate synthesis gold nanorod. The history of synthesis of spherical gold particles day back before. The most common method followed for gold nanorod preparation is by citrate reduction process, where aspect ratio can be maintained by adjusting the ratio between the citrate and the gold salt. However the synthesis of gold nanorods appears to be very complicated and only the successful and reliable method of GNR synthesis was achieved in the past decade. There are so many methods have been developed and commonly followed, some of them are follows.

2.9.1. Seed-mediated growth method

Among the various methods of gold nanorod preparation reported seed mediate method are most popular, due to its simplicity of experiment and high yield nanorods and easy to control the size and flexibility of structure modification.

The seed mediate growth approach for gold nano rod preparation was first demonstrated by Jana *et al.*(2001a). In this method first seed solution was prepared by reduction of gold salt by $NaBH_4$ in the presence of sodium citrate, which leads to the development of a citrate capped GNS about diameter of 3-4 nm and after that seed solution was added into the growth solution which was contain containing HAuCl4, Cetyltrimethylammonium bromide (CTAB, as the template), ascorbic acid (as reducing agent) and $AgNO_3$ (for shape induction) to allow the growth of GNRs to occur.

Jana *et al.* (2001b) modified the method and introducing three step protocol in absence of silver ion to grow the gold nanorod with bigger size. However the biggest

drawback of this method, part of the GNR some amount of GNS is also produced, which ultimately hamper the Plasmon resonance.

Nikoobakht and El-Sayed.(2003) create a significant modification, first they replace the sodium citrate with CTAB, that give the stronger stability to the seed solution, next controlled the silver ion concentration in a growth solution to control the aspect ratio (AR) of the gold nanorods (GNRs). To synthesize gold nanorod with highest aspect ratio they introduce additional surfactant benzyl dimethyl hexa decyl ammonium chloride (BDAC) to growth solution. By adjusting the concentration of silver ion in growth solution, GNRs of aspect ratio 10 had been achieved.

Murphy and his coworker in 2005 reported an improved method of gold nanorod synthesis by using a seed mediated growth and aromatic additive and low concentration of CTAB. This approach was shown to produce GNRs with a broad LPB tune able range, but lower leveled of impurities. However, while preparing the growth solution, an appropriate amount of sodium salicylic acid can give the LPB less than 700 and addition of organic acid give LPB (longitudinal plasmon band) more than 700 nm (Ye *et al.*, 2007).

Danielle and Brian (2008) conduct a study where they taken different company of CTAB for the synthesis of GNRs and the result found yield of nanorod strictly depend upon the purity of the CTAB.

The effect of iodide impurities within the CTAB is significant. Research have found when CTAB contains iodide impurities, GNRs do not form (Smith *et al.*, 2008).

The effect of the addition of silver nitrate is as critical as the use of CTAB in the synthesis process. The addition of silver nitrate allows for the stabilization of the synthesis process and increases the yield significantly (Li *et al.*, 2008).

Christopher *et al.*(2014) reported that HAuCl4 concentration during seed solution preparation significantly affect the morphology of the final rod morphology. Higher concentration resulted in larger seed and smaller aspect ratio of nanorods.

Wang *et al.*(2015) reported the facile green synthesis of gold nanorod by using epigallocatechingallate (EGCG) extract from tea leaves as a reducing agent and stabilizing agent in aqua solution to produce gold nano structure through seed mediate approach.

Mahmoud *et al.* (2016) reported stability of gold nanorods solution upon exposure to the human skin. They found positively charged nanopartical form aggregation when it comes to the contact of the human skin as compare to the negatively charged gold nanorod. They analysed its happened due to the secretion protein from the dermis layer of the skin.

2.9.2. Electrochemical method

Despite the seed mediated growth method, the most commonly followed for gold nanorod synthesis. The preparation of high yielding gold nanorod solution first demonstrated by electrochemical method, this being seen as the precursor of the seed mediate growth method (Yu *et al.*, 1997).

Chang *et al.* (1999) reported the electrochemical method of gold nanorod synthesis, where gold metal plate is used an anode and platinum plate as a cathode are immersed into an electrolytic solution containing a rod-inducting cationic surfactant CTAB and a cationic co-surfactant tetra dodecyl ammonium bromide (TCAB) and a small amount of acetone and cyclohexane was added before starting of a electrolysis. During electrolysis bar gold anode consumed as an AuBr4 −, which then form complexes that are migrating to cathode where reduction process going on.

Jian *et al.*(2003) conducted an experiment and they have taken absorption spectra at different time interval of the electrolysis process, demonstrated that gold nanorods began to grow after the finishing of the electrolysis process.

2.9.3. Template method

The template method GNRs based on the electrochemical deposition of gold within the nonporous etched polycarbonate or an alumina membrane template. Martin and coworkers in 1996 first employed this method for synthesis of Goldnanorods. The template was prepared by anodization of almonium in an acidic solution which result formation of pore of diameter of 5-100 nm. The gold nanorod formed by electrochemical deposition in the pore and followed by chemical etching of anode to release the GNRs.

The length of the rod created it coincide with the width of the nanorod, that the length of the nanorod could be controlled by varying the concentration of the gold that is used (Van der Zande *et al.*, 2000). The disadvantages of this method were reported to include a variation in the length of nanorod, due to the uneven deposition of gold and further low yield of gold nanorod (Vigderman *et al.*, 2012).

2.10. Estimation of aspect ratio(AR)of gold nanorods

The aspect ratio (AR) is defined as the length over the diameter of the GNR. The shape of the particle causes the absorption and light scattering spectrum to have two peaks: longitudinal and transverse. The interaction between the two dipoles causes the longitudinal peak to be observed in the visible to NIR region. The longitudinal peak is much more intense than the transverse peak and can be tuned by changing the aspect ratio of the gold nanorods (Jun *et al.*, 2006). As the AR of the gold nanorods is increased, the longitudinal peak shifts into the NIR (Near infrared region) region

The optical properties of GNRs have successfully been modeled using Gans theory and Discrete Dipole Approximation (DDA) (Jain et *al.*, 2008). Two equations were derived using the two theories to output the longitudinal peak wavelength by inputting a given aspect ratio(AR).

The two equations can predict the AR of GNRs using the absorption data collected from the UV-Vis spectrophotometer. The equations can give preliminary estimation of the AR before observing the particles on a TEM.

2.11. Functionalization of GNRs

Li and Rothberg. (2004) reported the bi-layer of a CTAB which cover the GNRs, it act a surfactants for maintaining the stability and direct the growth of a GNRs.

Connor *et al.* (2005) reported GNSs covered with citrate are immensely stable in aqua solution and it can be directly used for binding of DNA with neutral pH. But in case GNRs capped with CTAB are only stable, when it suspended with CTAB and with low pH value.

In various studies, it was found that excess CTAB on a solution are cytotoxic to human cells, but however those are attached to the GNRs is non cytotoxic. Despite that free CTAB molecule can be removed by centrifugation, those are sticking to GNRs will leads to serious accumulation and settle down of GNRs which ultimately hamper the conjugation process. Hence, it is necessary to switch the surface of the GNRs, so as to increase the application like biosensing, imaging, and drug delivery (Huang *et al.*, 2006).

2.11.1. Surface covering

Aims to introduction of an supplementary coating on the cover of the CTAB, make positively charged GNRs to negatively charged molecule with the help of an anionic polyelectrolyte's through electrostatic absorption such as example, poly

(sodium- 4-styrenesulfonate) (PSS), which not only switches the positively charged molecule to negative, but it can also enable antibody to attach to GNRs (Durr *et al.*, 2004)

Poly acrylic acid (PAA) is another polyelectrolyte agent which can cover the CTAB capped GNRs and COOH group of PAA allows protein and other amine terminate biomolecules to covalently bind to the GNRs via EDC/NHS (1-ethyl-3-(3-dimethylaminopro-pyl) carbodiimide, N-hydroxysuccinimide) linking chemistry (Gole and Murphy, 2005).

Surface changes of GNRs by the hard organic material such as silver, copper can achieve. Gorelikov (2008) reported attachment of a silver ion on the surface of the GNRs by reducing of the silver chloride solution with the hydroxylamine in presence of GNRs.

Perez-Juste *et al.* (2009) coating the citrate capped GNSs and CTAB capped GNRs with the silica molecule. The silica coating is done by simple addition of sodium silicate to a solution consist of gold nano spheres or GNRs with 3-mercaptopropyltrimethoxysilane (MPTMS) which ultimately changes in the peak position of a GNRs in UV-Vis spectra.

Xu *et al.* (2012) used EDC/NHS linking chemistry for conjugation of antibody to GNRs. GNRs first mixed with MPA(3-Mercaptopropinic acid)solution under constant sonication at 50 °c and after that use of EDC/NHS linking chemistry antibody can be conjugate and result found that considerable red shift from the original unmodified GNRs.

Wang and Tang (2013) reported that CTAB capped on the GNRs attracted the negatively charged protein which leads to nonspecific absorption of a protein, that's why there was a need to completely replace the CTAB caped on the GNRs.

2.11.2. Ligand exchange

Ligand exchange is a one more surface modification technique where, CTAB capped on the outer surface of the GNRs is to replaced by thaiol-terminate molecule. The advantage of this method includes a reduction of the cellular toxicity and influence biocompatibility GNRs when it binds to biological molecules.

Yu *et al.* (2007) reported that after binding of an antibody to GNRs a longitudinal shift of about 60 nm by physiological absorption of an antibody to GNRs.

The thiol terminate PEG is one of the common molecule that can used as a ligand for the modification of a GNRs (Pierrat *et al.,* 2007; Liao and Hafner, 2005).

The one of the most advantages of thiolated PEG is, it was a water soluble molecule, so the ligand exchange can be possible in aqueous solution. Such PEG modified gold particles are highly stable and it can possible to use directly for biomedical application. One of the disadvantages of this thiolated PEG molecule is due to larger size of the molecule it creates distance between an analyte and a PEG modified gold nanoparticle that can reduce the sensitivity when it was used as LSPR biosensors (Rostro-Kohanloo *et al.*, 2009).

In addition to the advantages, if the thiolated PEG with bi functional group, it can be used for the binding of protein and antibody to the GNRs (Vigderman *et al.*, 2009).

MUA is a another molecule which are frequently used in so many studies for which exchange the CTAB by layer on the surface of the GNRs. MUA molecule has a COOH group which are desirable for bio cognition of NH_2 terminate biomolecules like protein and antibody. However, unlike like the thiolated PEG it not dissolved in water. It dissolved only in an organic substance.

The hydrophobic nature of the MUA making a significant challenges to the surface modification of the GNRs with MUA because in the presence of organic solvent cause severe aggregation. Cao *et al.*(2012)reported the entire removal of CTAB with the help of a MUA. MUA-ethanol and GNRs solution kept under constant ultrasonication to prevent the aggregation of GNRs, along with temperature maintain at 50 ^{0}C for removal of CTAB bounded on a GNRs surface.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Development of a nano biosensor for detection of *Bract mosaic virus* in banana (*Musa* spp.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), Kerala Agricultural University and Centre for Materials and Electronics (C-MET) Mulakunnathukavu, Thrissur during 2014-2016. The objective of this study was to develop antibody-based nano biosensor for quick detection of *Banana Bract mosaic virus* (BBrMV) in banana. Materials used and methodologies adopted for the studies were described in this chapter.

3.1. MATERIALS

3.1.1.Collection of diseased and healthy sample

Banana bract mosaic disease (BBrMD) was recently described viral diseases of banana infect most of the cultivars of banana, which leads yield loss up to 5-36 percent. For the present study, the diseased and healthy samples were collected from the Banana research station, Kannara, Thrissur, Kerala. The samples showing characteristic symptoms green, reddish-brown spindle colour shaped streak along the petioles and pseudostem and mosaic pattern on leaves were stored in -80°C for long term use. The details of cultivars studied are presented in (Table 2).

3.1.2. Laboratory chemicals and glass wares

All the chemicals used for the experiment were in extra pure form and were purchased from the company like Sigma-Aldrich, Merck India Ltd, Alfa-Aesar, Nice and SRL. Hydrogen tetra chloroauratehydrate (HAuCl4. 3H2O) 99.999 % trace metal basis, 3-Mercaptopropinic acid (MPA), 1-ethyl-3-(3-dimethylaminopro- pyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), (3-mercaptopropyl) trimethoxysilane (MPTMS)was purchased from Sigma-Aldrich. Sodium phosphate dibasic (Na_2HPO4; 99%),Cetyltrimethylammonium bromide (CTAB; 99%),Sodium chloride(NaCl; 99%), Potassium phosphate monobasic(KH_2PO4; 99%) and

Potassium chloride(KCl; 99%)procured from Merck India Pvt. Ltd in an extra pure form, Silver nitrate (AgNO3; 99%) from Nice, and Sodium borohydride (NaBH4; 99%), Ascorbic acid, Ethanol ($C_2H_5O_6$; 99%), Sulfuric acid (H_2SO_4;99%), PVP, Diethyl amine purchased from SISCO Research Laboratories Pvt. Ltd. For ELISA Twin-20, p-NPP substrate and BSA purchased from Bangalore GeNei ^{TM} Pvt. Ltd. The glass wares which were used for the experiments purchased from Borosilicate Pvt. Ltd.and96 well plates ELISA micro titer plates were obtained from Tarsons Products Pvt. Ltd. Commercial monoclonal antibody of *Banana Bract mosaic virus* was procured from Agdida, USA. Pvt. Ltd. All the experiments were carried out at room temperature unless otherwise noted and MilliQ water was used in different experiments.

Table 2. Details of banana varieties studied

SI.NO	Variety	Genome	Place of collection
1	Nendran	AAB	BRS, Kannara, KAU
2	Mysore Poovan	AAB	
3	Rasthali	AAB	
4	Red banana	AAA	
5	Dwarf Cavendish	AAA	

3.1.3. Laboratory equipments

The equipment available at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture and Centre for Materials and Electronics (C-MET) were used for this study. The characterization of Gold nanorods (GNRs) was carried out by UV-Vis spectrophotometer and Hitachi H-7650 transmission electron microscopy (TEM) available at C-MET was used. Nanodrop ND-1000was used for checking the protein (antigen) concentration of a sample, BIORAD electrophoresis unit was used for SDS- PAGE analysis of protein and for ELISA VERSA max micro plate reader was used. Details of laboratory equipment used for the study are provided in (**Annexure I**)

3.2. Methods

3.2.1. Synthesis of Gold nanorods(GNRs)

Among the various methods of Gold nanorod synthesis, seed mediated method is mostly followed, where the seed solution was prepared by the reduction of the $HAuCl_4$salt in the presence of $NaBH_4$and surfactant (CTAB) which leads to a formation of gold nanospheres. This was followed by final addition of a suitable amount of a seed solution into a growth solution containing a mixture of $HAuCl_4$, CTAB, Ascorbic acid and Silver nitrate. The procedure described by Nikoobakht and El-Sayed. (2003) was followed with slight modification. The details of the procedure followed are given below (Fig 4).

A) Reagents used

CTAB- 0.1 M

CTAB- 0.2 M

$HAuCl_4$- 0.01 M

$NaBH_4$- 0.01 M

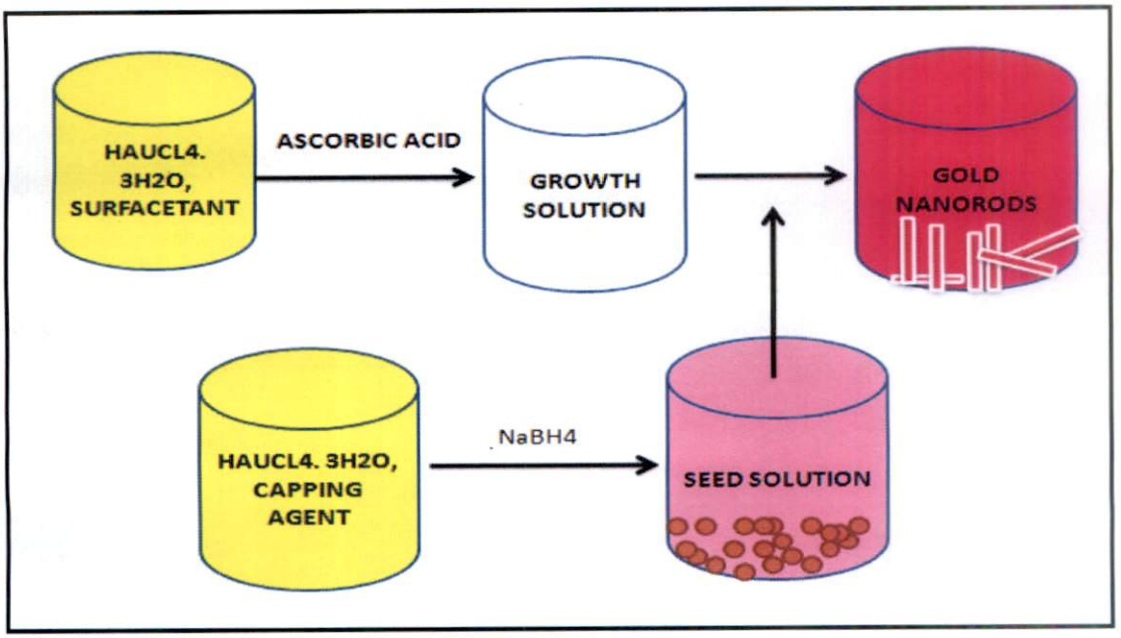


Fig 4. Schematic representation of gold nanorods (GNRs) synthesis

Ascorbic acid- 0.01 M

Silver nitrate-0.01 M

.B)Procedure

1. Seed solution was first prepared by adding 250 µl of $HAuCl_4$ into a 7.5 ml of CTAB and mixed vigorously.
2. Followed by the addition of 600 µl of ice-cold $NaBH_4$to this mixture under vigorous stirring for 2 min.
3. After stirring it was kept at 25 °C for at least 15 min before use.
4. After the synthesis of seed particles, the growth solution was prepared by mixing 50 ml of 0.2 M CTAB and 42.2 ml water and 5 ml of gold solution and mixed vigorously.
5. Followed by the addition of 600 µl of Silver nitrate and 5.5 ml of Ascorbic acid under gentle stirring at25 °C.
6. Followed by final addition of seed solution and kept for overnight for the growth of the gold nano rods (GNRs).**(Annexure II)**

3.2.2. Purification and characterization of GNRs

The primary growth solution was centrifuged at 15000g for 35 minutes in a 20ml centrifuge tube. The gravitational force on the particles causes the particles to sediment to the bottom of the tube. The difference in masses between the GNRs and the impurities results in the separation of the particles (Gao *et al.*, 2003). The GNRs move towards the side of the centrifuge tube and the gold nanospheres stay at the bottom of the tube. The impurities and the supernatant are removed and the purified GNRs were resuspended in ultrapure water.

Characterization by UV-Vis spectrophotometer

A UV-Vis spectrum of centrifuged GNRs were taken by Perkinz UV-Vis spectrophotometer at a wavelength of 200- 1100 nm, where cuvete was washed and dried prior to each measurement.

Characterization of gold nanorods using transmission electron microscope (TEM)

The centrifuged Gold nanorods solution of 10µl was taken in carbon coated TEM grid and dried for an hour. Element analysis was carried out by Hitachi2500 Delta scanning electron microscope. A total of 15 TEM images were recorded for each sample at different resolutions.

3.2.3. Estimation of Aspect Ratio (AR) of gold nanorods (GNRs)

The particle absorption is measured using a Perkinz UV-Vis spectrophotometer used for determination of aspect ratio of GNRs. The longitudinal peak wavelengths were calculated and put into the equation below for estimation of an aspect ratio. The method gives a quick estimation of the aspect ratio. The equations below are developed from GANS Theory(link *et al.*, 1999).

$$\mathrm{AR} = \frac{(Peakwavelength - 381.49)}{97.56}$$

The TEM images of the particles can calculate the size distribution and purity of the particles. TEM images, from a Hitachi H-7600 Transmission Electron Microscope, were analyzed using ImageJ software. The diameter and the length were measured for approximately 20 particles. The average of length, diameter and aspect ratio were calculated using this information.

3.2.4. Formation of Self assembly monolayer (SAM) on the surface of gold nanorods

The CTAB capped on the periphery of GNRs were completely replaced in this experiment, where layer of alkali-thiol group formed can act as a self assembly monolayer (SAM) for the binding of antibody (Song *et al.*, 2013).

A) Reagents used

MPA - 20 mM (3-Mercaptopropinic acid)

Gold nanorod solution- 1.5 ml

B) Procedure

1) From the stock solution about 5 ml of gold nanorod solution was taken and centrifuged for 30 min at 15000 rpm.
2) The supernatant was discarded and the rod-palate was dispersed in water.
3) From the centrifuged solution 1.5 ml of gold nanorods were taken and 40 µl of MPA was added.
4) The mixture of solution was kept for ultra sonication for 30 min at 50 °C.
5) The mixture of gold nanorods (GNRs) centrifuged for 30 min at 13000 rpm.
6) The MPA modified GNRs dissolved in pure distilled water.

3. 2. 5. Preparation of GNR-antibody conjugates

For covalent binding of an antibody to gold nanorods mainly EDC/NHS (1-ethyl-3-(3-dimethylaminopro-pyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS)) linking chemistry was applied, where a carboxylic acid group attached to the gold nanorods was covalently bound to the amine group of antibody. The Procedure followed was based on Song *et al.* (2013) with slight modifications.

A) Reagents used

EDC- 7.5 mM

NHS- 1.5 mM

MPA modified gold nanorods- 1.5 ml

B) Procedure

1. 1.5 ml of MPA modified gold nanorods were taken in a 2 ml Eppendorf tube
2. 100 µl of a mixture of EDC/NHS was added to this tube
3. The solution was incubated for 1 hour under constant sonication at 50 °C
4. 100 µl of antibody with different dilution (1:100; 1:200; 1:300) was added to this solution and kept for 2 hours incubation for specific binding.
5. After that 30 min of sonication was given for removing the non specific binding and GNRs were blocked using 0.02 % BSA with washing and the product are resuspended in a PBS buffer. **(Annexure III)**

3. 2.6. Immobilization of gold nanorod onto the substrate

The immobilization of GNRs on to a glass surface was done according to previous procedures with slight modifications (Huang *et al.*, 2011).The glass substrate was first modified by thiol-terminated saline group. Subsequently, the saline modified glass substrate was incubated with gold nanorods solution to form a monolayer of gold nanorods (GNRs).

A) Reagents used

Glass slides - (2.2cm×1.2 cm)

MPTMS- 5% ethanol

Piranha solution- 3 part of H_2O_2 and 7 part of H_2SO_4

Procedure

1. Glass slides were cleaned with a Piranha solution under an extreme condition at 70 °C for 30min.

2. After cooling at room temperature, the slides were washed thoroughly and sonicated for 10 min.
3. The slides were subsequently washed with ethanol and incubated with 5 % MPTMS ethanol solution for 5 hours.
4. The slides were washed with ethanol and water sequentially and air dried in room temperature for further modification.
5. The mercaptosilane-modified glass slides were finally incubated into a gold nanorod solution for 1 hour for the development of a gold nanorod chip.
6. The gold nanorod chip was characterized by UV- Vis spectroscopy.

3.2.7. Isolation of Antigen (Protein)

Isolation of antigen is a pre-requisite for the detection of BBrMV infected plant. Antigen from the infected leaf was extracted by using method developed by Gonsalves (1986) with slight modifications.

Reagents used

1. Extraction buffer
   Phosphate buffer-0.5M pH 7.4
   EDTA-0.01M
   β- Marceptoethanol-0.1%
2. Resuspention buffer
   Phosphate buffer-0.5M (pH-7.4)
   EDTA-5mM
   Urea- 0.5M
3. Acetone

Procedure

1. 100gm of fresh infected leaf samples were collected and stored at -80^0 C for 1 hour.
2. Midrib from the leaf had been removed and pulverized with liquid nitrogen in a pre-chilled mortar and pestle.
3. The pulverized tissues were then mixed with extraction buffer (1:1.5). The Homogenate was squeezed through a double layer muslin cloth and the debris was re-extracted with one volume of same buffer and transferred to a 2ml centrifuge tubes.
4. The filtrate was then clarified by centrifugation at 5000g for 15 minutes at 4^{0}C
5. The supernatant was collected in a 2 ml centrifuge tube and centrifuged again at 5000g for 10 min.
6. Finally supernatant was mixed with equal volume of acetone and kept in a refrigerator for precipitation of protein.
7. Precipated protein was collected and suspended in suspension buffer.

3.2.8. Quality analysis of antigen by SDS-PAGE

The quality of antigen in the isolated protein was determined through SDS-PAGE. The 12% resolving gel was used for separating the protein based on the molecular weight and 5% stacking gel was used for aligning the protein over one another. The detailed procedure is given below.

A) Reagents used

1. Tris (pH-6.8 and 8.8)
2. 10% SDS
3. 10% APS
4. TEMED
5. 30 per cent Acrylamide, Bis-acrylamide solution
6. Double autoclaved distilled water

7. Protein loading dye
8. 1X SDS-electrophoresis buffer (running buffer)
9. Staining solution
10. Developer solution
11. Fixer solution

B) Procedure

1. Sufficient amount of resolving gel and stacking gel solution was prepared separately and poured into the casting plate and finally allowed to polymerase at room temperature.(Table 3)
2. After polymerization of the gel the comb was removed carefully and the trays were assembled in the SDS-PAGE electrophoresis unit containing 1X running buffer
3. Freshly isolated protein samples were prepared by mixing 10 µl of protein with 5 µl of gel loading dye.
4. The mixture was heated at 95^0C for 3 minutes and immediately cooled in ice.
5. 20 µl of each protein ladder and samples were loaded onto the gel.
6. The cathode and anode were connected to power pack and the gel was run at constant voltage of 70 volt for stacking the proteins and 100 volt for resolving the proteins.
7. The gel was then carefully removed from the plates and placed into the fixer solution for 10 minutes.
8. Then the gel was washed twice with distilled water and then placed into the staining solution for 8-10 minutes in dark condition. The gel was then washed with distilled water twice and placed into the developer solution for 10-15 minutes for visualizing the band.

9. The gel was removed from the developer solution and placed into the fixer solution for 2-3 minutes.
10. Finally the gel was removed from the fixer solution and then placed in distilled water and the desired size band was observed. **(Annexure IV)**

Table 3. Composition of different reagents used for the preparation of SDS gel

SI.No.	Reagent	Composition	
		For 5 per cent stacking gel	**For 12 per cent resolving gel**
1.	Distilled water	4.1 ml	3.3 ml
2.	4X Tris (pH-6.8)	0.75 ml	-
3.	4 X Tris (pH-8.8)	-	2.5 ml
4.	Acrylamide-Bis Solution(10%)	1 ml	4 ml
5.	10 per cent SDS	60 µl	100 µl
6.	TEMED	20 µl	40 µl
7.	10 per cent APS	60 µl	100 µl

3.2.8.2. Quantification of antigen by Spectrophotometer

The samples which were giving 38 kDa band in the SDS-PAGE were further analyzed to determine the quality and quantity of the isolated protein using Nanodrop® ND-1000 (Nanodrop technologies Inc., USA). Before taking the reading in the Nanodrop the paddle stand of the instrument was wiped properly and then 1µl of resuspention buffer was used as blank to set the instrument to zero, after that 1 µl of the sample was loaded. OD260/OD280 ratio was observed to determine the purity of the isolated viral protein. A sample whose OD260/OD280 ratio is less than 1.8 shows the good quality protein without any contamination of DNA and RNA.

3.2.9. Monitoring of antigen-antibody interaction (Detection of virus)

In the detection stage, a hour of incubation performed to allow probe specific target binding to reach to an equilibrium stage, which leads to dramatic aggregation of gold nanorod and as well as change in the colour of the solution. Then the absorption spectra between 200 to 1100wave length and corresponding peak shift was recorded. Each peak shift was recorded from three parallel experiments and expressed as a mean ± standard deviation. The general scheme for the detection process illustrated in (Fig 5).

For the detailed study of the antigen-antibody interaction, eight different concentrations of antigen were taken and the peak shifts were monitored for gold nanorods-antibody conjugate. The five different varieties of banana leaf samples which were collected from the banana research station Kannara were evaluated through nanobiosensor.

3.2.10. Monitoring antigen–antibody interaction through micro titer plate reader

The novel metallic nanoparticles possess a special plasmonic optical property, has shown to be potential applications in miniature sensor devices. Gold nanoparticles of 10-50 nm diameters have a remarkably strong extinction coefficient and distance-dependent optical phenomena as a result of plasmonic coupling. The colour changes of solution due to particle – particle coupling as well as aggregation between particles is one of the most powerful methods of nano biosensing. This method demonstrated a sensitive method of biological sensing and it can be accomplished without using complex instrument.

So, in this particular method of detection, colour changes that were observed after the addition of different concentration of antigen were quantified using ELISA reader.

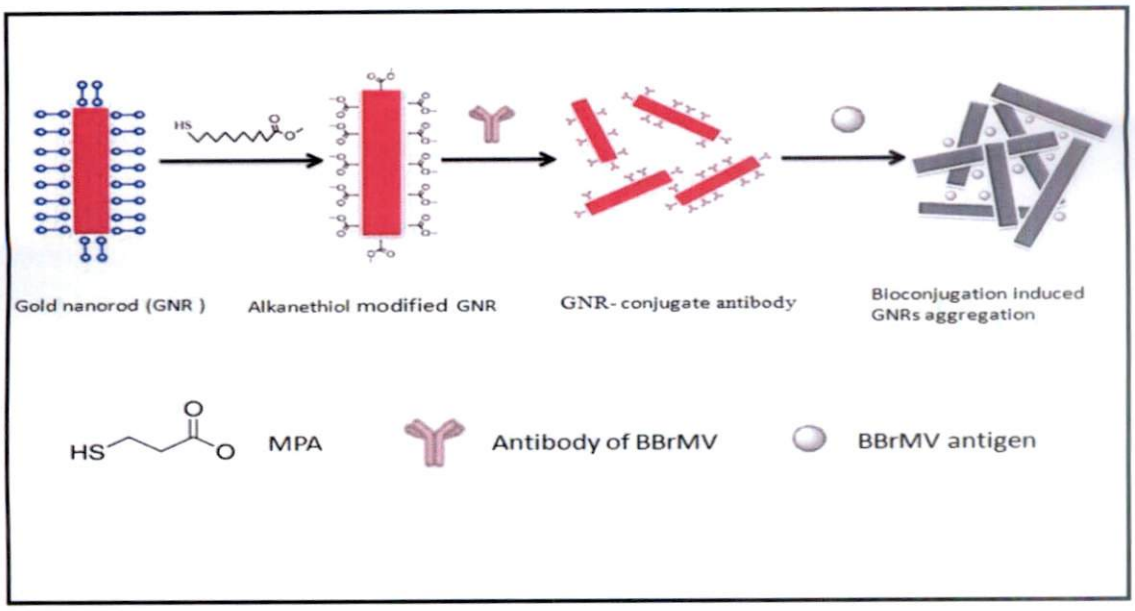


Fig 5.Schematic representation of bio-recognition based immunoassay for detection of BBrMV

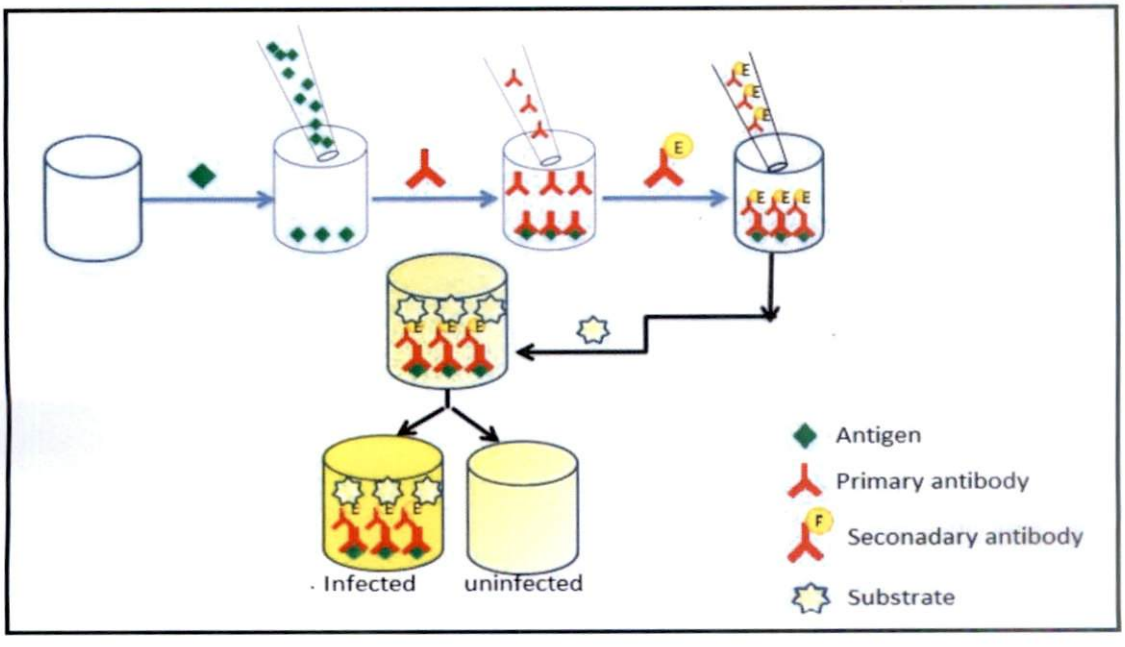


Fig 6. Schematic diagram of a DAC- ELISA

3.2.11. Kinetics of antibody labeled gold nanorod-antigen interactions.

The kinetics' of antibody labeled gold nanorod with antigen was studied in order to determine optimum time for getting a maximum peak shift and also for determining the aggregation behavior of a gold nano probe with different concentration of antigen. The data obtained from the UV-Vis result of an individual sample was used to plot the kinetics of each sample.

Each sample was monitored up to 100 min at 5 min intervals for getting maximum peak shift and for measuring absorbance of gold nanorods- antibody conjugate at different concentrations of antigen.

3.2.12. Determination of antibody titer for BBrMV

One gram of infected leaf from cultivar Nendran without midrib was homogenized in the presence of coating buffer contain 2 % (W/V) PVP in a pre-chilled condition. Healthy plant extract was prepared by using a leaf from tissue culture plant.

The homogenized product was centrifuged for 10 min at 15000 rpm in 4◦C. The supernatant was collected and 100 µl samples were added into an each well. After incubation of 2 hours at 37◦C plate were washed with PBS-T buffer three times. Blocking was done adding 100 µl one percent BSA for 30 min at 37°C. After incubation blocking agent was removed from the plate and washed with PBS-T buffer three times. After that 100 µl antibody that are purchased was added to each well and incubated overnight at 4°C. An antibody dilution of 1: 100; 1:250; 1: 500; 1:1000 were used for titer determination. Three replications were maintained for each treatment and incubated at 4°C. The plates were washed with PBS-T and treated with 100 µl of alkali- phosphate conjugated secondary antibody for 2 hour at 37 °C. Wells were washed with PBS-T buffer as before and the p-NPP was added to each well and incubated for one hour at 37 °C. Absorbance reading was taken at 405 nm.

3.2.13. DAC- ELISA (Direct antigen Coating -Enzyme Linked Immuno Assay)

Enzyme linked Immuno Sorbent Assay, commonly known as ELISA is one of the most widely used serological test for the plant virus diagnostics quantification and comparison owing to its simplicity, sensitivity and adaptability. The amount of virus present is proportional to the amount of enzyme – labeled antibody bound with the antigen forms the basis of the test. In the case of DAC-ELISA antigen were directly coated on the surface of the micro titer plate followed by the addition of the primary antibody and enzyme conjugated secondary antibody subsequently. Then substrate is added for colour development which could easily differentiate the infected from healthy sample. The details of procedure are given below (Figure 6)

A. Reagents used

1) PBS Coating buffer (pH-7.4)

NaCl - 137 mM

KCl - 2.7 mM

Na_2HPO_4- 10 mM

KH_2PO_4 - 1.8 mM

PVP- 2 %

2) Wash buffer (pH-7.4)

PBS (PH-7.4)

Twin-20: 0.005%

3) Blocking buffer 1X (pH-7.4)

PBS 1X (PH-7.4)

Twin-20: 0.005%

BSA- 0.2 %

4) Antibody dilution buffer

PBS (PH-7.4)

Twin-20: 0.005%

PVP – 2 %

BSA- 0.2 %

5) Substrate buffer (pH-9.8)

Substrate -p-NPP(p-nitro phenyl phosphate)

B) **Procedure**

1. 1gm of leaf samples was ground with 5 ml of coating buffer in a pre-chilled mortar pester.
2. The homogenized sample was transferred into a 2 ml Eppendorf tube and centrifuged for 15 min at 15000 rpm at 4 ˚C.
3. The supernatant was collected and transferred into a new centrifuge tube and stored in refrigerator until it used
4. ELISA micro titer plate was coated with 100µl of antigen at different concentration of antigen along with control and positive samples and incubated at 37 ˚C for 2 hour
5. After 2 hours incubation micro titer plate was washed with washing (PBS-T) buffer at least for three times.
6. After that the residual sites of each well were blocked using blocking buffer by incubating for 1 hour at 37 ˚C.
7. After incubation with blocking buffer plate are washed with washing buffer and 100 µl primary antibodies(1: 200 dilutions) was added into each well.
8. Incubated at 4 ˚C for overnight.
9. Then in the following day plate was washed with washing buffer for three times
10. 100 µl of secondary antibody was added into each well and incubated for37˚C for 2 hours.
11. Washed the plate at least for 3 times for removing the unbound secondary antibody and followed by addition of 100 µl of substrate and incubated at room temperature in a dark condition for 30 min.

12. The absorbance was taken at 405 nm wavelength and development of colour in healthy and diseased sample was visualized. (**Annexure-V**)

3.2.14. DOT-IMMUNO BINDING ASSAY (DIBA)

The assay was based on the absorption of a protein on the nitrocellulose membrane and visual observation of chromogenic product that are precipitated on the nitrocellulose membrane. The method followed with slight modifications of Banttari and Goodwin (1985) and Hibi and Satio (1985).

Reagents

1. Coating buffer- pH 7.4
2. PBS buffer – pH 7.4
3. Washing buffer – PBS-T pH 7.4
4. Blocking solution- PBS-T + 0.2 BSA
5. Antibody buffer- PBS-T + 0.2 BSA
6. Primary antibody (1: 200 dilution)
7. Alkali phosphates labeled antibody (1:200 dilution)
8. Substrate buffer- pH 9.8

Procedure

1. 2 µl of sample containing crude antigen was spotted on the nitrocellulose membranes and allowed to dry for 15 minutes at room temperature.
2. The nitrocellulose membranes were treated with 0.2 % blocking solution and kept for 1hour incubation.
3. The nitrocellulose blot was washed three times with PBS-T buffer at 5 min interval.
4. Nitrocellulose blot was incubated with primary antibody solution for 2 hours at room temperature.
5. Then it was washed three times with PBS-T buffer 5 min each.

6. Membrane was incubated with alkaline phosphates conjugated secondary antibody for 1 hr.
7. The membrane was washed three times (5 min each) with PBS-T buffer

9. The substrate was added for colour development.

10. The reaction was stopped by adding distilled water.

Results

4. Results

Banana bract mosaic virus causing banana bract mosaic disease locally known as a *Kokkan* disease is a serious threat to banana cultivation. The result of the different experiments carried out for the "Development of a nano biosensor for detection of *Bract mosaic virus* in banana (*Musa* spp.)" in Centre for plant biotechnology and molecular biology (CPBMB) and Centre for material and electronics (C-MET) during 2014-2016 are presented in this chapter. Salient findings of the study were given below.

4.1. Synthesis of gold nanorod (GNRs)

The synthesis of gold nanorods was carried out by the procedure described by a Nikoobakht and El-Sayed. (2003). The seed solution is a mixture of hydrogen aurium chlorotetrahydrate, CTAB and sodium borohydride was added to the growth solution containing aurium chlorohydrate, CTAB, silver nitrate and ascorbic acid, which turn the growth solution into a pink colure solution due to the formation of GNRs (Plate 1).

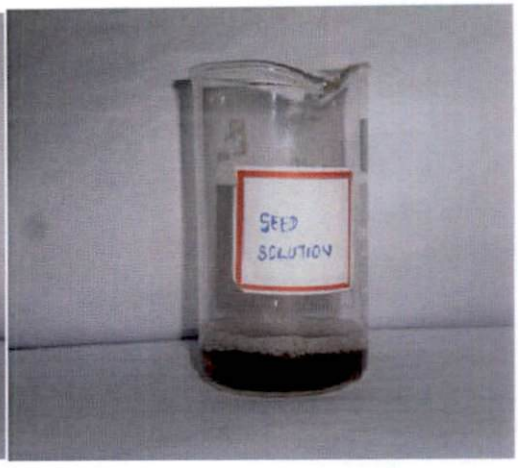
The colour differences were observed between two types of GNRs solution due to addition of different quantity of silver nitrate solution (Plate 2).

4.2. Characterization of gold nanorod

The absorption peak of the gold nanorods solution was measured at a wavelength of 200 to 1100 in a UV-Vis spectrophotometer and the result showed transverse Plasmon peak at 521 nm and other longitudinal Plasmon peak at 710 nm, which are the typical characteristic absorptive peak of gold nanorods (GNRs) (Fig 7).

The role of silver nitrate in the growth of GNRs is very important. In the study, it was found that with the increase in the volume of silver nitrate in the growth solution creates a measurable shift in peak from (710 to 740) nm (Fig-8).

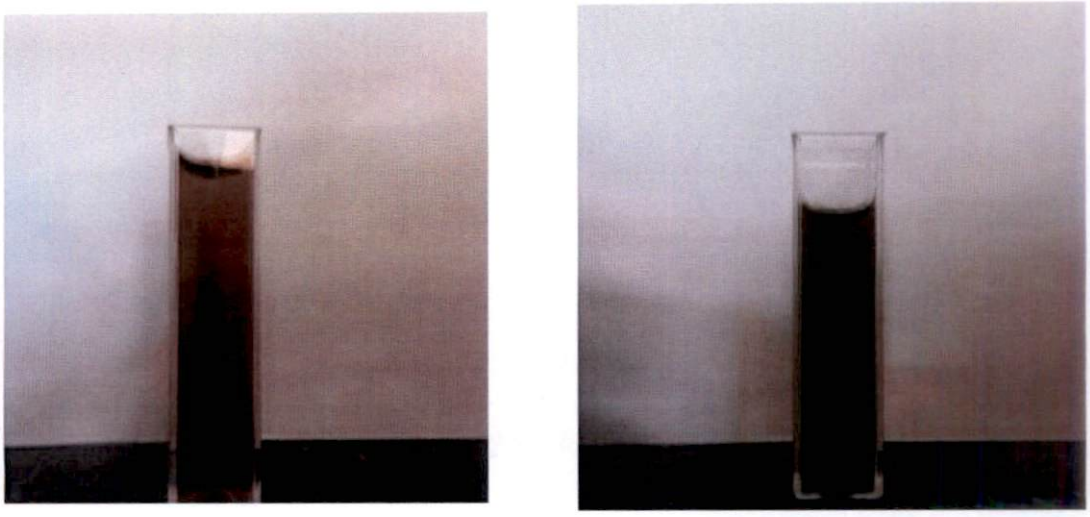
Gold solution



Seed solution

Growth solution

Plate 1. Preparation of gold nanorods (GNRs) solution



(A) (B)

Plate 2. Gold nanorods solution with different volumes of silver nitrate solution (A. GNRs with 100 µl seed solution; B. GNRs with 120 µl seed solution)

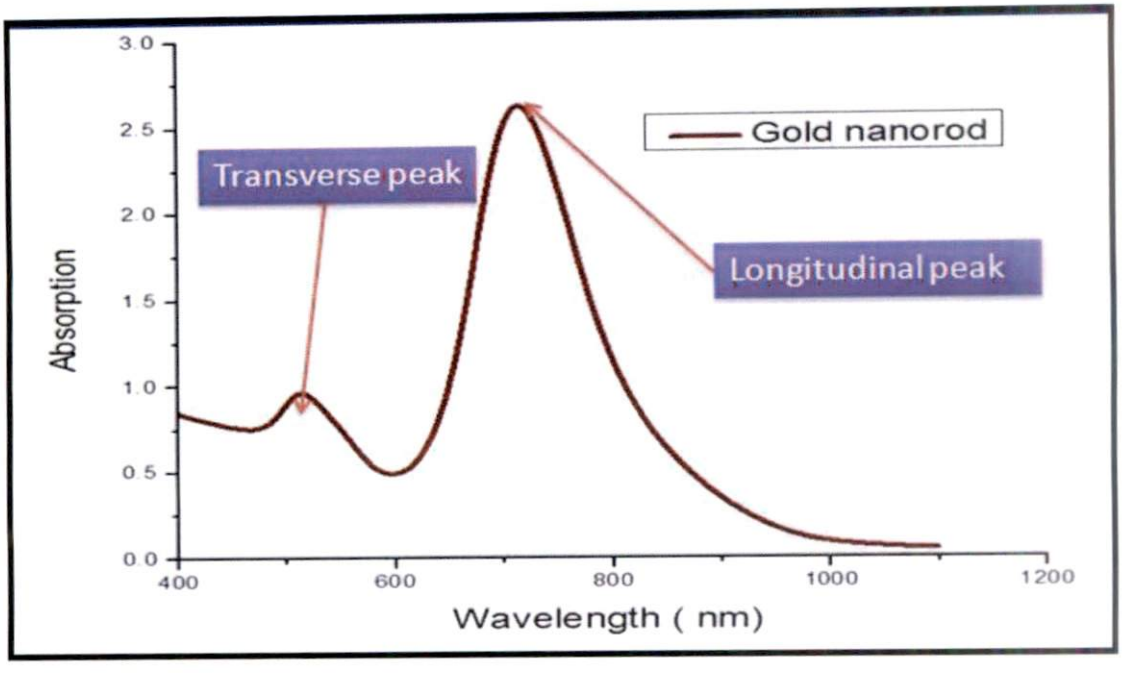


Fig 7. UV-Vis spectra of GNRs with 0.6ml of silver nitrate solution

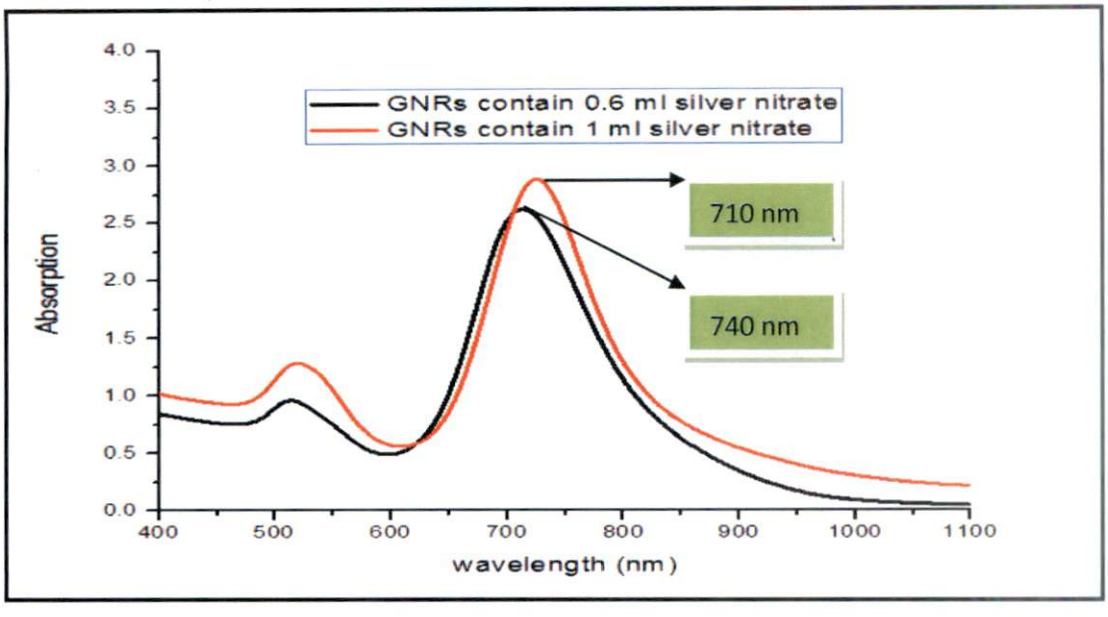


Fig 8.UV-Vis spectra of gold nanorods solutions with different volume of silver nitrate solution

UV-Vis spectra of GNRs revealed importance of seed solution on the growth of GNRs. It was found that in case of 100 µl of seed solution yield of GNRs is more compared to 120 µl of solution, which was clearly indicated by absorption peak of GNRs where both transverse and longitudinal peaks came down due to the addition of 120 µl of seed solution (Fig 9)

The TEM image of GNRs revealed that, the average length and diameter of GNRs were (42±3) nm and (14±1.9) nm respectively (Plate-3&4).It was also observed that if the volume of silver nitrate solution is increased in the growth solution, aspect ratio of GNRs increased from (3.03±0.36) to (3.75±0.49) Table (4&5).

4.3. Estimation of the Aspect Ratio

TEM images, taken from a Hitachi H-7600 Transmission Electron Microscope, were analyzed using ImageJ software. The diameter and the length are measured for approximately 20 particles. The average aspect ratio, diameter, and length are calculated using this information table (4&5).

In this experiment, it was observed that concentration of silver ion play an important role for increasing the aspect ratio as well as size of GNRs. In the result, it shows aspect ratio of GNRs increased from 3.03 to 3.75 due to addition of more amount of silver nitrate (0.6 to 1) / 50 ml in the growth solution.

Absorption data showed a wide range of optical properties of the synthesized particles. The experimental values calculated using the TEM images was compared with the theoretical values obtained using the Gans theory and made a comparison between theoretical values and experimental values and showed that average aspect ratio (3.38)from Gans theory correlated well with the overall experimental data (3.18)(Table 6).

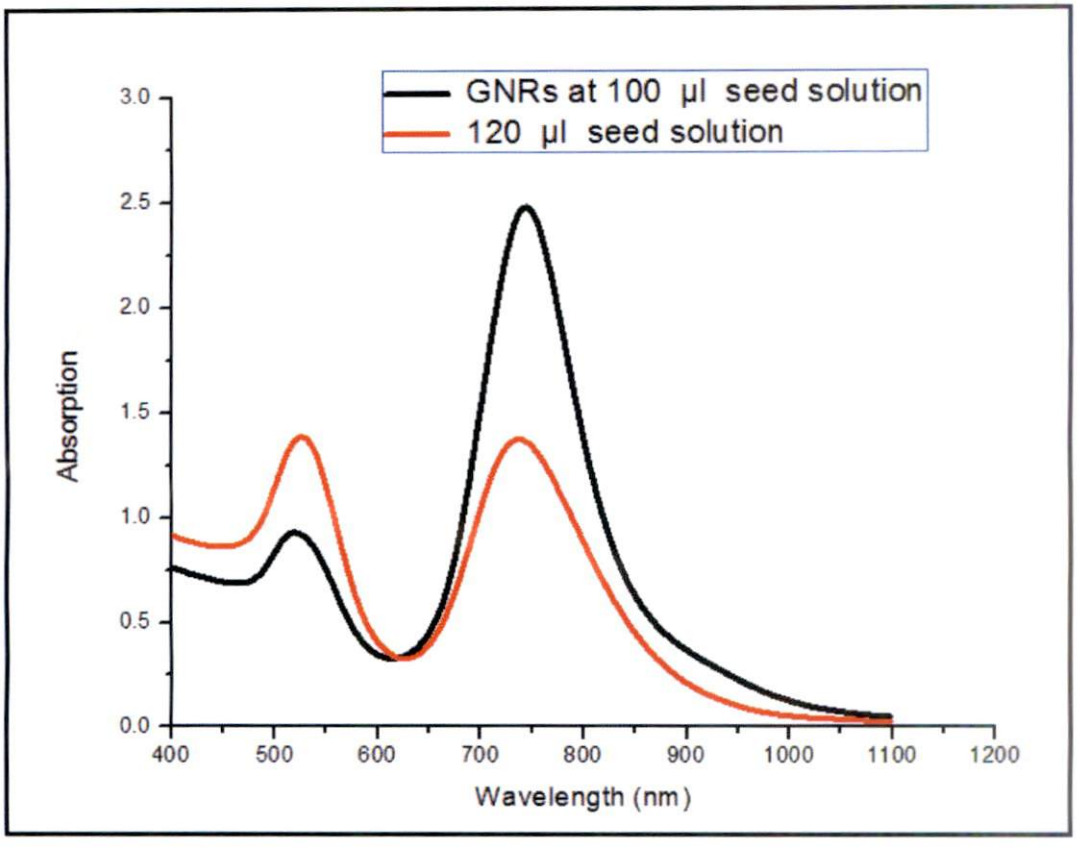


Fig 9.Effect of seed solution on the growth of gold nanorods (GNRs)

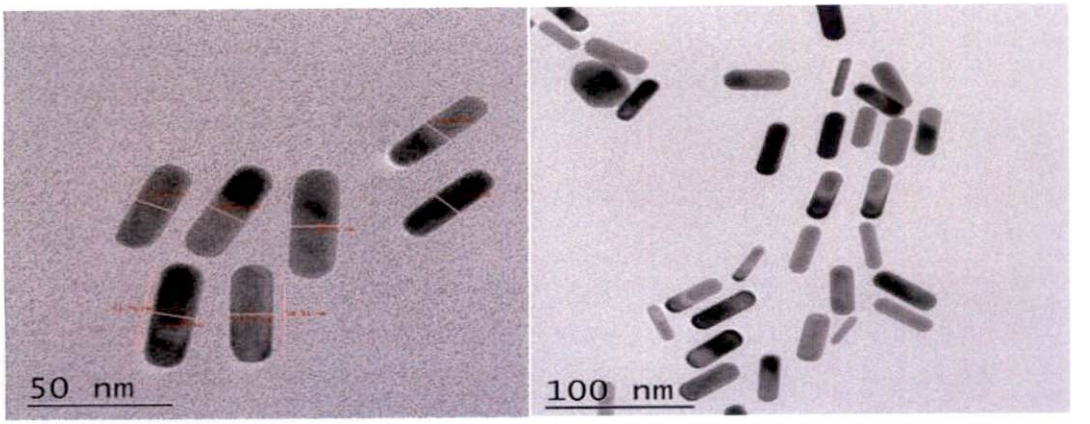


Plate 3. TEM image of GNRs with0.6 ml of silver nitrate solution

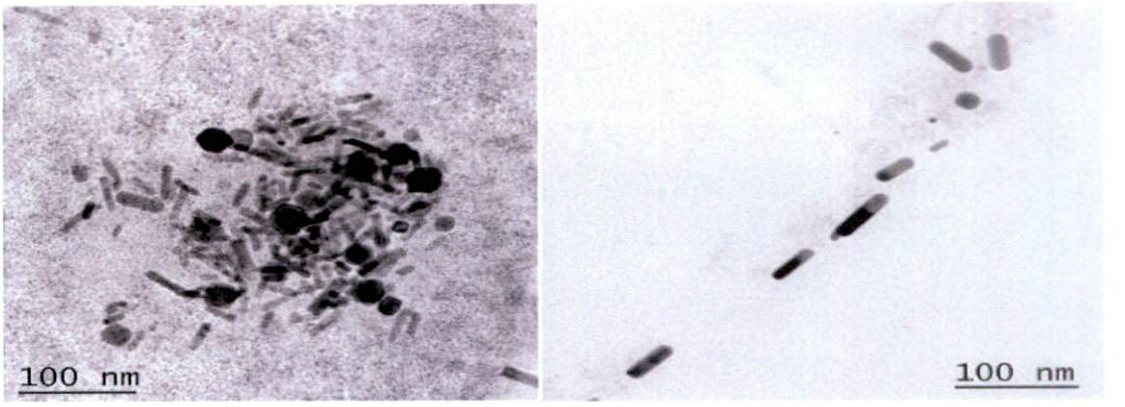


Plate 4. TEM image of GNRs with 1ml of silver nitrate solution

Table 4: Dimension of GNRs with 0.6 ml of silver nitrate solution

SI.NO	Length (nm)	Diameter(nm)	Aspect ratio (nm)
1	47	17	2.7
2	39	13	3
3	44	15	2.9
4	37	12	3.08
5	41	15	2.7
6	42	11	3.8
7	43	12	3.5
8	39	13	3
9	41	11	3.7
10	39	13	3
11	42	15	2.8
12	44	13	3.38
13	46	17	2.70
14	49	18	2.72
15	44	14	3.14
16	37	16	2.3
17	44	13	3.38
18	46	15	3.06
19	42	14	3
20	44	14	3.14
Average	42.28	14.04	3.03
Std. Dev	3.21	1.9	0.36

Table 5: Dimension of GNRs with 1ml of silver nitrate solution

SL. NO	Length (nm)	Diameter (nm)	Aspect ratio
1	63	11	5.72
2	43	11	3.90
3	47	12	3.92
4	45	18	2.5
5	48	16	3
6	54	15	3.6
7	56	16	3.5
8	55	16	3.43
9	61	18	3.3
10	59	17	3.47
11	50	16	3.25
12	51	14	3.64
13	48	13	3.69
14	50	14	3.57
15	56	12	4.66
16	52	14	3.71
17	48	12	4
18	52	13	4
19	45	12	3.75
20	49	11	4.45
Average	51.6	14.05	3.75
Std. Dev	5.4	2.32	0.49

Table 6: Comparison between aspect ratios of GNRs obtained from experimental data and Gans theory.

SL. NO	Aspect ratio (nm)	
	TEM image	Gans theory
1	2.7	3.2
2	3	2.9
3	2.9	3
4	3.08	3.1
5	2.7	2.8
6	3.8	3.5
7	3.5	3.78
8	3	3.67
9	3.7	3.89
10	3	3.5
Average	3.18	3.38
Std. Dev	0.39	0.37

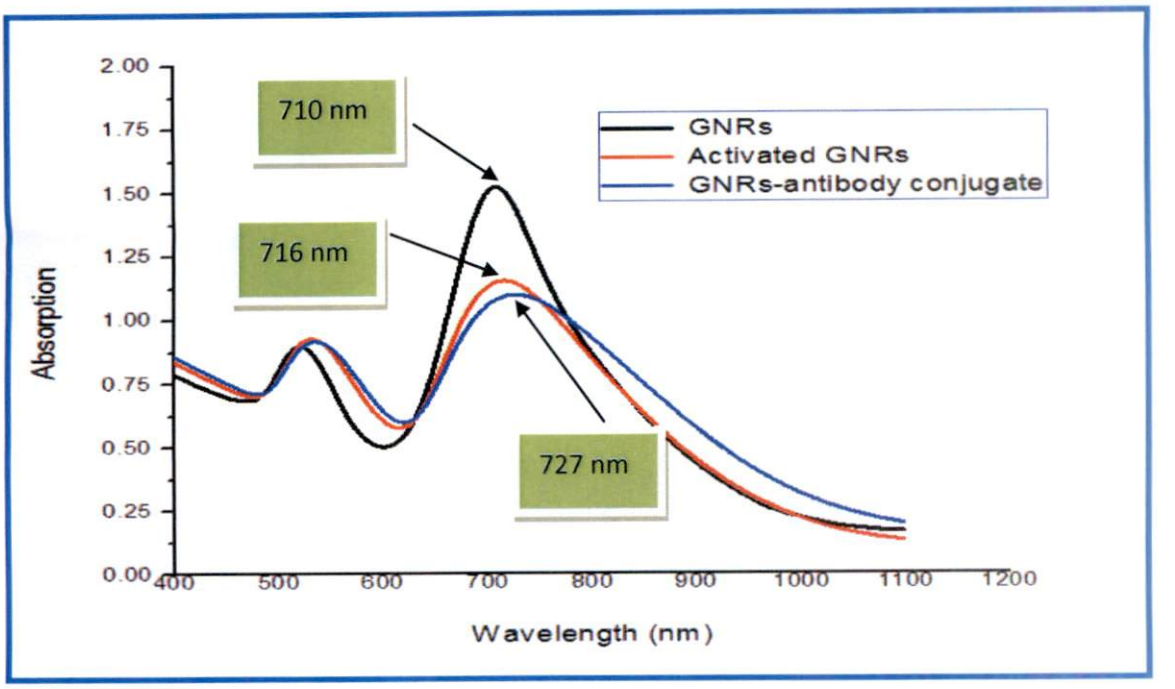


Fig 10.UV-Vis spectra of GNRs after addition of antibody

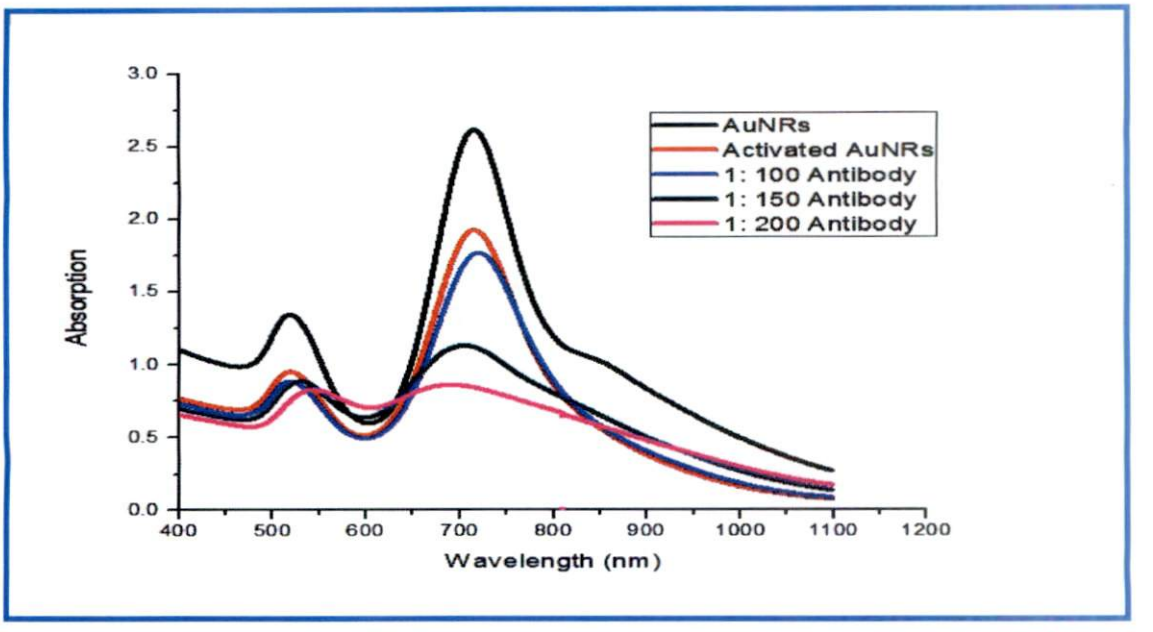


Fig 11. UV-Vis spectra of GNRs at different dilutions of antibody

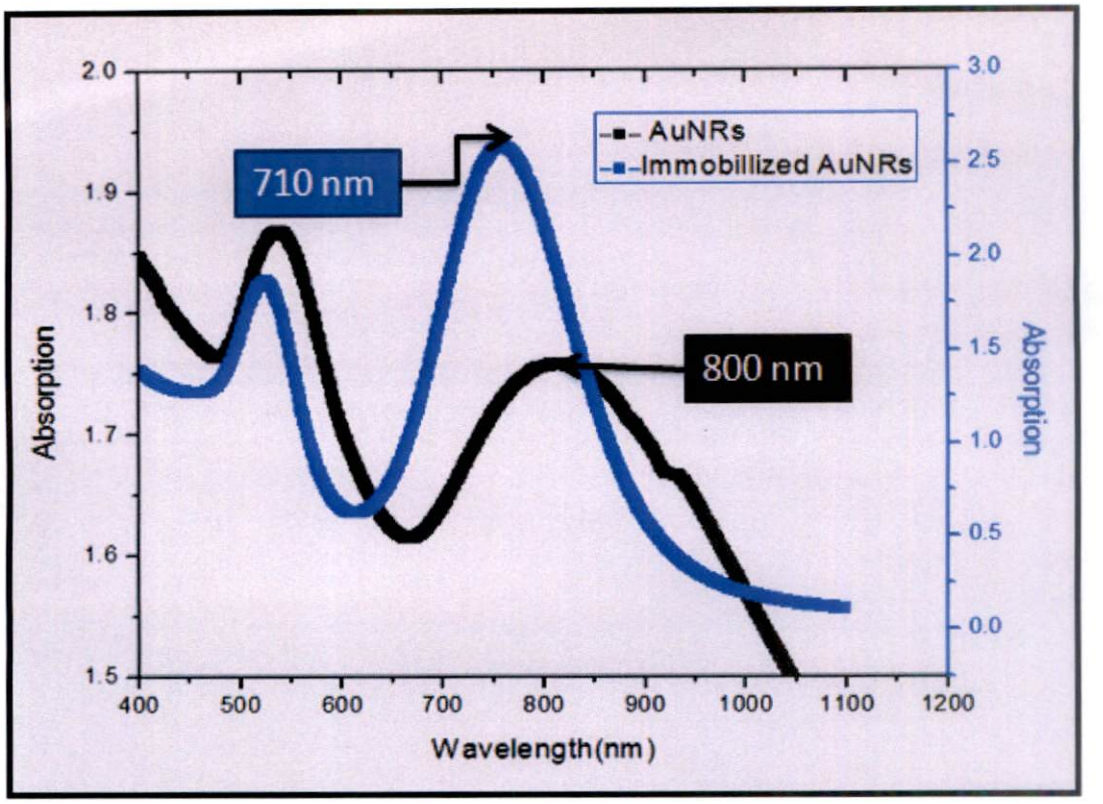


Fig 12.UV-Vis spectra of gold nanorods immobilized on glass substrate

4.4. Preparation of GNR-antibody conjugates

Extra CTAB in the GNRs solution was removed by repeated centrifugation. SAM (Self Assembly Monolayer) on the surface of GNRs was created by the addition of analkalithiol molecule (MPA) (3-Mercapto propanoic acid), so that GNRs will easily bind to an antibody. The efficiency of binding of antibody with GNRs is characterized by UV-Vis spectra and it was observed due to formation of SAM (self assembly monolayer) the peak position of a GNRs shift from 710 to716 nm(Fig 10).

Local reflective index changing on the surface of the GNRs due to bimolecular interaction is one type of phenomena, which can be monitored by red shift of peak position. As shown in the (Fig 10), the peak position of a GNRs shift from 716 to 727 nm due to binding of antibody to self assembly monolayer of GNRs

In this experiment, the stability of GNRs with different dilution of antibody was also studied and the result shows the GNRs are stable at (1:100) dilution (Fig 11).

4.5. Immobilization of gold nanorods onto the substrate

An attempt was made for immobilization of GNRs on to a glass substrate, Glass substrate with thiolated layer was used for immobilization of GNRs. After immobilization of GNRs onto a glass substrate, the glass substrate was characterized by UV-Vis spectrophotometer and peak shift was observed from (710-800) nm, which indicate binding of GNRs on to a Glass substrate.(Fig 12)

It was found that GNRs were simply washed out from the glass substrate in the subsequent steps for the binding of antibody to gold nanorods immobilized on glass substrate. Hence the attempt was failed.

4.6. Isolation of antigen from BBrMV infected banana plant

Isolation of the BBrMV was carried out from the infected young unopened leaves of banana, which is the primary host of the virus. The Plants showing characteristic symptoms (Plate 5) were mainly chosen for isolation of antigen. The

method adopted by Gonsalves *et al.* (1986) found to be unsuccessful, resulted in a low concentration of virus (antigen) in the samples along with a high concentration of host protein contaminants.

However, in the present study with modified method of Gonsalves *et al.* (1986) clear viral particles (coat protein) was obtained with less aggregation of virus particles.

4.7. SDS – PAGE analysis of isolated antigen

SDS-PAGE of isolated antigen of *Banana Bract mosaic virus* performed under a denatured condition in presence of SDS. This is a widely used for checking the viral specific coat protein based on the molecular mass of a viral coat protein. The molecular mass of the viral coat protein can be estimated by the mobility of the marker protein of known molecular weight.

SDS-PAGE analysis of isolated antigen on 12 percent SDS gel revealed the presence of a band with an approx molecular weight of 38 KDa (Plate 6).

4.8. Detection of BBrMV through nanobiosensor

Bio recognition induced gold nanorod aggregation and change in the colour of the solution as well as changes of peak position in UV-Vis spectra was used as an analytical tool for detection of BBrMV based on the specific reaction between captured antibody and antigen.

In this study, for the detection of *Banana bract mosaic virus* (total protein) or antigen was added to a nanoprobe gold solution and it was observed by visually that colour of the nanoprobe solution turn purple to blackish colour due to an interaction between antigen and antibody labeled GNRs (nanoprobe) and along with different concentration of antigen changes in colour also varied (Plate 7).

A B

Plate 5. (A). Bract mosaic symptoms in a bract of male bud; (B) Characteristic reddish brown spindle coloured streak on pseudo stem

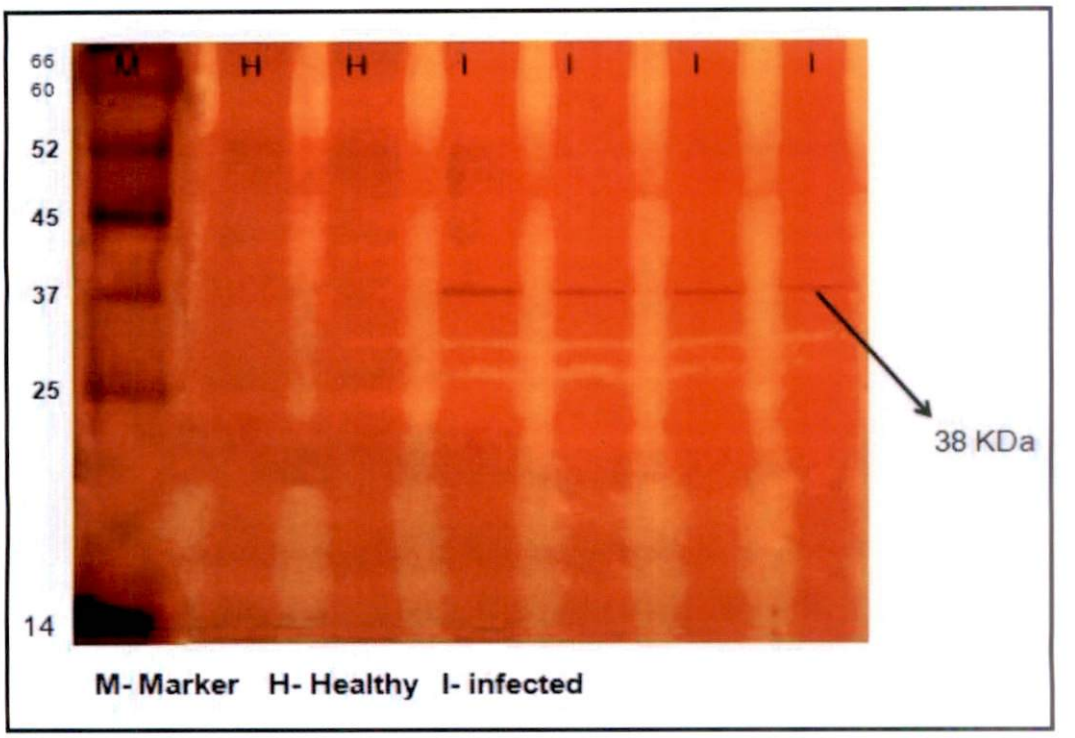
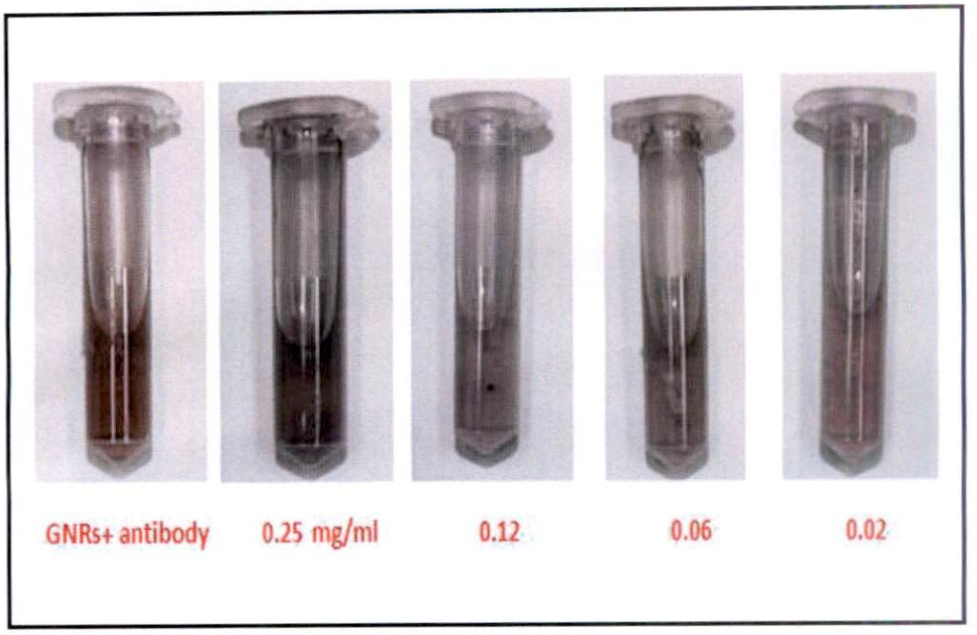
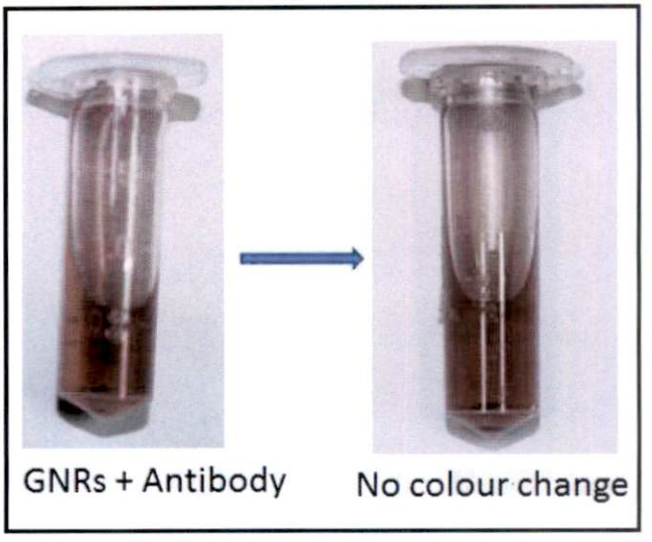


Plate 6. SDS-PAGE analysis of isolated antigen from BBrMV infected plant leaf

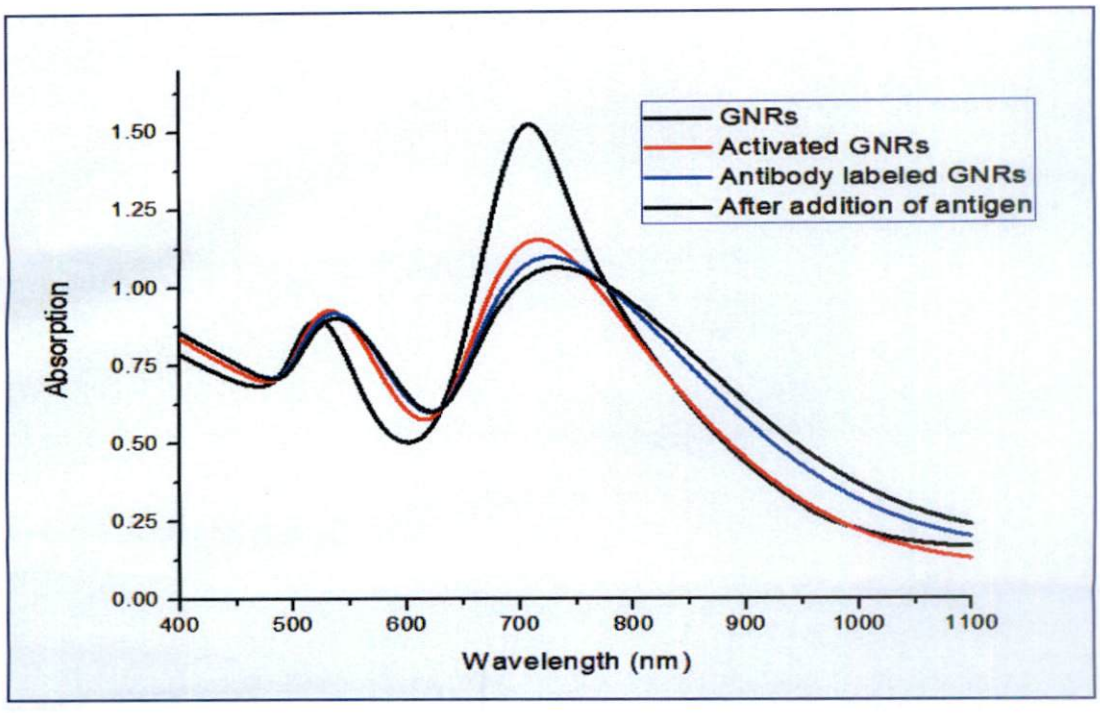


A. Infected sample

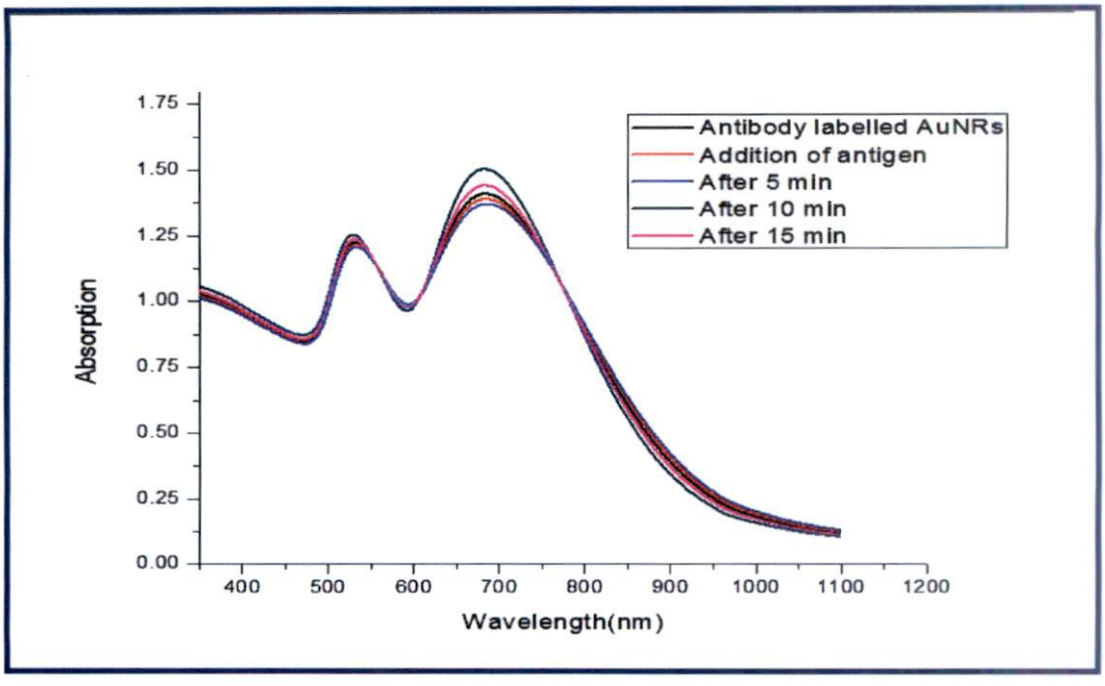


B: Healthy sample

Plate 7. Detection of BBrMV through colorometric sensing



Infected sample



Healthy sample

Fig 13. Detection of BBrMV through UV-Vis spectrophotometer

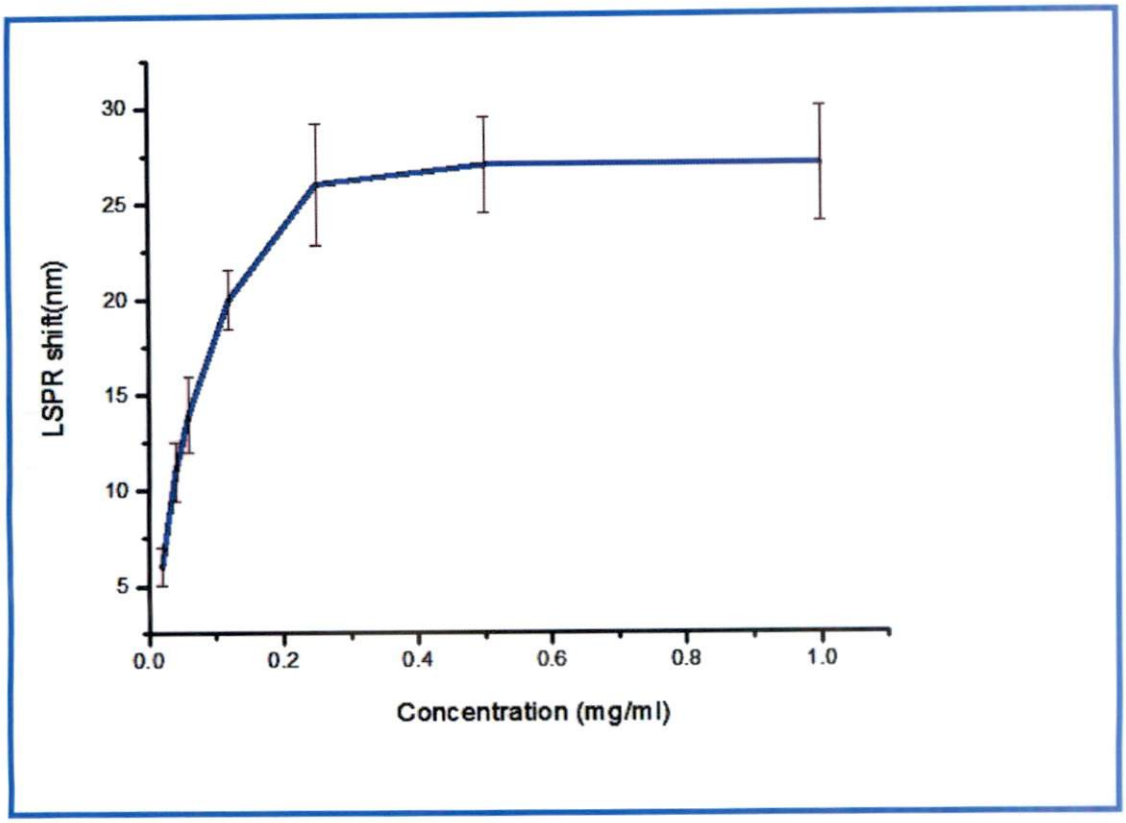


Fig 14. A plot of longitudinal LSPR peak shift over a different range of antigen concentrations

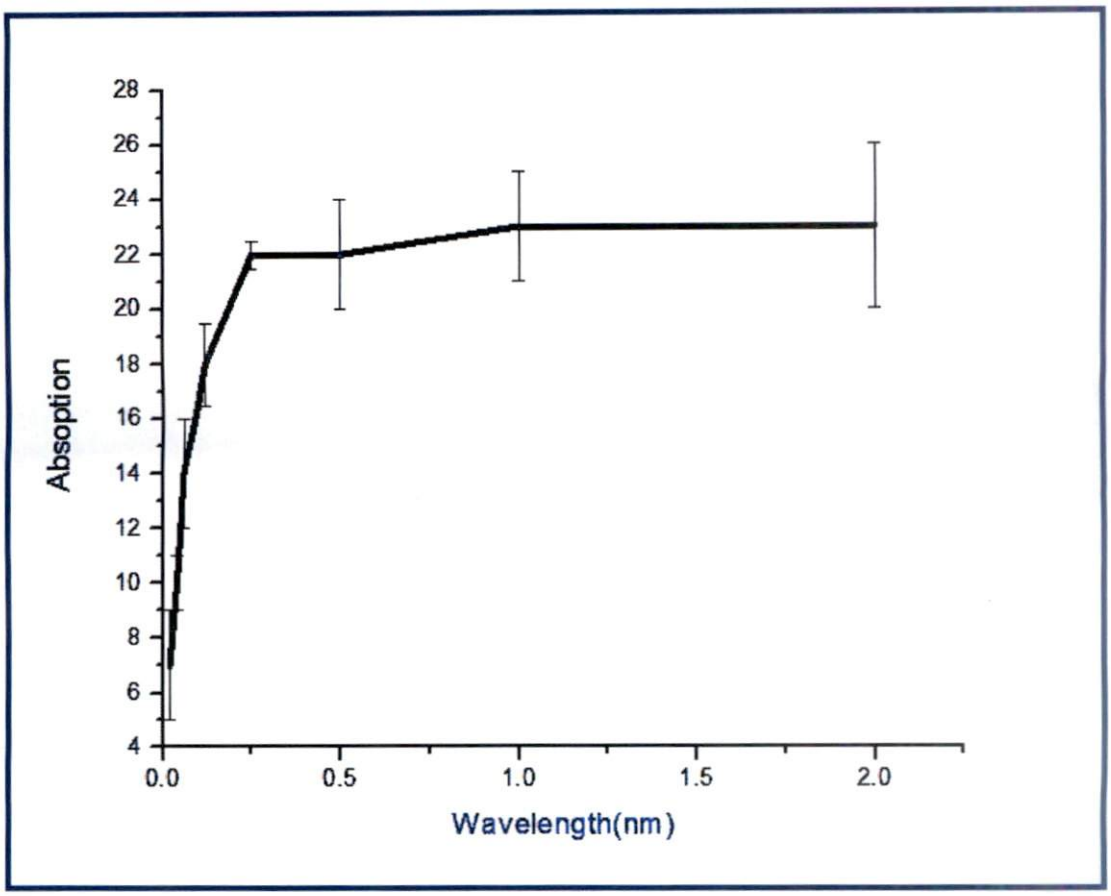


Fig 15. A plot of transverse LSPR peak shift over a different range of antigen concentrations

UV-Vis spectra of a GNRs revealed that due to addition of antigen to antibody labeled GNRs both transverse and longitudinal peak of GNRs shift 7nm (533-540 nm) and 10nm (727-737nm) respectively.

But in case of healthy sample no colour changes were observed and very little changes in peaks shift observed compared to infected sample (Fig 13). It was also observed that up to certain limit (0.25 mg/ml) changes in the LSPR peak shift was observed after that if the concentration of antigen increased also peak remained unchanged. Details of the changes in the transverse and longitudinal peak shift is given in (Table7, Table 8 and Fig 14, Fig 15).

4.9. Monitoring antigen–antibody interaction through ELISA reader

One of the unique properties of GNRs is when particle-particle coupling as well as aggregation of nano materials happen the colour of the solution change, due to its unique optical property. Thus it can be used as a colorometric sensor for detection of virus. ELISA reader, detect virus by reading the colour absorbance. Similar way, in this particular experiment different concentration of antigen were added to antibody labeled GNRs and the result shows that (Table 9) with increasing concentrations absorbance's also increases up to0.25mg/ml concentration after that not much increase in the absorbance, if the concentration of antigen is increased.

4.10. Kinetic behavior of antibody labeled gold nanorod to different concentrations of antigen

The Kinetic curves of antibody labeled nanorods were studied to find out the aggregation pattern of GNRs along with peak shifts at different concentrations of antigen.. The data were extracted from UV-Vis spectra for obtaining the kinetics curves of GNRs (Table10). In this experiment clear differences were observed between the control and the infected sample.

Table 7:LongitudinalLSPR peak shift of GNRs-antibody conjugate at different concentrations of antigen

Concentration (mg/ml)	LSPR peak shift (nm)				
	Sample	Sample	Sample	Average	Std. Dev
2	26	24	27	26	3.05
1	24	27	30	27	3.00
0.5	25	30	28	27	2.51
0.25	23	29	28	26	3.21
0.12	19	22	20	20	1.52
0.06	12	14	16	14	2.00
0,04	13	11	10	11	1.52
0.02	6	5	7	6	1

Table 8: Transverse peak shift of GNRs- antibody conjugates at different concentrations of antigen

Concentration (mg/ml)	LSPR peak shift (nm)				
	Sample	Sample	Sample	Average	Std. Dev.
2	27	24	20	23	3
1	26	21	24	23	2
0.5	23	19	24	22	2
0.25	21	22	22	22	0.5
0.12	20	18	17	18	1.5
0.06	15	16	12	14	2
0.04	10	9	11	10	1
0.02	9	5	7	7	2

Table 9: Detection of BBrMV through ELISA reader

Sample	AuNRs	AuNRs+ antibody	Healthy	Concentration of antigen (mg/ml) (infected)							
Absorbance at 405 nm											
1				**2**	**1**	**0.5**	**0.25**	**0.12**	**0.06**	**0.04**	**0.02**
2	0.212	0.18	0.192	0.330	0.310	0.295	0.254	0.231	0.19	0.191	0.192
3	0.212	0.189	0.202	0.293	0.296	0.300	0.232	0.198	0.186	0.203	0.197
	0.219	0.169	0.164	0.316	0.310	0.290	0.210	0.204	0.228	0.199	0.185
Average	**0.214**	**0.179**	**0.186**	**0.313**	**0.305**	**0.295**	**0.232**	**0.211**	**0.201**	**0.197**	**0.191**

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Table 10: Changes in the LSPR longitudinal peak shift of antibody labeled gold nanorods with different concentrations of antigen at different time interval

Concentration(mg/ml)		LSPR peak shift(nm)			
		5 min	**10 min**	**15 min**	**20 min**
0.06	**Infected**	3	10	13	15
0.12		5	15	18	19
0.25		8	20	25	25
Control	**Healthy**	2	2	2	2

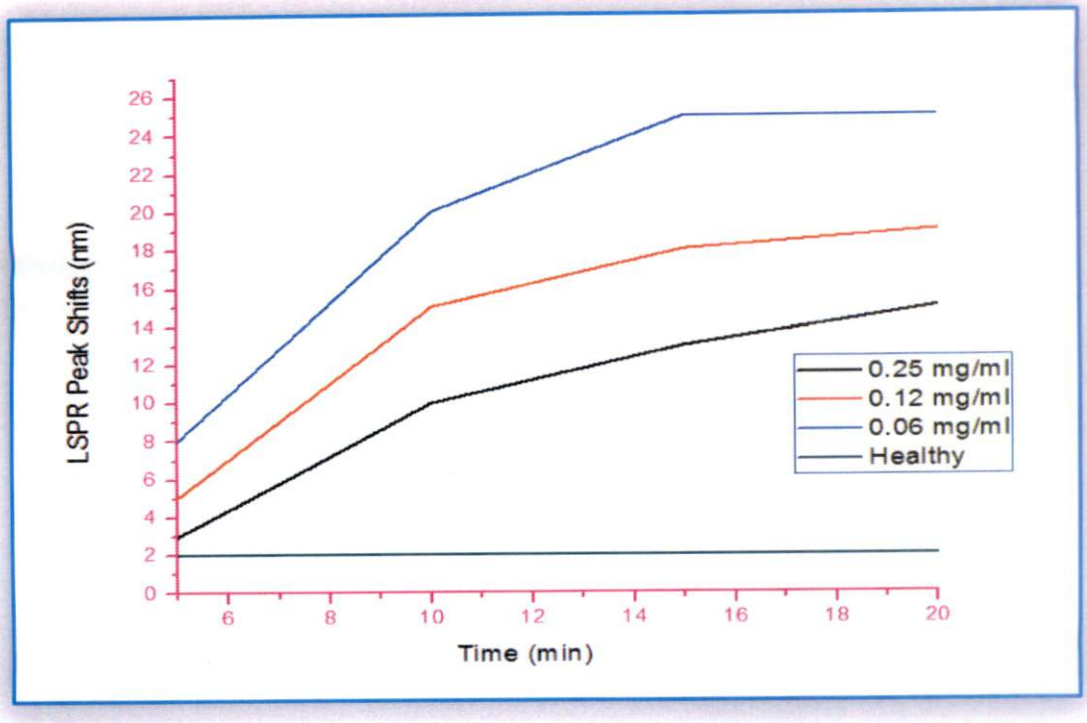


Fig 16. Kinetic curves of longitudinal LSPR peak shift of antibody labeled GNRs at different concentrations of antigen

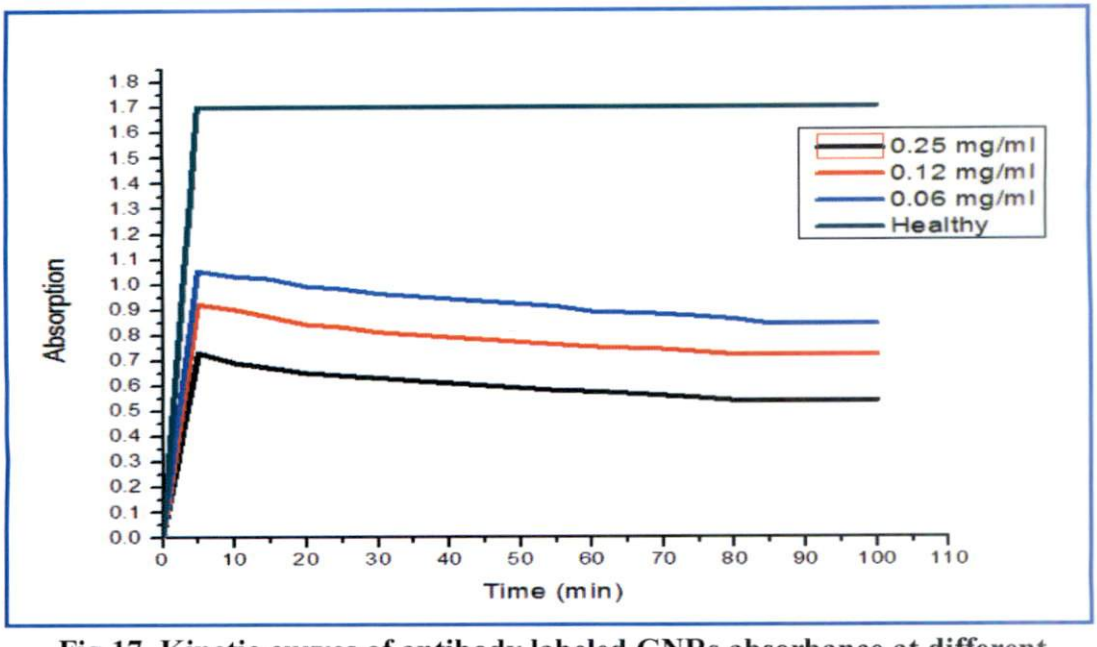


Fig 17. Kinetic curves of antibody labeled GNRs absorbance at different concentration of antigen

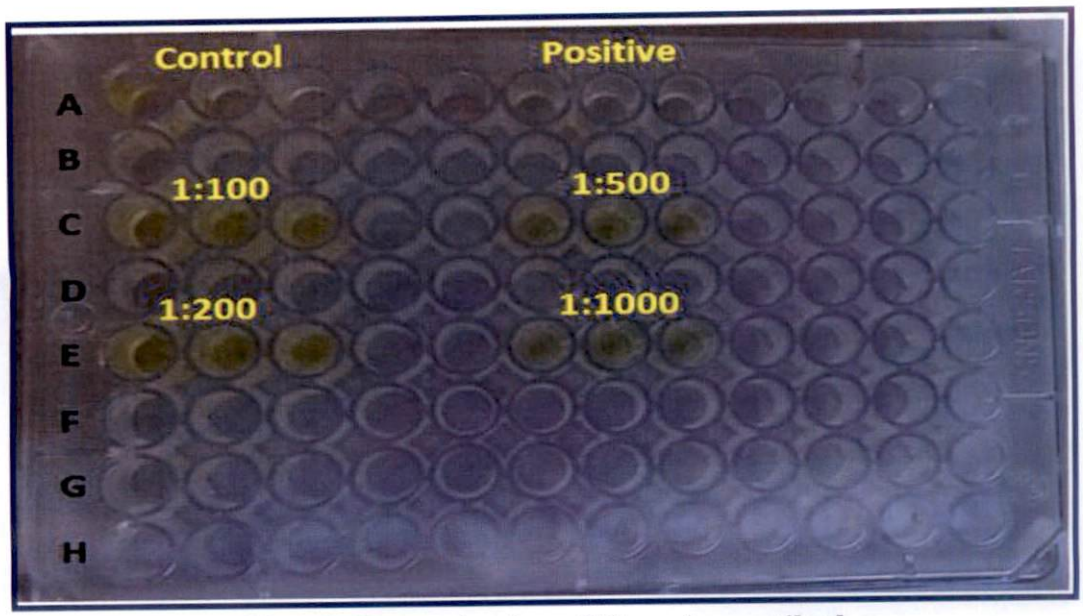
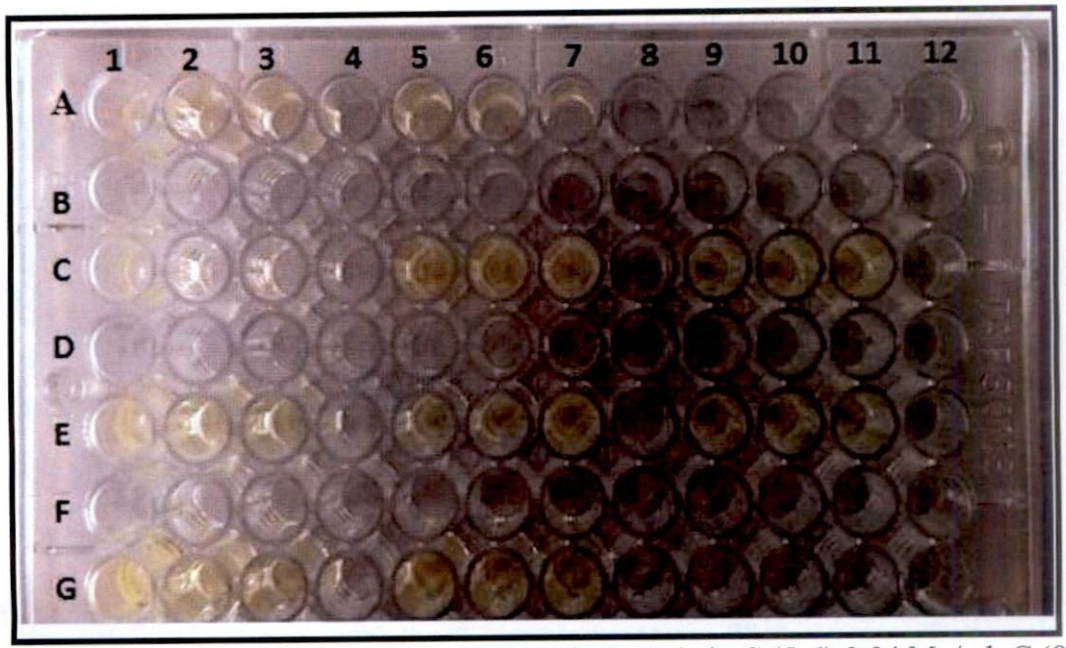


Plate 8. Determination of titer of an antibody



A(1- 3) Control ; A (5-6) Healthy: C (1-3) 0.02 Mg/ml; C (5-6) 0.04 Mg/ml; C (9-10) 0.06 Mg/ml; E (1-3) 0.12 Mg/ml; E(5-7) 0.25 Mg/ml; E (9-11) 0.50 Mg/ml; G(1-3) 1 Mg/ml; H(5-7) Mg/ml.

Plate 9. Detection of BBrMV at different concentrations of antigen

In case of LSPR peak shift the kinetics curved of an infected sample remain relatively flat after 15min and remained stable until the end of the observation(Fig 16) and the absorbance of GNRs continuously decreased up to 80 min and after that no changes were observed in the kinetics curve of an absorbance of GNRs (Fig 17).

In contrast kinetic curve of a healthy sample shows no changes in the absorption along with the LSPR peak shift and the curve remain flat from the beginning itself.

4.11. Determination of titer of monoclonal antibody

DAC-ELISA was carried for the determination of titer of an antibody with different dilution of (1:100; 1:200; 1:500; 1:1000) antiserum that was purchased from Agdida, U.SA. The absorbance value was recorded using a VESAMAX micro titer plate reader. The results obtained 30 minutes after addition of substrate are presented in Table 11 and Plate 8.

In the present investigation, it was found that BBrMV could be best detected in 1:200 primary antibody dilution along with 1:500 alkali-phosphatase conjugated secondary antibody dilution.

4.12. DAC- ELISA (Direct antigen Coating -Enzyme Linked Immuno assay)

Antiserum was purchased from Agdida, USA and Samples were collected from both infected and healthy was used to perform DAC-ELISA. The titer of antiserum used for the study was 1:200 which was previously determined by DAC-ELISA. The result of the experiment revealed that the BBrMV specific antibody gave higher reactivity and it can give clear differences is between healthy and infected sample (Plate 9; Table12).The study also revealed that 0.02 mg/ml concentration of antigen is the least concentration of antigen that could be detected by DAC-ELISA.

Table 11: Determination of titer of BBrMV specific antibody

Sample	Absorbance at 405 nm					
	Control	Healthy	1: 100	1:200	1:500	1:1000
1	0.109	0.157	0.590	0.620	0.563	0.402
2	0.114	0.168	0.560	0.590	0.569	0.475
3	0.125	0.170	0.570	0.640	0.550	0.403
Average	0.116	0.165	0.573	0.616	0.560	0.426

Table 12: Absorbance value of infected sample at different concentrations of antigen

Concentration(mg/ml)	Absorbance at 405 nm								
	Healthy			Control			Infected		
0.02	0.212	0.180	0.192	0.209	0.202	0.198	0.202	0.189	0.202
0.04							0.629	0.580	0.600
0.06							0.880	0.994	0.885
0.12							1.203	1.302	1.195
0.25							1.312	1.345	1.295
0.5							0.995	0.913	0.950
1							0.900	0.880	0.850
2							0.834	0.879	0.990

4.13. Dot immuno binding assay (DIBA)

DIBA is a simple serological method of detection, widely followed for detection of a virus. Antigen isolated from an infected leaf sample was blocked onto a nitrocellulose membrane and virus was detected with virus specific antibody.

To know the minimum quantity antigen detected by this method a range of antigen concentration from (2-0.2) mg/ml was taken and the result shows virus were detected at a concentrations ranges from(2 to 0.12) mg/ml and no precipitation in healthy sample were observed(Plate 10).

4.14. Comparison among DAC-ELISA, DIBA and NANOBIOSENSOR

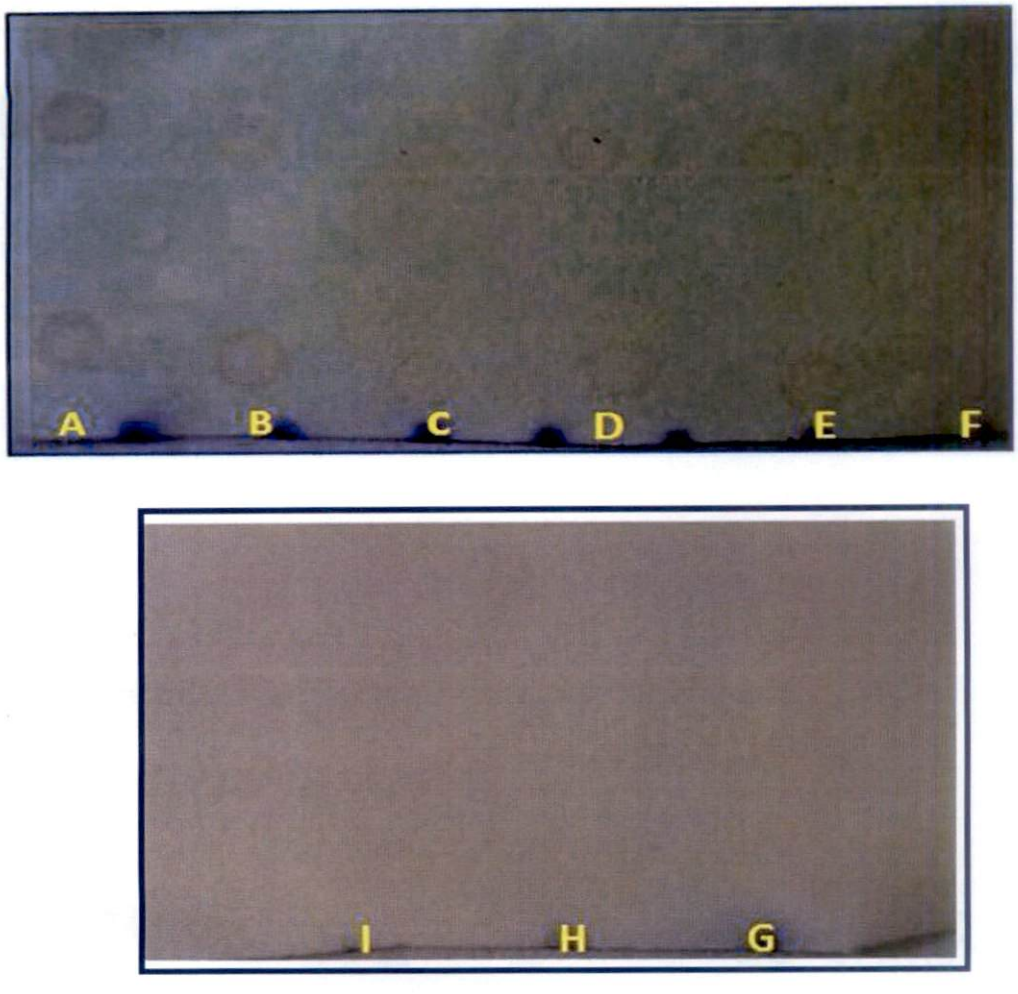
The binding of an analyte or target to virus-specific antibody labeled GNRs has-been monitored through changes in the LSPR peak position of a GNRs in UV-Vis spectrophotometer, which can be used for fabrication of nanobiosensor that can detect a wide range of concentrations of an analyte.

In this experiment, it was found that BBrMV can be detected at a range of (2-0.02) mg/ml by using a nanobiosensor but in the case of ELISA, BBrMV cannot be o detected at 0.02 mg/ml concentration of antigen.(Table13).

Table13: Comparison between ELISA, DIBA and Nanobiosensor

Experiment	**Concentration (mg/ml)**								
	2	1	0.05	0.25	0.12	0.06	0.04	0.02	
Nanbioosensor	+	+	+	+	+	+	+	+	
ELISA	+	+	+	+	+	+	+	_	
DIBA	+	+	+	+	+	_	_	_	

+ - Infected; - Healthy



A-2 mg/ml ; B- 1 mg/ml ; C- 0.5 mg/ml ; D-0.25 mg/ml ; E- 0.12 mg/ml

F-0.06 mg/ml; G-0.04 mg/ml :

H-0.02 mg/ml; I- Healthy

Plate 10. Dot blot analysis isolated antigen at different concentrations of antigens

4.15. Evaluation of different varieties of Banana for BBrMV infection

A novel GNRs based nanobiosensor was fabricated for the detection of a BBrMV based on the shift in LSPR(longitudinal localized surface plasmon resonance) peak. It was found that this assay could very well differentiate infected and healthy samples in a wide range of concentrations. Here the different varieties of banana which were infected by BBrMV collected from natural field condition was evaluated through fabricated nanobiosensor and it was found that cv. Nendran more seriously affected by BBrMV compared to other selected varieties of banana.(Table 14 and Fig 18).

Table 14: Evaluation of different varieties of Banana for BBrMV infection

Variety	Longitudinal LSPR peak shift (nm)			
	Replication 1	**Replication 2**	**Replication 3**	**Healthy**
Nendran	30	28	27	3
Mysore Poovan	18	20	17	2
Rasthali	24	25	22	2
Red banana	20	21	26	4
Dwarf Cavendish	23	19	22	1

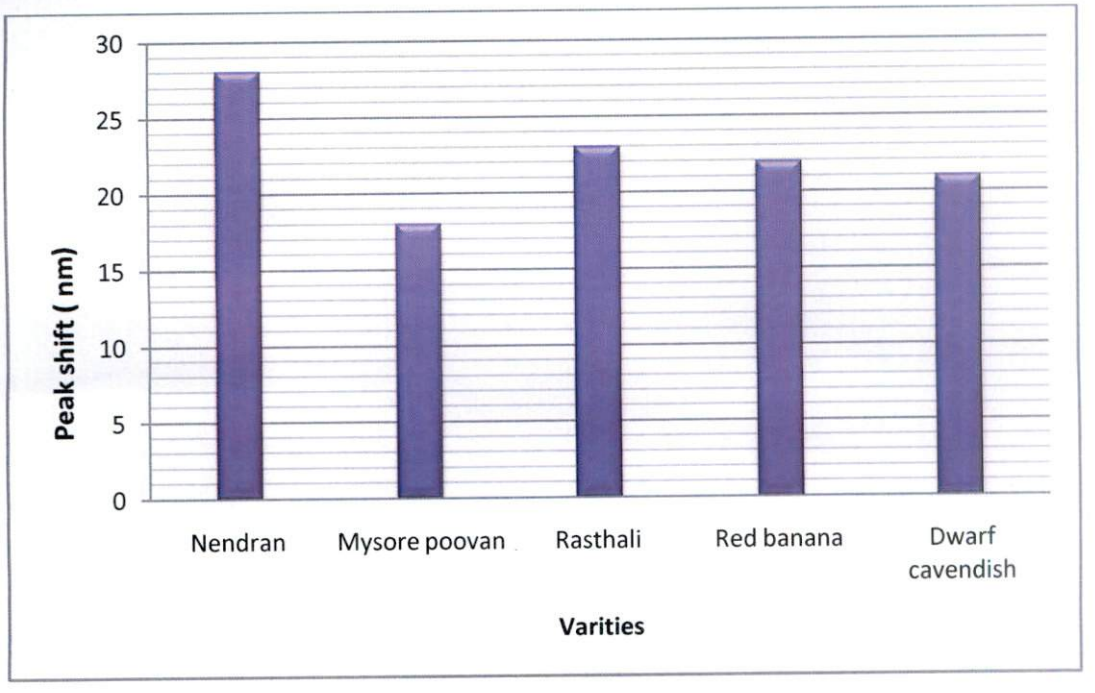


Fig 18. Evaluation of different varieties of Banana for BBrMV infection

Discussion

5. Discussion

Banana is the second most important horticultural crop in the world, which is grown as a staple food items and a significant cash crop in the tropic and sub tropic regions of the world. Banana is mainly propagated vegetatively either by means of sucker or through tissue culture plant which resulted in the infestation of pest and diseases, especially viral diseases, with 100 % yield reduction in banana production. *Banana bract mosaic virus* (BBrMV) is one of the economically important viral diseases which contributed to about 40% yield reduction. It was first reported Magnaye (1979) in Philippines. Later in India the disease was first reported in Kerala by Samraj *et al.* (1966).

The virus often exists as a symptomless carrier which is very difficult to identify the infected plants. In India there are many serological and molecular methods of detections are followed, but none of the methods are cost effective sensitive and reliable for easy and quick detection of virus. Hence, there is an urgent need of reliable detection technique. In recently novel metallic nanoparticles was used for fabrication of nanobiosensor which can detect very low concentration of virus. The development of nanobiosensor comprises synthesis and characterization of GNRs, conjugation of antibody to GNRs and characterization of GNRs-antibody conjugate, then finally addition of antigen to GNRs-antibody conjugate solution for detection of virus. In this chapter each steps discussed according to methodologies adapted for the development of a nano biosensor for detection of *Bract mosaic virus* in banana (*Musa* spp.).

5.1. Synthesis and characterization of gold nanorods (GNRs)

In the recent years biosensor based on the novel metallic nanoparticle gains more importance due to their novel plasmonic property. Even though there are several nanoparticles existing but GNRs are most commonly used now a days due to

its LSPR(Localized Surface Plasmon Resonance) property and easy synthesis in different sizes which, exhibits different colour due to its optical property at near infrared region (Kissinger,2005)

The successful development of LSPR based sensors requires a reliable and accurate method of synthesis. The most commonly used method for synthesis of GNRs is seed mediated method. In this method for synthesis of GNRs a modified protocol of Nikoobakht and El-Sayed. (2003) was followed and GNRs of average (42±3) nm length and diameter of (14±1.9) nm was developed, where gold nanorods of dimension (55.18±5.53) nm in length and (23.25±3.67) nm was reported.

Silver ion is known to play an important role in the growth of nanoparticles especially for growth of GNRs. There was a report by (Li *et al.*, 2008)that concentration of silver ion affects the yield and stability of GNRs. In this experiment two different volume (0.6 and 1 ml) of silver nitrate solution was added to 50 ml of growth solution for synthesis of GNRs and found that GNRs synthesized with 0.6 ml of silver nitrate solution yielded higher quantity of GNRs. The result was confirmed by TEM image analysis of GNRs. This result very well correlated with earlier report by (Li *et al.*, 2008).

The synthesis procedure described by Lin *et al.* (2014) indicated that plasmonic longitudinal peak of GNRs was observed at 780 nm where as in the current study the longitudinal peak was found at 710 nm. The red shift in longitudinal peak from 710 to 780nm was due to the higher concentration of silver ion used in the current study for synthesis procedure. The concentration of gold nanospheres (seed particles) of 5-10 nm will act as a template for further attachment of gold ion to produce GNRs of optimum size which is very important in the synthesis of GNRs. It was reported by Malik *et al.* (2013)that to a fixed concentration of growth solution, (mixture of CTAB, ascorbic acid, silver nitrate and aurium chlorides) if more seed solution is added, yield of GNRs will decrease. In the current study when 120 µl of

seed solution was added to 50 ml growth solution the yield of GNRs was reduced. 100 µl of seed solution was required for 50 ml of growth solution for obtaining maximum yield of GNRs which was indicated by UV-Vis spectral analysis.

5.2. Comparison of Aspect ratio of GNRs using theoretical and experimental data.

The aspect ratio of GNRs is defined by length of GNRs divided by diameter of GNRs. The GNRs are rod shaped and are covered by positive charges when light falls, dipole movement is created due to electron oscillation, which results in the formation of two peaks. The interaction between two dipole causes the longitudinal peak to be observed in the visible to near infrared region (NIR). The longitudinal peak of the GNRs is important than the transverse peak, the peaks can be shifted by changing the aspect ratio of GNRs, which in turn depends upon the size of the GNRs.

The size of the GNRs was experimentally measured by TEM image analysis of GNRs and theoretically the size can also be determined using the equation described by Jain *et al.*(2008). In this study a comparison was made between theoretical and experimental data. It was found that the results obtained by experimental data i.e. TEM image was on par with the theoretical data. This indicated GNRs of optimum size was synthesized using a modified method of Nikoobakht and El-Sayed. (2003).

5.3. Immobilization of GNRs on to glass substrate.

Surface modification of glass with thiol-terminals is critically important for self-assembly of gold nanostructures. This was carried out by using saline chemical MPTMS (3-mercaptopropyl) trimethoxysilane). The silane groups are hydrolyzed to liberate silanols, which then condenses with the surface residues to form siloxane linkages. The thiol (SH) groups in MPTMS forms Au-S covalent bonding with GNRs (Wang and Tang. 2013). UV-Vis characterization of glass substrate after coating with

GNRs shows measureable shift in longitudinal LSPR peak and reduction in the intensity of absorbance.

The effective immobilization of GNRs on to the glass substrate depends upon the optimum concentration of CTAB in the GNRs solution, The excess amount of CTAB adversely affect assembly of GNRs on to the glass substrate due to electrostatic shielding to stabilize the colloidal gold solution. Hence the attraction of nanoparticles to the substrate becomes secondary and therefore prevents assembly of GNRs to the glass substrate. Therefore in the present study it was found that GNRs could not effectively bind to a glass substrate. Hence in the subsequent procedures GNRs was washed away from the glass substrate and the attempt failed and other methods were tried according to (Huang *et al.*, 2009).

5.4. Bioconjugation of GNRs with antibody of BBrMV

Gold nanorods are elongated nanoparticles with distinct optical property which depends upon the shape of the rod. It has several advantages compared to other metallic nanoparticles. The biocompatibility and optical property of GNRs make it a versatile and reliable nanoparticle for industrial application.

(Nikoobakht and El-Sayed 2011) reported that CTAB can interfere the effective binding of antibody which is a biological molecule with GNRs. In this study excess CTAB was completely removed from GNRs solution by repeated centrifugation for effective binding of antibody with GNRs. Mercaptopropanoic acid (MPA) acid was used to create a self assembly monolayer (SAM), which can act as a linker molecule between antibody and GNRs in the conjugation process (Song *et al.*, 2013).

Non covalent conjugation of antibody to the GNRs can be induced by electrostatic and hydrophobic interaction, with the advantages of simple and time effective. However the ionic environment and pH of the solution could be easily

changed due to electrostatic and hydrophobic interaction which resulted in the aggregation of GNRs, which make them difficult to be stable in this solution (Wang *et al.*, 2012).

The chemistry behind this conjugation process in covalent binding of antibody to GNRs is that the chemical EDC/NHS activated the COOH group of SAM, which covalently, links to the NH_2 group of an antibody Song *et al.* (2013). In this study bio conjugation of antibody to GNRs was carried out by covalent binding. For affective detection of analyte there was a need to characterize the binding of an antibody to GNRs. It was reported that when the GNRs are functionalized with any bimolecular (antibody) there is a change in the absorption pattern of the GNRs and as well as changes in the peak shift of GNRs Huang *et al.* (2009). Accordingly in the current study characterization of antibody labeled GNRs was carried out using UV-Vis spectral analysis and the result indicated that there is considerable amount of peak shift in the GNRs conjugated with antibody solution. This confirms there is effective binding of antibody to the GNRs.

There was a report by Yu *et al.* (2007) due to non covalent binding of an antibody to GNRs a longitudinal shift of about 60 nm is observed where as in the current study a longitudinal peak shift of 15nm was only observed due to covalent binding of an antibody to GNRs.

5.5. Isolation and characterization of an antigen (BBrMV virus particles)

In several experiments a number of procedures were followed for isolation of antigen but none of the protocol yielded good quality antigen. The procedure described by Gonsalves *et al.* (1986) for the isolation of an antigen gave low yield. In the current study modified protocol was followed to maximize the yield of antigen.

The most common problem associated with the isolation of antigen is aggregation of viral particles mainly in the initial stages of isolation, which leads to reduction of yield during low speed centrifugation.

Triton X-100, a non ionic detergent in the extraction buffer and in a resuspention buffer counters the aggregation of viral particles. The Urea in both also reduced aggregation considerably.

Phenolic oxidation of an enzyme polyphenol oxidase is another problem associated with the reduction in the yield of an antigen. In order to overcome this in the current study PVP 2% and β-mercapto ethanol 0.1 % were used in extraction buffer for inactivating the activity of polyphenol oxidase. The reduction in the yield of a viral particle due to Phenolic oxidation was reported in *Solanum nodedum virus* and barley *Bean Yellow Mosaic Virus* (Chu and Franki, 1983; Ehlers and Paul, 1986).

SDS-PAGE is an excellent method to identify the protein with desired size. Staining with CCB (Coomosie brilliant blue) is an ideal method of Visualization of a protein band with high concentration of protein. In previous procedure described by (Lakshmi, 2004) used CCB stain for identification of a BBrMV coat protein band and result found desired 38 KDa band. In the current study silver staining was followed for visualizations of protein band as the concentration of protein in the sample was low. Further the result confirmed the presence of 38 kDa protein of viral particle as reported in the previous studies.

As per the earlier reports BBrMV coat protein of Potyvirus group ranges 33 to 38 KDa (Sukhla *et al.*, 1994). The viral isolates of 33.4 kDa viral particle comprises of 900 nucleotides and about 300 amino acid sequences. The predicted molecular weight of 38 kDa in our present investigation is slightly smaller than the 39 kDa fusion protein observed by Rodeni *et al.* (1990) as well as 37 and 38 kDa protein reported by Thomas *et al.* (1997).

5.6. Detection of BBrMV through nanobiosensor.

The aggregations of GNRs induced by the biological molecules are utilized here for colorimetric detection of BBrMV, which is further confirmed by UV-Vis

spectrophotometer analysis. Antibody of BBrMV is covalently link to GNRs acts like nanoprobe. When the target/antigen (viral particles) when added to a nanoprobe solution, due to immunological reaction a complex structure was formed between antigen and antibody labeled gold nanorods (nanoprobe). This leads to severe aggregation of a nanoparticles and changes in the plasmonic property, which are visualized by changes in the colour of a GNRs-antibody conjugation solution. The result obtained very well correlated with the result reported by Xu *et al.* (2012). There is binding of analyte (antigen) to antibody which resulted in cross linking of GNRs followed by its aggregation.

Stewart *et al.* (2008) reported that changes in the absorption of GNRs nanoprobe solution after the addition of target is completely depends upon the concentration of an analyte/antigen. The concentration of analyte in a sample was increased absorption decreased due to aggregation of nanoparticles. The result obtained in the presence study indicated by kinetics curves analysis of GNRs with different concentration of antigen also gave the same result.

LSPR peak shift of GNRs in UV-Vis spectra is another method for detection of a target or analyte. The changes in the peak position of GNRs both in transverse and longitudinal peak and along with reduction of absorption after the addition of an analyte is due to plasmonic coupling between particles and the property of aggregation. In our study it was found that over a time with different concentrations of antigen binding with capture probe reached to a saturated condition and after that no changes were observed both in transverse and longitudinal peak shift. If the concentration of an antigen increases graph indicated a sigmoid pattern of curve. Wang *et al.* (2012) reported only longitudinal peak shift during antibody antigen interaction.

5.7. Kinetics of interaction of antigen with nanoprobe solution

Kinetics is the study to determine the behavior of a chemical reaction going on in a particular process. In this particular study the kinetics of antigen and antibody conjugated GNRs were monitored and validated by determination the rate of reaction and the extend of aggregation of antigen with nanoprobe solution the result showed the kinetics curve of aggregation initially increases and then it remained relatively constant. Gold nanoprobe is having several binding site for covalent linking of analyte, after a particular time interval binding between gold nanoprobe and an analyte decreased due to saturation of binding site Lim *et al.* (2008). The result obtained in the present study well correlated with the above report.

Song *et al.* (2013) demonstrated the kinetic curves of longitudinal LSPR peak shift increased over a certain time and curve remained relatively flat as the reaction progresses as similar to our experiment.

5.8. Determination of titer of BBrMV specific antibody

Antibody titer is a measurement of how much antibody is produced by an organism that recognized a particular epitope expressed in terms of a dilution. In most of the cases DAC-ELlSA was followed for the determination of titer of an antibody and in some of the cases ODD test was also followed for determination.

In this experiment it was found, out of the four dilutions 1: 200 dilution give the best result compared to other dilutions. The antibody titer reported by Dasanayaka *et al.* (2004) was 1: 500.The titer reported by Laksmisithal (2004) for DAC-ELISA was 1:200.

5.9. Serological method of detection

5.9.1. ELISA (Enzyme linked immunosorbant assay)

Serological methods of detection, which were based on the interaction between antigen and antibody, are routinely used for checking the sanitary status of a plant. Even though many serological methods of detection have been reported, ELISA is most commonly followed for virus detection. In present study DAC-ELISA was carried out for the detection of virus using antibody purchased from Agdida, USA and also for making a comparison between ELISA and nanobiosensor. DAC-ELISA was performed with various concentrations of antigen and results showed up to a range of concentration (2 to 0.02 mg/ml) detection is possible with nanobiosensor, where as Gonsalves *et al.* (1986) reported that for the detection of *Cardamom mosaic virus,* can be detected at a dilution ranging from 10^5-10^6 by DAC-ELISA.

Nanobiosensor based on the LSPR peak shift is a novel detection technique which can detect a wide range of analyte within minutes. The study conducted by Lin *et al.* (2014) for the detection of orchid virus by nanobiosensor could detect virus at a concentration 1 to 0.01 mg/ ml, which is not possible by ELISA. They also conducted a study for screening of different infected samples where the virus load very less and detection was carried out using nanobiosensor where ELISA was not sensitive. In the current study also confirmed ELISA could detect only up to a concentration of 0.04 mg/ml and where as nanobiosensor was sensitive up to 0.02 mg/ml.

5.9.2. DIBA (Dot blot immuno binding assay)

DIBA is an immunological technique used to detect the biological molecules or virus particles on a nitrocellulose membrane based on the interaction between antigen and antibody. In the presence study test samples was positive for virus infection and healthy samples were negative by DIBA. The similar results were

reported by Selvarajan (2000). The study also compared the sensitiveness DAC-ELISA over DIBA. The result of the present study also confirmed ELISA is more sensitive than DIBA and could detect a concentration of 0.04 mg/ml of antigen where as DIBA could detect up to 0.12 mg/ml. but in the case of fabricated biosensor it could detect up to 0.02 mg/ml antigen concentration, which showed its sensitiveness over other serological techniques.

5.6. Evaluation of different varieties of Banana for BBrMV infection

Diseases caused by various types of viruses have become deliberate constraints to banana production in the world. The four major viruses that infect bananas were *Banana streak virus* (BSV) *Cucumber mosaic virus* (CMV), *Banana bunchy top virus* (BBTV) and *Banana bract mosaic virus* (BBrMV). Banana bract mosaic virus causes characteristics spindle colour shaped streak on pseudostem and mosaic pattern on leaves which causes reduction in yield up to 40 per cent, where as in case of severe infection reduction of yield up to 100 per cent. The result of this study revealed cultivar Nendran is more seriously affected by BBrMV than other cultivar which correlates with earlier report by Cherian *et al.* (2004).

Summary

6. Summary

The study entitled "Development of a nano biosensor for detection of *Bract mosaic virus* in banana (*Musa* spp.)" was conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture and Centre for materials and electronics, Thrissur during the academic year of 2014-2016. The objective of the study was to develop a reliable and quick diagnostic method for detection of *a Bract mosaic virus* in banana.

The salient finding of this study summarized in three different sub heads.

Synthesis of Gold nano rods (GNRs) and conjugation with antibody

- Gold nanorods synthesized using seed mediated method showed characteristic 710 nm longitudinal band and a 520 nm transverse band.
- TEM image of GNRs indicated that, GNRs were uniformly dispersed into a solution with (42±3) nm length and diameter of (14±1.9) nm.
- Effect of Silver nitrate on the growth of GNRs was studied and result indicated aspect ratio of GNRs increased from 3.03 to 3.75. UV-Vis spectra of GNRs also indicated with increasing concentration of silver nitrate solution in the growth solution peak of the GNRs shifted from 710 to740 nm.
- With increasing volume of seed solution to growth solution showed decrease in the yield of GNRs. The optimum volume of seed solution was 100 µl to every 50ml of growth solution.
- The UV-Vis characterization of antibody bound GNRs showed about 11 nm peak shifts of a GNRs and absorbance also decreased due to binding of antibody to GNRs.

Detection of BBrMV through nanobiosensor and Comparison with ELISA and DIBA

- SDS- PAGE was done for isolated protein and confirmed the presence of 38 kDa coat protein band.

- Nanodrop spectrophotometer analysis of protein revealed that concentration of the antigen in the sample was 3mg/ml.
- Diagnosis of BBrMV infection in healthy and diseased sample was carried out visually by observing the colour changes in the GNRs solution by the addition of total protein isolated from both sample and it was found that the addition of protein from infected samples changes the colour of GNRs solution from red to black and in healthy sample colour of the GNRs solution remained same.
- The result was further confirmed by UV-Vis spectral analysis. A measurable peak shift from 7 to 25nm was observed based on the concentrations of antigen in the sample.
- It was observed that after an addition of particular concentration (0.25 mg/ml) of antigen no changes in the LSPR peak shift and also after 15 min of addition of antigen no changes were observed in LSPR peak shift.
- The sensitiveness of nanobiosensor compared with the other immunological techniques (ELISA and DIBA), indicated nanobiosensor was best compared to other detection methods.
- The detection of a target (BBrMV) was also carried out by ELISA reader, using antibody captured GNRs, after the addition of an antigen the colour changed from red to black in infected sample which was further confirmed by measurement of absorbance in healthy and diseased samples using ELISA reader. The data generated by ELISA reader showed variations in absorbance based on the concentrations of antigen in the sample.

Evaluation of different varieties of banana infected by BBrMV

- Five different varieties of banana plant were evaluated for the extent of BBrMV infection through nanobiosensor. Result indicated that among the five varieties Nendran cultivar was severely affected by BBrMV compared to other varieties.

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Future line work

In that light of the above information the future studies could be done on the following aspects:-

1. Development and fabrication of a prototype of nanobiosensor.
2. Digitization and commercialization of the final product

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Annexure

ANNEXURE I

List of laboratory equipment used for the studies

Electron microscope	:	Hitachi H-7650
UV-Vis spectrophotometer	:	Perkinz USA
Laminar air flow	:	Labline industries,Kochi
Shaker incubator	:	JEIO Tceh, Korea
ELISA reader	:	VERSA max, USA
Centrifuge	:	KUBATOO
Magnetic shaker	:	IKA laboratory technology

ANNEXURE II

Chemical composition used for Goldnanorods (GNRs) synthesis

Chemical	Concentration (M)	Quantity(gm)	Volume(ml)
CTAB	0.1	0.2733	7.5
CTAB	0.2	3.640	50
$NABH_4$	0.01	0.0037	10
HAuCl4	0.01	0.027	7
$AgNO_3$	0.01	0.008	10
Ascorbic acid	0.01	0.01	6

ANNEXURE III

Chemical composition used for conjugation of antibody to Goldnanorods (GNRs)

1) **MPA (20 mM)** - 200 µl

   Make up volume dissolved in a 9.8 ml of ethanol for making 10 ml of solution

2) **EDC(7.5 mM)** – 0.007 gm mix with 5ml of water

3) **NHS (1.5 mM)**- 0.008 gm mix with 5ml 0f water

ANNEXURE IV

Chemical composition used for SDS-PAGE analysis of protein (antigen)

1) Acrlamide stock solution

Acrylamide	29.2 g
N,N-methyl bis-acrylamide	0.8 g
Distilled water	Upto 100 ml

2) SDS (10 %)

SDS	10 g
Distilled water	Upto 100 ml

3) Ammonium per sulphate (APS) -10%

APS	0.1 g
Distilled water	Upto 1 ml

4) Protein loading dye (1X)

Tris-HCl	50mM
β-Mercaptoethanol	0.1%
SDS	10%
Bromophenol blue	0.1%
Glycerol	10%

5) Electrode buffer or Tank buffer (5x) pH-8.3

Tris-base	15 g
Glycine	72.0 g
SDS	5.0 g
Distilled water	Upto 1000 ml

6) Resolving gel Buffer (1.5 M Tris-HCl, pH-8.8)

Tris-base	18.15
Distilled water	Upto 100 ml

7) Stacking gel buffer (1 M Tris-HCl, pH-6.8)

Tris-base	12
Distilled water	Upto 100 ml

8) Fixer solution and Stop solution

Ethanol	150 ml
Acetic acid	7.5 ml
Distilled water	Upto 1500 ml

9) Staining solution

Silver nitrate	2.25 g
Formaldehyde	2.25 ml
Distilled water	Upto 1500 ml

10) Developer solution

Sodium hydroxide	22.5 g
Formaldehyde	2.25 ml
Distilled water	Upto 1500 ml

ANNEXURE V

Buffer used for DAC-ELISA

1. **Coating buffer (PBS - pH 7.4)**

Sodium chloride	8.0 g
Potassium di hydrogen phosphate	0.2 g
Disodium hydrogen phosphate	1.1 g
Potassium chloride	0.2 g
Water 1000 ml	

2. **Washing buffer(PBS-T)**

PBS buffer (pH- 7.4)	1000 ml;
Twain 20 (0.05%) 0.5 ml	

3. **Substrate solution (pH 9.8)**

Diethanolamine	97 ml
Sodium azide	0.2 g
Water 800 ml Add HCl to give pH 9.8	

4. **Blocking solution**

PBS-T	100 ml
BSA (0.2%)	0.2 gm

5. **Antibody dilution buffer**

PBS-T	100ml
BSA (0.2%)	0.2gm
PVP (2%)	2gm

DEVELOPMENT OF A NANO BIOSENSOR FOR DETECTION OF BRACT MOSAIC VIRUS IN BANANA (*Musa* spp.)

by

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ABSTRACT OF THE THESIS

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Abstract

Banana bract mosaic virus (BBrMV) is a recently described virus of banana which contributed to yield reduction by 5 to 36 per cent and is a barrier to international exchange of germplasm. There is a no effective measure to control this virus, only by routine virus indexing of planting material can protect the spreading of this virus. Currently ELISA and real time PCR is effectively used for diagnosis but the protocol is time consuming and expensive.

In the recent years biosensor based on the novel metallic nanoparticle gain much importance for industrial applications and efficient detection of viruses. The study entitled "Development of a nano biosensor for detection of *Banana bract mosaic virus* in banana (*Musa* spp.)" was carried out in the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture and Centre for Electronics and Materials(C-MET), Thrissur during the academic year 2014-2016. The objective of this study to develop an antibody based nanobiosensor for quick detection of *Banana bract mosaic virus*.

Goldnanorods (GNRs) were fabricated through seed-mediated procedure and UV-Vis spectra of GNRs solution indicated characteristic longitudinal and transverse band at 710 nm and 520 nm respectively. The transmission image of electron microscope revealed that solution contain rod shaped gold nanoparticles with length and diameter (42±3) nm (14±1.9) nm respectively. The aspect ratio of GNRs was measured through ImageJ software and found that aspect ratio of GNRs was 3.03.

The effect of silver nitrate solution on the growth of GNRs was studied and found that with increasing silver ion concentration in a growth solution longitudinal peak shift was observed from 710-740 nm and also aspect ratio of GNRs also increased from 3.03 to 3.75. In order to detection of analyte (BBrMV) surface of a GNRs activated with complete replacement with alkalithiol molecule for covalent attachment of an antibody. UV-Vis spectra of activated GNRs indicated that due to formation of SAM (Self assembly monolayer)position of a peak shifted from 710-

716 and also due to binding of an antibody to SAM layer again peak position was changed from 716-727nm.

SDS-PAGE and Nanodrop spectrophotometer analysis were carried out for the BBrMV antigen to check the quality and quantity of protein (antigen). The results had shown 38KDa band coat protein specific band of virus in a gel and concentration of antigen was 3mg/ml.

Bio-recognition induced gold nanorods aggregation here takes as an analytical tool for detection of a BBrMV. In this case due to addition of antigen to antibody labeled GNRs solution. Colour of the solution changed red to black and notable peak shift of (7- 25) nm was observed both in transverse and longitudinal peak of GNRs in UV-Vis Spectra. Antigen concentration up to 0.25 mg/ml and above shows stability in the Peak shift and colour change in infected sample compared to control sample. In healthy sample no colour changes were observed and with only minimum peak shift was there. The positive result was also obtained in a micro titre plate where ELISA reader clearly differentiated healthy and infected samples with different concentrations of antigen.

In case of longitudinal peak shift, the kinetics curve of an infected sample remained relatively flat and after 15min the shift remained stable until the end of the observation and the absorbance of GNRs continuously decreased up to 80^{th} min and after that no changes were observed in the kinetics curves of an absorbance.

For determining the accuracy and sensitiveness of a nanobiosensor, results of the different serological techniques (ELISA, DIBA) were compared with the result of the fabricated solution based nanobiosensor and found that nanobiosensor could detect the viral protein at a very low concentration (2-0.02) mg/ml, whereas in the case of other techniques the detection was possible up to 0.12 mg/ml of antigen concentration.

The developed gold nano rods based nanobiosensor was evaluated for detection of a BBrMV in five varieties of banana and it was found that cv. Nendran

was more affected by BBrMV compared to other varieties of banana due to the high concentration of viral load in the infected samples. The solution based gold nano rod based biosensor is sensitive, cost effective and easy for virus indexing of tissue culture plants and planting materials compared to other methods currently in use. Further investigations and refinement could lead to the fabrication and development of nanobiosensor on a commercial scale.