Chapter 8
Nanotechnology for the Detection and Diagnosis of Plant Pathogens

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Abstract Rapid detection technologies with high sensitivity and selectivity for plant pathogens are essential to prevent disease spread with minimal loss to crop production and food quality assurance. Traditional laboratory techniques such as microscopic and cultural techniques are time-consuming and require complex sample handling. Immunological and molecular techniques are advanced but have some issues related to rapidity and signal strength. In this context, integration of immunological and molecular diagnostics with nanotechnology systems offers an alternative where all detection steps are done by a portable miniaturized device for rapid and accurate identification of plant pathogens. Further, nanomaterial synthesis by utilizing functionalized metal nanoparticles as a sensing component offer several desirable features required for pathogen detection. The sensitive nature of functionalized nanoparticles can be utilized to design phytopathogen detection devices with smart sensing capabilities for field use. This chapter provides an overview of the application of nanotechnology in the field of microbial diagnostics with special focus on plant pathogens.

Keywords Agriculture • Detection • Diagnosis • Nanosensor • Nanotechnology • Pathogen • Quantum dots

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8.1 Introduction

Plant diseases are major limiting factor in sustainable crop production. It is estimated that about 20–30 % of the field crops are annually lost due to infection of diseases (Nezhad 2014; Sankaran et al. 2010; Mann et al. 2008). Although, combined infestation of pests and diseases in plants could result up to 82 % losses in attainable yield in case of cotton and over 50 % losses for other major crops (Pan et al. 2010; Thind 2012). Further, if we combine these losses with post-harvest spoilage and deterioration in quality, these losses become more critical particularly for resource poor countries like India. Usually, the bacterial, fungal, and viral infections, spread over larger area in crops, groves and plantations through accidental introduction of vectors or through infected seed or plant materials. Another route for the spread of pathogens is through ornamental plants that act as hosts. These plants are frequently sold through mass distribution before the infections are known. In this context, early detection of diseases is of key importance to prevent disease spread with minimal loss to crop production (Sankaran et al. 2010; Martinelli et al. 2014).

Traditional methods for identifying plant pathogens rely on the interpretation of visual symptoms and/or the isolation, culturing and laboratory identification of the pathogen. These techniques suffered from some major drawbacks such as lack of sensitivity, time-consuming and costly etc. Additionally, the accuracy and reliability of these assays depend largely on the experience and skill of the person making the diagnosis (Sankaran et al. 2010; Kashyap et al. 2011; Alvarez 2004). Table 8.1 provides comparative analysis of conventional (culture-based), immunological, nucleic acid based-assays and nanotechnological tools for detection and diagnosis of plant pathogens. From past two decades, several attempts have been devoted to the development of methods for detecting and identifying plant pathogens based on enzyme-linked immunosorbent assay (ELISA), biochemical assays based on specific protein and toxins, nucleic acid probe technology and polymerase chain reaction (PCR) amplification of nucleic acid sequences (McCartney et al. 2003; Sundelin et al. 2009; Kumar et al. 2013; Kumar and Kashyap 2013; Kashyap et al. 2013a; Singh et al. 2014).

Table 8.1 Comparison of diagnostic methods used for the detection of plant pathogens

Method	Assay duration (h)	Detection limit	Time before result	Specificity	On-field portability	Sensitivity
Plating technique	>72	1 cfu ml^{-1}	1–3 days	Good	Poor	Poor
Immunological technique	1–3	1 pg/mL	1–2 h	Moderate	Very Good	Moderate
Nucleic acid-based technique	1–3	10^3 cfu ml^{-1}	6–12 h	Very good	Moderate	Good
Nanotechnology-based techniques	0.30–1.0	1 fmol l^{-1}	0.30–1.0 h	Excellent	Excellent	Very good

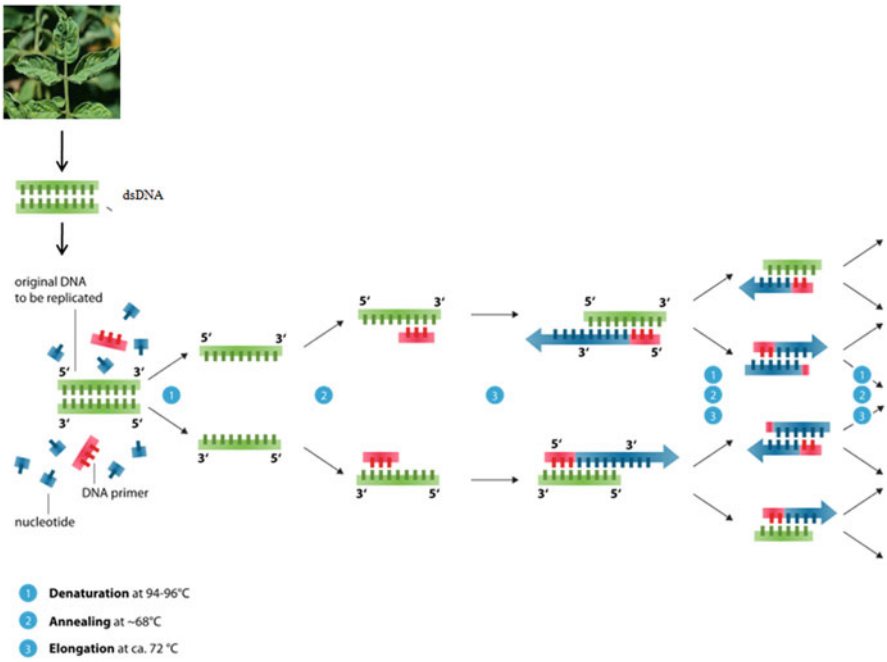

Fig. 8.1 Schematic illustration of Polymerase chain reaction (PCR). PCR is used to amplify, or create millions of identical copies of a particular DNA sequence within a tiny reaction tube. Prior to the initiation of each new round for DNA amplification, the DNA is denatured, two sets of DNA primers anneal to the denatured complementary strand. Then, primers lead DNA synthesis by the DNA polymerase. All reactions occur sequentially in template dependent manner

PCR has been widely used for the detection of plant diseases caused by fungi, bacteria, viruses and phytoplasma (Fang and Ramasamy 2015; Kashyap et al. 2011). It takes 5–24 h in detection that depends on the specific PCR variation used and this does not include any previous enrichment steps. Figure 8.1 illustrates the PCR method, the extracted and purified DNA followed by annealing of specific primer and an extension phase using a thermostable polymerization enzyme. Then each new double stranded DNA acts as target for a new cycle and exponential amplification is thus obtained. The presence of the amplified sequence is subsequently detected by gel electrophoresis. In addition to the basic PCR, variants of PCR methods such as reverse-transcription PCR (RT-PCR) has also been used for plant pathogen having RNA as a genetic material (López et al. 2003; Jeong et al. 2014). Multiplex PCR was proposed to enable simultaneous detection of several pathogens in a single reaction (López et al. 2003). Real-time PCR platforms, loop-mediated isothermal amplification (LAMP), isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) and microarray have also been used for on-site, rapid diagnosis of plant diseases based on the fungal, bacterial and viral nucleic acids (Jeong et al. 2014; DeBoer and Lopez 2012; Kashyap et al. 2013). Although these nucleic acid-based techniques and biochemical assays are very sensitive,

accurate, and effective for confirming visual scouting, they are unreliable as screening tests to monitor plant health status before the appearance of symptoms. They require detailed sampling procedures, expensive infrastructure, and may feign the real status of pathogen infections. Unfortunately, these assays can be only effectively used for a restricted number of plants pathogens. Still, most of these methods cannot be applied for on-site pathogen detection in the agricultural fields. Furthermore, the high price and short shelf-life of molecular biology reagents, such as enzymes and primers, limit the application of molecular methods. As a result, developing low-cost methods to improve the accuracy and rapidity of plant pathogens diagnosis is needed. Recent advances have led to the development of functional nanoparticles (electronic, optical, magnetic, or structural) that can be covalently linked to biological molecules such as peptides, proteins, and nucleic acids.

One of the most promising nanomaterials is quantum dots (QD), which have been widely used in a broad range of bio-related applications including rapid detection of a particular biological marker with extreme accuracy (Kashyap et al. 2015). Biosensor, quantum dots, nanostructured platforms, nanoimaging and nanopore DNA sequencing tools have the potential to raise sensitivity, specificity and speed of the pathogen detection, facilitate high-throughput analysis, and can be used for high-quality monitoring and crop protection (Khiyami et al. 2014). Furthermore, nanodiagnostic kit equipments can easily and quickly detect potential plant pathogens, allowing experts to help farmers in the prevention of epidemic diseases. Currently, a vast library of nanostructures has been synthesized and documented, with different properties and applications (Savaliya et al. 2015; Khiyami et al. 2014). Figure 8.2 illustrates potential applications of nanotechnology in detection and diagnosis of plant pathogens. Briefly, the present article discusses the various applications of nanotechnology in plant pathogen detection for quicker, more cost-effective and precise diagnostic procedures of plant pathogens. Such an accurate technology may help to frame an effective integrated disease management system which may modify crop environments that adversely affect crop pathogens.

8.2 Nanotechnology-Based Diagnostic Systems

The integration of molecular diagnostics and nanotechnology is a promising technology for rapid and accurate identification of plant pathogens. Presently, several nanodevices and nanosystems have been used in diagnostics as well as sequencing single molecules of DNA. Assays with the use of nano-size devices to investigate DNA sequences and diagnose disease are becoming faster, more flexible and more sensitive. It is worth mentioning here that newly developed nanomaterials with special nanoscale characteristics offers tremendous breakthrough in plant pathogen detection and diagnosis technology (Khiyami et al. 2014). Table 8.2 provides an overview of major developments in nanotechnology-based systems for detection and diagnosis of plant pathogens. Besides this, nanotechnology is also driving the

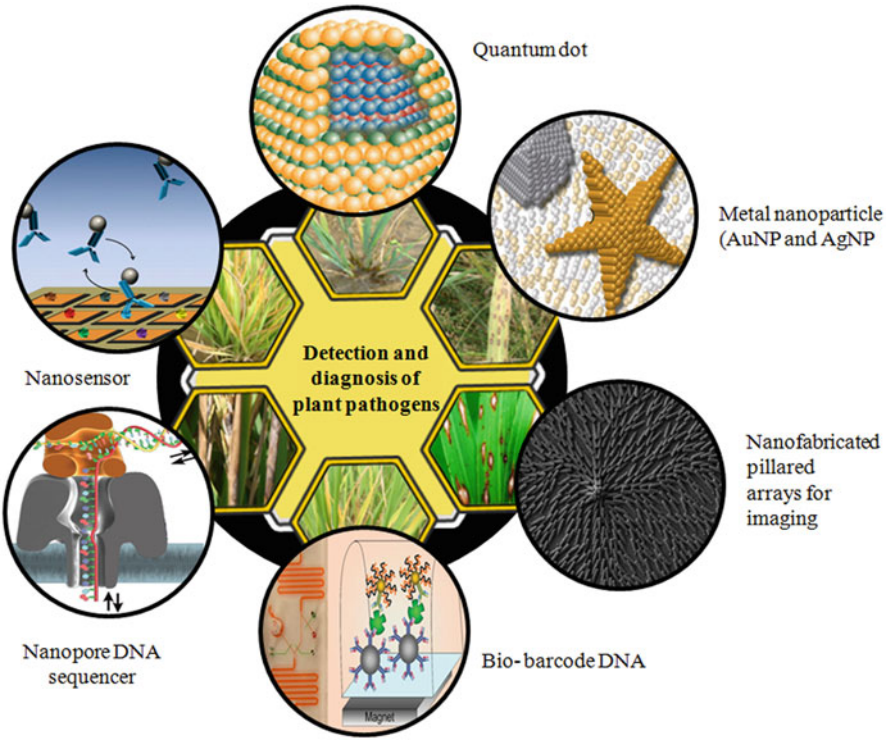

Fig. 8.2 Application of nanotechnology and currently synthesized nanomaterial for detection and diagnosis of plant pathogens. These include metallic, semiconductor and organic molecule nanomaterials of a variety of shapes, sizes and structures

development of lab-on- chip systems for detecting pathogens, toxicity in water, observing nutrients in irrigation water and controlling the quality in food products.

8.2.1 Functional Quantum Dot Nanoparticles Based Diagnostic System

Quantum dots (QD) are a class of luminescent semiconductor nanocrystals that emit light of specific wavelengths, in which the size of the nanoparticle determines the wavelength; the larger the size, higher the wavelength of the infra-red light emitted (Edmundson et al. 2014). They offer several advantages over organic dyes based broad excitation spectra. The quantum dots have narrow defined tunable emission peak, longer fluorescence lifetime, resistance to photobleaching and 10–100 times higher molar extinction coefficient. These properties of quantum dots allow multicolor quantum dots to be excited from one source by common fluorescent dyes without emission signal overlap and results in brighter probes comparing to

Table 8.2 Major breakthroughs in the development of nanotechnology-based systems for detection of plant pathogens

Year	Breakthrough(s)	Nanomaterial used	References
2009	Fluorescence silica nanoprobe as a biomarker for rapid detection of *Xanthomonas axonopodis* pv. *vesicatoria*, responsible for bacterial spot disease in tomatoes and peppers	Fluorescent silica nanoparticles (FSNP) combined with antibody molecules	Yao et al. (2009)
2010	Electrocatalytic oxidation of phytohormone salicylic acid at copper nanoparticles-modified gold electrode and its detection in oilseed rape infected with fungal pathogen *Sclerotinia sclerotiorum*	Copper nanoparticles-modified gold electrode	Wang et al. (2010)
2010	Surface plasmon resonance based immunosensor for Karnal bunt (*Tilletia indica*) diagnosis based on the experience of nano-gold based lateral flow immune-dipstick test	Nano-gold particles	Singh et al. (2010)
2012	Development of a quantum dots FRET-based biosensor for efficient detection of *Polymyxa betae*, a vector of beet necrotic yellow vein virus (BNYVV) responsible for *Rhizomania* disease in sugar beet	Tioglicolic acid-modified Cadmium-Telluride QD	Safarpour et al. (2012)
2012	Detection of *Candidatus Phytoplasma aurantifolia* with a quantum Dots FRET-based biosensor	Tioglicolic acid-modified cadmium-telluride quantum dots (CdTe-QD)	Rad et al. (2012)
2013	Synthesis of CuO nanoparticles and fabrication of nanostructural layer biosensors for detecting *Aspergillus niger* fungi	CuO nanoparticles and nanostructural layer biosensors	Etefagh et al. (2013)
2013	Development of a fluorescence resonance energy transfer (FRET)-based DNA biosensor for detection of synthetic oligonucleotide of *Ganoderma boninense*, an oil palm pathogen	Modified QD that contained carboxylic groups	Bakhori et al. (2013)
2013	Polypyrrole nanoribbon based chemiresistive immunosensors for viral plant pathogen detection	Nanoribbon	James (2013)
2014	Direct detection of orchid viruses using nanorod-based fiber optic particle plasmon resonance immunosensor	Nanorod	Lin et al. (2014)
2014	Electrochemical detection of p-ethylguaiacol, a fungi infected fruit volatile using metal oxide nanoparticles	TiO_2 or SnO_2 nanoparticles on screen-printed carbon electrodes	Fang et al. (2014)

(continued)

Table 8.2 (continued)

Year	Breakthrough(s)	Nanomaterial used	References
2014	Plant diseases detection using nanowire as biosensor transducer	Nanowire	Ariffin et al. (2014)
2015	Development of a helicase-dependent isothermal amplification (HDA) in combination with on-chip hybridization for the detection of selected *Phytophthora* species	Silver nanoparticle	Schwenkbier et al. (2015)

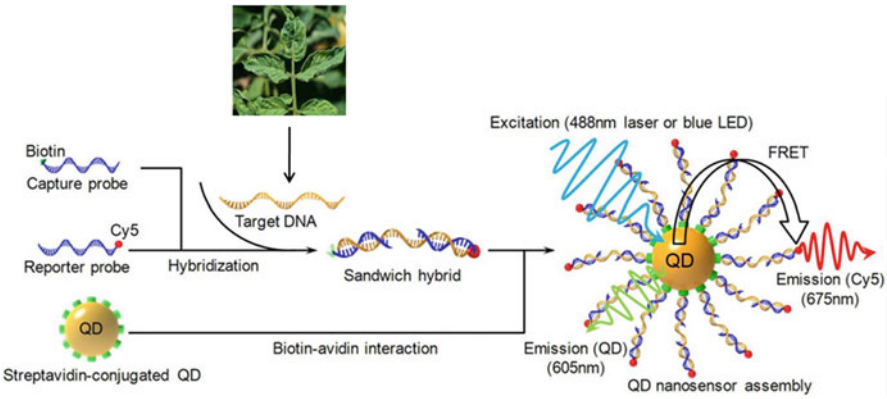

Fig. 8.3 Schematic illustration of quantum dot fluorescence resonance energy transfer (QD-FRET) sensors. Two probes labeled with biotin and Cy5 respectively hybridize to the target DNA (pathogen infected plant sample) and form a sandwich hybrid. The hybrids self assemble onto the quantum dots surface to form a QD-FRET nanosensor (Source: Chen et al. 2013)

conventional fluorophores (Zhao and Zeng 2015). Due to these advantages, QD-FRET-based nanosensors gained a wide spread popularity in agriculture and allied sectors. Figure 8.3 describes the schematic illustration of QD-FRET nanosensor. These sensors are most frequently applied in the domain of nucleic acid and enzyme activity detection (Stanisavljevic et al. 2015).

The mycosynthesis of semiconductor nanomaterials was first reported in unicellular yeast, which was capable of producing cadmium sulphide (CdS) crystallites in response to cadmium salt stress (Dameron et al. 1989). Different microbes have also been used for the biosynthesis of CdS (Yadav et al. 2015), however, limited studies have focused on its fluorescent properties. A proficient myco-mediated synthesis of highly fluorescent CdTe quantum dots was accomplished by *Fusarium oxysporum* when reacted with a mixture of $CdCl_2$ and $TeCl_2$ (Jain 2003; Kashyap et al. 2013b; Alghuthaymi et al. 2015). Knudsen et al. (2013) have shown that QD-based nanosensors are capable of probing multiple enzyme activities simultaneously. Recently, CdTe quantum dots has been used as biosensors by coating them with specific antibodies against *Polymyxa betae* specific glutathione-S-transferase (GST) protein (Safarpour et al. 2012). The mutual affinity of antigen and antibody brought the

CdTe quantum dots and rhodamine together close enough to allow the resonance dipole-dipole coupling required for fluorescence resonance energy transfer (FRET) to occur. The constructed immunosensor showed a high sensitivity, specificity and was successfully used for high-throughput screening of plant samples with consistent results within 30 min. On parallel lines, Rad et al. (2012) also developed a quantum dot (QD)-based nano-biosensor for highly sensitive detection of phytoplasma (*Candidatus Phytoplasma aurantifolia*) in infected lime trees. The developed immunosensor showed 100 % specificity with a detection limit of 5 *Ca. P. aurantifolia* μl^{-1}. Recently, an optical DNA biosensor based on fluorescence resonance energy transfer (FRET) utilizing synthesized quantum dot (QD) has been developed for the detection of specific-sequence of DNA for *Ganoderma boninense* (Bakhori et al. 2013). Modified quantum dots (5–8 nm) that contained carboxylic groups was conjugated with a single-stranded DNA probe (ssDNA) via amide-linkage.

Hybridization of the target DNA with conjugated QD-ssDNA and reporter probe labeled with Cy5 allows the detection of related synthetic DNA sequence of *Ganoderma boninense* gene based on FRET signals. The developed biosensor has shown high sensitivity with detection limit of 3.55×10^{-9} M. This approach is also capable in providing simple, rapid and sensitive method for detection of plant pathogens. Moreover, quantum dots can be excited using UV light and fluorescence can be visualized with the naked eye, this technology can be transferred into the field for immediate use. Research is just beginning for the use of quantum dots for detecting plant pathogens and toxins on and in foods and plants. Work must continue for the optimization of assays to obtain an accurate signal for low levels of pathogens in complex systems, whether they are food, plants or insects. The opportunities are endless for the applications of functional quantum dot nanoparticles based diagnostic system in agriculture and allied sectors, the field is ever-expanding and scientists are trying to keep up with the latest technologies that can be used to protect agricultural crops and food commodities from plant pathogens.

8.2.2 Metal Nanoparticles Based Diagnostic System

Metal nanoparticles have been applied in biosensors as marker tags to replace enzymes as the label. Striping voltammetry as an electrochemical technique can be applied to detect the metal nanoparticles directly making the assay simple to perform. Gold (AuNP) and silver nanoparticles (AgNP) can be used in these methods including different inorganic nanocrystals (ZnS, PbS and CdS) for analyte detection (Upadhyayula 2012). The unique physical and chemical properties of nanoparticles such as colloidal gold can provide excellent application in a wide range of biosensing techniques (Rosi and Mirkin 2005; Khan and Rizvi 2014). AuNPs have high surface-to-volume ratios and can be functionalized to detect specific pathogen targets, offering lower detection limits and higher selectivity than conventional strategies. AuNP system is composed of two types of AuNP. Each type of AuNP is coated

with different thiol-oligonucleotides. The last 15 nucleotides are complementary to one-half of a target DNA sequence. When the target DNA is introduced into the system, the two types of AuNP both bind to the DNA and aggregate. This results in a color change from red to blue, which is a well-known behavior of AuNPs. Further, there is still a wide scope to improve the signal amplification efficiency by exploring the effects of the loading of detector probes and the number of gold nanoparticles in colour development. Figure 8.4 illustrates the schematic diagram of oligonucleotide-gold nanoparticle aggregation assay and detection strategy. This AuNP based technology is an excellent example of exploitation of the tunability of AuNP surface chemistry to optimize performance under real field conditions. Several products are available in the market such as Oxanica (UK) Quantum dots and MultiPlxBeads™ from Crystalplex Corporation, USA (Tothill 2011).

Nanoparticles can also be exploited in conductivity based sensors where they can induce a change in the signal upon the attachment of the nanoparticles tagged antibody with the antigen captured on the sensor surface (Servin et al. 2015). Various strategies such as antibody-antigen, adhesion receptor, antibiotic and complementary DNA sequence recognitions have been developed for a specific detection between target phytopathogenic cells and bio-functionalized nanomaterials (Conde et al. 2014). Gold nanoparticles are excellent markers to be used in biosensors due to ease in alternation of their optical or electrochemical procedures to identify pathogens. A number of nanoparticle-based experiments have been performed to develop biomolecular detection with DNA- or protein functionalized gold nanoparticles, which are used as the target-specific probes (Thaxton et al. 2006). These detection methods include conductive polymer nanowires (Pal et al. 2008), carbon nanotubes (Poonam and Deo 2008), nanoporous silicon (Yang et al. 2008) and gold nanoparticles (Wang et al. 2010). Singh et al. (2010) used nano-gold based immunosensors that could detect Karnal bunt disease in wheat (*Tilletia indica*) using surface plasmon resonance (SPR).

Wang et al. (2010) exploited indirect stimulus to develop a sensitive electrochemical sensor, using modified gold electrode with copper nanoparticles, to monitor the levels of salicylic acid in oil seeds to detect the pathogenic fungus, *Sclerotinia sclerotiorum*. They successfully and accurately measured salicylic acid using this sensor. Research on similar sensors and sensing techniques needs to be expanded for detecting pathogens, their by products or monitor physiological changes in plants due to infections. Dubertret et al. (2001) noticed the ability of gold nanoparticles to act as fluorescence quenchers and, therefore, it could be used to solve major drawbacks in molecular biology experiments. For instance, a DNA oligonucleotide could be synthesized, fluorescently labelled at its 5′ end and conjugated at the 3′ end with gold nanoparticles. The successful application of these oligonucleotides has been reported in the diagnosis of the phytoplasma associated with the flavescence dorée (FD) of grapevine (Firrao et al. 2005). Fan et al. (2003) reported that the gold nanoparticles efficiently quench the fluorescence of light harvester polymers, such as polyfluorene, and will open new perspectives in the development of optical performances of nanobiotransducers for diagnostic purposes. Furthermore, diagnostic probe made of a specific oligonucleotide bearing a fluorescein at its 5′ terminal and

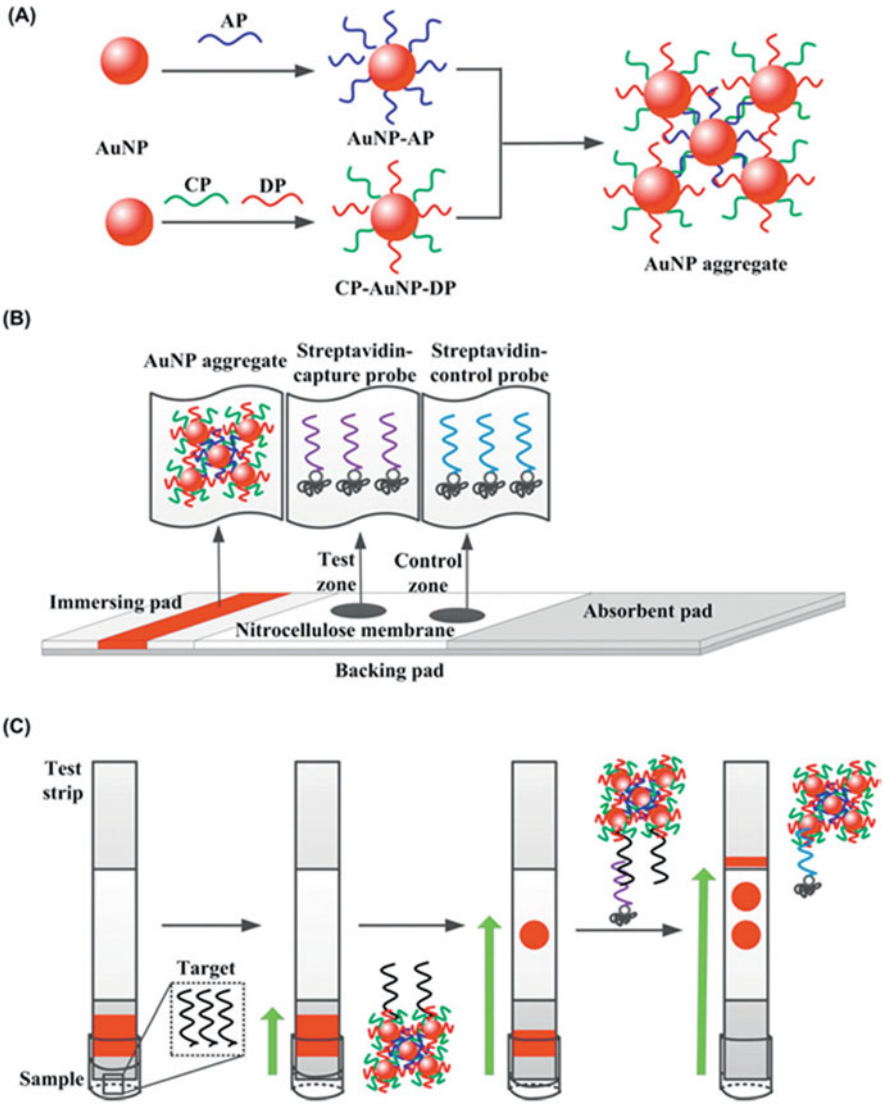

Fig. 8.4 Qligonucleotide-gold nanoparticle aggregation assay and detection strategy. (**a**) Preparation of oligonucleotide-linked gold nanoparticle (AuNP) aggregates. (**b**) Design of nucleic acid lateral flow (NALF) test strips. (**c**) Principle of the AuNP aggregates based NALF assay. *AP* amplification probe, *CP* complementary probe, *DP* detector probe. AP and CP are complementary to each other (Source: Hu et al. 2013)

gold nanoparticles (2-nm) at its 3′ terminal act as a nanobiotransducer in DNA hybridization. It produces a stronger fluorescence signal when hybridized to target DNA (Firrao et al. 2005).

The bio-barcode assay is another ultrasensitive method of amplification and detection of nucleic acids or proteins. They are characterized by their ultrahigh sensitivity and multiplexing capability for the detection of oligonucleotides or proteins. In case of oligonucleotide targets, Oligo-AuNP probes are hybridized to oligonucleotide-functionalized magnetic microparticle (MMP) probes using the target sequence as a linker. These complexes are then separated magnetically for subsequent release of the oligonucleotides from the Oligo-AuNP probes. These released biobarcodes are quantitatively analyzed by the scanometric assay (Fig. 8.5). The assay is also promising by allowing the quick detection of nucleic acids at high-zeptomolar levels (Nam et al. 2004) and protein targets at low-attomolar concentrations under optimized conditions (Goluch et al. 2006). Schwenkbier et al. (2015) developed a helicase-dependent isothermal amplification (HDA) in combination with on-chip hybridization for the detection of *Phytophthora* species. This approach allows efficient amplification of the yeast GTP-binding protein (Ypt1) target gene region at one constant temperature in a miniaturized heating device. The assay's specificity was determined by on-chip DNA hybridization and subsequent silver nanoparticle deposition. The silver deposits serve as stable endpoint signals that enable the visual as well as the electrical readout. These advancements, point to the direction of a near future on-site application of the combined techniques for a reliable detection of several kinds of plant pathogens.

8.2.3 Nanostructured Platforms Based Diagnostic System

Advancement of nanotechnology and biotechnology has prompted utilization of nanostructure as a novel sensing platforms, owing to their ultra-high surface area to volume ratio, size dependent electrical properties, and possibility of device miniaturization (Prieto-Simon et al. 2007; Sertova 2015). The principal application of such nanostructures is to decrease the time for pathogen detection. Nanomaterials, such as carbon nanomaterials (carbon nanotubes and graphene), nanowires, nanocomposites, and nanostructured metal oxide nanoparticles are playing an increasing role in the design of nanosensing platforms for pathogen and mycotoxin determination (Malhotra et al. 2014). Other types of nanostructure platforms are based on microfluidics systems, which can also be used to detect pathogens efficiently in real time and with high sensitivity (Baeummer 2004). A major advantage of such systems is their miniature format and their potential to detect compounds of interest in minute sample volumes with rapidity (García et al. 2010). Food spoilage due to fungal and bacterial microbes can be detected by several kinds of nanostructured platforms. For instance, Bhattacharya et al. (2007) designed an array of thousands of nanoparticles to be visualized in different colours in contact with food pathogens. Kaushik et al. (2009) fabricated nanoSiO2 and chitosan based nanobiocomposite film on an ITO substrate to co-immobilize r-IgGs and BSA for OTA (*Aspergillus ochraceus*) detection. They concluded that BSA/r-IgGs/CH-NanoSiO2/ITO immunoelectrode can be used for OTA detection with improved sensing characteristics.

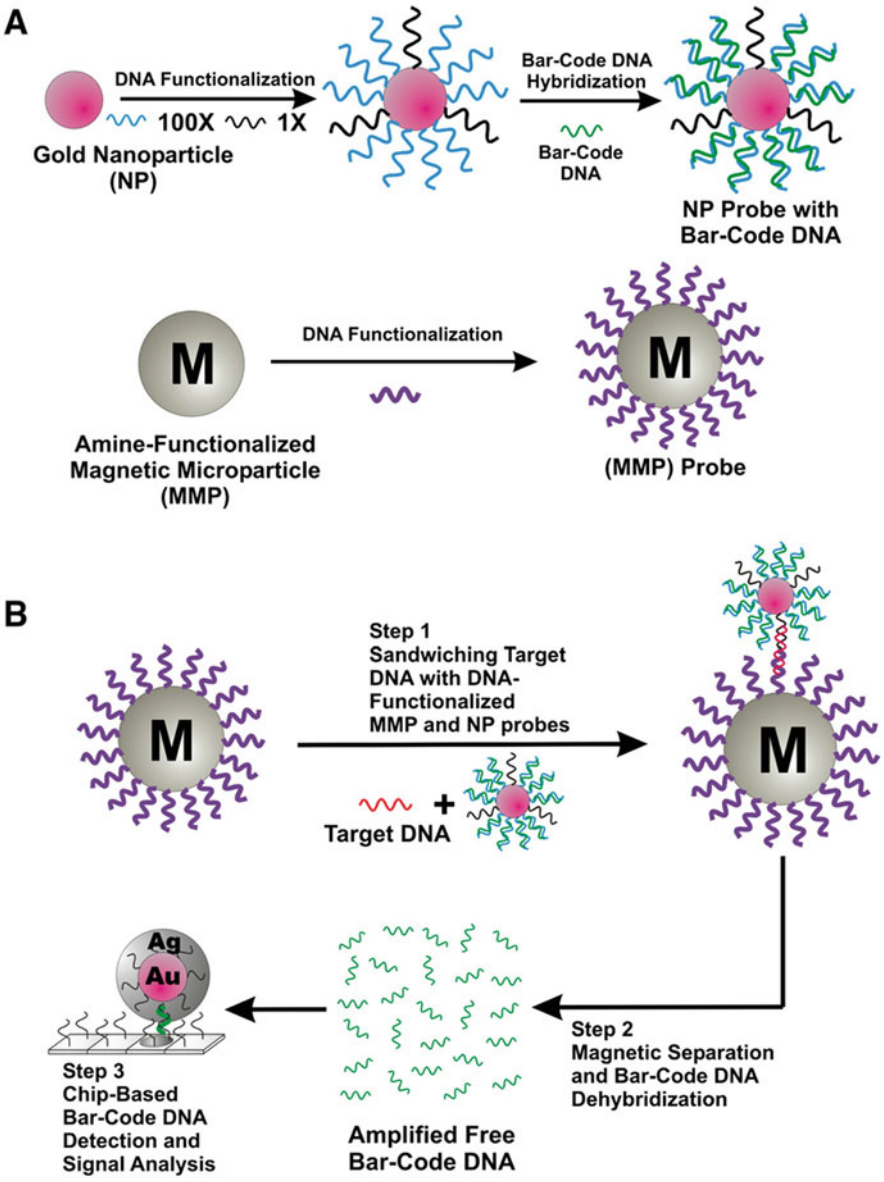

Fig. 8.5 A bio-barcode-amplification based assay for target DNA detection. (**a**) Preparation of gold nanoparticle and magnetic microparticle probes. Gold nanoparticles are functionalized with hybridized barcode DNA and capture strands for the target. Magnetic nanoparticles are functionalized with capture strands for the target. (**b**) Biobarcode based amplification (BCA) technique coupled with scanometric detection (Source: Nam et al. 2004)

Horseradish peroxidase (HRP) biosensors have been applied without any pre-treatment to determine OTA (*A. ochraceus* and *Penicillium viricatum*) in spiked beer samples and roasted coffee (Alonso-Lomilloa et al. 2010). Paniel et al. (2010) used magnetic nanoparticles to improvise electrochemical immunosensor for the detection of ultra-trace quantities of AFM1 (up to 0.01 ppb) produced by *A. flavus* in foodstuffs. Hervas et al. (2011) described a 'lab-on-chip' strategy integrating an electrokinetic magnetic bead-based electrochemical immunoassay on a microfluidic chip for the quick, sensitive and discriminatory quantification of zearalenone produced by *Fusarium* sp. Further, Panini et al. (2010) developed an immunosensor that utilized multi-wall carbon nanotubes and a continuous-flow system for the rapid and sensitive quantification of zearalenone in corn silage samples. Ansari et al. (2010) demonstrated that sol–gel derived Nano-ZnO film can be used for the immobilization of r-IgGs and BSA for blocking nonspecific binding sites of r-IgGs to detect OTA with a detection range, 0.006–0.01 nM/dm^3. Similarly, Kaushik et al. (2013) developed a nanostructured cerium oxide film based immunosensor for the detection of food-borne mycotoxins. Rabbit-immunoglobulin antibodies and BSA have been immobilized onto sol–gel derived nanostructured cerium oxide film synthesized onto an indium tin-oxide covered glass plate for the detection of ochratoxin-A.

Mak et al. (2010) reported an ultra-sensitive magnetic nanoparticle immunoassay for detecting more than one mycotoxin. Using the magnetic nanoparticle as the solid phase allowed a significantly increased surface area for the immobilization of the reactants and their uniform distribution throughout the whole volume of the reaction medium, thereby eliminating the diffusion limitations of traditional ELISA. The application of a magnetic field separated the reactants simply and rapidly, and facilitated the wash steps that are also required in traditional microplate-based ELISA (Fig. 8.6). Using these advantages, the MNP-based immunoassay scheme was developed and implemented in ELISA microplate wells for detection of aflatoxin-B1, zearalenone and HT-2 mycotoxins. The ozonization and adsorption efficiency of modified nanodiamonds to detect the content of aflatoxin-B1 has been examined by Puzyr et al. (2010). Recently, Actis et al. (2010) described ultrasensitive method for the detection of mycotoxins by STING (signal transduction by ion nano-gating) sensors, with a detection limit up to 100 fg ml^{-1}. Silica and clays are most efficient in combination with smaller sized water molecules and smaller mycotoxins such as aflatoxins and ochratoxins. However, clays are less efficient in binding the larger mycotoxins such as fumonisin and deoxynivalenol (vomitoxin) because the distance among clay layers is not sufficient to accommodate the larger molecules (Jaynes et al. 2007). By using nano-sized clay, the gap between the layers of clay has been prolonged ten times. As a result, the nanoclay can bind the whole family of mycotoxins.

A rapid enzyme-linked immunosorbent assay (ELISA) was improvised by using superparamagnetic nanoparticles (Radoi et al. 2008). The assay was effective for detecting aflatoxin M1 (AFM1) with a detection limit of 4–250 ng l^{-1} (Radoi et al. 2008). Cysteamine functionalized-gold nanoparticles (C-AuNP) along with aflatoxin B1 antibodies (aAFB1) were immobilized on a 4-mercaptobenzoic acid-based

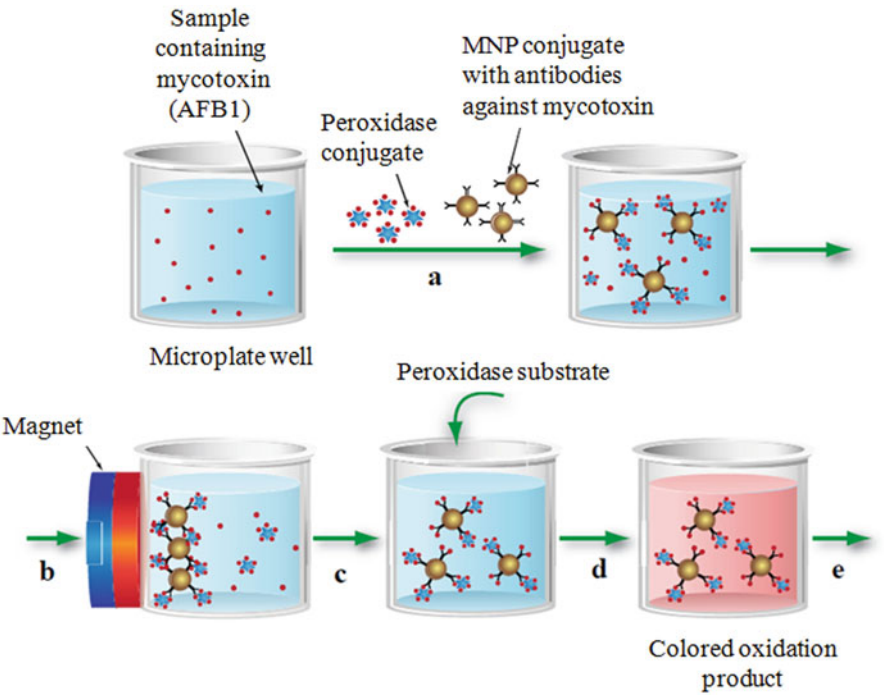

Fig. 8.6 Assay of mycotoxin (e.g. AFB1) detection using magnetic nanoparticles. (**a**) Free AFB1contained in the test sample competes with peroxidase-labeled AFB1 for binding sites on the antibodies adsorbed on the MNP surface; (**b**) A magnet separated magnetic nanoparticle (MNP) from unreacted components; (**c**) MNP are washed and, by removing the magnetic field, returned into solution; (**d**) the peroxidase substrate is added to the MNP suspension and; (**e**) development of colored oxidation product

self-collected monolayer on a gold electrode (MBA/Au) to fabricate a BSA/aAFB1-CAuNP/MBA/Au immunoelectrode. These electrodes were used to detect AFB1 in the range of 10–100 ng l^{-1} (Sharma et al. 2010). Recently, a moveable machine has been developed that can concurrently identify various bacterial, fungal toxins and pathogens in stored food (Yalcin and Otles 2010; Biswal et al. 2012). From all these reports, it seems that nanostructured platforms can be an exciting alternative to the conventional techniques for the detection of mycotoxins and pathogens spoiling food and agricultural crops.

8.2.4 Nanofabrication Imaging

Nanotechnology offers unique opportunities to precisely tune and control the chemical and physical properties of contrast materials in order to overcome problems of toxicity, useful imaging time, tissue specificity and signal strength. Nie (2013) reported that mesoscopic nanoparticles (5–100 nm diameter) have large surface

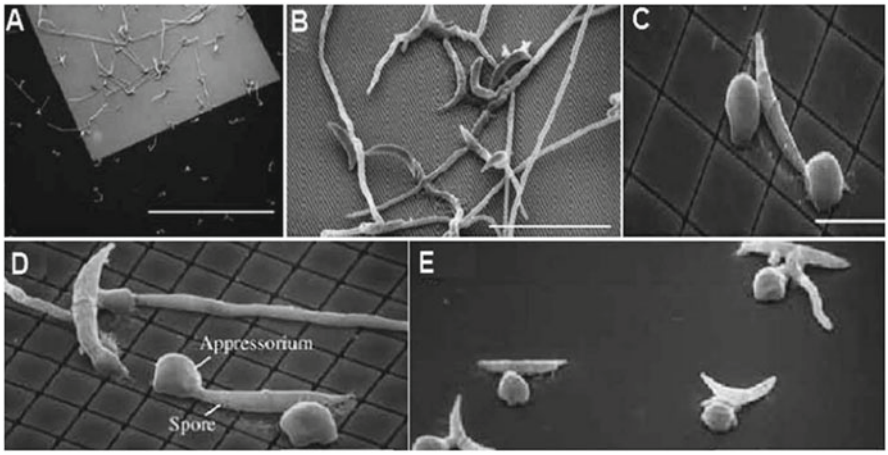

Fig. 8.7 Scanning electron micrographs (SEM) showing the fungus *Colletotrichum graminicola* grown on nanofabricated pillared arrays. When the individual pillars are very small (0.5 mm wide) and do not provide much surface contact (**a**, **b**), the spores of the fungus grow without forming 'appressoria'. When the pillars are wider (**c**, **d**) or when the surface is completely smooth (**e**), appressoria are formed quickly (Source: Mccandless 2005)

areas and ideal for conjugating functional groups in multiple pathogen diagnosis assays. Electron beam and photolithography techniques are also used to fabricate topographies that mimic leaf surface features as well as the internal plumbing of plants, and then nano-imaging technologies are used to study how pathogen invade and colonize the leaf tissue (Mccandless et al. 2005). Lithography was used to nanofabricate a pillared surface on silicon wafers. This lawn of miniature pillars (1.4 and 20 mm wide) was used to examine the movement across the surface by the fungus that mimicked some of the characteristics of the host plant. Images of the *Colletotrichum graminicola* crawling across the nanofabricated surface assisted the researchers to determine that the fungus needs to make a minimum contact (at least 4.5 mm) prior to initiation of appressoria formation (Fig. 8.7). To develop disease resistant cultivars, the infection process and behaviour of *Xylella fastidiosa* causing Pierce's disease inside grapevine xylem were studied using nanofabrication methods (Meng et al. 2005). The application of carbon-coated magnetic nanoparticles and microscopy methods at different levels of resolution to visualize the transport and deposition of nanoparticles inside the plant host was reported by González-Melendi et al. (2007). Further, Szeghalmi et al. (2007) investigated nanostructured surface-enhanced Raman scattering (SERS) substrates for imaging applications at high-spatial resolution (1 μm). They performed SERS imaging of dried fungal hyphae grown on commercially available nanostructured gold-coated substrates and concluded that this type of nanofabrication techniques offer a well-characterized and reproducible substrate for *in-situ* or *in-vivo* imaging studies of plant pathogen interactions. Rispail et al. (2014) evaluated the behavior of quantum dots and superparamagnetic nanoparticles on *Fusarium oxysporum* and indicated that both

nanomaterials rapidly interacted with the fungal hypha labeling the presence of the pathogenic fungus, although, they showed differential behavior with respect to internalization. This work represents the first study on the behavior of quantum dots and superparamagnetic particles on fungal cells, and constitutes the first and essential step to address the feasibility of new nanotechnology-based systems for early detection and eventual control of pathogenic fungi.

8.2.5 Nanobiosensor Based Diagnostic System

Nanosensors with immobilized bioreceptor probes that are selective for target analyte molecules are termed as nano biosensors. They offer the advantages of being small, portable, sensitive with real-time monitoring, precise, quantitative, reliable, accurate, stable, reproducible and robust to identify potential and complex disease problems. At present, these systems were employed to detect and quantify minute amounts of contaminants such as viruses, bacteria, fungi, toxins and other biohazardous substances in the agriculture and food systems (Srinivasan and Tung 2015). Therefore, these nanosensors may have a huge impact on the precision farming methods (Rai and Ingle 2012). Moreover, early on-site detection of plant pathogens with portable nanobiosensors will enable to design the strategies to control the spread of diseases and will also help the study of disease epidemiology. These sensors can be linked to a GPS and distributed throughout the field for real-time monitoring of disease, soil conditions and crop health (Nezhad et al. 2014). The combination of biotechnological and nanotechnological approaches in bio-sensors can be used to construct equipment with increased sensitivity, allowing an earlier response to ecological changes and disease prevalence. Nanosensors will allow us to identify plant diseases before visible symptoms appear and thus facilitate their control. Precision farming will be improved by using nanosensors by providing precise data, helping growers to make better decisions, thus enhances agriculture production and productivity (Rai and Ingle 2012). Hashimoto et al. (2008) developed a new biosensor system for the rapid diagnosis of soil-borne diseases, consisting of two biosensors. The system was constructed using equal quantities of two different microbes, each individually immobilized on an electrode. Taking into consideration the particular optical properties of silver nanoparticles, the interaction between silver nanoparticles and sulphurazon-ethyl herbicide was investigated by Dubas and Pimpan (2008). They found that silver nanoparticles are sensitive to increase concentrations of herbicide in a solution and induced a variation in colour of the nanoparticles from yellow to orange red and finally to purple. This approach is useful for detecting contaminants, such as organic pollutants and microbial pathogens in water bodies and in the environment (Dubertret et al. 2001). Fluorescent silica nanoparticles (FSNP) combined with antibody molecules successfully detected plant pathogens such as *Xanthomonas axonopodis* pv. *vesicatoria* which

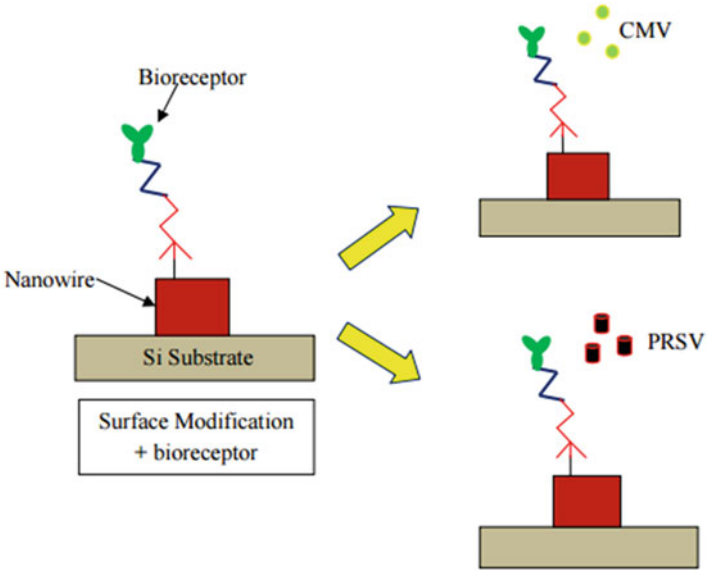

Fig. 8.8 Plant diseases detection using nanowire as biosensor transducer. Nanowire undergo surface modification with amino group solution and enzyme applied on the nanowire surface and thus, bioreceptor binds with nanowire structure which it used for grab plant viruses such as *Cucumber Mosaic Virus* (*CMV*) and *Papaya Ring Spot Virus* (Source: Ariffin et al. 2014)

causes bacterial spot disease in tomatoes and peppers (Yao et al. 2009). Copper oxide (CuO) nanoparticles and nanolayers were synthesized by sol–gel and spray pyrolysis methods, respectively. Both CuO nanoparticles and nanostructural layer biosensors were used for detecting *Aspergillus niger* fungi (Etefagh et al. 2013).

Furthermore, the highly ordered nanowires array combined with multiple biorecognition holds the promise of developing multiplexed nanobiosensors. Nanowire biosensors are a class of nanobiosensors, of which the major sensing components are made of nanowires coated by biological molecules such as DNA molecules, polypeptides, fibrin proteins, and bacteriophages. Since their surface properties are easily modified, nanowires can be decorated with virtually any potential chemical or biological molecular recognition unit, making the wires themselves analyte independent. The nanomaterials transduce the chemical binding event on their surface into a change in conductance of the nanowire in an extremely sensitive, real time and quantitative fashion. They will be very useful for high-throughput diagnosis and screening. By employing this concept, Ariffin et al. (2014) used nanowire as biosensor transducer for the detection of *Cucumber Mosaic Virus* (CMV) and *Papaya Ring Spot Virus* (PRSV) (Fig. 8.8). These results clear indicated that the nanowires are a good candidate material for fabricating nanoscale biosensors for making remote-controlled nanbiosensors for future applications in crop health-care testing, disease diagnostics and environmental monitoring.

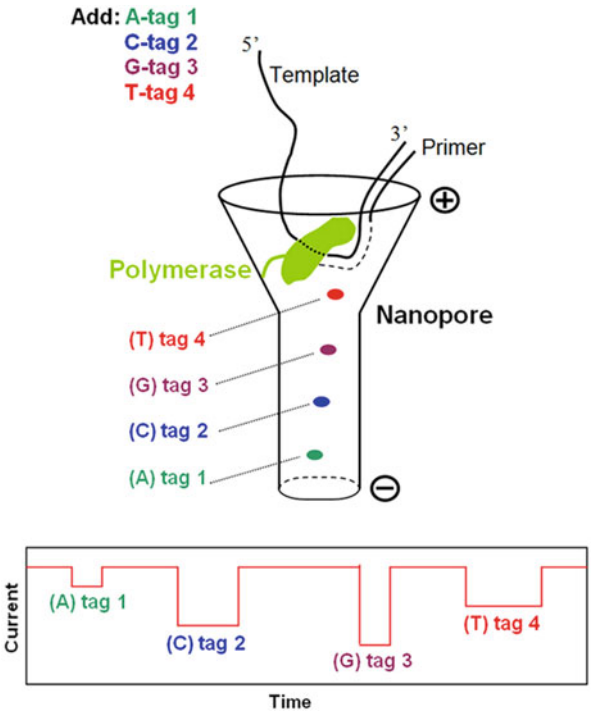

Fig. 8.9 Working flow of single molecule DNA sequencing by a nanopore with phosphate-tagged nucleotides. Each of the four nucleotides will carry a different tag. During sequencing by synthesis (SBS), these tags, attached via the terminal-phosphate of the nucleotide, will be released into the nanopore one at a time where they will produce unique current blockade signatures for sequence determination. A large array of such nanopores will lead to high throughput DNA sequencing (Source: Kumar et al. 2012)

8.2.6 Nanopore System

Nanopore systems are based on electronic detection of DNA sequence and have the potential of low sample preparation work, high speed, and low cost (Branton et al. 2008). Nucleotide identification using nanopore system is based on the measurement of conductivity changes across a lipid membrane while a DNA fragment is pulled through a nano-scale pore by an electric current. Conductivity changes are nucleotide-specific, enabling the identification of nucleotides as they cross the pore (Egan et al. 2012). The protein nanopore is inserted in a polymer bilayer membrane across the top of a microwell. Each microwell has a sensor chip that measures the ionic current as the single molecule passes through the nanopore. However, the speed at which the DNA strand travels through the nanopore is too fast for accurate identification (Clarke et al. 2009). It is worth mentioning that nanopore sequencing platform models have the potential to rapidly generate ultra long single molecule reads. Recently, Kumar et al. (2012) reported nanopore-based sequencing by synthesis (Nano-SBS) approach can accurately distinguish four DNA bases by detecting four different sized tags released from 5′-phosphate-modified nucleotides at the single molecule level for sequence determination.

The basic principle of the Nano-SBS strategy is described in Fig. 8.9. Another new sequencing technology developed by IBM and Roche together is ‘DNA

transistor' technology, which could potentially record the nucleotide sequence as the template is pulled through the nanopore sensor (Zhang et al. 2011). Recently, a portable DNA sequencing machine (MinION) was launched by U.K.-based Oxford Nanopore Technologies (Hayden 2015). This tool is able to sequence 10 kb of a single sense and anti-sense DNA strand and will make next-generation sequence (NGS) within the reach of many research groups. This system offers on the spot data, a special feature that can help scientists to quickly determine the cause of an epidemic outbreak, epidemiology of a disease or catalog the rare and exotic species. Further, it can discriminate between closely related bacteria, fungi and viruses, read complex portions of the genome, and differentiate between the two versions of a gene that are carried on each chromosome pair. Therefore, nanopore platform implemented within current diagnostic equipment has the potential of analyzing the entire genome in minutes instead of hours. From agricultural point of view, this technology can be applied to analyze plant and pathogen genomics and gene functions in addition to pathogens detection and prediction in agricultural crops.

8.2.7 Nanodiagonastic Kit Based Equipment System

Nanodiagonastic kit also known as 'lab in a box' refers to packing sophisticated measuring devices, reagents, power supply and other features that take up laboratory space into a parcel no larger or heavier than a briefcase (Khiyami et al. 2014). This type of a diagnostic kit can easily and quickly detect potential serious plant pathogens in fields, allowing experts to help farmers in prevention of disease epidemics from breaking out (Pimentel 2009; Nezhad 2014). For instance, 4mycosensor is a tetraplex competitive antibody-based assay in a dipstick format for the real-time detection of ZEA, T-2/HT-2, DON and FB1/FB2 mycotoxins on the same single strip for corn, wheat, oat and barley samples at or below their respective European maximum residue limits (MRLs) (Lattanzio et al. 2012). Nanodiagonastic kit based on immunoassay is fast, cheap, easy-to-use and suitable for the purpose of quick detection and screening of mycotoxins in cereals. However, there are many challenges which must be addressed before nanodiagonastic kit based equipment systems are truly ready for use in agriculture and allied sectors. These include the discovery and selection of effective antigen, antibody and nucleotide targets, which are required to improve the specificity of the diagnostic kits and permit strain differentiation. Furthermore, universal standards for the assessment of tests and levels of detection must be set so that studies of detection limit can be compared. In addition, for genomic target detection of a particular pathogen, strategies to simplify the purification and isolate genes of interest are vital. So far, the advances of nanotechnology have not been fully applied to pathogen disease detection in agricultural crops, nanotechnology can potentially address many of the challenges outlined earlier for effective on-site real time diagnostics of crop diseases.

8.3 Conclusion

Nanotechnology presents a wealth of potential tools for researchers involved in the detection, identification, and monitoring of plant pathogens. Indeed, the recent reports on the applications of portable diagnostic equipment, nanoparticle-based bio-barcoded DNA sensor, quantum dots, nanostructured platforms, nanoimaging and nanopore DNA sequencing tools have prompted virtually unqualified speculation as to the coming profusion of cheap, rapid, and accurate means of identifying and diagnosing complex disease problems. The promise for the development of portable handheld nano-devices for *in situ* field identification, has further whetted the appetite for nanodiagnostic technologies. Nanodiagnostics is an area of huge interest and future research will focus on the multimodality of nanoparticles. Research in this area is just at infancy stage for detecting plant pathogens and toxins in agricultural crops and various food commodities. There is a need to continue the pace for the optimization of nanodiagnostic assays to obtain an accurate signal for low levels of pathogens to solve plant disease-complex mysteries existing in the farmers' fields. The opportunities are endless for potential use of nanotechnology in disease diagnosis. The field is ever expanding and scientists are trying to keep up with the latest technologies that can be used to protect agricultural crops to achieve millennium nutrition and food security agenda.

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