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Assessing Stability of Gold Nanoparticles in Presence of Two Enzymes, RNaseA and RNaseH, Using Colorimetric Detection

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ABSTRACT

Gold nanoparticle-based diagnostic methods have attracted much attention due to their simplicity, high sensitivity and low cost. These methods are mostly used for early and efficient detection of various pathogenic factors and assessment of gene expression and nanoparticles stability. In this study, melon plants were cultured *in vivo* and then, RNA was extracted from leaf explants. A 40 bp probe was designed based on the *Actin* gene sequence and applied to investigate the gene expression by colorimetric detection. Color variation of the nanoparticles, from red to blue was observed in presence of the target molecules. The maximum observed changes was at 550-650 nm. Moreover, the activity and the effect of two enzymes, RNaseA and RNaseH, on the stability of gold nanoparticles were investigated. The results showed the effect of the RNaseA enzyme in the initial time period along with the RNaseH enzyme over the time on the stability of gold nanoparticles. Such colorimetric techniques, which are based on the stability of the gold nanoparticles could be used for rapid assessment and recognition of gene expression and the factors affecting the stability of the gold nanoparticles. These could also be considered as a useful technique for screening other enzymes and medicinal molecules.

Keywords: RNaseA enzyme, RNaseH enzyme, *Actin* gene, Colorimetric assay, Gold nanoparticles

INTRODUCTION

Nanobiotechnology is one of the most promising areas of nanoscience and nanotechnology in the modern age. This technology is emerging in various fields of science, including chemistry, biology and materials science [1]. Nanoparticles are among the most common elements of science and nanotechnology and because of their interesting properties, they have obtained numerous applications in the chemical, medical, pharmaceutical, agricultural industries as well as electronics. Gold nanoparticles are different sized particles ranging from one to several hundred nanometers [2]. These particles represent specified chemical, physical and optical features in relation to the size, shape and surface [3,4]. The very small size of nanoparticles enable them to react with biomolecules [5,6].

Gold nanoparticles can also be regarded as a scaffold that has a high surface to volume ratio, since they can react with 100 small molecules or 30 stranded oligonucleotides [7]. Specific light with a wavelength longer than the nanoparticles causes bipolar fluctuations in Au and in turn, leads to transfer of free electrons from one orbit to the surface of the nanoparticles [8]. In a particular frequency, the magnitude of oscillation reaches its maximum limit. This frequency, known as the Surface Plasmon Resonance (SPR) is responsible for color variation of colloidal Au [9,10].

The solution of gold nanoparticles is red when is dispersed, whereas after deposition, it turns blue [11,12]. As a colorimetric sensor, this diagnostic method, which is based on gold nanoparticles, is used for a variety of molecules, ions and small organic molecules [13]. Other advantages of nanoparticles are good biocompatibility, easy synthesis and connection with different bimolecular ligands (*e.g.*, DNA, RNA and proteins), antibodies, and other molecules. These have made them proper to be used as

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biochemical and diagnostic biosensors to detect single nucleotide polymorphism, mutations, pathogens and gene expression [14,15] as well as medical diagnostics [16]. The interactions between oligonucleotide and nuclease enzymes are important because of its significant role in gene regulation [17]. DNA-AuNPs are also commonly used in gene diagnosis and regulation [18]. RNaseA and RNaseH enzymes are among endonuclease enzymes, RNaseA breaks down RNA single and double stranded molecules and contributes with DNA purification, while RNaseH enzyme crops RNA single stranded in RNA-DNA hybrid [19].

The aim of this study was to evaluate the *Actin* gene expression in melon plant and to understand how the two endonuclease enzymes, RNaseA and RNaseH, affect the stability of gold nanoparticles using nanoparticle-based colorimetric assay. Since identification of factors affecting the stability of nanoparticles is of particular importance, it would be useful to realize the way the enzymes affect gold nanoparticles and their stability.

MATERIALS AND METHODS

RNA Extraction

Seeds of melon plants were cultured in vitro and then RNA was extracted from leaves using GeneAll kit. 0.1 g of the plant sample was grinded with liquid nitrogen and transferred to a 1.5 ml tube. Next, the extraction protocol was performed according to the kit manufacturer instructure. Finally, the extracted RNA was placed at -80 °C until use.

Probe Designed Based on the Sequence of *Actin* Gene

Exon area of the *Actin* gene (Genbank accession number AB033599.1) was identified using the NCBI database and a part of it was used to design the 40bp oligonucleotide probe. The specificity of the probe was evaluated using the NCBI database. The sequence of the designed probe is shown in Table 1.

Synthesis of Gold Nanoparticles

Gold nanoparticles were synthesized using sodium citrate [20]. A 95 ml solution containing 5 mg of HAuCl4 was mixed with 5 ml of 1% sodium citrate solution, then the mixture was heated and stirred until the solution turned red. Final solution was kept at room temperature in dark condition. The structure and dispersion of synthesized particles was evaluated using the absorption spectrum of the nanoparticles, TEM, and FT-IR spectrum.

Determining Relative Quantity and Quality of Extracted RNA Using Gold Nanoparticles

In order to determine the relative quantity and quality of the extracted RNA using gold nanoparticles, first, 0.2 µl RNA at concentration of 230 µg ml^{-1} was mixed and vortexed with 10 µl of 0.02 mM phosphate. Next, 10 µl of 0.25 M NaCl added to the mixture in the tube. Then, it was vortexed and spinned. Finally, 120 µl of the gold nanoparticles was added to it and the colorimetric changes were studied.

Actin Gene Expression Using Gold Nanoparticle Probes

To confirm *Actin* gene expression in melon plant, 2 µl of RNA at concentration of 230 µg ml^{-1} with 10 µl of 0.02 mM phosphate buffer was mixed and vortexed. Then, 5 µl of probe 10 pM along with 10 µl of 0.25 M NaCl were added and vortexed. The tube was placed at 65 °C for 25 min to perform probe and RNA hybridization. After cooling at room temperature, 120 µl of gold nanoparticles were added to it. Color change was monitored and absorption was read by a spectrophotometer. The negative control used in this study is the watermelon plant.

Comparison of RNaseH and RNaseA Performance

In order to evaluate the performance of two enzymes, RNaseH and RNaseA, first 2 µl of RNA at concentration of 230 µg ml^{-1} was mixed and vortexed with 15 µl of 0.02 mM phosphate buffer. Then 10 pM probe and 10 µl of 0.25 M NaCl were added to the tube and vortexed. For hybridization, the tube was put at 65 °C for 25 min. In the next step 0.5 µl of RNaseH or RNaseA was separately added to the tube and the tube was placed at 42 °C for 30min. Finally, 120 µl of the gold nanoparticles (OD equal to 0.82) were added to it and color variation was studied.

RESULTS

RNA Extraction

Result of gel electrophoresis confirmed the accuracy and

Table 1. Probe Sequence Designed for *Actin* Gene

Probes	Sequence
PA	- 5´TGGCCCATCCCAACCATAGCACCAGTATGACGAGGTCGGAC´3

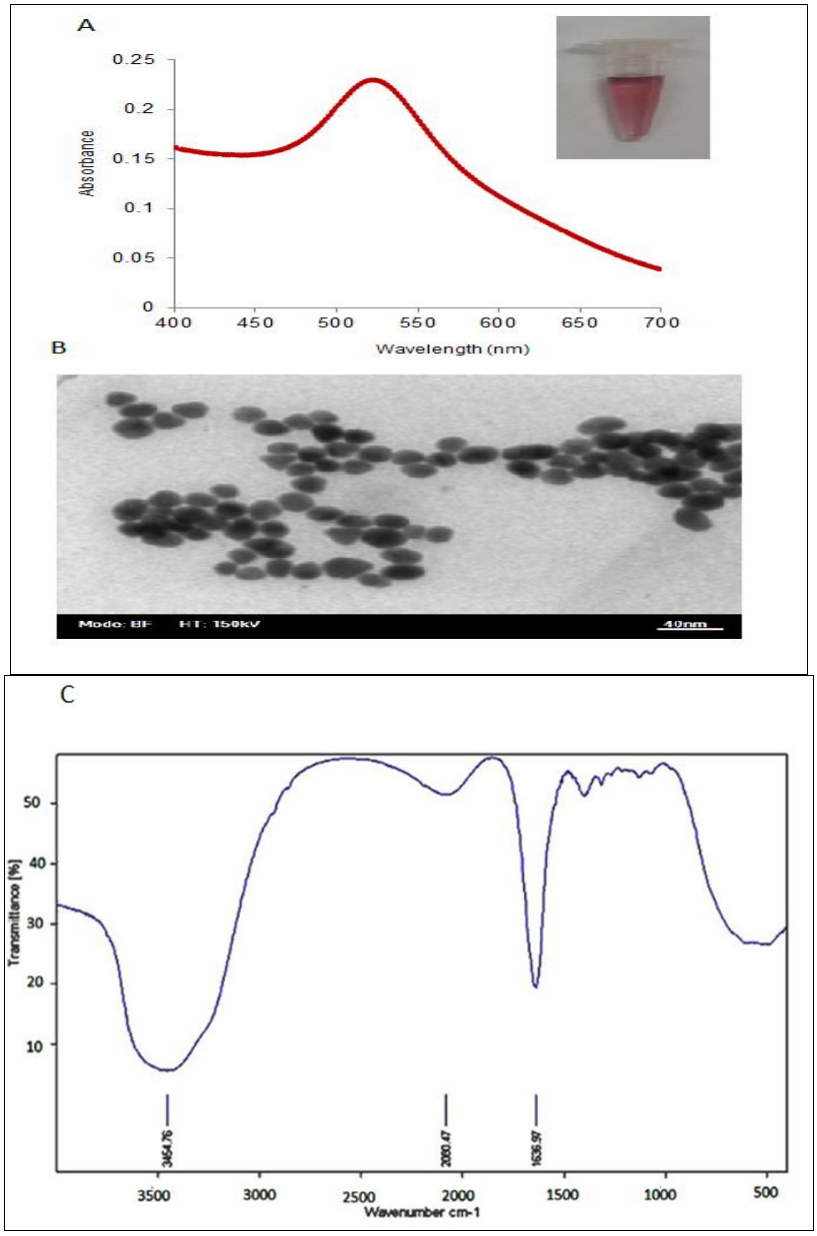

Fig. 1. A) Absorption spectrum of gold nanoparticles and maximum absorption at 528 nm, B) TEM image of synthesized gold nanoparticles with sodium citrate at a magnification of 140000, C)FT-IR spectrum of synthesized gold nanoparticles.

integrity of the extracted RNA. Determination of the quality of total RNA was evaluated using spectrophotometry at the wavelengths of 260 nm/280 nm and the ratio of 1.8 was achieved.

Gold Nanoparticles Synthesis

Red color of prepared gold nanoparticles solution confirmed the formation of the GNPs. the absorption spectrum of the synthesized nanoparticle was measured using a spectrophotometer and the maximum absorption spectrum was at 528 nm (Fig. 1A). TEM results indicated spherical shape and uniform size of around 20 nm (Fig. 1B). Moreover, FT-IR was used to ensure the presence of citrate ion on the surface of gold nanoparticles and the synthesis of these particles. As is seen in the spectrum, the peak was obtained in the range of 1636 cm^{-1}, which represents the functional group C=O. The presence of the peak in the range of 3454 cm^{-1} is also due to the hydroxyl functional group. The peak available in the range of 1380- 1400 cm^{-1} is also due to the presence of functional group, $-\overset{O}{\overset{\|}{C}}-O$. The obtained results confirmed the presence of the citrate group as the main factor in gold revival (Fig. 1C).

Confirmation of the *Actin* Gene Expression Using Gold Nanoparticle Probe

The results of the aggregation of gold nanoparticles and changes of their wavelength and color in the presence of the probe and RNA (as shown in Fig. 2) were confirmed the connection of the oligonucleotide and RNA.

Comparison of RNaseA and RNaseH Performance on Stability of Gold Nanoparticles

After hybridization between the probe and RNA, RNaseA enzyme breaks down RNA completely in the presence of gold nanoparticles and NaCl, hence the RNA ability for binding to the surface of the nanoparticles was enhanced and the nanoparticles get out of NaCl reach. Therefore, no color change was observed in the reaction tube (Fig. 3).

To ensure the accuracy of the assay, two-stranded cDNA was used as negative control in the presence of the nanoparticle solution containing RNaseA enzyme and the probe. Since RNaseA enzyme just cuts the single-stranded RNA molecules, it does not act in the presence of cDNA instead of RNA, therefore change in color and absorption spectra was observed in the presence of phosphate buffer and NaCl (Fig. 4).

RNaseH enzyme affects RNA-probe hybrid. This enzyme corps some parts of RNA that are formed with hybrid probe. In this reaction the probes attached to RNA were released to cover the surface of the nanoparticles. As a result, some gold nanoparticles were out of NaCl access. However, there were still a lot of nanoparticles to interact with NaCl available in the tube, so the color was changed (Fig. 5).

To ensure the accuracy of the assay on the impact of RNaseH enzyme, the RNA of watermelon plant was used as a negative control. Since RNaseH affects the probe-RNA hybrid, in the absence of a two-stranded hybrid, the enzyme did not work and no change was observed in color and spectrum (Fig. 6).

Also, analyzing the stability of gold nanoparticles in the presence of the RNaseH enzyme after two hours at 42 °C showed that over time RNA was more separated and the fragments resulted from RNA degradation could be play a role as probes and cover the surface of nanoparticles; consequently, in presence of NaCl and phosphate buffer, less changes in terms of color were observed in the reaction tube.

DISCUSSIONS

In recent years, application of the DNA-based diagnostic tests has expanded greatly. DNA-identification can be done using diagnostic DNA biosensors. Diagnostic biosensors have numerous functions such as DNA detection, gene analysis and rapid identification of pathogenic agents as well as criminal cases. Available DNA-based detection systems which depend on hybridization of a target DNA or its complementary probe, could be performed in solution or on a solid surface. Diagnostic methods which are based on gold nanoparticles can be used as colorimetric sensors for a variety molecules and ions [13,21,22], various types of bacteria, including *Salmonella* [23,24], *E. coli* [25], *Leishmania spp* [26], various viruses such as*hepatitis C* [27], *influenza A* [28], *Maize chlorotic mottle virus* [29] and cancer cells [30,31].

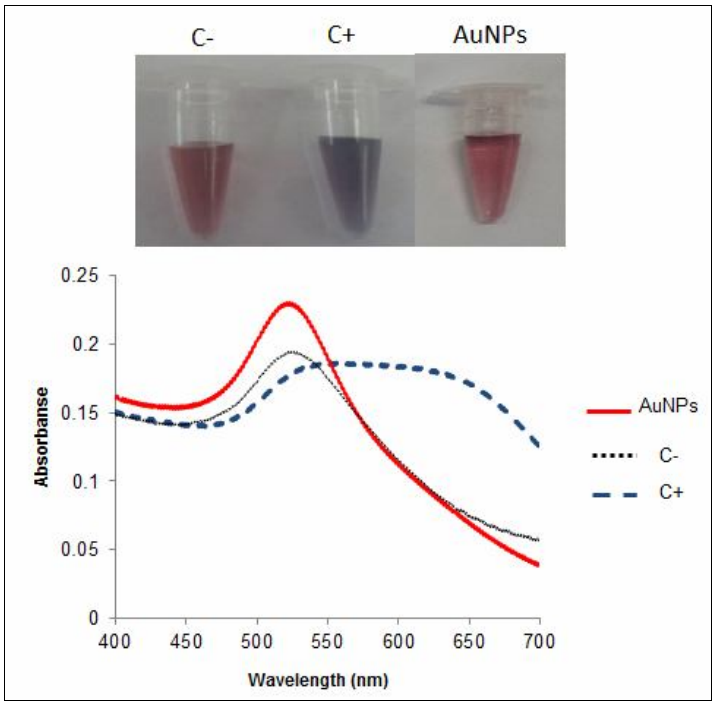

Fig. 2. Change in color and wavelength spectrum resulting from probe connected to target sequences in RNA (+) compared to the negative control (-).

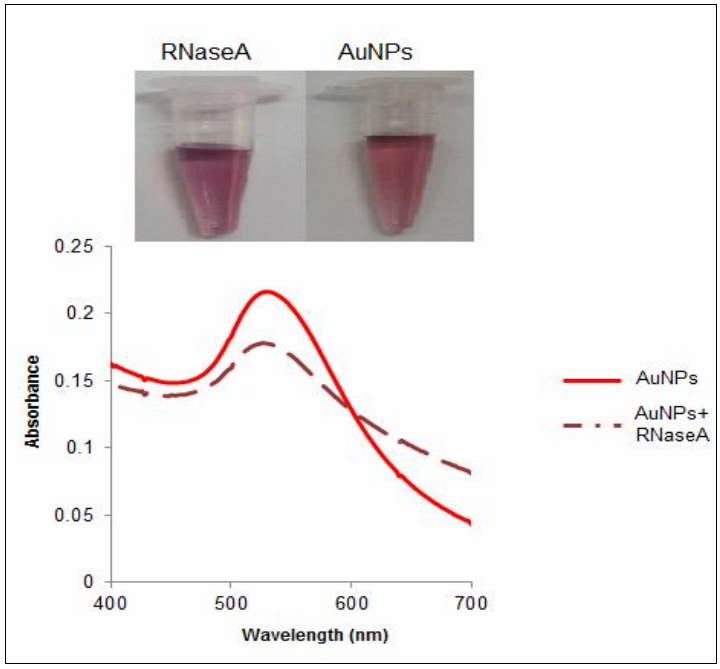

Fig. 3. Gold nanoparticles with no color change and with stability in presence of RNaseA enzyme.

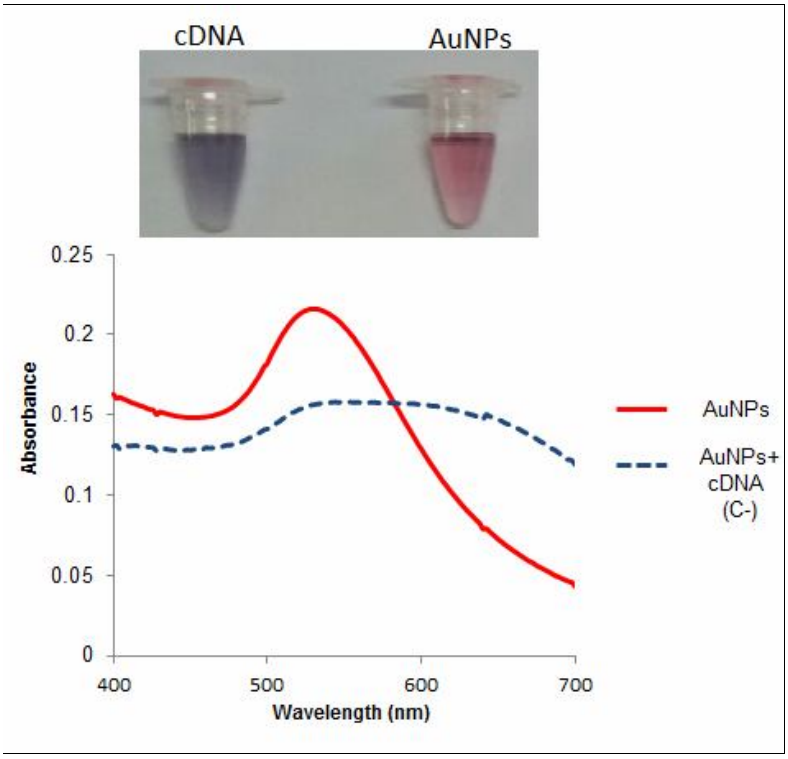

Fig. 4. Change in color and wavelength of gold nanoparticles in presence of cDNA as negative control.

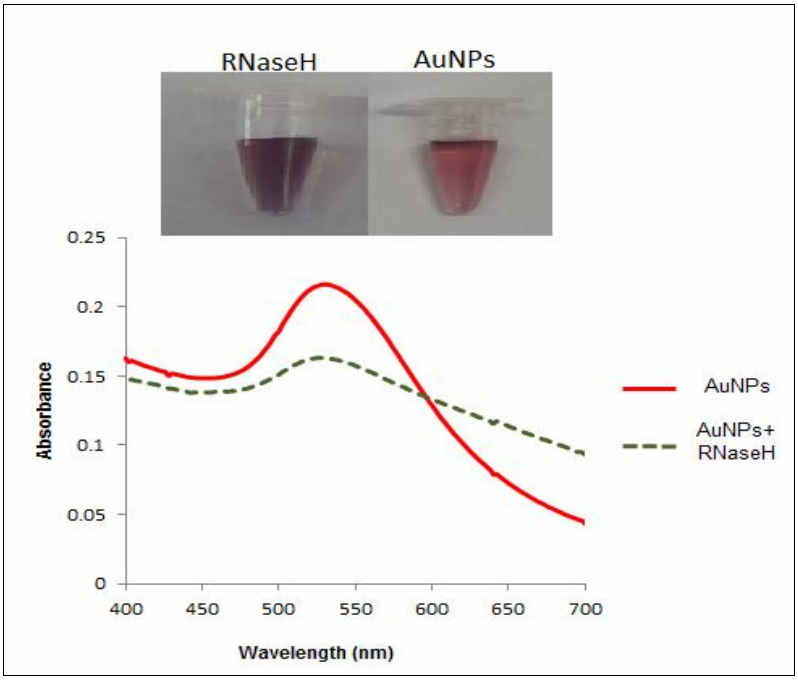

Fig. 5. Slight change in gold nanoparticles color and wavelength in presence of RNaseH enzyme.

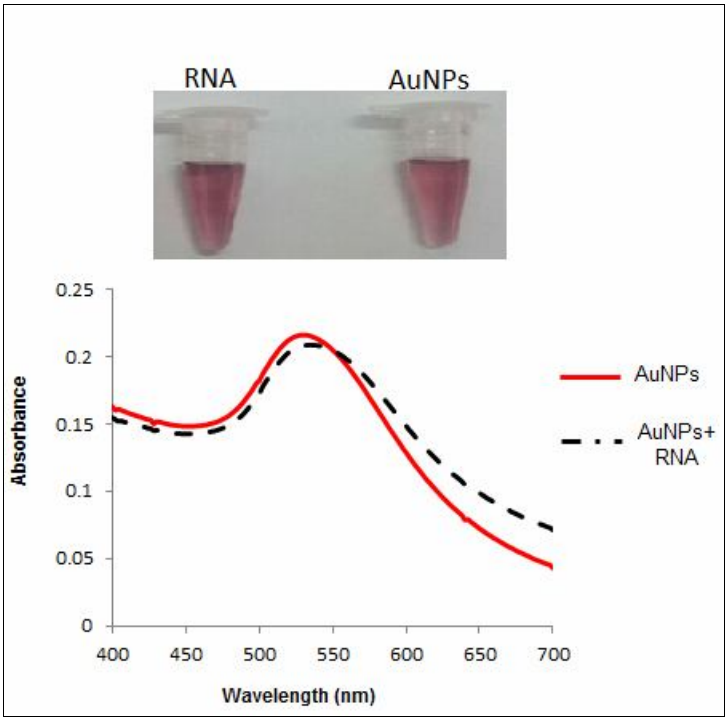

Fig. 6. Change in color and wavelength of gold nanoparticles in presence of RNA as negative control.

Evaluation of gene expression is conducted using conventional methods such as Real Time PCR [32], RT PCR [33], Northern blots [34] and RNase protection assay [35]. Although these methods are still used widely, most of them are time-consuming and costly. In this study, gold nanoparticle-based colorimetric techniques were used to investigate the expression of *Actin* probe. Hybridization of the extracted RNA and probe in presence of phosphate buffer and NaCl solution caused changing from red to blue. In addition, changes of wavelength were measured using a spectrophotometer and the greatest change of wavelength was observed at 550 to 650 nm confirming the interaction of the RNA corresponded to *Actin* gene expression with the probe. So this method can be introduced as a more rapid, economical, and accurate way than other diagnostic procedures used to verify and confirm gene expression. It also evaluated and compared the effects of the enzymes, RNaseA and RNaseH, on RNA-DNA duplex and stability of the gold nanoparticle solution.

Xie*et al*. detected H Ribonuclease enzyme of HIV virus using gold nanoparticle-based colorimetric technique [36]. They studied the effect of RNaseH enzyme on stability of gold nanoparticles and the methods used to analyze RNA and its binding to nanoparticles were colorimetric and PAGE techniques. They also evaluated three factors of time, temperature, and enzyme concentrations. The results showed that at 45 °C and after about 2 h, more than 90 percent of the connected RNA to RNA-DNA duplex was segregated, yet even adding the gold nanoparticles, no color change was observed in the environment. They also stated that the higher the concentration of the RNaseH enzyme caused the less color change in the nanoparticles. It is possible to increase the specificity of the interaction of the nuclease enzymes using DNA-AuNp connections. Using the modified nanoparticles in the presence of oligonucleotide, they showed that in presence of nanoparticles, RNaseH enzyme could break down the RNA molecules of RNA-DNA duplex and play an important role in creating stability of the nanoparticles [37].

In this study, following hybridization of RNA, resulted from the expression of *Actin* gene and the probe, by addition of RNaseA and RNaseH to the DNA-RNA duplex solution,

we observed that RNaseA enzyme was affected RNA to break it down into smaller fragments after 30 min at 42 °C. The fragments produced by RNA degradation could act as probes and cover the surfaces of gold nanoparticles; therefore, gold nanoparticles would be out of NaCl reach and no color change was observed in the reaction tube. Color change of the gold nanoparticles from red to blue in the presence of RNaseH enzyme increased due to cropping the hybrid areas between RNA and DNA at initial stage and over the time. As a result, the parts which gained the ability to bind to the surface of the gold nanoparticles due to RNaseH activity were fewer than those which obtained this binding ability due to RNaseA activities. So that color changes to blue was more observable in the presence of RNaseH compare to the RNaseA. After about 120 min, no color change was observed in the presence of the RNaseH enzyme, hence a greater amounts of RNA were attached to the nanoparticles leading to their stability. In accordance with the results of this study, the colorimetric technique used in this study can be considered as a proper detection technique, which can analyze gene expression within a short time, low cost and high precision. Moreover, using this technique, it would be possible to measure the effective factors in the stability of gold nanoparticles including two nuclease enzymes. Finally it seems that identification of the way that RNaseH enzyme affects the stability of the gold nanoparticles could be considered as a main factor for detection of the viruses containing this enzyme, such as retroviruses, since their proper and initial identification can greatly contribute to treatment and prevention of these infections.

CONCLUSIONS

This study represents a method to detect *Actin* gene expression in plants using gold nanoparticles and colorimetric techniques. In addition the effect of RNaseA and RNaseH on stability of gold nanoparticles was investigated. Colorimetric technique based on gold nanoparticles could be applied as a fast and inexpensive technique with no need to use special equipments for the detection. However in colorimetric technique it is very important to check the stability of gold nanoparticles in the presence of various enzymes to help better understanding of their impact on the stability of nanoparticles.

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