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Nanomolecular detection of human influenza virus type A using reverse transcription loop-mediated isothermal amplification assisted with rod-shaped gold nanoparticles

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Reverse transcription loop-mediated isothermal amplification (RT-LAMP) and rod-shaped gold nanoparticles (gold nanorods; GNRs) were employed for nanomolecular detection of human influenza virus type A RNA. After cDNA synthesis from the RNA, the primers targeting the M protein gene were used for LAMP amplification. A blue shift from red to purple from the GNR inserting into the LAMP–DNAs can be seen by the naked eye. Transmission electron microscopy revealed the formation GNR aggregates due to their interactions with LAMP DNA. One pg RNA (10^{-3} dilution of the viral cDNA) was detected using this colorimetric test. The nanomolecular test showed 100% sensitivity and 95.8% specificity in comparison to results by RT-PCR. Also, the test indicated 100% sensitivity and 100% specificity in comparison to results by RT-LAMP. The described nanomolecular test could detect human influenza virus type A RNA in nearly 1 hour.

1. Introduction

The influenza virus is an RNA virus that causes respiratory illness in humans.[1] Global epidemics of influenza have repeatedly occurred and many people have died as a result.[2] Since this virus is highly contagious, it can spread rapidly. Hence its early detection in the initial stages is very important for control of the disease.[2] Several molecular methods have been developed for diagnosis of human influenza viruses that employ different technologies such as nucleic acid amplification.[3,4] Loop-mediated isothermal amplification (LAMP) has been used for detection of the human influenza virus.[5] LAMP amplifies DNA in a unique manner (Fig. 1) and produces DNA amplicons with ladder shape behaviors with gel electrophoresis.[6,7] LAMP DNAs have been named cauliflower-like DNAs and their characteristics have previously been described.[7,8] In LAMP, large fragments of Bst DNA polymerase with strand-displacement activity are used, and loop and bumper primers are employed for DNA fabrications.[6–8]

LAMP technology has previously been reported for molecular detection of various microorganisms (*e.g.* human influenza virus type A).[8–10] The conventional method for analyzing LAMP DNAs was gel electrophoresis; however, the turbidity derived from magnesium pyrophosphate formation in the LAMP reaction introduced a way for visualization of the positive results.[11] Also, various methods using fluorescent or colorimetric metal indicators (*e.g.* calcein) were reported for visualization of the LAMP byproducts.[12]

Up to now there has only been one report using LAMP DNAs with gold nanoparticle probes;[13] however, rod-shaped gold nanoparticles have not been employed with LAMP for rapid nanomolecular assay. Hence, a nanodiagnostic colorimetric assay for LAMP DNA detection could improve the LAMP tests for nanomolecular diagnosis of microorganisms.

The rod-shaped gold nanoparticles are different from spherical nanoparticles because of their physical characteristics such as elongated shapes, surface charge, and unique optical properties. The Vis-IR spectra of GNRs have two peaks contained transverse and longitudinal plasmon bands.[14,15] The optical properties of GNRs have been utilised in therapy and diagnostics.[16] In this study, nanomolecular detection of human influenza virus type A was to be accomplished *via* LAMP–DNA interactions with GNRs (Fig. 2). This event has been shown to bring about changes in the surface plasmon resonance of GNRs[17] thus it could be employed in the design and development of a novel colorimetric test by rod-shaped gold nanoparticles.

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2. Results and discussion

2.1. Vis-IR spectra

The spectra of the gold nanorods before and after the interaction with the cauliflower-like DNAs are shown in Fig. 3. The spectra shown is between 425 and 1075 nm. Two localized

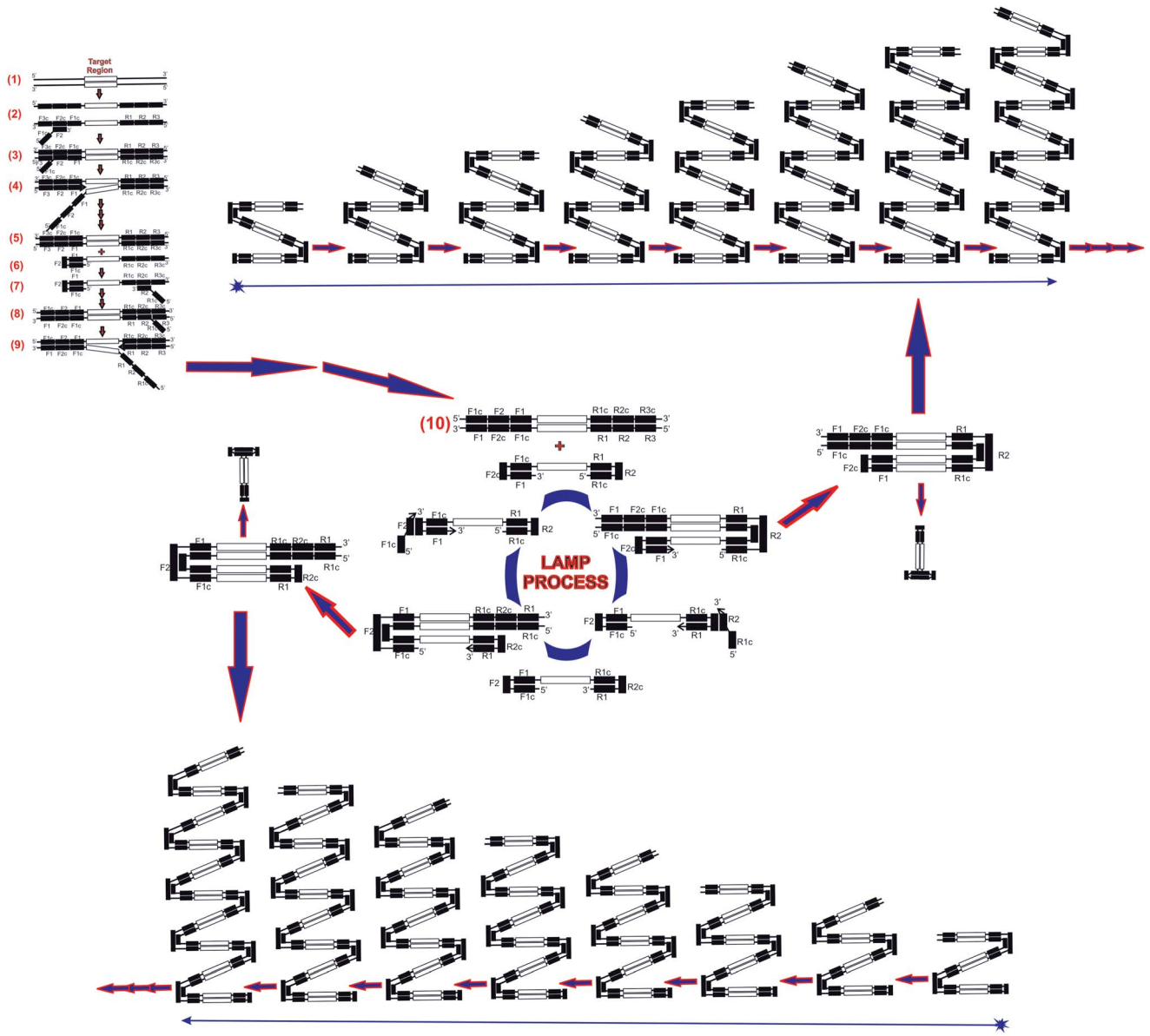

Fig. 1 Scheme of loop-mediated isothermal amplification: suitable lengths of DNA sequences are present in the target region for LAMP. Each of the arbitrary sequences is preferably targeted so that a terminal loop structure forms. Strand displacement-type synthesis of DNA is carried out starting from the annealed F3 primer. The complementary chain separates the strand from the template. A sequence is formed into a single strand by displacement. Synthesis of the DNA strand is then started from the R1 primer. A primer containing sequence R3 is annealed to R3c of the template. Strand displacement and synthesis of DNA is then conducted starting from the annealed R3 primer. The R1 primer is annealed to the portion R2c forming the hairpin loop in two sequences. Synthesis of a nucleotide chain containing a target sequence occurs, the asterisked arrows indicate the fabrication of various sizes of cauliflower-like DNA nanostructures *via* the incubation time.

surface plasmon resonance (LSPR) peaks were seen in the visible-infrared region. The peaks were typical for GNR characterization. The visible and infrared peaks of GNRs were seen at 545 nm and 915 nm before the interaction with cauliflower-like DNAs. The peaks were significantly reduced after the interaction with the LAMP DNAs. Also, the interaction resulted a positional shift of the visible peak at 525 nm; however, the infrared peak was kept at 915 nm.

The Vis-IR spectra indicated that the intensity of the second LSPR-peak was reduced significantly when compared to those results from unmodified GNRs (Fig. 1). The reason was explored *via* transmission electron microscopy of the nanoparticles after their interactions by the LAMP DNAs.

2.2. TEM micrographs

The TEM micrograph of the GNRs is shown in Fig. 4a. The nanoparticles are rod shaped and seem to be 25–30 nm in length and 5–10 nm in width. The aggregates of the gold nanorods after interaction with the LAMP DNAs have been indicated in Fig. 4b.

The interactions between LAMP DNAs and GNRs resulted in aggregations of the nanoparticles to cluster forms (Fig. 4b). It

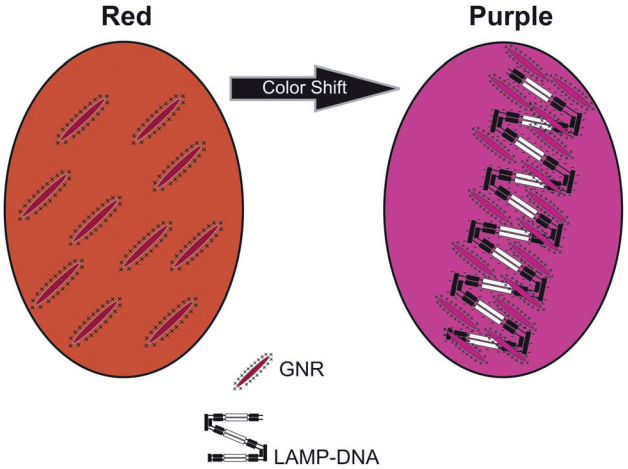

Fig. 2 The gold nanorod assembly and colorimetric assay. Rod-shape gold nanoparticles have positive charges on their surface in diH_2O due to the cetyltrimethylammonium bromide (CTAB) cap. LAMP DNAs have negative charges on their surface due to phosphate groups. The addition of GNRs to LAMP DNAs produces a purple color as they interact with the DNA amplicons. Conversely, for the GNRs before interaction to LAMP DNAs, a red color of separated gold nanorods was observed.

seems the electrostatic interaction between negatively-charged phosphate groups of DNAs and the positively-charged CTA^+ bilayer on GNRs made these nanoparticle complexes.[18]

2.3. Colorimetric detection of RT-LAMP DNAs

As shown in Fig. 5a, the sensitivity of the colorimetric assay was confirmed by loop-mediated amplification of ten-fold serial dilution RNAs obtained from *human influenza virus type A.* The viral RNA dilutions were also detected by RT-LAMP and gel electrophoresis stained with SYBRGold (Fig. 5b). The results show the same sensitivity for detection by LAMP assisted with

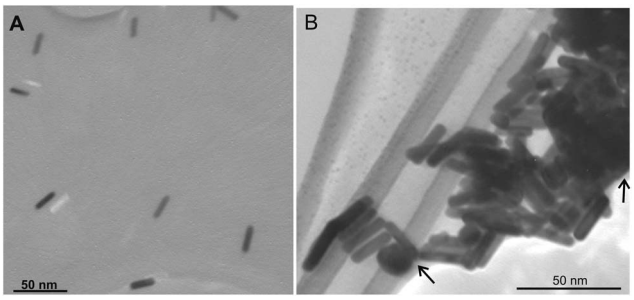

Fig. 4 TEM micrographs of rod-shape gold nanoparticles before (A) and after (B) interaction with LAMP amplicons.

GNRs and the conventional gel electrophoresis. The minimum dilution of RNAs that was detected by the RT-LAMP–GNR assay was 10^{-3}; however, the dilution was also detected by gel electrophoresis and SYBRGold stained agarose gel.

Hence, loop-mediated amplification and the GNRs meant that the viral cDNA could be colorimetrically detected. This was similar to the result from gel electrophoresis of the LAMP product; hence 10^{-3} dilution of RT-LAMP amplified viral RNA could be detected through a color change from red to purple of the GNR solution (Fig. 5a). There was no need for extra modifications of the gold nanoparticles and buffer changes for interaction with the LAMP mixture. Moreover, the visualization method could be comparable with the other colorimetric assays using reagents such as calcein and hydroxynaphthol blue that are employed for detection of pyrophosphates (as the byproducts) derived in LAMP reactions.

2.4. Clinical performance of the colorimetric assay

Clinical performance of the colorimetric assay was determined by comparing the GNR–RT-LAMP results to those of RT-PCR.

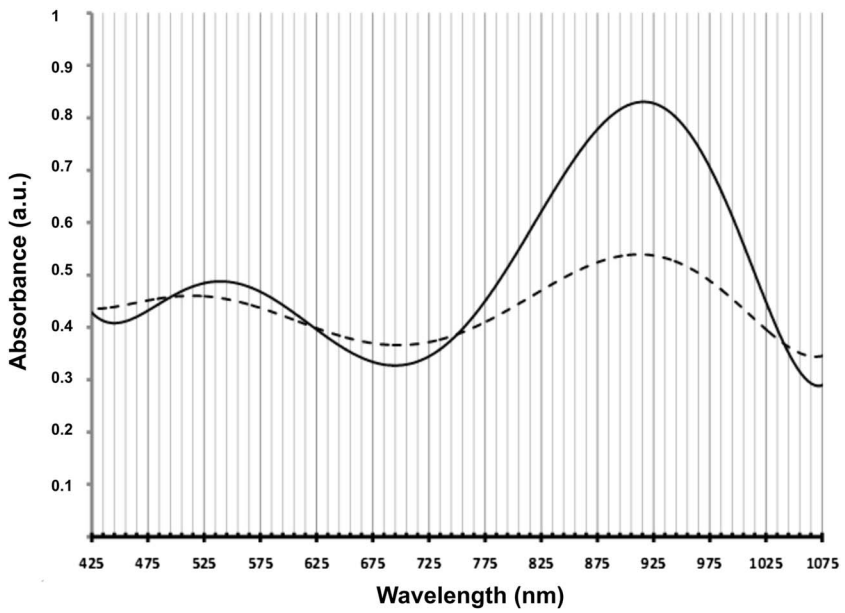

Fig. 3 Vis-IR spectra of the rod-shape gold nanoparticles before (—) and after (- - -) interaction with LAMP DNAs.

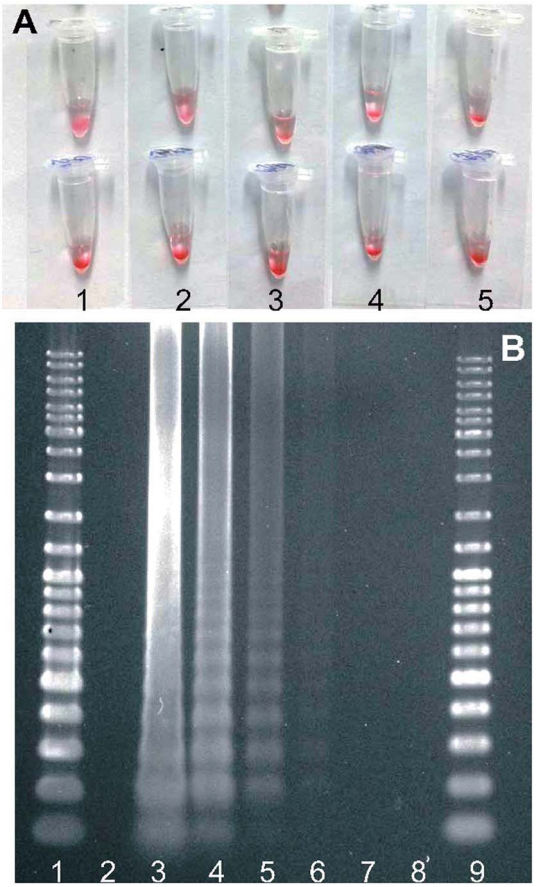

Fig. 5 (A) RT-LAMP–GNR results of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions of 1 ng viral RNA (top) and negative results (down) are determined in purple and red colors, respectively. (B) Gel electrophoresis of RT-LAMP results of 1 ng viral RNA with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions (lanes 3–7); lanes 2 and 8 are empty; lanes 1 and 9 contained GeneRuller 100–10 000 bp from Fermentas.

Included in the study, the 125 specimens from 125 patients received by the laboratory for initial diagnosis by RT-PCR. The overall results presented in Table 1 indicate that the colorimetric assay has a sensitivity of 100% and specificity of 95.8% in comparison to those of RT-PCR. In addition, 100% sensitivity and 100% specificity were obtained by GNR–RT-LAMPs when compared to the conventional RT-LAMPs.

3. Experimental

3.1. Chemicals and instruments

Isothermal conditions for the LAMP reaction were set using a cool-hotter dry bath incubator (Kiagen, Iran). Transmission electron microscopy was done using a Philips EM028 transmission electron microscope. UV-vis spectra were measured using a Carry-100 Varian UV-vis double beam spectrophotometer. The large fragments of Bst DNA polymerase were purchased from New England Biolabs (Beverly, MA, USA). The RNA extraction kit was purchased from Roche Applied Sciences (Germany). The cDNA synthesis kit was obtained from Thermo Scientific. SYBRGold nucleic acid gel stain was purchased from Molecular Probes (USA). GeneRuler DNA ladder mix was from Fermentas Life Sciences (Lithuania). Oligonucleotides (Table 2) were synthesized by Sigma-Aldrich. Gold nanorods were purchased from Sigma-Aldrich.

Table 1 Clinical performance of GNR-LAMP in comparison to RT-PCR

		RT-PCR			
Test and result		Positive	Negative	Sensitivity (%)	Specificity (%)
GNR-LAMP	Positive	29	4	100	95.8
	Negative	0	92		

3.2. Clinical specimens

The 125 samples were collected from 125 nasopharyngeal swabs and analyzed from patients admitted to the referenced influenza laboratory in Golestan University of Medical Sciences, Gorgan, Iran. Initially the RNA was extracted from clinical nasopharyngeal swabs by using a Roche RNA extraction kit. Subsequently, cDNA was synthesized by use of a Thermo Scientific cDNA synthesis kit.

3.3. LAMP process

Reaction mix [12.5 μl; 2× reaction mix; 40 mM Tris–HCl, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betain, and 2.8 mM each deoxyribonucleic acid phosphates, pH 8.8], 2.5 μl primer mix (1.6 μM FIP, 1.6 μM BIP, 0.2 μM F3, 0.2 μM B3), 8 U Bst DNA polymerase, and 7 μl distilled water were dispensed in each microtube up to 25 μl.[7,8] Influenza cDNA was synthesized from 1 ng RNA sample according to the manufacturer's protocol and added to the master mix. For the negative-control reaction, 2.0 μl distilled water was added to the microtubes. The solutions were mixed by pipetting and then spun down. The tubes were set in the block of the cool-hotter dry bath incubator and incubated at 60 °C for 60 min separately. Then, Bst DNA polymerase was inactivated by incubating the mixture for 5 min at 80 °C to terminate the reaction.[7,8]

3.4. Oligonucleotides

Primers were selected from published influenza A virus M gene nucleic acid sequences encoding the matrix protein (Gene bank accession no. JX239989), synthesized by Sigma-Aldrich. These primers amplified a 219 nucleotide fragment of the target cDNA that includes the species-specific region to identify the influenza A virus (Table 2).

3.5. Colorimetric assay *via* gold nanorods

In a typical colorimetric experiment, a 20 μl RT-LAMP–GNR mix contained 5 μl of GNR solution. After the LAMP reaction, the solution immediately was analysed with a UV-vis double beam spectrophotometer.

3.6. Transmission electron microscopy

Transmission electron microscopy (Philips EM028) was used to determine the size and morphology of the gold nanorods,

Table 2 Oligonucleotide sequences

F3	5′ TGGTGC(A/G)CTTGCCAGTTG 3′
B3	5′ CCAGCCATTTGCTCCATAGC 3′
FIP[a]	5′ TGCTGGGAGTCAGCAATCTGTTACAG(G/A)ATGGGGGCTGT(A/G)ACC 3′
BIP[b]	5′ AGGCAAATGGTG(G/A)CAACAACCTGTAGTGCTGGCCA(A/G)AACC 3′

[a] FIP, forward internal primer. [b] BIP, backward internal primer.

before and after interaction with the LAMP products. For this purpose, the samples were immobilized by syringe spraying on agar scientific holey carbon film with 300 mesh $Cu_{(50)}$.

3.7. Analytical validations

True positives (TP) and true negatives (TN) were determined by culture results, LAMP results, and the clinician's diagnosis with false positives (FP) and false negatives (FN) attributed to findings from the capillary-LAMP assay. Sensitivities and specificities were determined as follows: sensitivity = TP/TP + FN and specificity = TN/TN + FP.[19]

3.8. Limit of detection (LOD) of colorimetric GNR–LAMP

For LOD validation, two sets of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) from 1 ng viral RNA were provided in diH_2O.[20] Diluted templates were amplified using LAMP. The first series of LAMP products contained GNRs and colorimetric detection was used. The second ones were detected by gel electrophoresis stained with SYBRGold.

4. Conclusion

To now there have been various reports on using gold nanoparticles and nucleic acid isothermal amplifications for identification of microorganisms.[21–24] Rod-shaped gold nanoparticles, so-called gold nanorods, are another form of gold nanoparticles but their uses with isothermal amplification technologies have not yet been introduced. These nanoparticles are different from the spherical gold nanoparticles particularly due to presence of a CTAB layer leading to positive charges on their surfaces.[25,26] On the other hand, the colorimetric assay *via* nucleic acids and GNRs has been described, previously.[27] The assay makes use of the attraction between opposite charges on nucleic acids and GNRs. In DNA, the negatively charges areas are due to the phosphate groups of nucleotides.[17]

In recent years, various nanotechnological methods have been reported for detection of molecular targets.[28–31] As the most known of nanoparticles, gold nanoparticle probes (GNPs) have been employed for colorimetric detection of several nucleic acid amplifications (NAAs) such as PCR,[32] NASBA (nucleic acid sequence-based amplification),[23] HDA (helicase-dependent amplification),[22] EXPAR (exponential amplification reaction),[33] and LAMP.[13] Unmodified gold nanoparticles have also been used for visualization of single- and double-stranded DNAs *via* the electrostatic interaction with colloidal gold nanoparticles in salt solution.[34] Both the gold nanoparticle-based detections led to color changes associated with gold aggregation.

Various reports have applied LAMP technology for molecular diagnoses of microorganisms.[8] Here, we described the application of GNRs for direct colorimetric detection of LAMP DNAs where no covalent functionalization of the gold nanoparticles, the probe, or the target DNA is required. Because the underlying adsorption mechanism is electrostatic, the colorimetric assay may be used simply for nanomolecular detection of target nucleic acids (*e.g.* M-gene sequence in human influenza virus type A). The phenomenon produces color changes observable without instrumentation and it needs no electrophoresis or turbidimetry experiments for assessment of the LAMP results.

In conclusion, adapting rod-shape gold nanoparticles for the rapid nanomolecular detection of the amplified nucleic acids *via* isothermal amplifications would simplify the diagnosis of the genomic materials of pathogenic microorganisms. Hence, the nanomolecular detection of viruses could alternatively be introduced for direct detection of amplified DNAs using the instrumental-based measurements such as fluorescent-dyed gel electrophoresis,[18] turbidimetry,[11] and enzyme-linked immunosorbent assay[35] of LAMP products.

Author contributions

P. Gill conceived and designed the experiments. H. Nikbakht and A. Niazi performed the experiments. P. Gill and A. Tabarraei analyzed the data. P. Gill contributed reagents/materials/analysis tools. P. Gill and H. Nikbakht wrote the paper.

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