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Development of Quantum Dot-based Nanobiosensors against *Citrus Tristeza Virus* (CTV)

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Abstract: Citrus tristeza is one of the most important diseases of citrus in the world. To avoid the destructive effect of the disease, early detection of infected plants is crucial. Therefore, simple and sensitive diagnosis tools are decisive. The main objective of the present study was developing nanobiosensors for detection of citrus tristeza based on the florescence emission of cadmium telluride quantum dots (CdTe-QDs). To achieve that, CdTe-QD particles were initially synthesized and effectively conjugated to CTV coat protein (CTV-CP) corresponding antibody. In a parallel reaction, rhodamine dye molecules were attached to the purified recombinant CTV-CP. Two independent approaches were explored for detection of the infected plants. First, in a fluorescence resonance energy transfer (FRET) based assay, the quenching ability of rhodamine molecules was applied for altering the QDs light emission. More specifically, donor-acceptor complexes (Ab-QD+CP-Rd) were created based on the affinity of antibody-antigen molecules. The resulting assembly brought Ab-QD (the donor) and the Rd-CP (the acceptor) into a close proximity and resulted in a substantial decrease in the intensity of QD light emission. Addition of free antigen into the solution resulted in the replacement of CP-Rd with free CP and a subsequent increase in the emission of QDs. In the second approach, a non-FRET based assay was performed through the addition of free antigen to the Ab-QD solution, which led to the aggregation of the Ab-QD conjugates and consequently a significant increase in the light intensity emission of the QD. To the best of our knowledge, this is the first time that the non-FRET based assay developed herein is being reported.

Keyworlds: Biosensor, Citrus, FRET, Quantum dot, Tristeza.

1. Introduction

Citrus tristeza is the most economically-destructive and widely-distributed viral disease in citrus plants worldwide [1]. The disease is caused by a flexuous rod shaped Citrus tristeza virus (CTV) [2]. The viral particles of 2000 nm in length and 12 nm in diameter, are naturally transmitted by aphids in a semi-persistent manner [3]. The CTV consists of a positive sense single-stranded RNA genome of approx. 19296 bp including 12 open reading frames encoding at least 17 proteins [4]. The viral particle consists of two

capsid proteins identified as the major (p25) and minor (p27) proteins with molecular weights of 25 kDa and 27 kDa, respectively [5]. The disease symptoms are highly variable depending on some factors including virulence of the viral strain, host species, and environmental conditions. In general, the viral strains are mainly responsible for three kinds of symptoms including decline (quick and slow), stem pitting, and seedling yellows [3, 4].Protective control of CTV is mainly based on severe quarantine regulations and certification programs by grafting of virus-free scions onto CTV-tolerant rootstocks [6, 7]. Early detection is a significantly critical strategy for effective control of CTV by removing infected plants in nursery gardens and by preventing the transportation of infected plants into the clean area. Conventional detection methods suffer from a number of drawbacks and, therefore, there is an urgent need for simple, rapid, sensitive, and specific screening techniques to detect CTV at very early stages of infection. At present, the most common methods used for detection of CTV is enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). These methods have some disadvantages limiting their application, as a case in point, ELISA based methods are time consuming and cannot detect low amounts of pathogens which is crucial for the detection of the disease in the preliminary stages of the infection [8, 9]. On the other hand, DNA based-methods are time consuming and not able to distinguish live pathogens from the dead ones [10]. More sensitive diagnostic approaches such as nanobiosensors have been proposed as alternating techniques to the conventional methods [8, 9, 11-13]. Nano-materials owing to their unique features including stable emissions and size-dependent properties have attracted a great deal of attention as transducers. Among these nanomaterials, fluorescent semiconductor nano crystals, also known as quantum dots (QDs), have been most widely used for disease diagnosis [12, 14-17]. Attractive properties of QDs as unique fluorescent label include high signal brightness, stability, size-tunable light emission, and resistance to photo-bleaching [18, 19]. These properties have been the driving reason behind the wide interest in using QDs in the development of biosensors and immune-histochemical kits [19, 20].

The present article describes two specific and sensitive QD-based biosensors for rapid detection of CTV infected plants.

2. Material and Methods

2.1. Plant Materials

Plant samples including shoots, fully expanded leaves, and peduncles were collected from different citrus trees growing in different orchards in northern part of Iran, i.e. Sari region in Mazandaran province. Young leaves were collected from four different locations around the canopy, placed in marked plastic bags, and were transferred to the laboratory on ice.

2.2. Initial Detection of Infected Plants

The presence of CTV in the collected plant samples were confirmed by a commercial DAS-ELISA kit (Bioreba, Switzerland) as described [21]. Briefly, a 96-wells microtiter plate was coated with purified anti-cp antibody diluted to 1/1000 in PBS and incubated at 37 °C for 2 h. The extraction of the plant sap from healthy and infected citrus trees was carried out by crushing 0.1 g leaves in liquid nitrogen followed by suspension in 1 ml extraction buffer (Tris buffer pH7.4 containing 137 mM NaCl, 3 mM KCl, 2 % PVP 24 kDa, 0.05 % Tween20 and 0.02 % NaN_3). The plant extracts and purified protein control (CP positive control) were added to the plate and incubated overnight at 4 °C. Subsequently, the alkaline-phosphatase conjugated anti-CP polyclonal antibody at a dilution rate of 1:1000 was added to the sample and incubated at 37 °C for 30 min. Finally, the absorbance values were read at 405 nm. The sample was positively identified if the mean DAS-ELISA (A405nm) value of the samples exceeded at least twice the mean values of the healthy control.

2.3. Preparation of CdTe-QDs

The preparation and synthesis of CdTe-QD particles was performed using the optimized protocol described earlier [22]. Briefly, in the presence of thiolglycolic acid (TGA), a fresh solution of Sodium hydrogen telluride (NaHTe) was added steadily to N_2-saturated $CdCl_2.2.5H_2O$. The mixture was then heated at 92 °C and stirred in a reflux system under nitrogen atmosphere. To remove the excess amount of Cd2+and TGA, the solution was washed three times with ethanol and spun at 4000×g for 15 min. The pellet was re-suspended again in 250 mL double distilled water and refrigerated.

2.4. Antibodies Preparation

To prepare specific antibodies against the CTV particles, purified recombinant CTV-CP was used for immunization of rabbits. The recombinant protein was prepared and affinity-purified as described in our previous report [23]. Two-month-old female white New Zealand rabbits were injected intramuscularly with the antigen. The first injection contained about 100 µg of the recombinant CP (20 µl) protein and 500 µl of Freund's complete adjuvant, mixed with 480 µl PBS 1× (137 mm NaCl, 2.7 mm KCl, 10 mm Na_2HP0O_4, 2mM KH_2Po_4 pH7.4) while incomplete Freund's adjuvant was used for the subsequent injections with 100 µg purified recombinant IMP diluted by an identical volume of Freund`s adjutant. Antibody purification from the serum was performed using protein A spin column according to the manufacturer's instructions (AbD serotec,UK). Concentration of the purified antibody was estimated on a SDS-PAGE.

2.5. Conjugation of Antibodies with QDs

In order to conjugate CTV antibodies and QD particles (Ab-QDs), 200 µg of QDs and 50 µg of the specific purified CTV immunoglobulins were mixed, pH 7.4 and the volume of solution was increased to 100 µl. After that, 150 *µ*L of the freshly-prepared EDC solution (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (4.2 mg/mL) was added to the mixture and stirred 2 h at room temperature in the dark. The solution was then kept at 4 °C overnight. The prepared Ab-QDs conjugate subsequently separated by centrifugation at 20,000×g for 20 min. The pellet containing Ab-QDs conjugates was then dispersed by 1.5 mL of 1× PBS solution pH 7 and was stored at 4 °C in the dark.

2.6. Conjugation of CTV-CP with Rhodamine

The recombinant coat protein of the CTV was conjugated to rhodamine 123 molecules *via* aldehyde intermediates (CP-Rd). To achieve this, around 100 µl CP (5 µg/ml) was gently added to 360 µl Glutaraldehyde 10 %. Subsequently, 2 µl of dinitroprydin was added to the mixture under stirring and mixed for 30 min. Then 1 mg of $NaBH_4$ was added and the mixture was stored for 1 h at room temperature. The CP-Rd conjugates were then separated from the excess antigen *via* dialysis using 1× PBS pH 7. In fact, rhodamine 123 was used as a quencher in developed nanosensing system.

2.7. The Fluorometry Assay

A UV-VIS Spectrophotometer MultiSpec-1501 (Shimadzu, Japan) was used for fluorometric assays. The excitation of the CdTe QDs was adjusted at 380 nm. To cover the emission wavelength of the fluorophore molecules, the emission spectra were adjusted at between 500 and 640 nm. The bandwidth of the device was fine-tuned at 5 nm.

2.8. Nanobiosensor Fabrication and Evaluation

The major components of the developed nanobiosensor included the bio-conjugates of Ab-QD and CP-Rd. The presence of the antigen in the solution was estimated *via* two independent FRET- and non-FRET based approaches. In the first method, defined amounts of Ab-QD (2 µl) and CP-Rd (10 µl) diluted in 1×PBS were added into a 100 µl fluorometer well. The obtained spectrum showed that the baseline emission of QD particles was quenched by the rhodamin molecules. For detection of the native and/or recombinant antigen in the solution, 2 µl of the plant extract and/or recombinant CP was then added to the same fluorometer well and the second spectrum was acquired. In the presence of the foreign antigen, the emission intensity of the QD would be increased and the baseline would be recovered.

In the second non-FRET based approach, there was no need for the presence of CP-Rd, and the mixture only contained defined amounts of Ab-QD (2 µl) and antigen (2 µl) diluted in 96 µl of PBS. The sample was loaded in a fluorometer well and the presence of antigen was detected by improving of the light emitted by the QD particles. In the both mentioned approaches, the samples were marked as negative when no baseline shift was observed.

3. Results

3.1. Preparation of Antibody

A rapid immunoassay method employing QDs for detection of citrus tristeza is described herein. First, for preparation of specific polyclonal antibody against CTV particles, immunization of two rabbits was performed by intramuscularly injecting the purified recombinant CTV-CP. The antibody titer was determined after each boosting, and after 6 weeks when the antibody titer exceeded 1:65000, blooding was performed and the whole serum was obtained. An affinity column containing staphylococcus protein A was used for purification of the IgG molecules. The IgG purity and integrity was monitored by SDS-PAGE in which distinct bands with molecular weights of around 25kDa and 50kDa pertaining to the light and heavy chains, respectively, were observed (Fig. 1). Furthermore, the concentration of the IgG was measured at approx. 1mg. ml^{-1} by direct comparison with known amounts of a standard protein, i.e., BSA.

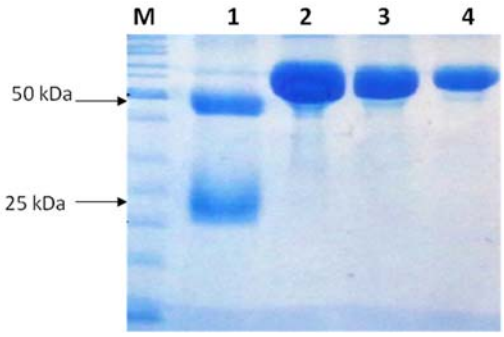

Fig. 1. SDS-PAGE analysis of the affinity purified immunogloboline. M: unstained protein molecular weight marker SM0431 (Fermentas, Vilnius, Lithuania); 1: purified antibody; 2: BSA 3500µg ml-1, 3: BSA 1700 µg ml-1, 4: BSA 750 µg ml-1.

The feasibility of the prepared antibody for detection of infected plants was analyzed by a serological approach. The results proved a high specificity against the recombinant CTV-CP protein as well as against the native virus particles within the infected plant samples (data not shown).

3.2. Construction of Biosensors

For developing of nanobiosensors, the QD particles were used as fluorophore and were conjugated to IgG molecules. For efficient coupling of Ab-QD, the surface of the CdTe QD particles were initially surface modified by thioglycolic acid (TGA). Then, purified IgG molecules against CTV were immobilized on their shells. This was accomplished thanks to the hydrophilic behavior of the QD particles resulting in the attraction of IgGs onto their surfaces [24]. The rhodamine 123 molecules were used as an acceptor for quenching the light emitted from QD particles. For this aim, the purified recombinant CTV-CP was used for conjugation to rhodamine *via* aldehyde intermediate using the 27 free amine groups (lysine) of the CTV-CP. Rhodamine 123 as a fluorescent molecule can be detected easily and inexpensively with fluorometers. The absorption and emission spectra of the pure solution of CdTe QDs and rhodamin are shown in Fig. 2. As presented, the maximum emission peak of the QDs takes place at 522 nm while the maximum emission peak of rhodamine is at 580 nm. Therefore, adddion a mixture of Ab-QD and CP-Rd into a same well would lead to a significant decrease in the emission light of the Ab-QD conjugates (Fig. 2 and Fig. 3.A). This is due to the quenching effect of rhodamine on the emitted light of QDs occurring in a FRET system where Ab-QD and CP-Rd are involved in a specific antigen-antibody interaction.

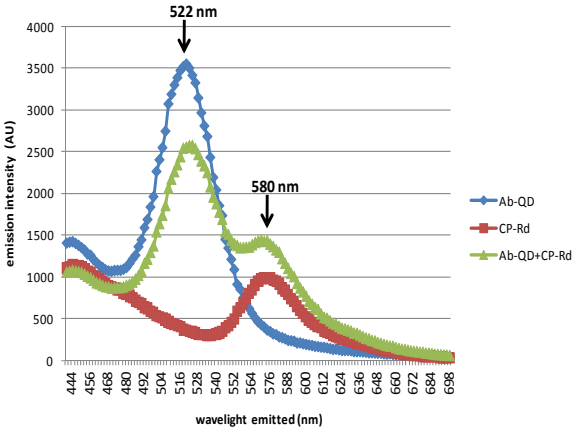

Fig. 2. Developing of FRET assay by applying of Ab-QD and CP-Rd. The excitation is done by a light with 390 nm wavelength and maximum value of emission obtained in 522 nm. The Y axis shows emission intensity by fluorescence arbitrary units (AU).

Ab-QD: The spectrum derived from excitation of Ab-QD particles.

CP-Rd: The spectrum derived from excitation of CP-Rd.

Ab-QD+CP-Rd: The spectrum derived from excitation of solution containing Ab-QD particles and CP-Rd.

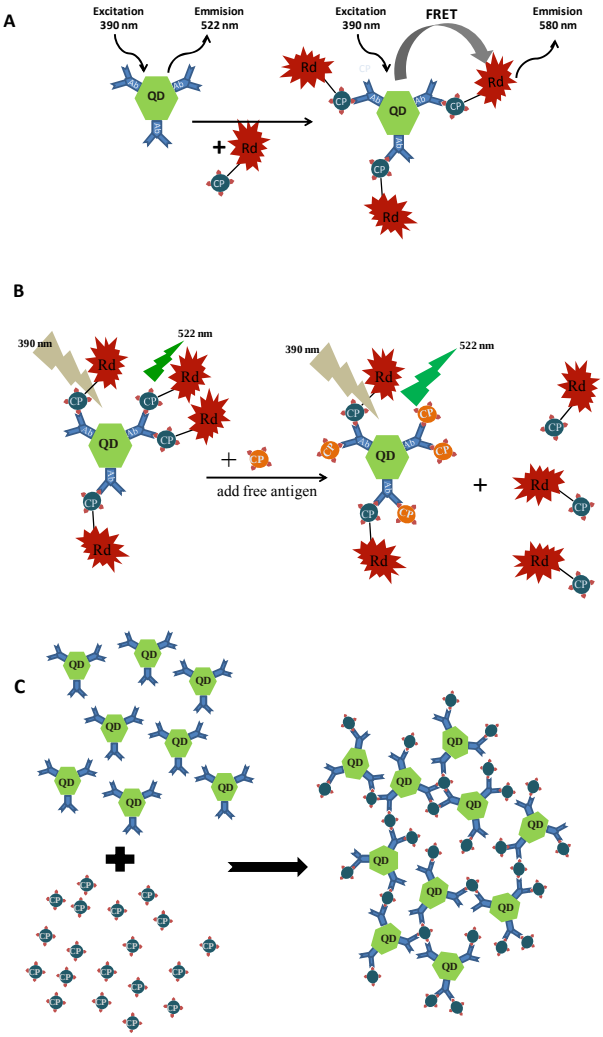

Fig. 3. Schematic presentation of specific CTV nanobiosensor: (A) Occurring of FRET in presence of Ab-QD and CP-Rd, (B) the FRET-based mechanism for detection of infected CTV plant, (C) Non-FRET based mechanism for detection of infected CTV plant.

3.3. The FRET based Nanobiosensor

In the first approach for detection of CTV, the FRET-based nanobiosensor was used in which the emission intensity of the Ab-QDs alone was measured at around 5700 AU; however, after the addition of Rd-CP, this value showed a downward shift peaking at 2200 AU (Fig. 4) and this was considered as base line spectra. Subsequently, the addition of the recombinant or native antigen (CTV-CP) led to a significant increase in the emission intensity from the base line curve. This was due to the loss of the quenching effect of rhodamine on QD particles (Fig. 3.B). In better words, this increase was ascribed to the separation of the CP-Rd from the Ab-QD in response to the presence of free antigens.

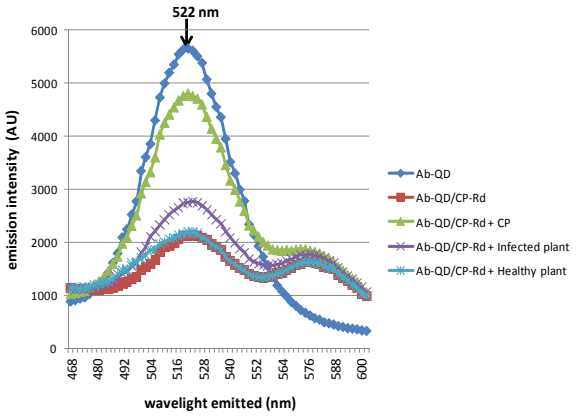

Fig. 4. Detection of infected plant by FRET based nanobiosensor. The excitation is done by a light with 390 nm wavelength and maximum value of emission obtained in 522 nm. The Y axis shows emission intensity by fluorescence arbitrary units (AU).

Ab-QD: The spectrum derived from excitation of Ab-QD particles alone

Ab-QD/CP-Rd: The spectrum derived from excitation of a solution containing Ab-QD particles and CP-Rd.

Ab-QD/CP-Rd + CP: The spectrum derived from excitation of a solution containing Ab-QD particles, CP-Rd and recombinant CTV-CP.

Ab-QD/CP-Rd + Infected plant: The spectrum derived from excitation of a solution containing Ab-QD particles, CP-Rd and extract of infected plant.

Ab-QD/CP-Rd + Healthy plant: The spectrum derived from excitation of a solution containing Ab-QD particles, CP-Rd and extract of healthy plant.

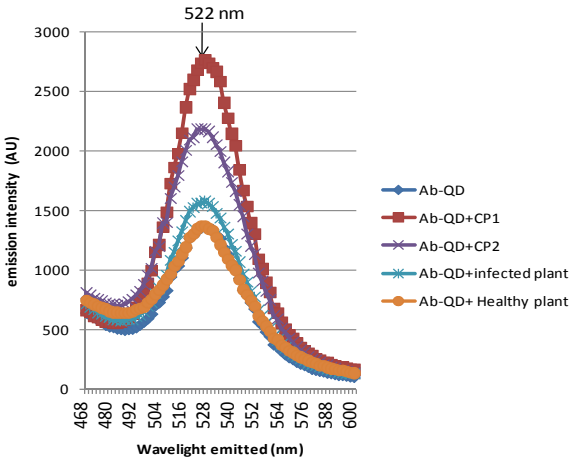

Fig. 5. Detection of infected plant by non-FRET based nanobiosensor. The excitation is done by a light with 390 nm wavelength and maximum value of emission obtained in 525 nm. The Y axis shows emission intensity by fluorescence arbitrary units (AU).

Ab-QDs: The fluorometric peak of Ab-QDs conjugates alone.

Ab-QD+CP1: The fluorometric peak of Ab-QDs conjugates and recombinant CTV-CP (700ng/ml) complex.

Ab-QD+CP2: The fluorometric peak of Ab-QDs conjugates and and recombinant CTV-CP (500ng/ml) complex.

Ab-QD+infected plant: The fluorometric peak of Ab-QDs conjugates and extract of infected plant.

Ab-QD+ Healthy plant: The fluorometric peak of a solution containing extract of healthy plant alone.

3.4. The Non-FRET based Nanobiosensor

In a separate assay, the non-FRET based nanobiosensor, i.e., the capability of the Ab-QD particles alone for the detection of infected plant samples without applying CP-RD, was evaluated. Herein, the detection was mainly based on the aggregation of Ab-QD particles by the addition of antigens. In this approach, the addition of the recombinant CTV-CP to Ab-QD solution would lead to aggregation of Ab-QD particles by CP molecules. Under this situation, the aggregates emitted lights of a higher intensity when exited at 390 nm (Fig. 3.C). Applying the recombinant CP and crude extract of the infected plants confirmed the specificity of the nanobiosensor for the detection of CTV infected plants (Fig. 5).

The comparative analysis of two above described approaches showed that the non-FRET based method is more sensitive for detection of CTV-CP in the solution. The limit of detection (LOD) was calculated based on LOD= 3S0/ K equation, here S0 is the standard deviation of blank measurements (n=6) and K is the slope of calibration curve. The detection limit of FRET and non-FRET based methods was estimated around 198 ng/ml and 246 ng/ml of purified CTV-CP, respectively. Based on our knowledge, this non-FRET based detection approach is firstly described here for detection of specific antigen in a solution.

4. Discussion

Present article describe construction of two specific and novel nanobiosensor s against CTV that could be easily used for efficient detection of infected plants. The bio-receptor part of these sensors comprised of specific antibody against the CTV-CP that is able to detect presence of cognate antigen, CTV, in a solution. It is believed that the attachment of antibody-antigen molecules are completed by several non-covalent interactions. These types of attachments are not so strong and in a distinct antibody-antigen complex, the antigen moiety could be replaced by free antigen molecules in the solution (Safarnejad et al., 2011).

Herein, the major part of the constructed biosensors is cadmium telluride quantum dot particle. These luminescent colloidal semiconductor nanocrystals are well-suited for sensing and biotechnological applications. These worth full particles do not suffer from current limitations of organic dyes and exhibit size-dependent tunable, having broad absorption with narrow fluorescence emission spectra. [25, 26].

The first developed nanobiosensor comprised of CP-Rd and Ab-QD which sense presence of foreign antigen, CTV, on a FRET-based mechanism (Fig. 2 and Fig. 3.A). More specifically, when a sample containing free coat proteins of CTV in a solution was

added to the mixtures of pre-bound CP-Rd/Ab-QD, the CP-Rd moiety was replaced by the free CP in the investigated sample. This led to a decrease in the quenching ability of the CP-Rd against the Ab-QD leading to a recovery of the fluorimeteric curve in comparison with the curve previously obtained for the Rd-CP/Ab-QD conjugates. This could be explained through the optical quenching mechanism of the QD-Ab domain by the CP-Rd domain based on the Forster dipole–dipole interaction model [24]. In other words, the inorganic dye, i.e., rhodamine (a fluorescence acceptor) conjugated to the antigen (CTV-CP), occupied the peptide binding pocket of the antibody, i.e., anti-CTV polyclonal antibody. Therefore, when the free CTV-CP derived from the pathogenic agent was added, it displaced the rhodamine-CP domain in the Rd-CP/Ab-QD conjugates, resulting in a increase of fluorescence emission by the displaced Ab-QD which was no longer quenched by the CP-Rd molecules (FRET) (Fig. 2 and Fig. 3.A). Moreover, higher free native CTV-CP concentrations in the infected sample would be translated into fluorimeter curves peaking at higher photoluminescence (PL) intensity. FRET has been successfully applied for detection of several important plant pathogens and toxins [8, 27-30].

In the non-FRET based detection approach, the addition of the free antigen molecules into the reaction solution would lead to self-assembling of Ab-QD molecules into microscale aggregates in the presence of free CP subunits through antibody-antigen molecular recognition. This would lead to a higher intensity of the light emitted by the QD particles. Soman and Giorgio [31] used this approach in a flow cytometry assay for rapid and simultaneous detection of Angiopoietin-2 (Ang2) and vascular endothelial growth factor A (VEGF).

5. Conclusion

This designed nanobiosensor showed a high performance for detection of the infected plants. No cross reaction was detected by applying healthy citrus plants as well as those infected with other diseases such as witches broom disease of lime (WBDL) and citrus bacterial cankers. The developed nanobiosensor showed a complete accuracy in detecting the infected samples carrying the CTV particles. In general, the method described herein showed several advantages over the conventional detection methods, including simplicity and higher sensitivity in the detection of the pathogens in plant.

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