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Direct detection of unamplified hepatitis C virus RNA using unmodified gold nanoparticles

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ABSTRACT

Background: Gold nanoparticles (AuNPs) exhibit a unique phenomenon known as Surface Plasmon Resonance, which is responsible for their intense red color. This color changes to blue upon aggregation of AuNPs.

Objective: This work aims to develop a rapid, simple and cheap assay for direct detection of unamplified HCV RNA extracted from clinical samples using unmodified AuNPs.

Methods: Serum samples were collected from healthy volunteers (n = 45) and chronic HCV patients (n = 30). Extracted RNA, hybridization buffer containing PBS, and a primer targeting the 5′UTR of HCV were mixed. The mixture was denatured, annealed, and then cooled to room temperature for 10 min followed by addition of AuNPs.

Results: Salt, primer, AuNPs concentrations and annealing temperature and time were all optimized. In HCV positive specimens, the color of the solution changed from red to blue within 1 min. The assay has a sensitivity of 92%, a specificity of 88.9%, and a detection limit of 50 copies/reaction.

Conclusions: To our knowledge, this is the first assay that allows the detection of unamplified HCV RNA in clinical specimens using unmodified AuNPs. The developed assay is highly sensitive, has a turnaround time of 30 min, and eliminates the need for thermal cycling and detection instruments.

Introduction

According to the World Health Organization, there are around 200 million people worldwide infected with hepatitis C virus (HCV), with 3 to 4 million newly infected patients annually [1]. HCV is a small enveloped single-stranded RNA virus that belongs to the *Flaviridae* family, *Hepacivirus* genus, which comprises a group of highly variable strains or isolates. HCV is a blood borne virus and infection has different clinical outcomes ranging from acute resolving hepatitis to chronic liver disease, including liver cirrhosis and hepatocellular carcinoma [2–4]. The acute viral infection resolves in 15% of infected patients but progresses in 85% of patients to chronic infection [5].

Currently HCV is detected using immunoassays and confirmed by molecular assays. Immunoassays such as enzyme linked immunoassays (EIAs) and recombinant immune blot assays (RIBA) are used for detection of anti-HCV antibodies [6]. Conventional RT-PCR is used for qualitative detection of HCV RNA while quantitative detection is achieved using real-time RT-PCR and/or branched DNA-based assays [7]. Despite the high sensitivity and specificity of these methods, they are time-consuming, labor intensive, expensive, and require specialized equipment. Therefore, there is a great need to develop a low-tech assay for the direct detection of unamplified HCV RNA with acceptable sensitivity and specificity, short turnaround time, and cost-effectiveness. Such an assay would be critical to control HCV in developing countries with limited resources and high infection rates, such as Egypt.

Nanoparticles have been recently proposed as promising tools to develop the next generation of diagnostic assays. Because of their unique properties and ability to interact with biomolecules on one-to-one basis, various nanoparticles show great promise to meet the rigorous demands of the clinical laboratory for sensitivity and cost-effectiveness, and can be used in the future in point-of-care diagnosis [8]. Gold nanoparticles (AuNPs) are spheres with a typical diameter of approximately 2–50 nm. They exhibit a unique phenomenon known as Surface Plasmon Resonance (SPR), which is responsible for their intense red color. This color changes to blue upon aggregation of AuNPs [9]. The addition of salt shields the surface charge on the AuNPs, which are typically negatively charged owing to adsorbed negatively charged citrate ions on their surfaces, leading to aggregation of AuNPs and red-to-blue color shift [10]. SPR is also responsible for the large absorption and scattering cross-sections of AuNPs which

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, Hepatitis C virus; EIA, Enzyme Linked Immuno-assaysl; RIBA, Recombinant immune blot assays; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction; SNPs, Single Nucleotide Polymorphisms; AuNPs, Gold Nanoparticles; SPR, Surface Plasmon Resonance.

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are 4–5 orders of magnitude larger than those of conventional dyes [11]. These unique optical properties have allowed the use of AuNPs in simple and rapid colorimetric assays for clinical diagnosis offering higher sensitivity and specificity than current detection techniques [12,13].

Li et al. developed a colorimetric assay using unmodified citrate-coated AuNPs [10,13]. This method is based on the fact that single-stranded DNA (ssDNA) adsorbs on citrate-coated AuNPs. This adsorption increases the negative charge on the AuNPs leading to increased repulsion between the particles, thus preventing aggregation. The adsorption of ssDNA on AuNPs occurs due to the fact that ssDNA can uncoil and expose its nitrogenous bases. The attractive electrostatic forces between the bases and the AuNPs allow adsorption of the ssDNA. On the other hand, double-stranded DNA (dsDNA) does not adsorb on AuNPs due to the repulsion between its negatively-charged phosphate backbone and the negatively-charged coating of citrate ions on the surfaces of the AuNPs. Therefore, when AuNPs are added to a saline solution containing the target DNA and its complementary unlabeled primer, AuNPs aggregate (since the primers are not free to stabilize the AuNPs) and the solution color changes to blue. However, in the absence of the target or the presence of a non-complementary target, the primers are free to stabilize the AuNPs thus preventing their aggregation and the solution color remains red. This method has been used to detect single nucleotide polymorphisms in PCR-amplified genomic DNA extracted from clinical samples [10]. Moreover, AuNPs are capable of quenching fluorescent dyes and this property has been used for detection of synthetic HCV sequences with high sensitivity and selectivity [14,15].

In this study, AuNPs-based colorimetric method has been used to directly detect unamplified HCV RNA extracted from clinical specimens (Fig. 1). The colorimetric assay is simple, rapid, and sensitive. In addition, the hybridization of the unlabeled probe to the target takes place in a separate tube before addition of the AuNPs and therefore allows hybridization to take place under the desired optimum conditions without affecting the stability of the gold colloid.

Materials and methods

Synthesis of AuNPs

A colloidal solution of AuNPs with a diameter of 15 nm ± 2 was prepared by citrate reduction of hydrogen tetrachloroaurate (III) ($HAuCl_4 \cdot 3H_2O$) as described elsewhere [16]. Briefly, the reflux system was cleaned by aqua regia and then rinsed with ultrapure water, and blown out with N_2. An aqueous solution of $HAuCl_4 \cdot 3H_2O$ (1 mM, 100 mL) was brought to reflux while stirring, then 10 mL of 1% trisodium citrate (38.8 mM) were added quickly. This resulted in consequent change in solution color from yellow to clear to black to purple to deep red. Afterwards, the solution was refluxed for an additional 15 min and then allowed to cool to room temperature. The colloidal solution was then filtered through 0.45 μm acetate filter, and transferred into a clean storage glass bottle.

Characterization of AuNPs

Size and distribution of the prepared AuNPs were characterized using field emission scanning electron microscopy (Model: Leo Supra 55). One drop of the AuNPs solution was added onto a silicon slide that was allowed to air dry before examination. The λ_{max} for AuNPs was measured using UV spectrophotometer (Jenway 6800). The concentration of the prepared AuNPs was calculated as described previously [17].

Serum samples

Seventy five serum samples were collected from healthy volunteers (n = 45) and chronic HCV patients (n = 30). All samples were negative for hepatitis B surface antigen and hepatitis B antibody. All positive samples had elevated ALT and AST levels. Rapid HCV test was performed on all the samples. Viral load of HCV positive samples was determined by real-time PCR (Artus kit; Qiagen).

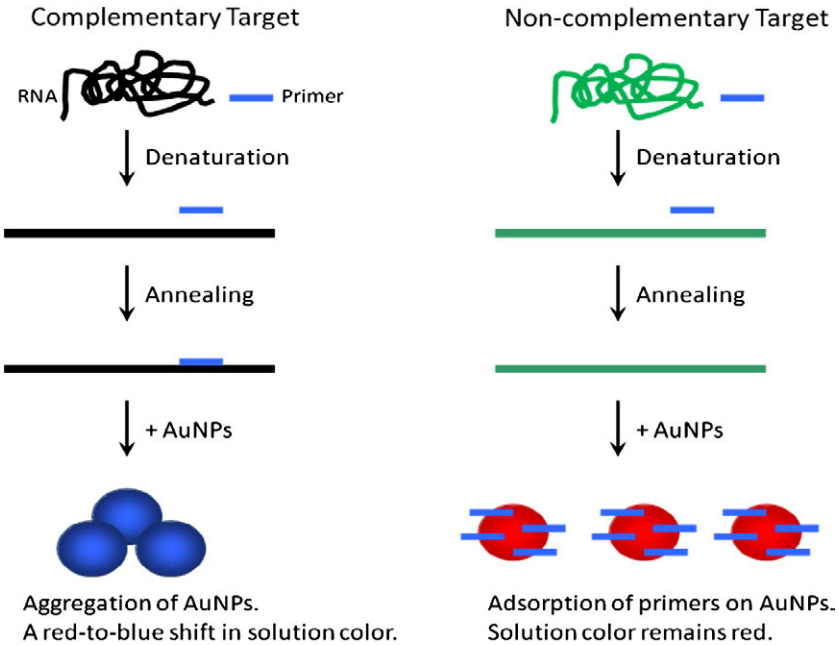

Fig. 1. Schematic diagram of a colorimetric assay based on unmodified AuNPs for detection of full length HCV RNA. First, the tertiary structure of the target RNA is denatured and the complementary primer hybridizes to the target forming double strands. Upon adding AuNPs they will aggregate since there are no free primers to stabilize the AuNPs, and the solution color changes from red to blue. In the presence of a non-complementary target RNA, the primers will be free to adsorb onto and stabilize the AuNPs thus preventing their aggregation and the solution color remains red. Modified with permission from the American Chemical Society (Reference 11).

HCV RNA extraction

Extraction of RNA from serum samples was assessed using three different kits: QIAamp Viral RNA kit (Qiagen; Cat. No. 52904), Absolute RNA Miniprep Kit (Stratagene; Cat. No. 400800) according to standard manufacturer's instructions, and SV total RNA isolation system (Promega; Cat. No. Z3100) according to the modified manufacturer's protocol for HCV RNA isolation [18].

Cell-free DNA may fragment and may interfere with the assay. These fragments can lead to false negative results due to their adsorption onto the AuNPs. Therefore, the samples were treated with DNase to exclude cell-free DNA and/or genomic DNA in the sample. Qiagen viral RNA extraction kit, as stated in the kit's instruction manual, does not guarantee the absence of DNA in the final eluted RNA. The Stratagene total RNA kit and Promega RNA extraction kit both include a DNase treatment step.

RT-PCR and real time RT-PCR

Amplification of RNA was done by using Qiagen one step RT-PCR enzyme mix (Cat. Number 210210) using primers targeting the 5′UTR region, forward primer **5′GTGAGGAACTACTGTCTTCACG3′**, and the reverse primer **5′ACTCGCAGGCACCCTATCAGG3′**. The thermal cycling protocol was 50 °C for 30 min (reverse transcrcriptase reaction), 95 °C for 15 min (Taq activation), and 40 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C; 10 min at 72 °C, and then held at 4 °C yielding a product of 265 bp. Real-time RT-PCR was done by AgPath ID One Step RT-PCR kit (cat # AM1005; Ambion) where 8 μL of the sample were taken and completed to 10 μL with ultra pure water. Amplification was done using a Stratagene Mx3005P.

Colorimetric AuNPs assay for detecting full length HCV RNA in clinical specimens

One primer targeting 5′UTR of HCV RNA was added to 10 mM phosphate buffer saline solution (PBS, pH = 7.0; hybridization buffer). Different concentrations of NaCl in PBS buffer and primer concentrations were tested to determine the optimum concentrations for performing the assay. Hybridization buffer was prepared using 0.53 M NaCl and 6.66 μM (6.6 pmol/μL) primer. Different volumes of the AuNPs were tested, and 10 μL of the prepared AuNPs (10 nM) was selected for use in the final assay. As for the primer used in the assay, two primers were tested, both targeting the HCV RNA 5′UTR. The first primer was 22 nucleotides long and the second one was 27 nucleotides long. The second primer was finally used in this study due to its high specificity to all HCV genotypes and subtypes. In addition, it is not complementary to any human mRNAs as verified by blasting the primer using NCBI database.

The assay was performed as follows, 7 μL of the extracted RNA were placed in a sterile PCR tube and 3 μL of the hybridization buffer were added and mixed well (final concentration of the primer and NaCl after addition of AuNPs was 1 μM and 0.08 M, respectively). The mixture was then denatured at 95 °C for 30 s, and annealed at 59 °C for 30 s and then cooled to room temperature for 10 min. 10 μL of colloidal AuNPs were then added to the mixture, and the color was observed within 1 min. For a permanent record of the results, 1 μL of the final mixture was spotted onto a silica plate.

Detection limit measurements

HCV positive samples with known viral load, as determined by real-time PCR, were used to determine the detection limit of the colorimetric assay. Serial dilutions of the sample (25–2000 HCV RNA copies) were tested using the developed method to determine the detection limit of the assay.

Results

Size distribution and surface plasmon band of the prepared AuNPs

Scanning electron microscope image of AuNPs (Fig. 2) was analyzed using the Image 1.41 J software package (Wayne Rasband, National Institutes of health, USA. Http//:rsb.info.nih.gov/ij/Java1.6.0_05). The AuNPs were well dispersed as shown in Fig. 2 and the mean diameter was found to be 15 nm (Fig. 3). The absorption spectrum of the prepared AuNPs displayed a single peak in the visible region with λ_{max} at 518–520 nm.

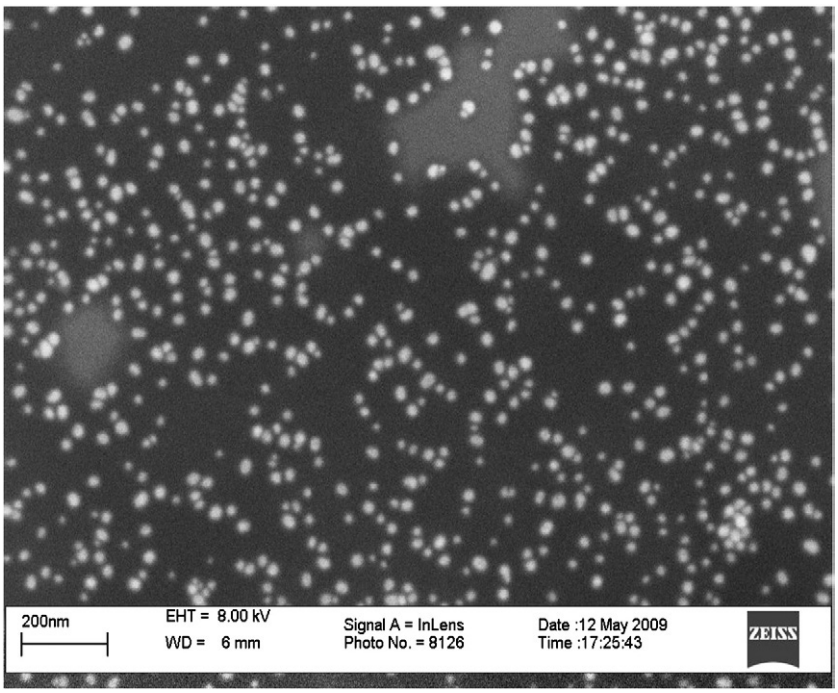

Fig. 2. Scanning electron monographs of the prepared AuNPs. One drop of AuNPs was placed on silicon slide and left to dry then examined using field emission scanning electron microscopy (Model: Leo Supra 55).

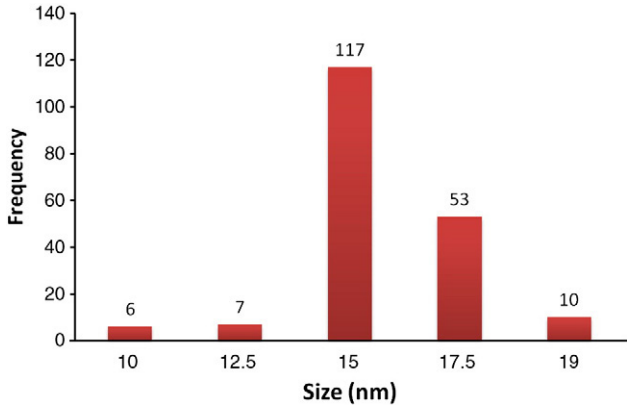

Fig. 3. Analysis of AuNPs size distribution. The scanning electron microscope image in Fig. 2 was analyzed by Image J 1.4 software Wayne Rasband, National Institutes of Health, USA. http://rsb.info.nih.gov/ij/java 1.6.0_05.

Suitability of RNA extraction method

Serum samples may contain some lymphocytes, which contain human mRNA or genomic DNA. Moreover, serum also contains cell-free DNA, and nucleoproteins [19]. Cell free DNA may fragment and adsorb on AuNPs leading to false negative results. Samples were treated with DNase to exclude cell-free DNA and/or genomic DNA. Extracting HCV RNA using Qiagen viral RNA extraction kit has lead to false negative results even in samples containing very high viral titers. On the other hand, reproducible results were obtained using RNA extraction kits that employ DNase treatment (Stratagene total RNA kit and Promega RNA extraction kit).

Colorimetric AuNPs assay: development and optimization

The color of AuNPs colloidal solution is affected by four main factors which should be adequately optimized for best results. These are concentrations of NaCl, AuNPs, and primer used, and the assay temperature.

AuNPs were prepared using the citrate reduction method which produces negatively charged nanoparticles due to citrate coating on their surfaces. This negative charge prevents their aggregation and a red color is obtained. Salt induces aggregation leading to a red-to-blue shift in solution color. The optimum final concentration of NaCl used was 0.08 M, which was sufficient for aggregation of AuNPs and visual detection of the color change, and at the same time, sufficient for proper annealing of the primer to its target.

Although ssDNA primers adsorb on AuNPs and prevent their aggregation, the concentration of the primers should be optimized. This is because, in the absence of the target, a very low primer concentration will not be sufficient to prevent aggregation leading to a false positive result. On the other hand, in the presence of the target, a very high primer concentration will prevent aggregation leading to a false negative result. In this study, at a final salt concentration of 0.08 M, a primer concentration less than 0.2 μM was unable to prevent aggregation of 10 nM 15 nm AuNPs in the absence of the target. On the other hand, a final primer concentration more than 3 μM was too high for any aggregation to occur in the presence of the target. Consequently, the optimal primer concentration was found to be 1 μM in the total assay volume.

The concentration of 15-nm AuNPs used in the assay was 10 nM in a total assay volume of 20 μL. This concentration is sufficient for visual detection of the color change, together with the primer and salt concentrations discussed above. Change in solution color was not clear and false results were obtained when lower concentrations of AuNPs were used.

Performing the assay without heating (necessary for RNA denaturation and annealing of primers) lead to irreproducible results for most of the samples (positive and negative). Consequently, the denaturation and annealing steps were deemed necessary before the addition of the AuNPs to increase the specificity of the assay. It should be noted that the addition of AuNPs directly after removal of the tubes from the thermal cycler (while the tubes are still hot), resulted in false positive results.

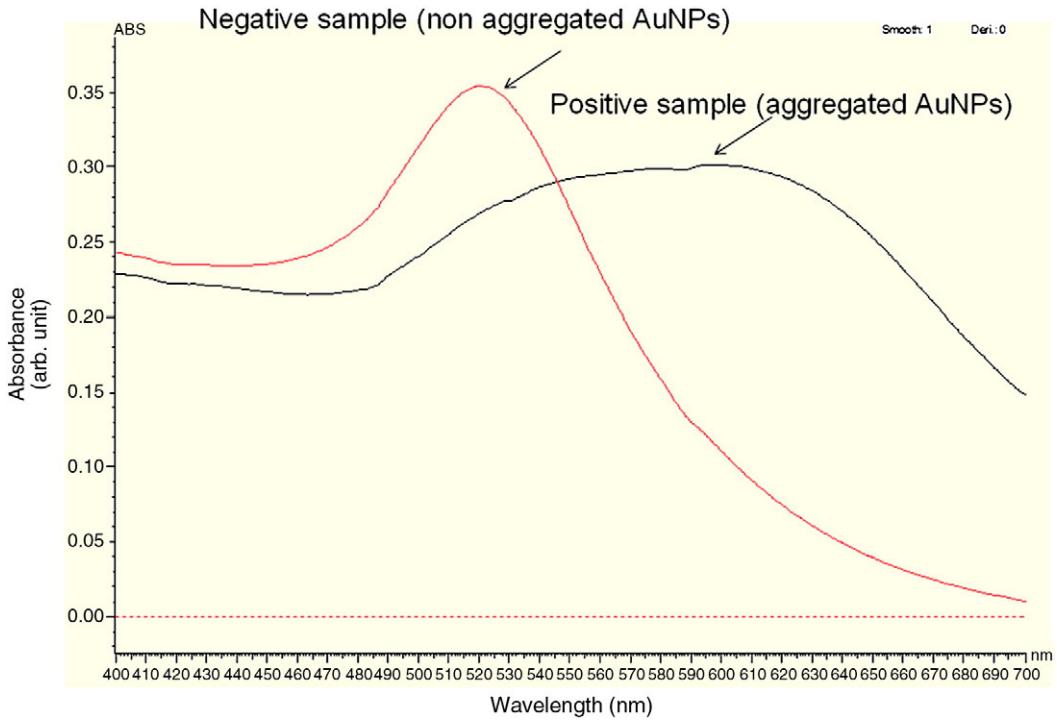

Fig. 4. Extinction spectra of positive and negative samples. The absorption spectra of positive sample (aggregated AuNPs, black) and negative sample (non-aggregated AuNPs, red). Note the red shift and broadening of the peak of the positive sample due to aggregation of AuNPs. For the negative sample, the λ_{max} was around 518–520 nm.

Therefore, the mixture must stand at room temperature for 10 to 15 min prior to addition of AuNPs in order to obtain reproducible results. Increasing the time of the denaturation and annealing steps also increases the percentage of false positive results. In our opinion, increasing the time of denaturation and annealing might increase the probability of the primer annealing non-specifically to some escaped nucleic acids other than HCV RNA that may be found in the nucleoprotein complexes in the serum.

In summary, after adding the hybridization buffer to the extracted RNA, the mixture was subjected to denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and then cooling to room temperature before the addition of the AuNPs. The change in color was visualized within only 1 min. Also, change in the AuNPs absorption spectra was measured (Fig. 4). 28 out of 30 HCV positive samples gave a blue color and 40 out of 45 HCV negative samples gave a red color (Fig. 5). Based on these results, the assay has a sensitivity of 93.3% and a specificity of 88.9%.

Real-time RT-PCR was used to calculate the HCV viral load in clinical specimens that ranged from 43 IU/mL to 12,000 IU/mL. Serial dilutions of the HCV RNA (25–2000 HCV RNA copies) were prepared and assayed using the AuNPs as described above. The assay was capable of detecting 50 HCVcopies/reaction.

The developed assay has been compared to commercial real-time RT-PCR method and in-house conventional RT-PCR method. Fifteen HCV positive samples and five negative samples were tested by realtime RT-PCR and the AuNP-based method. There was a 100% concordance (15/15) between the developed assay and TaqMan real-time PCR (Ambion) results for HCV positive samples. However, 4/5 samples were negative by both assays and one negative sample tested positive by the new assay. On the other hand, two samples that were borderline positive by conventional RT-PCR tested positive by the developed assay and the results were further confirmed by realtime RT-PCR. These results suggest that the new assay has comparable performance to conventional and realtime RT-PCR.

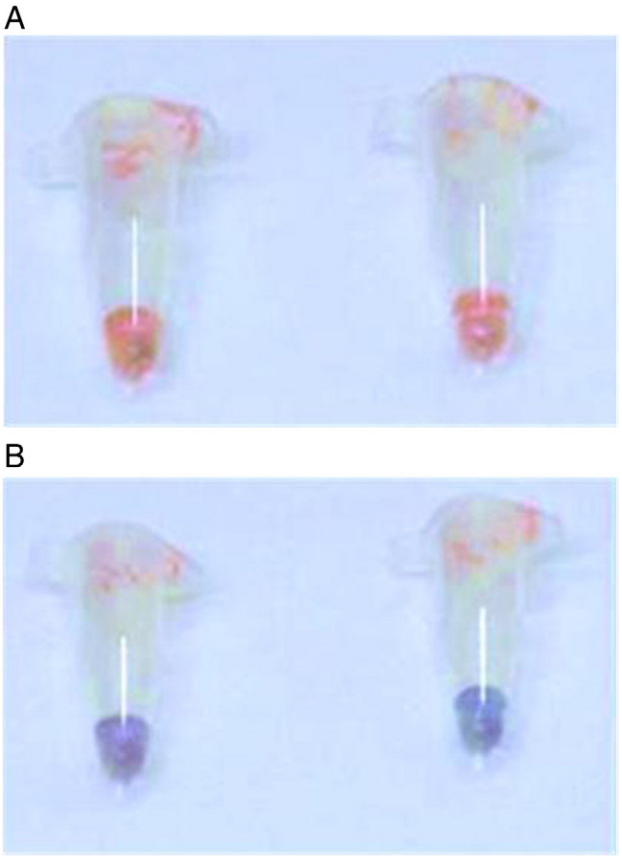

Fig. 5. Colorimetric HCV RNA assay using unmodified AuNPs. Each tube contains 7 μL sample, 1 μM primer and 0.08 M NaCl. The samples were denatured at 95 °C for 30 s and annealed at 59 °C for 30 s and then 10 μL of 15 nm AuNPs was added after cooling the mixture at room temperature for 10 min. The photographs were taken after 1 min from the addition of the AuNPs. (a) HCV RNA negative samples and (b) HCV RNA positive samples. Note the change in color from red to blue in the positive samples.

Conclusions

A colorimetric assay has been developed using unmodified AuNPs for the direct detection of unamplified HCV RNA in biological fluids without the need for RNA amplification. The assay has a detection limit of 50 copies/reaction. The detection limit of the developed assay is better than that of the commercially available HCV core antigen test that detects HCV RNA levels above 20,000 IU/mL (one IU is typically equivalent to 3–5 copies) [20]. The developed assay may be applied for tracking HCV replication even at low viral titers. The developed assay has several advantages including acceptable sensitivity, specificity, short turnaround time, and cost effectiveness. The cost of 1 g of gold chloride is about 200 euros that is enough to prepare 1 L of 15 nm gold nanoparticles where only 10 μL are needed per assay. Moreover, the use of AuNPs eliminates the need for expensive detection instrumentation. Furthermore, there is no need for functionalization of the AuNPs, the primer or the target. HCV RNA extraction is now easy and takes less than 20 min using commercial kits that are based on silica column extraction, magnetic beads, or organic extraction. The cost varies between kits but generally it is between 100 and 200 euros for 50 extractions.

The detection limit of the proposed assay could be improved by increasing the starting serum volume used for RNA extraction. Moreover, it could be further developed into quantitative tests by spectrophotometric quantification of the resulting blue color against a standard curve or developing a fluorometric version of the test by utilization of the size and distance nanoparticle surface energy transfer (NSET) properties of AuNPs. Also, this method may be further developed for detection of SNPs by manipulating the annealing temperature of the primers. This may have great implications for HCV genotyping, subtyping, and monitoring of viral factors that have been correlated to patient's response to interferon therapy [21]. To our knowledge, this is the first study done using unmodified AuNPs for direct detection of unamplified HCV RNA in clinical specimens. Upon further developments and optimization, the assay may soon compete with commercial immunoassays and RT-PCR methods as routine tests for management of HCV patients.

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