ردیابی سریع ویروس اس سیب‌زمینی با استفاده از نانوذرات طلای پوشش داده شده با آنتی‌بادی

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چکیده

علیرغم وجود روش‌های مختلف در تشخیص ویروس‌ها، زمان‌بر بودن آزمون‌ها یکی از مهمترین محدودیت‌های آنها است. از این رو، توسعه روش‌های سریع‌تر برای صدور گواهی در ایستگاه‌های قرنطینه و مدیریت ویروس‌های گیاهی اهمیت شایانی دارد. در این تحقیق، با استفاده از فناوری نانو، نانوذرات طلا (AuNP) برای شناسایی ویروس اس سیب زمینی (*Potato virus S*, PVS) به کار گرفته شد. AuNP از طریق احیاء سیترات تهیه شد و سپس آنتی‌بادی اختصاصی پروتئین پوششی PVS به آنها متصل شد. بهینه سازی واکنش اتصال با تغییر اسیدیته AuNPs و غلظت آنتی‌بادی انجام گرفت. با استفاده از نانوذرات پوشش داده شده با آنتی‌بادی، تشخیص آنتی‌ژن در غلظت‌های مختلف با طیف‌سنجی انجام شد. پس از اتصال آنتی‌بادی به نانوذرات طلا، بیشینه جذب نانوذره به علت افزایش اندازه ذرات به مقدار ٤-۵ نانومتر تغییر کرده و از ۵۲٤ به ۵۳۰ نانومتر رسید. اتصال آنتی‌ژن به نانوذره نیز موجب تغییر اندازه ذرات و افزایش بیشینه جذب از ۵۳۰ به ۵۳۹ نانومتر شد. طیف جذبی نانوذره در هنگام استفاده از PVY و CMV تغییر نکرد. این مطالعه اولین تلاش برای استفاده از نانوذرات در تشخیص سریع و آسان PVS است.

کلیدواژه: نانوذرات طلا، آنتی‌بادی، بیوسنسور، تشدید پلاسمون، ویروس اس سیب‌زمینی

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Rapid detection of *Potato virus S* using antibody-coated gold nanoparticles

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Abstract

In spite of existing of several methods for detection of viruses, time consuming is major limitation of them. So, developing faster and real time method is important to certificate the plant materials in quarantine stations and virus management. In the recent research, nanotechnology was employed to detect *Potato virus S* (PVS) using gold nanoparticles (AuNPs). Colloidal AuNPs were prepared through citrate reduction and conjugated to a specific antibody against PVS coat protein. Conjugation was optimized by changing the pH of AuNPs and antibody concentration. Antibody coated nanoparticles were used to detect different concentrations of antigen using spectrophotometry. After binding the antibody to the gold nanoparticles which cause an increase in particle size, the SPR peak was displaced in range of 4-5 nm and shifted from 524 to 530 nm. The binding of the antigen to the nanoparticle also caused a change in the particle size and an increase maximum absorbance from 530 to 539 nm. No spectral change were seen when PVY and CMV used as controls. This study is the first attempt of nanoparticle usage in quick and easy detection of PVS.

Keywords: Gold nanoparticles, Antibody, Biosensors, Plasmon Resonance, *Potato virus S*

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Introduction

Potato virus S (PVS) is a prevalent virus infecting potato plants, belongs to the genus *Carlavirus* in the family *Betaflexiviridae* (King *et al.* 2018) that cause moderate to high crop losses worldwide (Cox & Jones 2010). The genome consist of a positive-sense single-stranded RNA contains six Open reading frames (ORF) (Matousek *et al.* 2005). Different approach including biological, serological and molecular methods have been applying to detect plant viruses. ELISA and RT-PCR are two techniques commonly used to detect plant viruses for many years. However, ELISA have been frequently used for virus detection because of specificity and feasibility when testing of large number of samples are needed (Ling *et al.* 2000, Zimmermann *et al.* 1990). Beside the advantages, time consuming, labor intensive and requirements are the most limitation for ELISA.

There are many researches that have gone through to improve the detection methods in different aspects such as time and cost which nanotechnology has been recently helped to solve the problems. By the advent of nanotechnology, powerful, rapid and accurate methods have been developed to detect different antigens. Physical properties of nanoparticles (NPs) is one of interesting factor to design a new method to detect plant viruses (Thompson 2007). Several rapid and easy methods have been developed using nanomaterials to detect plant viruses (Danks & Barker 2000, Salomone & Roggero 2002, Salomone *et al.* 2002).

Nanotechnology has been encouraged researchers to use antibody-antigen binding activity to make highly efficient techniques based on immunosensores (Ali *et al.* 2004, Dong-Pham *et al.* 2012, Ricci *et al.* 2007, Saleh *et al.* 2011).

Using gold nanoparticle (GNP) which is synthesized easily and binds efficiently to biomolecules as a probe, makes it a good choice to develop biosensors in many industries including biotechnology, medicine and agriculture. Some steps such as incubation, washing and enzymatic reactions are not necessary in AuNPs probe systems. So, AuNPs based probes showed more stabile, faster and easier results compared to fluorescence-based and enzymatic-based detection systems (Xiulan *et al.* 2005). Antibodies coupling on the surface of AuNPs could be detected using UV–Vis spectrophotometry. Several factors such as shape, size, and chemical properties of metallic nanoparticles affect the position of surface plasmon resonance (SPR) peak (Perenboom *et al.* 1981). Spherical AuNPs with a size in range of 3–30 nm exhibit a plasmon band at ~520 nm which red-shifts upon agglomeration or change in media, but is more sensitive to agglomeration, hence the basis of this technique is based on the change in the absorption spectra position of SPR peak in AuNPs followed by color change (Perenboom *et al.* 1981). In the present study, using AuNPs coated by specific PVS antibody a rapid system for identification of PVS was developed.

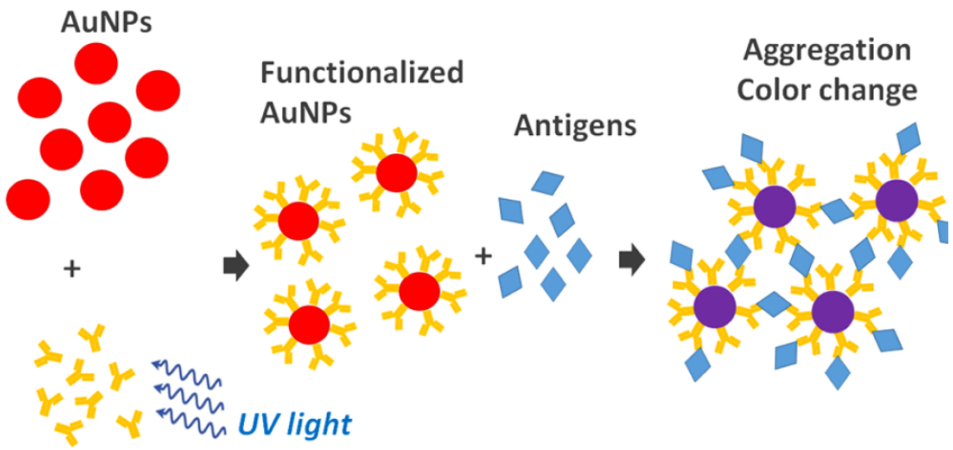

Fig. 1. Virus detection using antibody-labelled AuNPs. The heavy chain of virus-specific antibody is strongly anchored to AuNPs surface. The presence of multiple binding sites on AuNPs creates multiple binding sites for heavy chains and then clustering of antibodies on its surface takes place (form Larossi *et al.* 2018).

Materials and Methods

Materials

Except for Gold (III) chloride trihydrate 99.9% that is purchased from Aldrich, USA, the other materials including sodium citrate dehydrate ($Na_3C_6O_7$-$2H_2O$) and Bovine serum albumin (BSA) were received from Sigma. The PVS infected (isolate Ar 145) and healthy potatoes and PVS recombinant coat protein (isolate Dez 4, accession -number KY523839) (Masoudi *et al.* 2019) were received from Seed and Plant Certification and Registration Research Institute (Karaj, Iran). Commercial positive control and PVS-specific polyclonal antibody were received from Agdia (USA).

Preparation of AuNPs

The conventional reduction of tetrachloroauric acid by trisodium citrate was used to generate 20 nm AuNPs suspension (Turkevich *et al.* 1951) in which the concentration of citrate solution determine the size of nanoparticles. The magnetic stirrer used in the synthesis were thoroughly washed in aqua regia solution (HCl/HNO_3 3:1 v/v). A 0.25 mM $HAuCl_4$:$3H_2O$ solution (125mL) was boiled with vigorous stirring in a 250mL round bottom flask, then 40mM trisodium citrate solution (12.5mL) was added quickly to boiling solution. Color change from yellow to dark red indicated the formation of AuNPs. The solution was maintained for 10 min at boiling temperature and stirring was continued for another 15 min. This solution was cooled at room temperature and stored at 4 °C before use.

Characterization of AuNPs

UV–Vis spectrophotometry (APEL PD-3000UV, Japan) and SPR measurement were carried out to determine the optical characteristic of AuNPs. The morphological features and size of AuNPs were determined by transmission electron microscopy (80 KeV, Model EM 900, Zeiss, Jena, Germany). Sample preparation was done by coating a carbon-coated copper grid with a droplet of the colloidal AuNPs and consequent drying for 45 minutes.

Determination of optimal pH of AuNPs and antibody concentration for conjugation

The optimum concentration of antibody-coated AuNPs was estimated according to Slot & Geuze (1985). Antibody concentration and pH of nanoparticles can affect binding of antibody to AuNPs. For this purpose, colloidal AuNPs pH was adjusted at 7.5 and 8.5 using 0.2 M sodium carbonate. Aliquots of 16 different concentrations of PVS-specific antibody (0.15, 0.3, 0.6, 0.9, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 µg/ml) were prepared in 30 µl PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 X 2H2O, 1.5 mM KH2PO4; pH 7.4) and each concentration was added to 300 µl of AuNPs with different pH, separately. The plate were stirred for 60 minutes at room temperature and then 30µl of NaCl 10% was added to each tube. Addition of aqueous NaCl covers surface of non-coated AuNPs decrease inter particle distance and induces agglutination. The UV-visible absorbance was recorded and changes in absorption peak were measured.

Preparation and characterization of AuNPs-Ab ligands

100 µl antibody solution (10 µg/ml) were added to 500 µl AuNPs (pH 7.5), then were mixed for one hour at 25°C. The AuNPs-antibody solutions were then treated with 100 µl of 10% bovine serum albumin (BSA) and were incubated for 10 min at 25°C. BSA blocks nonspecific binding sites on AuNPs and contributes to better stabilization of the solution (Lai *et al.* 2015). Excess antibody was removed via centrifugation at 10000 rpm for 15 min at 4°C, followed by washing and re-suspending the pellet in 500 µl PBS buffer. Antibody-coated AuNPs was stored in 4°C.

Detection of PVS by antibody-coated AuNPs

Purified recombinant protein (Masoudi *et al.* 2019) and PVS infected potato extract (1:10 in the extraction buffer (PBS buffer containing 0.2% egg ovalbumine, 2% PVP40, 0.05% Tween-20 and 0.05% NaN_3)) were tested for virus detection using antibody-coated AuNPs. Four different concentrations of recombinant antigen (viz. 5, 2.5, 0.5 and 0.05 µg) were prepared in 20 µl PBS buffer and tested in immunoassay. Twenty microliters of each concentration of antigen were mixed with 50 µl of antibody-coated AuNPs solution and was incubated 40 min at room temperature. The aggregation process indicated a positive result that was

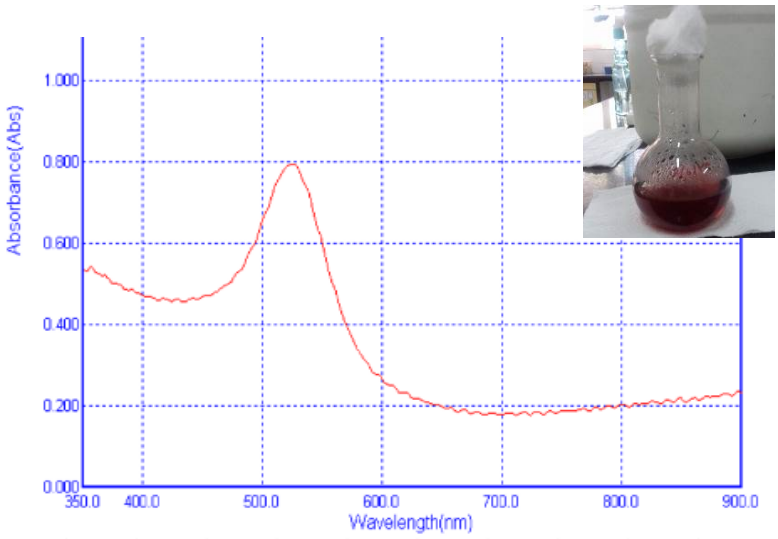

Fig. 2. UV-Vis spectrum of colloidal AuNPs. The absorption has been recorded from 350 to 900 nm. SPR peaks occurs at 524 nm.

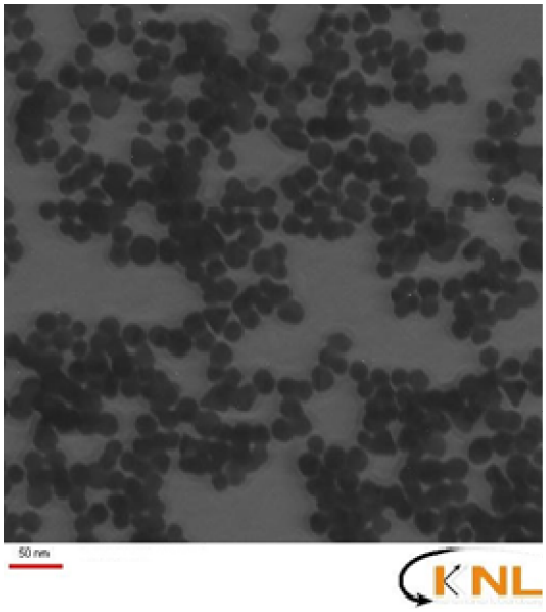

Fig. 3. TEM micrographs of AuNPs showing 20 nm spherical particles and uniform size of AuNPs.

confirmed by UV-visible spectrophotometry. Also, twenty microliters of PBS buffer and healthy plant extract mixed with antibody-coated AuNPs, was used as negative control.

Specificity of PVS antibody-coated AuNPs immunoassay

The specificity of antibody-coated AuNPs to detect PVS, was approved using *Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV). Extracts of infected plants (PVY infected potato and CMV infected cucumber) were added to antibody-coated AuNPs, as antigens, and were subjected to spectroscopy as for PVS antigens. Also, the leaf samples were homogenized (1:10) in the extraction buffer (PBS buffer containing 0.2% egg ovalbumine, 2% PVP40, 0.05% Tween-20 and 0.05% NaN_3).

Results and Discussion

Synthesis and characterization of AuNPs

Plasmon absorbance of the synthesized AuNPs was exhibited extinction peak at 524 nm (Fig. 2).

High resolution-TEM (HR-TEM) image of AuNPs (Fig. 3) indicated the spherical particles with 20nm size of AuNPs which demonstrated homogeneity and monodispersity of the particles.

The new findings were in line with previous studies which demonstrated the AuNPs absorption peak at 524 nm with Gaussian distribution that indicated the formation of spherical AuNPs with no aggregation (Huang 2006). This subject indicated the uniformity and excellent dispersion in colloidal AuNPs (Perezjuste 2005).

Optimization of AuNPs pH and antibody concentration for antibody-AuNPs ligand formation

Transparent red-colored colloidal AuNPs were obtained when appropriate pH was applied. Aggregation of the nanoparticles in solution happened

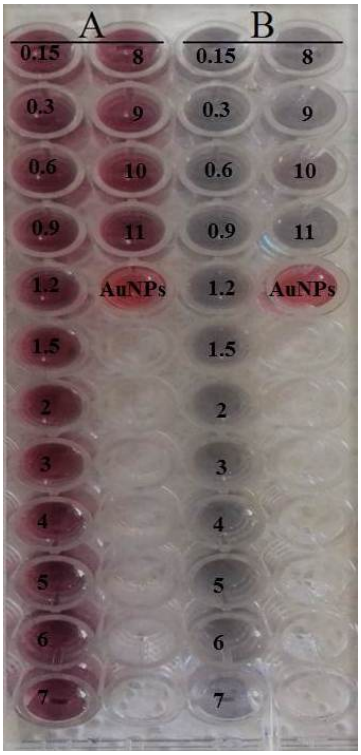

Fig. 4. Result of optimization of pH for binding of antibody to colloidal AuNPs. A and B represent pH 7.5 and 8.5, respectively. Sixteen concentrations of antibody (from bottom, 0.15, 0.3, 0.6, 0.9, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 µg/ml, respectively) were used for conjugation to AuNPs in two pH.

following the addition of NaCl 10% at pH 8.5, and then the color of solution was changed from red to blue immediately. The optimal pH for binding antibody to colloidal AuNPs was determined at 7.5 and no color change was shown (Fig. 4). pH of colloidal AuNPs adjusted to 7.5 and used for antibody conjugation.

Aliquots of 16 antibody concentrations were used to bind with AuNPs solution to determine the optimal concentration of antibody. The wavelength of citrate-stabilized AuNPs absorption peak was 524 nm but the absorption peak of conjugated AuNPs (with antibody) has been red-shifted several nanometers (Fig. 5). Since the NaCl was added, the AuNPs was aggregated in low concentrations of antibodies. The absorptions curves related to the aggregated AuNPs were shifted to the larger wavelengths. The solution maintained its stability against NaCl stress and color change and shift of absorption peak did not occur, when higher concentrations of the antibody were used. The amount of antibody which prevents discoloration of the solution and shifted peak position from 524 nm (AuNPs) to 530 nm (Ab:AuNPs), was considered as the appropriate antibody concentration (10 µg/ml) for conjugation. This change in wave length was result of slight increasing in AuNPs

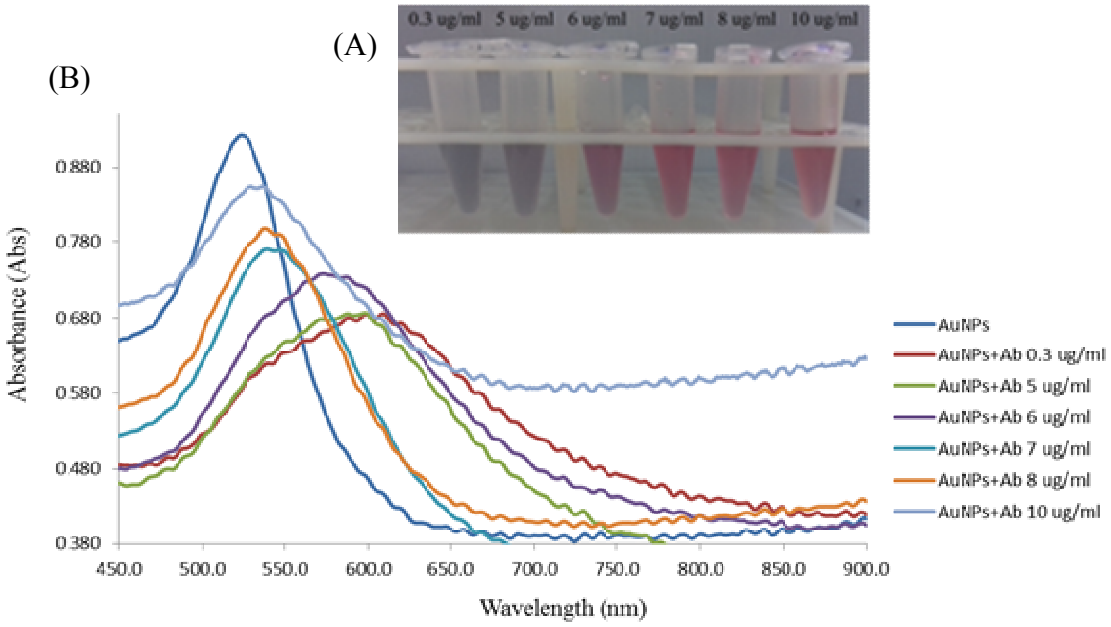

Fig. 5. A) Photograph of colloidal gold supplemented with different concentrations of antibodies. B) Spectral analysis of AuNPs incubated with six concentrations of antibodies.

size due to the PVS antibody binding. Increasing size of AuNPs makes a red shift in the absorption color (Choi *et al.* 2014). The absorptions curves of aggregated AuNPs are much flattened than the stabilized AuNPs and the absorption peak shifted to larger wavelengths (550 to more than 600 nm), while their absorption intensity was drastically decreased.

Detection of PVS by antibody-AuNPs ligands

After preparation of antibody-coated AuNPs, an antigen recognition test was performed by four concentrations of the PVS recombinant coat protein. Results revealed that intensity of SPR peak decreased in antigen concentrations up to 0.05 µg/ml in antibody-AuNPs ligands. Higher antigen concentrations (above 2.5 µg/ml), red shifted the UV–VIS absorption spectra of AuNPs (up to 9 nm) and decreased the SPR absorbance intensity (Fig. 6).

Shift in peak position and displacement towards the longer wavelengths resulted from binding of antigen to the Ab-AuNPs ligands which increased the particle size. By increasing antigen concentration, red-shift of peak position was increased and reached to 539 nm at the highest concentration (5 µg/ml). It is a fast (approximately 40 min) and simple method to detect of PVS infected plants.

To evaluate specificity of antibody-antigen interaction, the antibody-coated AuNPs solution was tested with two other plant viruses including PVY and CMV. In Fig. 7 the non-responding curves of PVS antibody conjugated AuNPs against PVY and CMV are shown. No change in peak intensity and peak position were observed when AuNPs, healthy plant extract and PBS buffer used as negative controls.

Increasing in antigen concentration (PVS CP) affects gradual reduction of AuNPs' SPR absorbance intensity and shift in peak position, which could confirm the interaction between antigen and antibody-coated AuNPs (Fig. 6).

It is known that, binding of specific analyte like antibody on surface of AuNPs can alter the native dielectric environment of AuNPs and results in a SPR peak shift that could be measured by UV-visible spectrophotometer (Petryayeva & krull 2011). This feature is used to detect antigens rapidly (Davatgaran *et al.* 2018, Fatemi *et al.* 2017). Spectrophotometer analysis showed after binding of antigens to antibody-coated AuNPs, SPR peaks are shifted towards larger wavelengths that are shown in the present study with PVS antibody as well.

Several detection methods on the basis of proteins, nucleic acids and bioassays are developed to detect viruses. Virus detection methods such as

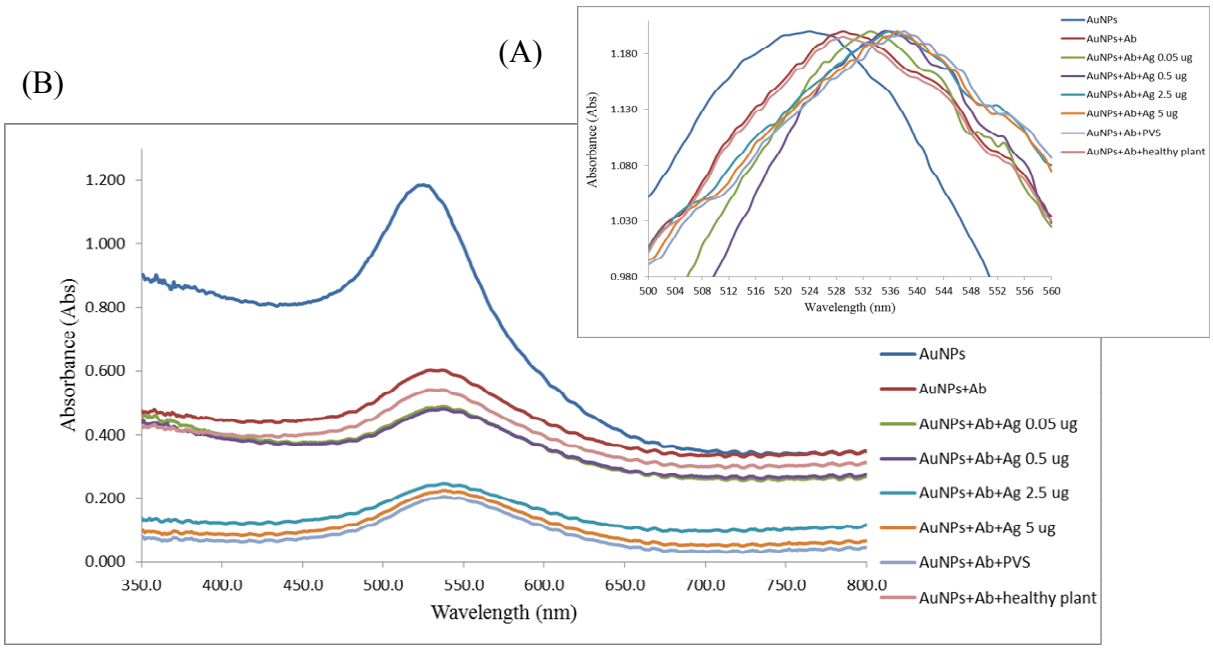

Fig. 6. Alteration in SPR peak (A) and absorbance intensity (B) of antibody-coated AuNPs incubated with four concentration of PVS recombinant coat protein, PVS infected potato and healthy plant extracts.

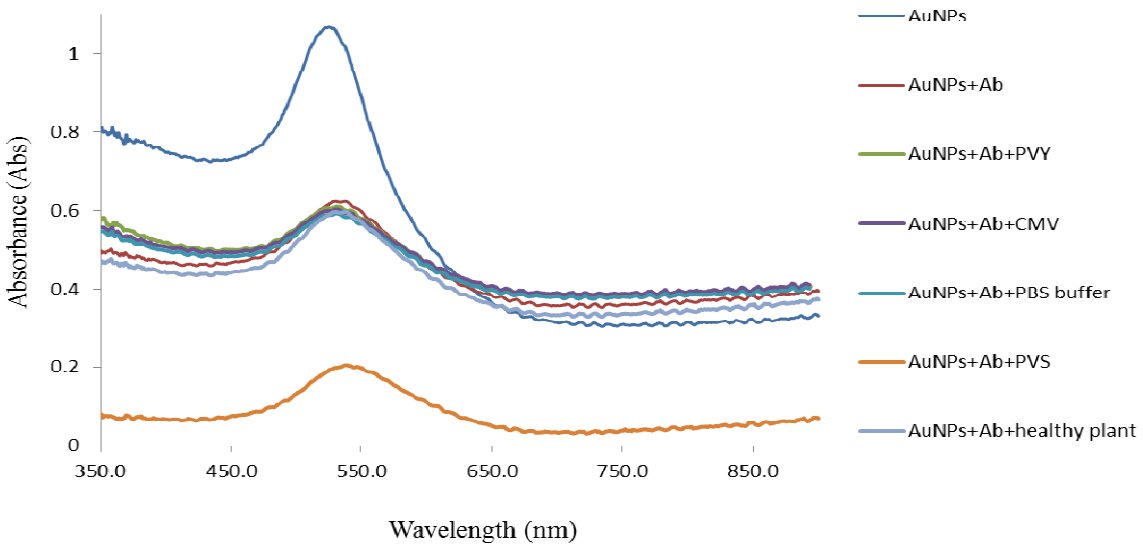

Fig. 7. Analysis of specificity of PVS antibody-coated AuNPs against PVY, CMV and PVS infected and healthy plant extracts.

ELISA require several steps of incubation and washing that are time-consuming and increase risk of contamination (O'Sullivan *et al.* 1979, Voller *et al.* 1976). In overall, an ELISA assay often take 24-48 hours for analysis and longer time of the assay is consumed in incubation steps. In the presented method, the virus identification process is one-step, fast and simple. The amount of antibody require for antibody-coated AuNPs is much lesser than ELISA that should worth to be noted.

In a same study, Liu *et al.* (2013) electrostatically attached 20 nm AuNPs into single-chain fragment variable antibody containing cysteine (scFv-cys), for detection of matrix methaloproteins-2 (MMP-2) (Liu *et al.* 2013). Springer & Homola (2012) covalently attached the AuNPs particles with 30 nm diameter to carcinoembryonic antigen, using the SPR system and had successfully used it to detect the target molecule.

Nanotechnology could improve the available virus detection methods to faster, cheaper and more accurate identification systems. Due to the importance of rapid detection of plant viruses to preventing spread of them, AuNPs coated by PVS antibody was used for rapid identification of PVS in infected potato plants. Results indicated that the AuNPs acidity and antibody concentration are the most critical factors in developing this technique.

The best pH for binding PVS antibody to AuNPs that did not affect the antibody function was 7.5. In this study, using PVS antibody coated-AuNPs a biosensor system was developed which could detect relatively low amount of antigen based on alteration in SPR of the nanoparticle. Additionally, the assay time and cost were significantly lowered compared to conventional ELISA. To our knowledge, the use of AuNPs to improve the detection of PVS is carried out for the first time.

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