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

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1 **Article Title**

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

4
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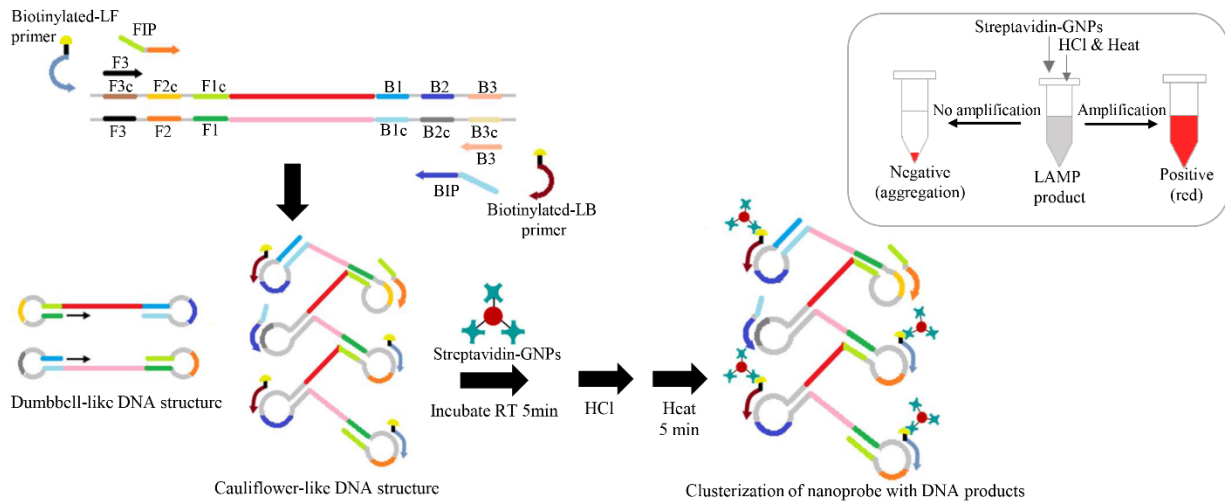
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37 Graphical abstract



38 Highlights

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

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/ μ L, 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.

63 **Keywords**

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

66

67 **1. Introduction**

68 Malaria is a worldwide life-threatening disease that requires prevention and, ideally, elimination. The
69 strategy of the World Health Organization (WHO) is to use practical prevention, rapid diagnosis, and
70 effective treatment to reduce disease and prevent deaths from malaria. In 2019, there were an estimated
71 405,000 deaths caused by *Plasmodium falciparum* infection globally [1]. In the early stage of malaria
72 infection, the initial symptoms (fever, headache, fatigue, and muscle pain) are consistent with other flu-
73 like diseases and can lead to confusion and misdiagnosis. Therefore, high-quality malaria diagnosis is
74 essential for discriminating among possible etiologies. All suspected malaria cases must be confirmed
75 using either malaria-specific rapid diagnosis tests (RDTs) or microscopy to identify malaria species before
76 treatment. Microscopy is considered the gold standard method for diagnosis because microscopists can
77 easily stain and directly identify stages and species of the parasite. However, the result depends on the
78 quality of reagents and microscope and on the experience of the microscopist [2]. To diminish these
79 problems, RDTs for malaria have been developed to detect parasite antigens and are used throughout
80 malaria-endemic countries. Although RDTs are rapid, inexpensive, noninvasive, and simple to perform and
81 have high sensitivity and specificity for *P. falciparum* detection, they cannot detect low levels of
82 parasitemia (≤ 200 parasites/ μL) or distinguish *P. falciparum* from other *Plasmodium* species [3-5]. Nested
83 polymerase chain reaction (nPCR) was developed in the late 1980s and is now considered another gold
84 standard method for malaria diagnosis because it can detect as few as 5 parasites/ μL of blood and
85 differentiate all human *Plasmodium* species. However, the use of nPCR is not practical in field clinics of
86 malaria-endemic areas because it requires sophisticated and well-maintained laboratory equipment and
87 has prolonged turnaround times. The detection time may require 8-12 hours or more and includes steps
88 of DNA extraction, amplification, and of DNA amplicon detection by electrophoresis [6-10].

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

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

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

130

131 **2. Experimental**

132 2.1. *P. falciparum* culture

133 *P. falciparum* strain TM267 was cultured in complete malaria culture medium (cMCM), which was
134 composed of 10.43 g RPMI 1640 medium with L-glutamine (Gibco, USA), 1.8 g D-(+)-glucose (Sigma, USA),
135 2 g sodium bicarbonate (Sigma, USA), gentamicin sulfate (50 mg/mL; GPO, Thailand), 1,000 mL sterile
136 distilled water (DW), AlbuMAX solution (final concentration at 5% w/v; Gibco, USA) and HEPES solution (1
137 M at pH 7.4; Sigma, USA). *P. falciparum* was incubated in cMCM with human erythrocytes (5% hematocrit)
138 in a CO₂ incubator (37 °C, 5% CO₂) [35]. After the culture growth had reached more than 5% parasitemia

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

141 2.2. Sample collection and DNA extraction

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

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

154 2.3. Nested PCR

155 In this study, nested PCR (nPCR) was the standard method for amplification of the 18S rRNA gene of *P.*
156 *falciparum* and was performed as described previously with slight adaptations [29]. In the first round of
157 nPCR, the reaction mixture (25 µL) consisted of 0.5 µM of each universal primer (P1 forward primer [5'-
158 ACGATCAGATACCGTCGTAATCTT-3'] and P2 reverse primer [5'-GAACCCAAAGACTTTGATTTCTCAT-3'])
159 (Bionics Co. Ltd., Seoul, South Korea), 200 µM of each deoxyribonucleic acid (dNTP) (Macrogen, Korea),
160 1.5 mM of MgCl₂ (Promega, USA), 5x Colorless GoTaq® Flexi Buffer (Promega, USA), 1 unit (U) of GoTaq®
161 DNA Polymerase (Promega, USA), and 2 µL of extracted DNA.

162 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,
163 and 72 °C for 1 min, and finished by a final extension at 72 °C for 5 min. Amplicons from the first round
164 were diluted 20-fold with DW. The reaction mixture of the second round was prepared for malaria species-
165 specific amplification as in the first round preparation, with the addition of 2 µL of the diluted amplicons
166 and using the P₁ forward primer combined with a species-specific reverse primer (*P. falciparum*, 5'-
167 CAATCTAAAAGTCACCTCGAAAGATG-3') Bionics Co. Ltd., Seoul, South Korea). The second round of nPCR
168 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
169 1 min, and finished by a final extension at 72 °C for 5 min. The final amplicons were visualized by
170 electrophoresis using a 1.5% agarose gel (Serva, USA). The amplicon sizes from the first and second rounds
171 of nPCR were expected to be 160 and 110 bp, respectively. Genomic DNA from healthy donors and DW
172 were used as negative controls.

173 2.4. Preparation of gold nanoparticles

174 In this study, gold nanoparticles (GNPs) were prepared in-house by citrate reduction of H₂AuCl₄·3H₂O as
175 previously described [36] with minor modifications. Briefly, a solution of H₂AuCl₄·3H₂O (20 mL, 0.5 mM)
176 (Sigma, USA) was vigorously stirred until boiling. Then, an aqueous solution with sodium citrate dihydrate
177 (1 mL, 38.8 mM) (Sigma, USA) was quickly added and vigorously stirred until the color of the solution
178 changed from pale yellow to wine red. The GNP solution was then cooled on ice and stabilized in a dark
179 container at 4 °C. These GNPs were then characterized by transmission electron microscopy (TEM; JEM-
180 2100 electron microscope, Japan), ultraviolet–visible (UV-Vis) spectroscopy (BioTek; Synergy HTX, USA),
181 and dynamic light scattering (DLS; Zetasizer Nano-ZS, UK).

182 2.5. Functionalization of GNPs

183 Prior to the functionalization of GNPs, commercial lyophilized streptavidin from *Streptomyces avidin* (SA;
184 Sigma, USA) was dissolved with sterile DW. Then, GNPs (optical density = 2.0 at 519 nm) in 2-(N-
185 morpholino) ethanesulfonic acid hydrate buffer (MES buffer, 25 mM, pH 6.0; Sigma, USA) were
186 functionalized with a solution of SA according to a previously reported protocol [37]. Various
187 concentrations of SA (from 5 to 20 µg/mL) were added to the GNP solution at a ratio of 1:1 and shaken
188 for 1 h. After that, the mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant was
189 collected for measurement of unbound SA. Simultaneously, the pellets of SA-GNPs were resuspended in
190 0.25% bovine serum albumin (BSA; Merck, USA) in DW and shaken for 30 min. The mixture was washed
191 thrice by centrifugation at 13,000 rpm for 15 min. Finally, the SA-GNPs were resuspended in DW and
192 stored in a dark container at 4 °C for further use. The SA-GNPs were also characterized by UV-vis
193 spectroscopy and DLS.

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

$$201 \text{ Binding efficiency (\%)} = [(P_i - P_u)/P_i] \times 100 \quad (1)$$

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

203 2.6. LAMP and condition optimization

204 The LAMP assay was performed in 25 µL of reaction mixture as previously described [38], with minor
205 modifications. First, LAMP primers were designed for *P. falciparum* detection based on the 18S rRNA gene
206 sequence as previously reported [30]. The nucleotide sequence of each primer is shown in Table 1. Each
207 reaction mixture consisted of 1x isothermal amplification buffer (New England Biolabs, USA), 6 mM
208 MgSO₄, 0.8 M betaine (Sigma-Aldrich, USA), 1.4 mM dNTPs, 1.6 µM of each inner primer (FIP and BIP;
209 Bionics Co. Ltd., Seoul, South Korea), 0.4 µM of each outer primer (F3 and B3), 0.8 µM of each loop primer
210 (biotinylated LF and LB), 8 U of *Bst* 2.0 WarmStart® DNA polymerase (New England Biolabs, USA), and 2

211 μL of template DNA. DW was used as a negative control in all LAMP reactions. These reactions were
212 performed on a thermal cycler (MJ Research PTC-200 Thermal Cycler; Bio-Rad, USA) at 60 °C for 40 min,
213 followed by heating at 80 °C for 5 min to inactivate the reaction. To optimize the temperature of the
214 LAMP, reactions were simultaneously performed at different temperatures (60 °C, 63 °C, and 65 °C) for
215 60 min and then heated at 80 °C for 5 min. To determine the optimal time for the LAMP, reaction mixtures
216 were amplified for 35, 40 or 45 min at 60 °C and then heated at 80 °C for 5 min. The LAMP amplicons were
217 confirmed by electrophoresis using a 1.5% agarose gel. Positive LAMP products were purified and
218 analyzed by DNA sequencing (Bionics Co. Ltd., Seoul, South Korea). The sequencing chromatograms were
219 validated using BioEdit, Version 7.0.1 and multiple aligned with the reference *P. falciparum* sequence from
220 GenBank.

221 2.7. LAMP detection using SA-GNPs (GNPs-LAMP)

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

228 The limit of detection of GNP-LAMPs was investigated using genomic DNA extracted from *in vitro* *P.*
229 *falciparum* culture at 2% parasitemia, equivalent to 10^5 parasites/ μL [39]. The DNA was diluted tenfold
230 from 10^5 to 10^{-2} parasites/ μL and analyzed by GNP-LAMP with different induction techniques. These were
231 compared to conventional LAMP with electrophoresis using a 1.5% agarose gel as a control technique and
232 SYBR® Green I (Invitrogen, USA) as a common color indicator.

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

237

238 3. Results and discussion

239 3.1. Preparation of SA-GNPs

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

244 3.2. Optimization of LAMP conditions for *P. falciparum* detection

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

254 3.3. GNP-LAMP assay

255 The SA-GNP solution (O.D. = 2.5) and the LAMP product were mixed at a ratio of 1:1 to obtain the best
256 hybridization and aggregation results. The concentrations of HCl solution (2 μ L) were in the range of 10-
257 1,000 mM. After spinning down, the 100 mM HCl solution clearly distinguished between positive (wine
258 red colloidal solution) and negative results (aggregation), while the difference with other concentrations
259 was less distinct (Figure 3). The higher HCl concentrations (500 and 1,000 mM) tended to provide false
260 negative results because the SA-GNPs were aggregated in both positive and negative controls. In contrast,
261 the lower HCl concentrations (10 and 50 mM) tended to produce false positives because aggregation did
262 not occur in the negative controls.

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

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

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

285 3.4. Sensitivity and specificity of GNP-LAMP assay

286 Of 60 human blood samples, 20 were identified as positive for *P. falciparum* by microscopic examination,
287 while 40 were negative for malaria parasites. Genomic DNA was extracted from all samples and tested in
288 parallel with GNP-LAMP and nPCR and compared to the microscopy results (Table 2). The sensitivity,
289 specificity, positive predictive value (PPV), and negative predictive value (NPV) of GNP-LAMP were 100%
290 (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
291 to 100.0), respectively. In addition, the nPCR results had 100% sensitivity (95% CI, 83.2 to 100.0), 100%
292 specificity (95% CI, 91.2 to 100.0), 100% PPV (95% CI, 83.2 to 100.0), and 100% NPV (95% CI, 91.2 to 100.0)
293 in comparison with those of microscopy. Thus, GNP-LAMP demonstrated significant results in that this
294 colorimetric assay was in almost perfect agreement with microscopy (P-value<0.001, Kappa = 0.927).
295 Despite the lower specificity of GNP-LAMP (95%), the LAMP amplicons of false positive results (n = 2) were
296 further sequenced and identical to the 18S rRNA gene of *P. falciparum*.

297 Remarkably, there were 2 cases in which the GNP-LAMP results were positive for *P. falciparum*, while
298 those of the reference (gold standard) methods were negative. Further analysis showed positive results
299 with genetic sequencing, suggesting that these 2 cases were true positive cases, consistent with a lower
300 detection limit of GNP-LAMP than those of nPCR (5 parasites/ μ L) and microscopy (50-100 parasites/ μ L)
301 [18]. Another explanation for the results in these 2 cases is that aerosol contamination (carry over) had
302 occurred and was detected by the extremely high sensitivity of the LAMP assay [40, 41]. Although the
303 workstations for DNA extraction, reagent preparation and postamplification analysis were physically
304 separated to minimize tube-to-tube cross contamination, contamination might occur in the
305 postamplification steps due to repeatedly opening the tube to detect the LAMP products. To reduce the
306 risk of contamination from postamplification steps, a closed-tube LAMP system for the visualization step
307 should be employed by using a wax-dye capsule as a container [42].

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

319

320

321 **4. Conclusion**

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

331

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345 **Conflict of Interest**

346 The authors declare no competing interests.

347

348 **References**

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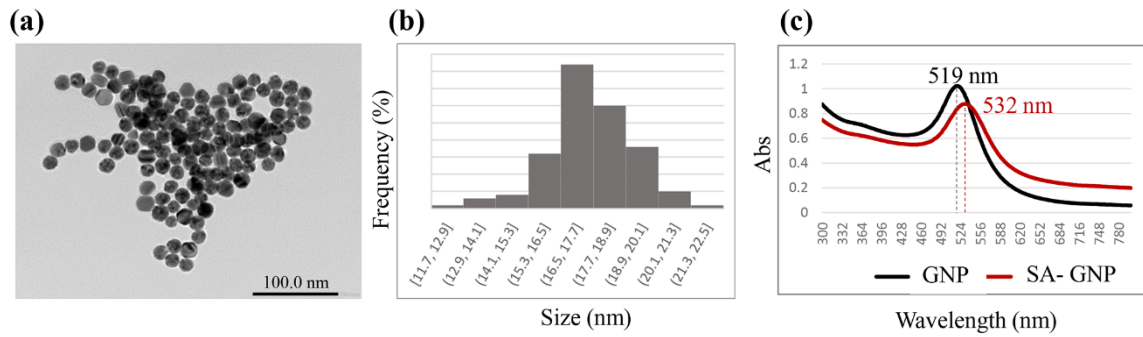
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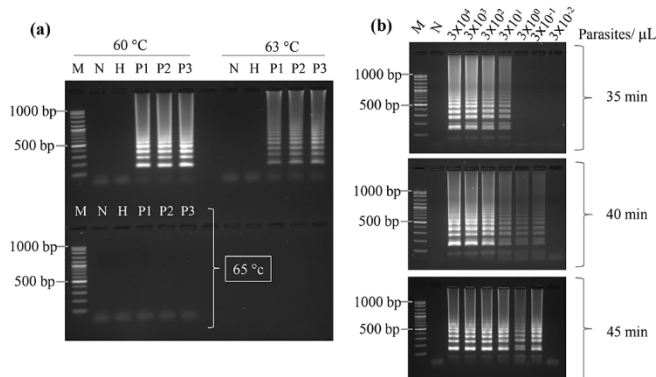
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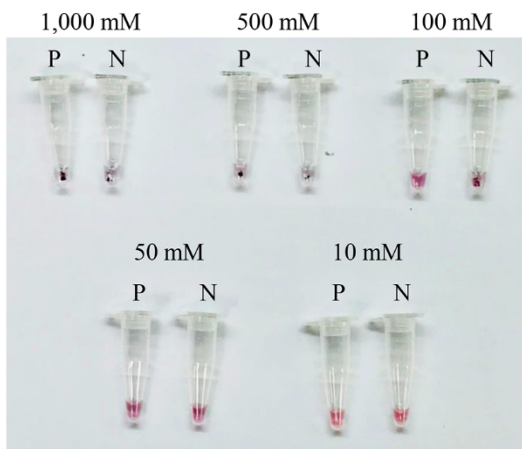


500
 501 **Figure 1.** Characterization of GNPs: (a) TEM imaging at the 100 nm scale, (b) size distribution analysis, and
 502 (c) absorbance spectra analysis of GNPs and SA-GNPs.

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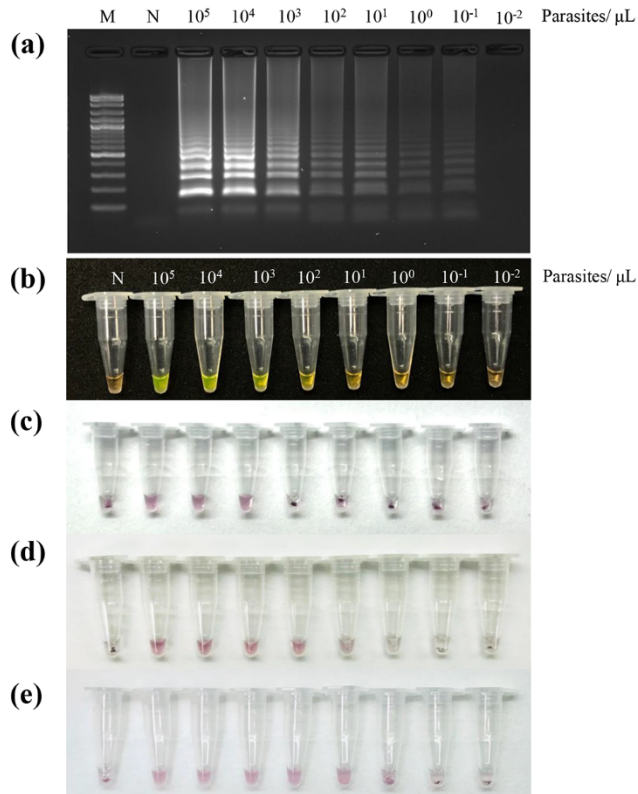


506
 507 **Figure 2.** (a) LAMP results with temperatures of at 60 °C, 63 °C, and 65 °C. M: DNA marker; N: negative
 508 control; H: human genomic; P1: *P. falciparum* strain 3D7; P2: *P. falciparum* strain TM267; P3: *P. falciparum*
 509 strain TM267 duplicate. (b) LAMP results with amplification times of 35, 40, and 45 min.

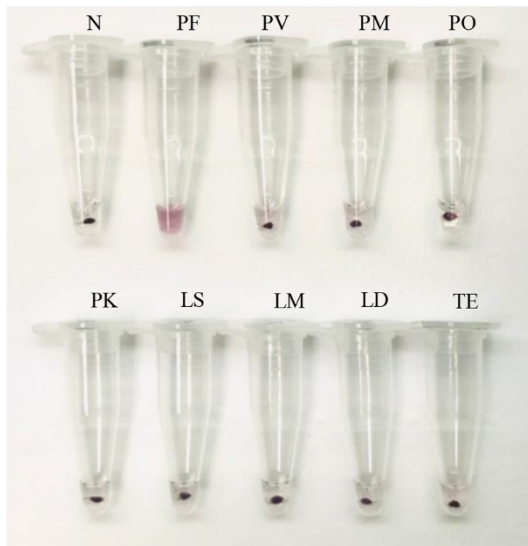


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511 **Figure 3.** Optimization of HCl concentration (10 to 1,000 mM at a fixed volume) from assessments in
512 paired reactions containing either the LAMP amplicons of *P. falciparum* (P) or the negative control (N).
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 530 **Figure 4.** Sensitivity of the GNP-LAMP assay for the detection of *P. falciparum* using 10-fold serial dilutions
 531 of DNA from cultured *P. falciparum*. (a) Analysis of the LAMP products using gel electrophoresis. Lane M:
 532 DNA marker; N: negative control. (b) Colorimetric results of SYBR Green I added to the LAMP products. N:
 533 negative control (DNA free). (c) Mixtures of GNP-LAMP amplicons and HCl solution after spinning down
 534 for 10 sec. (d) Mixtures of GNP-LAMP amplicons and HCl solution after heating at 60 °C for 5 min. (e) The
 535 mixtures of GNP-LAMP amplicons and HCl solution after incubating at room temperature for 40 min.
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538 **Figure 5.** Specificity of the GNP-LAMP assay evaluated using samples from human malaria species [*P.*
539 *falciparum* (PF), *P. vivax* (PV), *P. malariae* (PM), *P. ovale* (PO), and *P. knowlesi* (PK)], other blood protozoa
540 [*L. siamensis* (LS), *L. martiniquensis* (LM), *L. donovani* (LD), and *T. evansi* (TE)], and DW as a negative
541 control (N).

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558 **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)	TGTTGCAGTTAAAACGTTCTAGCCCAAACCAGTTTAAATGAAAC	45
LPF	Biotin- GCACCAGACTTGCCCT	16
LPB	Biotin- TTGAATATTAAGAA	15

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

Methods	No. (n) of samples with		(% Sensitivity (95% CI)	(% Specificity (95% CI)	(% PPV (95% CI)	(% NPV (95% CI)
	Microscopy 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				

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