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Detection of *Citrus tristeza virus* by using fluorescence resonance energy transfer-based biosensor

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ABSTRACT

Due to the low titer or uneven distribution of *Citrus tristeza virus* (CTV) in field samples, detection of CTV by using conventional detection techniques may be difficult. Therefore, in the present work, the cadmium-telluride quantum dots (QDs) was conjugated with a specific antibody against coat protein (CP) of CTV, and the CP were immobilized on the surface of gold nanoparticles (AuNPs) to develop a specific and sensitive fluorescence resonance energy transfer (FRET)-based nanobiosensor for detecting CTV. The maximum FRET efficiency for the developed nano-biosensor was observed at 60% in AuNPs-CP/QDs-Ab ratio of 1:8.5. The designed system showed higher sensitivity and specificity over enzyme linked immunosorbent assay (ELISA) with a limit of detection of 0.13 μg mL^{-1} and 93% and 94% sensitivity and specificity, respectively. As designed sensor is rapid, sensitive, specific and efficient in detecting CTV, this could be envisioned for diagnostic applications, surveillance and plant certification program.

1. Introduction

Citrus tristeza virus (CTV) as a phloem-limited *Closterovirus* is distributed all over the world and acts as a causal agent of one the most destructive disease named tristeza disease [1]. CTV infects almost all citrus species leading to a significant economic losses in the citrus industry [1]. Efficient seed and plantlet certification programs are the most important way to control spreads of CTV infection. Therefore, developing accurate and reliable detection techniques capable of detecting viruses in early stages of infection is of crucial importance. To date, different techniques have been used to detect CTV including biological indexing [2], serological and immunoassay techniques [3] as well as polymerase chain reaction (PCR)-based techniques [4]. These conventional detection techniques generally suffer from different drawbacks and therefore, there is a growing demand for developing simpler and more rapid, detection method which offers higher sensitivity and specificity as well [5].

By the introduction of nanosciences in the last decade, broad kinds of nanoscale materials with exclusive features have attracted a great deal of attention for disease diagnosis. From those nanoscale materials, quantum dots (QDs) owing to their unique characteristics such as easy synthesis and handling, long shelf life, high fluorescence yields, high resistance to photo-bleaching, and narrow symmetric emission spectrum have been widely used in fluorescence resonance energy transfer (FRET)-based sensors as an ideal donors for sensitive and rapid detection of different types of diseases [6–10]. It is worth quoting that QDs are generally composed of different semiconductor elements which have smaller sizes than the exciton Bohr radius [11].

On the other hand, different kinds of chemical materials and nanoparticles have been used as acceptor in the FRET-based sensors including rhodamine [6,8], TAMRA [7], gold nanoparticles [12,13], graphene oxide [14] and carbon nanotubes [15]. Among the above-mentioned materials, gold nanoparticles (AuNPs) offers unique optical properties and as a result, they have been used widely for detecting different

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analysts [12,16]. In fact, AuNPs have been considered as excellent FRET-based quencher because of their plasmon resonances in the visible range [17].

By considering the significance of the FRET phenomenon in clinical applications, great deals of theoretical and experimental researches have been performed to investigate the influence of metal surface on the emission spectra of fluorophore molecules [18,19]. In fact, a fluorophore molecule in the vicinity of a metal nanoparticle shows a wide-range of fluorescence dependency on the distance between fluorophore and nanoparticle [20].

Having elaborated that, the present study was set to take advantage of AuNPs as an acceptor placed in the vicinity of QDs to develop a sensitive, simple, and rapid FRET-based nanobiosensor for CTV detection. More specifically, a detection strategy was designed by labeling specific antibody against CTV's coat protein (CP) with QDs (QDs-Ab) while the target proteins (CP) were labeled with AuNPs (AuNPs-CP). The AuNPs-labeled CP and QDs-labeled antibody formed an immuno-complex. Therefore, the proximity of the QDs and AuNPs led to an energy transfer between the donor and the acceptor which consequently resulted in decreased fluorescence intensity of the QDs. At the detection stage and in the presence of target proteins in a given sample, the AuNPs-CP was competitively replaced by free CPs resulting in a recovery of the fluorescence intensity of the QDs.

2. Material and methods

2.1. Plant materials

Field samples were collected in January 2014 from different citrus trees growing in different orchards in Selangor and Pahang states of Malaysia. The young leaves from four different locations around the canopy pointing east, west, south, and north were collected in plastic bags, marked, and stored in a dry ice box and were then stored at −20 °C until use.

2.2. CTV presence confirmation

The presence and confirmation of CTV was performed at the plant protection department of the agricultural research institute by using reverse transcription polymerase chain reaction (RT-PCR).

2.3. Sap extraction from plant materials

The extraction of plants sap from healthy and infected citrus trees was carried out by crushing 1 g leaves in liquid nitrogen followed by suspension in 500 μL Tris-HCl buffer (70%, pH 7.5) (Thermo Fisher Scientific Inc.). The sap was then stored at −20 °C for future experiments.

2.4. ELISA procedure

The ELISA was performed as a standard test using a Bioreba ELISA Kit (Bioreba) following the manufacture protocol with some modification in order to compare with the sensitivity and specificity of the FRET-based nanosensor developed in the present study. Briefly, the 96-wells microtiter plate (Thermo Fisher Scientific Inc.) was coated with 100 μL of anti-CP antibody diluted to 1:500 in phosphate buffered saline (PBS) using carbonate coating buffer, and the plate was then incubated at 37 °C for 2 h. The plate was washed with PBST (PBS containing 0.05% (v/v) Tween-20) three times. The extracted sap samples obtained from the infected and healthy plant materials, as well as a known negative sample (as negative control), i.e., a sample infected with witches' broom disease of lime (WBDL), and purified CP protein (as positive control) were added to the plate in three replicates and the plate was left overnight at 4 °C. Then, it was washed with PBST as described above. Afterwards, diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (Abcam, UK) was added to each well and the plate was incubated at 37 °C for 2 h. Subsequently, the para-Nitrophenylphosphate (pNPP) (Sigma-Aldrich) as substrate was added to the plate followed by incubation for 30 min and finally, absorbance reading was performed.

2.5. Limit of detection (LOD) of ELISA

The LOD was estimated by analyzing ten samples with known CP concentrations (1 to 10 μg mL^{-1} CP) and a blank sample in six replications based on the procedure described above. Then, the standard deviation was calculated from the replications of the blank sample and the slope of the calibration curve was measured from the standard samples with the known concentrations. Afterwards, the LOD was estimated by using the following equation (Eq. (1)):

$$\mathrm{LOD} = 3S_0/K \tag{1}$$

where S_0 is the standard deviation of blank measurements (n = 6) and K is the slope of calibration curve.

2.6. Synthesis of thiolglycolic acid capped CdTe QDs

The water-soluble CdTe QDs were synthesized by using the procedure reported in our previous study [21]. In brief, 0.1 g Te powder was reduced by 0.3 g NaBH4 in 10 mL double-distilled water under stirring at 80 °C for 3 h. Then, a freshly prepared NaHTe solution was added gradually to N_2-saturated $CdCl_2 \cdot 2.5H_2O$ (Sigma-Aldrich) solution (0.4 g $CdCl_2$ in 100 mL double-distilled water) at pH 10 in the presence of 250 μL thiolglycolic acid (TGA) (Sigma-Aldrich). The mixture solution was heated at 92 °C and stirred in a reflux system under nitrogen gas. The crude solution was then precipitated and washed three times with ethanol and centrifuged for 15 min at 4000 × *g*. The precipitate was re-dispersed in 250 mL double distilled water and stored at 4 °C in the dark.

2.6.1. Labeling of antibody with QDs

In order to label the antibody with QDs, 400 μL of QDs and 200 μL of freshly prepared 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC) (Sigma-Aldrich) (6.4 mg mL^{-1}) and N-hydroxysuccinimide (NHS) (Thermo Fisher Scientific Inc.) (4.5 mg mL^{-1}) were mixed and incubated in a dark water bath at 37 °C for 1 h. Afterwards, 60 μL of the purified polyclonal antibodies (0.25 mg mL^{-1}) was added drop-wise to the solution in the dark and stirred gently at 4 °C for 2 h. Subsequently, 540 μL tris buffer (6 mg mL^{-1}, pH 7.2) was added to the solution and stirred under the above-mentioned condition for 1 h. The mixture was then centrifuged at 18,900 × *g* at 4 °C for 10 min. The lower phase was removed and the upper phase containing the QDs-labeled antibodies (QDs-Ab) were diluted by 1200 mL tris buffer and stored at 4 °C until use. To confirm successful conjugation of the QDs and the antibody, spectrophotometeric analysis was performed.

2.7. Synthesis of AuNPs

AuNPs were synthesized by the citrate reduction method by mixing 1 mL tri-sodium citrate (Sigma-Aldrich) solution (37.5 mg in 1 mL distilled water) to 5 mg $HAuC1_4 \cdot 3H_2O$ (Sigma-Aldrich) in 48 mL distilled water under stirring at 70 °C until the solution turned red.

2.7.1. Labeling of antigen with AuNPs

In order to label the antigen with the AuNPs, 400 μL of AuNPs and 200 μL of freshly prepared EDC (6.4 mg mL^{-1}) and NHS (4.5 mg mL^{-1}) were mixed and incubated in a water bath at 37 °C for 1 h. Afterwards, 30 μL of CP (0.25 mg mL^{-1}) were added drop-