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Article Title

Heat-enhancing aggregation of gold nanoparticles combined with loop-mediated isothermal amplification (HAG-LAMP) for *Plasmodium falciparum* detection

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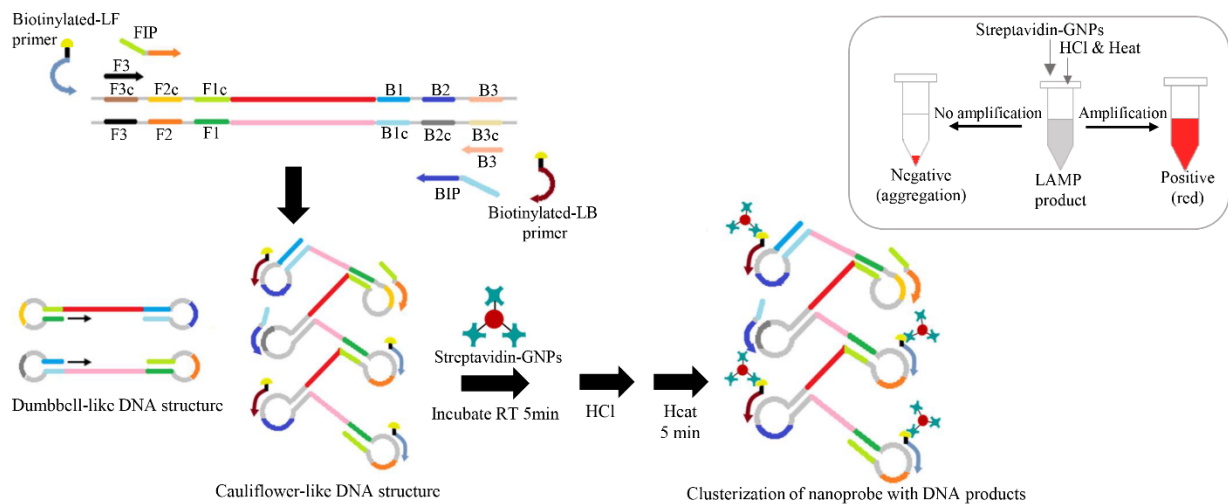
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37 **Graphical abstract**



38 **Highlights**

- 39
- 40 • A simple and sensitive method for detecting the 18S rRNA gene of *Plasmodium falciparum* based
 - 41 on loop-mediated isothermal amplification (LAMP) using biotinylated loop primers and
 - 42 visualization using colorimetric streptavidin-functionalized gold nanoparticles (SA-GNPs) is
 - 43 proposed.
 - 44 • The positive reactions remained wine red, and the negative reactions became colorless with
 - 45 partial aggregations induced by hydrochloric acid (HCl) under heat enhancement (60 °C).
 - 46 • The assay was completed within 50 min, its detection limit was 1 parasite/μL, and it was highly
 - 47 specific for *P. falciparum*.
 - 48

49 **Abstract**

50 Malaria infection represents a major public health and economic issue that leads to morbidity and

51 mortality globally. A highly effective and uncomplicated detection tool is required for malaria control in

52 geographical hotspots of transmission. We developed a simple and more sensitive novel approach for the

53 detection of the 18S rRNA gene of *Plasmodium falciparum* based on loop-mediated isothermal

54 amplification (LAMP) and visualization using colorimetric, streptavidin-functionalized gold nanoparticles

55 (SA-GNPs). Two loop primers of LAMP were biotinylated to produce biotin-containing products during

56 amplification. After the addition of SA-GNPs, clusters of avidin-biotin complexes were established in the

57 LAMP structure. While the positive reactions remained wine red, the negative reactions became colorless

58 with partial aggregations induced by hydrochloric acid (HCl) under heat enhancement (60 °C). All steps of

59 the assay were completed within 50 min, its detection limit was 1 parasite/μL, and it was highly specific

60 for *P. falciparum*. This effortless detection system with high sensitivity and specificity could provide an

61 alternative choice for malaria diagnostics in resource-limited regions.

Keywords

Loop-mediated isothermal amplification, Colorimetric system, Nanosensor, Gold nanoparticles, *Plasmodium falciparum*

1. Introduction

Malaria is a worldwide life-threatening disease that requires prevention and, ideally, elimination. The strategy of the World Health Organization (WHO) is to use practical prevention, rapid diagnosis, and effective treatment to reduce disease and prevent deaths from malaria. In 2019, there were an estimated 405,000 deaths caused by *Plasmodium falciparum* infection globally [1]. In the early stage of malaria infection, the initial symptoms (fever, headache, fatigue, and muscle pain) are consistent with other flu-like diseases and can lead to confusion and misdiagnosis. Therefore, high-quality malaria diagnosis is essential for discriminating among possible etiologies. All suspected malaria cases must be confirmed using either malaria-specific rapid diagnosis tests (RDTs) or microscopy to identify malaria species before treatment. Microscopy is considered the gold standard method for diagnosis because microscopists can easily stain and directly identify stages and species of the parasite. However, the result depends on the quality of reagents and microscope and on the experience of the microscopist [2]. To diminish these problems, RDTs for malaria have been developed to detect parasite antigens and are used throughout malaria-endemic countries. Although RDTs are rapid, inexpensive, noninvasive, and simple to perform and have high sensitivity and specificity for *P. falciparum* detection, they cannot detect low levels of parasitemia (≤ 200 parasites/ μL) or distinguish *P. falciparum* from other *Plasmodium* species [3-5]. Nested polymerase chain reaction (nPCR) was developed in the late 1980s and is now considered another gold standard method for malaria diagnosis because it can detect as few as 5 parasites/ μL of blood and differentiate all human *Plasmodium* species. However, the use of nPCR is not practical in field clinics of malaria-endemic areas because it requires sophisticated and well-maintained laboratory equipment and has prolonged turnaround times. The detection time may require 8-12 hours or more and includes steps of DNA extraction, amplification, and of DNA amplicon detection by electrophoresis [6-10].

A new generation of gene amplification based on isothermal amplification techniques is simpler and more rapid than conventional amplification. Among those techniques, loop-mediated isothermal amplification (LAMP) is outstanding and already has various applications in biomedical diagnostics [11]. Replication starts with DNA synthesis using *Bst* DNA polymerase and six primers under isothermal conditions (in the range of 65 °C). The six primers consisted of the forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3), backward outer primer (B3c), forward loop primer (LF) and backward loop primer (LB), which bound to 6 distinct regions on the DNA target. They amplify by strand displacement activity, producing up to 10^{10} DNA copies within 30-60 min [9, 12, 13]. Interestingly, the LAMP assay could be considered a point-of-care test, as a large number of amplicons can be isothermally amplified within an hour without the use of sophisticated equipment [14-16]. The large amounts of DNA amplicons and pyrophosphate ions lead to a white precipitate of magnesium pyrophosphate observable by the naked eye in the reaction tube and correlates with the amount of synthesized DNA [17]. However, if the levels of DNA amplicons are low, it is difficult to detect turbidity changes in the reaction mixture due to

interobserver differences [18]. Moreover, LAMP amplicons need to be confirmed by agarose gel electrophoresis, which requires a labor-intensive and skill-dependent readout process. Alternately, to avoid electrophoresis, the LAMP products can be traced with functionalized gold nanoparticles (GNPs) and analyzed by colorimetric detection [19-23]. GNPs can be conjugated to allow amplification of readout signals using various detection strategies [24]. GNPs exhibit surface plasmon resonance [22], that is, the fluctuation and interaction of electrons between charges at the particle surface, which gives rise to a sharp and intense absorption band in the visible range. The aggregation of GNPs through interaction between specific sites leads to a change in color from red to blue, which can be clearly observed by the naked eye. The color change can be attributed to the size and clustering of these particles [25-28]. These results can be used to confirm LAMP products in a field setting.

Based on the advantages of GNPs, providing a rapid, convenient and on-site readout technique for LAMP, we aimed to develop a sensing material for LAMP product analysis through aggregation of GNPs (GNP-LAMP). For malaria detection, six LAMP primers amplified the 18S ribosomal RNA (18S rRNA) gene of *P. falciparum* [29-31]. This is a multicopy gene located within strongly conserved regions and without cross-reaction to sequences from other pathogens. With this gene, parasite DNA can be easily amplified so that highly sensitive detection of human malaria parasites can be achieved. Moreover, the GNP-LAMP method has been reported previously with some modifications [32]. The accelerated primers, i.e., LF and LB primers, were conjugated with biotin at the 5' end, and the GNPs were functionalized with streptavidin (SA). In our study, LAMP products of the 18S rRNA gene of *P. falciparum* were added to SA-functionalized GNPs (SA-GNPs) and hydrochloric acid (HCl), and aggregation was induced by heating. The biotin-containing amplicons can bind SA-GNPs and form a cluster based on the formation of avidin-biotin complexes, resulting in no free SA-GNPs in the solution. In the case of no LAMP amplicon, HCl causes free SA-GNP aggregation, leading to the formation of sediment at the bottom of the tube that can be observed by the naked eye because the addition of salt into the GNP solution shields the surface charge of these GNPs, decreases interparticle distances, and leads to particle aggregation [33]. Moreover, the kinetic energy obtained by temperature can cause aggregation due to the bleaching of stabilizers [34]. The method was developed, and its performance was examined in blood samples. The results of LAMP were compared with nPCR, and the assay was evaluated for sensitivity, specificity and detection limit.

2. Experimental

2.1. *P. falciparum* culture

P. falciparum strain TM267 was cultured in complete malaria culture medium (cMCM), which was composed of 10.43 g RPMI 1640 medium with L-glutamine (Gibco, USA), 1.8 g D-(+)-glucose (Sigma, USA), 2 g sodium bicarbonate (Sigma, USA), gentamicin sulfate (50 mg/mL; GPO, Thailand), 1,000 mL sterile distilled water (DW), AlbuMAX solution (final concentration at 5% w/v; Gibco, USA) and HEPES solution (1 M at pH 7.4; Sigma, USA). *P. falciparum* was incubated in cMCM with human erythrocytes (5% hematocrit) in a CO₂ incubator (37 °C, 5% CO₂) [35]. After the culture growth had reached more than 5% parasitemia

(ring forms), synchronization using 5% sorbitol (Sigma, USA) was performed and ring-stage parasites were collected.

2.2. Sample collection and DNA extraction

P. falciparum malaria-infected blood samples (n = 20) were collected from malaria clinics in Mae Sot District, Tak Province, northwestern Thailand. In addition, malaria-negative samples (n = 40) were collected from healthy volunteers who neither lived in malaria endemic areas nor were exposed to malaria. Before blood collection, a consent form was signed by the participant. A batch of specific recording codes was used for the identification of volunteers' samples because their related information was confidential. Ethical review and approval were given by the Central Institutional Review Board (Reference no. MU-CIRB 2009/149.2605) of Mahidol University.

Genomic DNA was extracted from blood samples using the QIAamp® DNA Mini and Blood Kit (Qiagen, Germany) following the manufacturer's protocol. Using this kit, genomic DNA was extracted and purified from whole blood (200 µL) by silica membrane. The extracted DNA was stored at 4 °C for frequent usage or -20 °C for long-term storage. The DNA concentration was measured by a UV-Vis spectrophotometer at 260 nm (NanoDrop 2000c; Thermo Scientific, USA).

2.3. Nested PCR

In this study, nested PCR (nPCR) was the standard method for amplification of the 18S rRNA gene of *P. falciparum* and was performed as described previously with slight adaptations [29]. In the first round of nPCR, the reaction mixture (25 µL) consisted of 0.5 µM of each universal primer (P1 forward primer [5'-ACGATCAGATACCGTCGTAATCTT-3'] and P2 reverse primer [5'-GAACCCAAAGACTTTGATTCTCAT-3']) (Bionics Co. Ltd., Seoul, South Korea), 200 µM of each deoxyribonucleic acid (dNTP) (Macrogen, Korea), 1.5 mM of MgCl₂ (Promega, USA), 5x Colorless GoTaq® Flexi Buffer (Promega, USA), 1 unit (U) of GoTaq® DNA Polymerase (Promega, USA), and 2 µL of extracted DNA.

Template DNA was amplified at 94 °C for 10 min, followed by 35 cycles at 92 °C for 30 s, 60 °C for 1.5 min, and 72 °C for 1 min, and finished by a final extension at 72 °C for 5 min. Amplicons from the first round were diluted 20-fold with DW. The reaction mixture of the second round was prepared for malaria species-specific amplification as in the first round preparation, with the addition of 2 µL of the diluted amplicons and using the P₁ forward primer combined with a species-specific reverse primer (*P. falciparum*, 5'-CAATCTAAAAGTCACCTCGAAAGATG-3') Bionics Co. Ltd., Seoul, South Korea). The second round of nPCR was incubated at 94 °C for 10 min, followed by 20 cycles at 92 °C for 30 s, 60 °C for 1.5 min, and 72 °C for 1 min, and finished by a final extension at 72 °C for 5 min. The final amplicons were visualized by electrophoresis using a 1.5% agarose gel (Serva, USA). The amplicon sizes from the first and second rounds of nPCR were expected to be 160 and 110 bp, respectively. Genomic DNA from healthy donors and DW were used as negative controls.

2.4. Preparation of gold nanoparticles

In this study, gold nanoparticles (GNPs) were prepared in-house by citrate reduction of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ as previously described [36] with minor modifications. Briefly, a solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (20 mL, 0.5 mM) (Sigma, USA) was vigorously stirred until boiling. Then, an aqueous solution with sodium citrate dihydrate (1 mL, 38.8 mM) (Sigma, USA) was quickly added and vigorously stirred until the color of the solution changed from pale yellow to wine red. The GNP solution was then cooled on ice and stabilized in a dark container at 4 °C. These GNPs were then characterized by transmission electron microscopy (TEM; JEM-2100 electron microscope, Japan), ultraviolet–visible (UV-Vis) spectroscopy (BioTek; Synergy HTX, USA), and dynamic light scattering (DLS; Zetasizer Nano-ZS, UK).

2.5. Functionalization of GNPs

Prior to the functionalization of GNPs, commercial lyophilized streptavidin from *Streptomyces avidin* (SA; Sigma, USA) was dissolved with sterile DW. Then, GNPs (optical density = 2.0 at 519 nm) in 2-(N-morpholino) ethanesulfonic acid hydrate buffer (MES buffer, 25 mM, pH 6.0; Sigma, USA) were functionalized with a solution of SA according to a previously reported protocol [37]. Various concentrations of SA (from 5 to 20 µg/mL) were added to the GNP solution at a ratio of 1:1 and shaken for 1 h. After that, the mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant was collected for measurement of unbound SA. Simultaneously, the pellets of SA-GNPs were resuspended in 0.25% bovine serum albumin (BSA; Merck, USA) in DW and shaken for 30 min. The mixture was washed thrice by centrifugation at 13,000 rpm for 15 min. Finally, the SA-GNPs were resuspended in DW and stored in a dark container at 4 °C for further use. The SA-GNPs were also characterized by UV-vis spectroscopy and DLS.

To evaluate whether the GNPs were functionalized with SA, the unbound SA in the first supernatant was measured by the Bio-Rad protein assay for dye reagent concentration (Bio-Rad, USA). Triplicates of BSA standards were prepared at 0, 1.25, 2.5, 5, 10 and 20 µg/mL. Bradford reagent (160 µL) was thoroughly mixed with each concentration of BSA (40 µL) in a 96-well microtiter plate. The plate was covered and incubated at 25 °C for 5 min, followed by OD measurement at 595 nm. The result was calculated and set as a standard curve for the calculation of leftover SA in the supernatants. The binding efficiency of the SA-GNPs was calculated by using **equation 1** as follows:

$$\text{Binding efficiency (\%)} = [(P_i - P_u)/P_i] \times 100 \quad (1)$$

where P_i is the initial concentration of SA and P_u is the concentration of unbound SA in the supernatant.

2.6. LAMP and condition optimization

The LAMP assay was performed in 25 µL of reaction mixture as previously described [38], with minor modifications. First, LAMP primers were designed for *P. falciparum* detection based on the 18S rRNA gene sequence as previously reported [30]. The nucleotide sequence of each primer is shown in Table 1. Each reaction mixture consisted of 1x isothermal amplification buffer (New England Biolabs, USA), 6 mM MgSO_4 , 0.8 M betaine (Sigma-Aldrich, USA), 1.4 mM dNTPs, 1.6 µM of each inner primer (FIP and BIP; Bionics Co. Ltd., Seoul, South Korea), 0.4 µM of each outer primer (F3 and B3), 0.8 µM of each loop primer (biotinylated LF and LB), 8 U of *Bst* 2.0 WarmStart® DNA polymerase (New England Biolabs, USA), and 2

μL of template DNA. DW was used as a negative control in all LAMP reactions. These reactions were performed on a thermal cycler (MJ Research PTC-200 Thermal Cycler; Bio-Rad, USA) at 60 °C for 40 min, followed by heating at 80 °C for 5 min to inactivate the reaction. To optimize the temperature of the LAMP, reactions were simultaneously performed at different temperatures (60 °C, 63 °C, and 65 °C) for 60 min and then heated at 80 °C for 5 min. To determine the optimal time for the LAMP, reaction mixtures were amplified for 35, 40 or 45 min at 60 °C and then heated at 80 °C for 5 min. The LAMP amplicons were confirmed by electrophoresis using a 1.5% agarose gel. Positive LAMP products were purified and analyzed by DNA sequencing (Bionics Co. Ltd., Seoul, South Korea). The sequencing chromatograms were validated using BioEdit, Version 7.0.1 and multiple aligned with the reference *P. falciparum* sequence from GenBank.

2.7. LAMP detection using SA-GNPs (GNPs-LAMP)

To optimize the detection of LAMP products, the reaction was carried out with a variation of the hybridization step. In brief, LAMP products (5 μL) were directly mixed with SA-GNPs (5 μL). The mixture was incubated at room temperature for 5 min, followed by induced aggregation of GNPs using 2 μL of HCl solutions of different concentrations (10⁻¹-1,000 mM), and spun down at room temperature for 10 sec. The aggregation of GNPs and the color changes were evaluated by the naked eye. The optimized condition was subsequently used as our standard condition.

The limit of detection of GNP-LAMPs was investigated using genomic DNA extracted from *in vitro* *P. falciparum* culture at 2% parasitemia, equivalent to 10⁵ parasites/μL [39]. The DNA was diluted tenfold from 10⁵ to 10⁻² parasites/μL and analyzed by GNP-LAMP with different induction techniques. These were compared to conventional LAMP with electrophoresis using a 1.5% agarose gel as a control technique and SYBR® Green I (Invitrogen, USA) as a common color indicator.

The specificity of GNP-LAMP was assessed by analysis of the following human malaria species and other blood protozoa: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*, *Leishmania siamensis*, *L. martiniquensis*, *L. donovani*, and *Trypanosoma evansi*. All parasite genomic DNA was extracted, amplified and reacted with SA-GNPs.

3. Results and discussion

3.1. Preparation of SA-GNPs

TEM images of SA-immobilized GNPs showed uniformity in size and shape (Figure 1a). The average size of GNPs (n = 120) was in the range of 15-20 nm with a polydispersity index equal to 1 (Figure 1b). The maximum absorbance peak of GNPs was 519 nm, while that of SA-GNPs shifted to 532 nm (Figure 1c). The binding efficiency of the immobilization was approximately 64.2%

3.2. Optimization of LAMP conditions for *P. falciparum* detection

To optimize the LAMP conditions for rapid detection of the 18S rRNA gene of *P. falciparum*, different incubation temperatures and times were observed. The amplification of LAMP products exhibiting a mixture of stem-loop DNAs with various stem lengths was assessed using gel electrophoresis, and the highest yield of LAMP amplicons was observed at 60 °C (Figure 2a). Then, a 10-fold serial dilution of genomic DNA of *P. falciparum* was used at 60 °C to determine which of three incubation times (35, 40, and 45 min) amplified the largest yield of LAMP products. The results showed that the lowest incubation time, with sensitivity down to 3×10^{-1} parasite/ μ L, was 40 min (Figure 2b). Thus, the GNP-LAMP assay was performed at 60 °C for 40 min in this study. Nevertheless, this method is still limited by the requirement for a post amplification step that may cause aerosol contamination from tube opening.

3.3. GNP-LAMP assay

The SA-GNP solution (O.D. = 2.5) and the LAMP product were mixed at a ratio of 1:1 to obtain the best hybridization and aggregation results. The concentrations of HCl solution (2 μ L) were in the range of 10–1,000 mM. After spinning down, the 100 mM HCl solution clearly distinguished between positive (wine red colloidal solution) and negative results (aggregation), while the difference with other concentrations was less distinct (Figure 3). The higher HCl concentrations (500 and 1,000 mM) tended to provide false negative results because the SA-GNPs were aggregated in both positive and negative controls. In contrast, the lower HCl concentrations (10 and 50 mM) tended to produce false positives because aggregation did not occur in the negative controls.

To investigate the detection limits of the GNP-LAMP assay, visualization of the LAMP products using SA-GNPs was compared to that using SYBR Green I and 1.5% gel electrophoresis. While the detection limit of gel electrophoresis was found to be 10^{-1} parasite/ μ L (Figure 4a), those of the LAMP assay with SYBR Green I (Figure 4b) and the GNP-LAMP assay using HCl solution and spinning down for 10 sec (Figure 4c) were both found to be 10^3 parasites/ μ L. However, the detection of the LAMP product SYBR green I was enhanced by fluorescent excitation and resulted in a reduction in the detection limit to 10^{-1} parasite/ μ L (data not shown). Moreover, the mixture of GNP-LAMP amplicons and the HCl solution was also tested for aggregation-inducing time and temperature. The detection limit was found at 1 parasite/ μ L by heating the mixture at 60 °C for 5 min, although the GNPs were slightly aggregated at both 10^1 and 1 parasite(s)/ μ L (Figure 4d). The detection limit of the SA-GNPs was enhanced by incubating them with the LAMP product at 60 °C for 5 min, instead of spinning down, to influence the kinetic energy of the GNPs and stimulate their aggregation [34]. Unaided eye observation of the SA-GNPs with a heating step was one log higher than that using SYBR Green I. Furthermore, after incubating the mixture at room temperature for 40 min, the detection limit was found to be 10^1 parasites/ μ L, without any false-negative aggregations (Figure 4e).

To investigate the specificity of GNP-LAMP with other blood protozoa, SA-GNPs were tested for cross hybridization with the extracted DNA from samples of *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*, *L. siamensis*, *L. martiniquensis*, *L. donovani*, and *T. evansi*. No cross hybridization occurred with DNA from other blood-borne protozoa. The SA-GNPs only provided positive detection (resulting in a red solution) with the LAMP products of the *P. falciparum* sample (Figure 5). Using the same primer set, our validation results covered more species than those of a previous study [30]. The color of the SA-GNPs

remained stable over 24 h after the addition of HCl solution in both positive (red) and negative (colorless with particle aggregations) results.

3.4. Sensitivity and specificity of GNP-LAMP assay

Of 60 human blood samples, 20 were identified as positive for *P. falciparum* by microscopic examination, while 40 were negative for malaria parasites. Genomic DNA was extracted from all samples and tested in parallel with GNP-LAMP and nPCR and compared to the microscopy results (Table 2). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of GNP-LAMP were 100% (95% CI, 83.2 to 100.0), 95% (95% CI, 83.1 to 99.4), 90.9% (95% CI, 70.8 to 98.9) and 100% (95% CI, 90.7 to 100.0), respectively. In addition, the nPCR results had 100% sensitivity (95% CI, 83.2 to 100.0), 100% specificity (95% CI, 91.2 to 100.0), 100% PPV (95% CI, 83.2 to 100.0), and 100% PPV (95% CI, 91.2 to 100.0) in comparison with those of microscopy. Thus, GNP-LAMP demonstrated significant results in that this colorimetric assay was in almost perfect agreement with microscopy (P-value<0.001, Kappa = 0.927). Despite the lower specificity of GNP-LAMP (95%), the LAMP amplicons of false positive results (n = 2) were further sequenced and identical to the 18S rRNA gene of *P. falciparum*.

Remarkably, there were 2 cases in which the GNP-LAMP results were positive for *P. falciparum*, while those of the reference (gold standard) methods were negative. Further analysis showed positive results with genetic sequencing, suggesting that these 2 cases were true positive cases, consistent with a lower detection limit of GNP-LAMP than those of nPCR (5 parasites/μL) and microscopy (50-100 parasites/μL) [18]. Another explanation for the results in these 2 cases is that aerosol contamination (carry over) had occurred and was detected by the extremely high sensitivity of the LAMP assay [40, 41]. Although the workstations for DNA extraction, reagent preparation and postamplification analysis were physically separated to minimize tube-to-tube cross contamination, contamination might occur in the postamplification steps due to repeatedly opening the tube to detect the LAMP products. To reduce the risk of contamination from postamplification steps, a closed-tube LAMP system for the visualization step should be employed by using a wax-dye capsule as a container [42].

The process time of GNP-LAMP to detect *P. falciparum* was rapid at approximately 50 min (40 min for LAMP, 5 min for hybridization, and 5 min for heat-inducing aggregation), which was faster than agarose gel electrophoresis LAMP at 90-120 min and nPCR at 3-5 h. When compared with traditional nPCR and microscopic examination, GNP-LAMP can be an alternative method for screening and confirming malaria infection, as it is relatively more rapid, has comparable sensitivity, and is available for large-scale detection in a simple laboratory setting. It was interesting that rapid GNP-LAMP can be developed as point-of-care testing by combination with lateral-flow readout analysis [14-16]. However, the method was restricted by the complexity of preanalytical sample preparation, especially in DNA extraction. To provide a fully practical system, simple and rapid DNA extraction should be combined into a microfluidic device [43, 44]. Further studies will be focused on the possibility of DNA extraction, DNA amplification, and visualization of amplified products by the combination of GNP-LAMP and microfluidics on chip.

4. Conclusion

In summary, we developed a novel GNP-LAMP method that amplified the 18S rRNA gene of *P. falciparum* by LAMP and visualized the products through HCl-induced and heat-enhancing aggregation of GNPs. The results were generated within 50 min, including amplification of the target DNA within 40 min, reaction between streptavidin and biotin for 5 min, and heat-inducing postamplification analysis with identification by an unaided eye within 5 min. Colorimetric detection using SA-GNPs resulted in high sensitivity (detection limit of 1 parasite/ μ L) and specificity (detection was of *P. falciparum* only) and demonstrated the efficiency of the primers and visualization system. Accordingly, GNP-LAMP is an excellent candidate for use as a diagnostic method in the screening and monitoring of malaria infections, especially where very low parasitemia is suspected.

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Conflict of Interest

The authors declare no competing interests.

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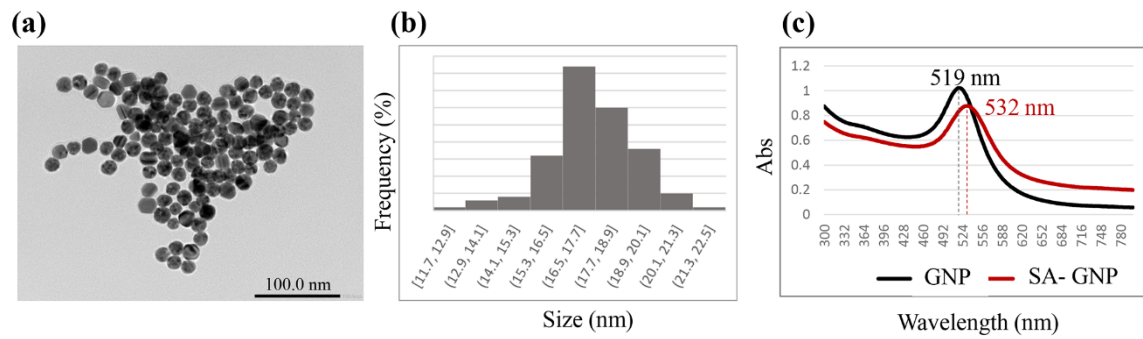


Figure 1. Characterization of GNPs: (a) TEM imaging at the 100 nm scale, (b) size distribution analysis, and (c) absorbance spectra analysis of GNPs and SA-GNPs.

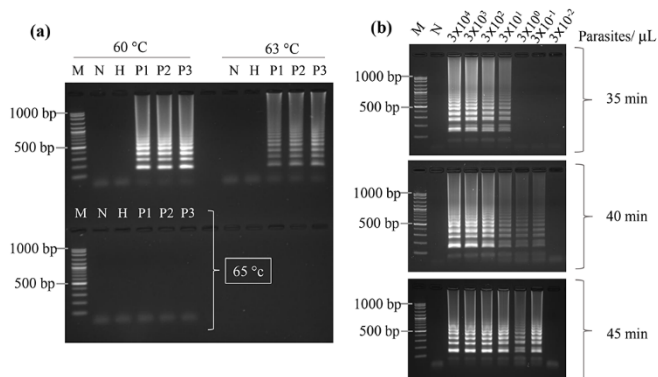


Figure 2. (a) LAMP results with temperatures of at 60 °C, 63 °C, and 65 °C. M: DNA marker; N: negative control; H: human genomic; P1: *P. falciparum* strain 3D7; P2: *P. falciparum* strain TM267; P3: *P. falciparum* strain TM267 duplicate. (b) LAMP results with amplification times of 35, 40, and 45 min.

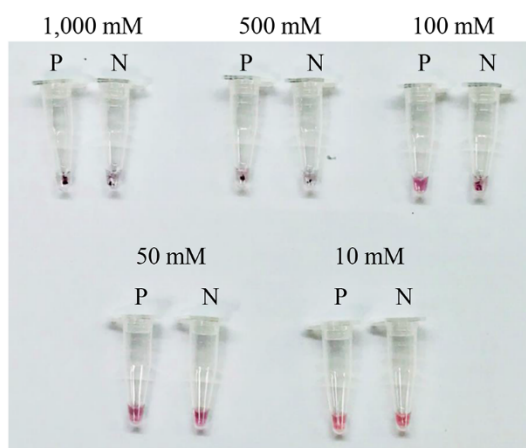


Figure 3. Optimization of HCl concentration (10 to 1,000 mM at a fixed volume) from assessments in paired reactions containing either the LAMP amplicons of *P. falciparum* (P) or the negative control (N).

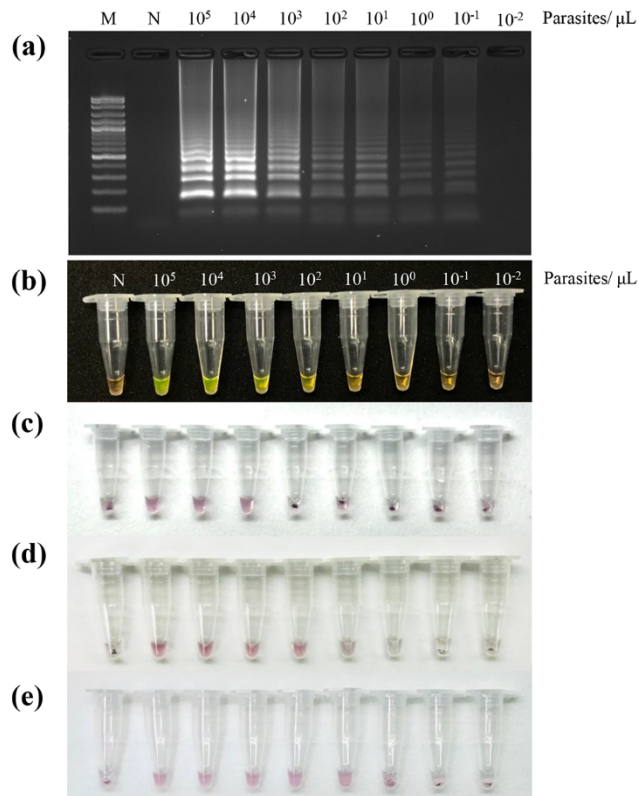


Figure 4. Sensitivity of the GNP-LAMP assay for the detection of *P. falciparum* using 10-fold serial dilutions of DNA from cultured *P. falciparum*. (a) Analysis of the LAMP products using gel electrophoresis. Lane M: DNA marker; N: negative control. (b) Colorimetric results of SYBR Green I added to the LAMP products. N: negative control (DNA free). (c) Mixtures of GNP-LAMP amplicons and HCl solution after spinning down for 10 sec. (d) Mixtures of GNP-LAMP amplicons and HCl solution after heating at 60 °C for 5 min. (e) The mixtures of GNP-LAMP amplicons and HCl solution after incubating at room temperature for 40 min.

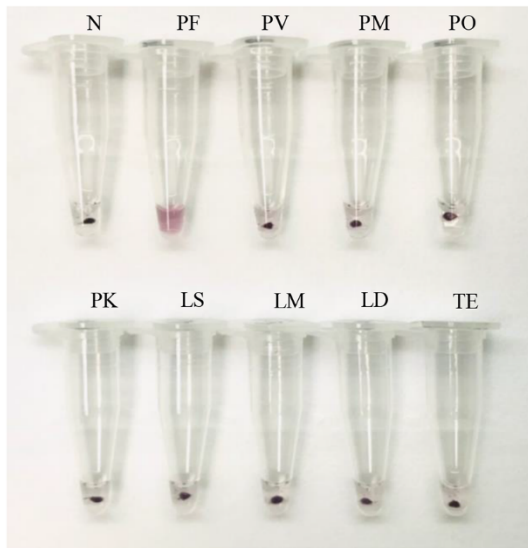


Figure 5. Specificity of the GNP-LAMP assay evaluated using samples from human malaria species [*P. falciparum* (PF), *P. vivax* (PV), *P. malariae* (PM), *P. ovale* (PO), and *P. knowlesi* (PK)], other blood protozoa [*L. siamensis* (LS), *L. martiniquensis* (LM), *L. donovani* (LD), and *T. evansi* (TE)], and DW as a negative control (N).

Table 1. Primer set for LAMP amplification

Primer name	Sequence (5'→3')	Length [15]
F3	TGTAATTGGAATGATAGGAATTTA	24
B3c	GAAAACCTTATTTTGAACAAAGC	23
FIP (F1c-F2)	AGCTGGAATTACCGCGGCTGGGTTCTAGAGAAACAATTGG	41
BIP (B1-B2c)	TGTTGCAGTTAAACGTTTCGTAGCCCAAACCAGTTTAAATGAAAC	45
LPF	Biotin- GCACCAGACTTGCCCT	16
LPB	Biotin- TTGAATATTAAAGAA	15

Table 2. Sensitivity and specificity of the GNP-LAMP assay to detect 18 s rRNA gene of *P. falciparum* among patients with *P. falciparum* infection.

Methods	No. (n) of samples with Microscopy		(%) Sensitivity (95% CI)	(%) Specificity (95% CI)	(%) PPV (95% CI)	(%) NPV (95% CI)
	Positive	Negative				
<u>nPCR</u>						
Positive	20	0	100 (83.2-100.0)	100 (91.2-100.0)	100 (83.2-100.0)	100 (91.2-100.0)
Negative	0	40				
<u>GNP-LAMP</u>						
Positive	20	2	100 (83.2-100.0)	95 (83.1-99.4)	90.9 (70.8-98.9)	100 (90.7-100.0)
Negative	0	38				