Capping Gold Nanoparticles to Achieve a Protein-like Surface for LAMP Acceleration and Ultrasensitive DNA Detection

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Abstract

Loop-mediated isothermal amplification (LAMP) is a popular DNA amplification method. Gold nanoparticles (AuNPs) were reported to enhance the efficiency of LAMP, although the underlying mechanism remained elusive. Since AuNPs strongly adsorb a range of ligands, pre-adsorbed ligands cannot be easily displaced. In this work, we systematically investigated the effect of surface-modified AuNPs on LAMP by varying the order of mixing of AuNPs with each reagent in the LAMP system (Mg^{2+}, template DNA, dNTPs, primers, and polymerase). Mixing the AuNPs with the primers delayed the LAMP based on SYBR Green I fluorescence, while other orders of mixing had little effect, all accelerated the reaction. We then tested other common ligands including polymers (polyethylene glycol and polyvinylpyrrolidone), inorganic ions (Br^-), proteins, glutathione (GSH), and DNA (A_{15}) on AuNP-LAMP. The boosted AuNP performance on LAMP was most obvious when the AuNPs can form a protein-like surface. Finally, using GSH-capped AuNPs, a detection limit of around 100 copies μL^{-1} of target DNA was achieved. This work has identified a ligand-capped AuNP strategy to boost LAMP and yielded a higher sensitivity in DNA sensing, which also deepens the understanding of AuNP-assisted LAMP.

Keywords: NanoPCR, gold nanoparticles; LAMP; biosensors; proteins; GSH

Introduction

Using nanomaterials to improve the efficiency and specificity of polymerase chain reaction (PCR), coined nanoPCR, has been reported for many years since Fan's pioneer work.[1-4] With the advancement of nanotechnology, a range of inorganic,[5-10] organic,[11-12] and carbon-based[13-15] nanomaterials have successfully improved the efficiency and specificity of PCR. Several hypotheses have been put forward to explain such a behavior of nanomaterials, including single-strand DNA binding protein (SSB)-like mechanism,[16-17] heat transfer effect,[7] DNA polymerase adsorption,[6, 16] PCR reagents condensation,[11] and catalytic activities.[18] Nevertheless, the mechanism of nanoPCR remained elusive because of the complexity of the PCR system. None of the proposed mechanisms can elucidate all experimental observations. In addition, most of the reports mixed nanomaterials directly with little understanding on the order of mixing.

Among the various types of nanomaterials, gold has received the most attention since gold nanoparticles (AuNPs) were the first nanomaterial reported for this effect.[1] In addition, AuNPs have excellent optical properties allowing for convenient monitoring. Finally, AuNPs have well-established surface chemistry. Functionalization of AuNPs with molecules containing various functional groups not only affects the colloidal stability but also the surface chemistry of AuNPs, which further influences its molecular recognition, adsorption, and signaling.[19-20] Recently, Vanzha et al studied the surface chemistry of AuNPs on PCR amplification.[21] They found that only the citrate-capped AuNPs improved the PCR, whereas AuNPs capped by polymers such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) had no effect. When a cationic ligand such as CTAB was used, an adverse effect to PCR was observed. This work offered an interesting perspective that tuning the surface chemistry of nanoparticles can modulate PCR amplification as well.

Since PCR requires repeated heating and cooling cycles, it's more difficult for mechanistic studies. In contrast, loop-mediated isothermal amplification (LAMP) is an isothermal DNA amplification method and nanomaterial-assisted LAMP was also reported.[22-25] For example, Ye et al showed AuNP-mediated LAMP with enhanced specificity and attributed it to the hot-start property of AuNPs.[26] Gao et al studied the effect of AuNPs and

AgNPs on LAMP, where AuNPs in a certain concentration and diameter range accelerated the LAMP, but the AgNPs always inhibited it.[27] Furthermore, Ma et al studied the effect of the morphology of ZnO on LAMP and the flower-like ZnO nanoparticles exhibited an optimal performance.[28] Within this context, we aimed to carefully explore the mechanism of AuNPs on LAMP with a focus on its surface chemistry.

Herein, we systematically evaluated the effect of AuNPs on LAMP using real-time fluorescence and gel electrophoresis analysis. AuNPs were found to influence LAMP directed by its concentration, incubation time and temperature, mixing order of the LAMP reagents, and surface ligands. Based on results from mixing orders and various surface ligands, the acceleration effect of AuNPs appeared to be mainly mediated by surface interactions. Finally, GSH capped-AuNPs were chosen to demonstrate highly sensitive DNA detection.

Materials and methods

Materials

Bst 2.0 DNA polymerase, 10×isothermal amplification buffer, dNTPs, $MgSO_4$, and single-stranded DNA template (M13mp18) were purchased from New England Biolabs. The four primers for LAMP amplification of M13mp18 include BIP: 5′-CGACTCTAGAGGATCCCCGGGTACTTTTTGTTGTGTGGAATTGTGAGCGGAT-3′, FIP: 5′-ACAACGTCGTGACTGGGAAAACCCTTTTTGTGCGGGCCTCTTCGCTATTAC-3′, B3: 5′-ACTTTATGCTTCCGGCTCGTA-3′, F3: 5′-GTTGGGAAGGGCGATCG-3′, and they were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). SYBR Green I was purchased from ThermoFisher (Waltham, MA, USA). L-glutathione reduced (GSH), bovine serum albumin (BSA), polyvinylpyrrolidone (MW: 40,000), sodium bromide (NaBr), catalase and pyruvate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). PEG (MW: 20,000) and deoxyribonuclease were purchased from Alfa Aesar. Hydrogen tetrachloroaurate(III) ($HAuCl_4$) and sodium borohydride were from Sigma-Aldrich (St. Louis, MO).

Synthesis of AuNPs

Citrate-capped AuNPs with a diameter of 13 nm were synthesized following a previously

reported method.[29] 2 mL $HAuCl_4$ (50 mM) was added to 98 mL water in a two-neck flask. After heating the sample to reflux, 10 mL sodium citrate (38.8 mM) was added. Then, the solution was maintained reflux for 20 min and then cooled to room temperature. The stock AuNPs (13 nm) was about 10 nM based on its absorbance at 520 nm (extinction coefficient: 2.7×10^8 $M^{-1}cm^{-1}$).[30] Citrate-capped AuNPs with a diameter of 5 nm were prepared following the sodium borohydride method.[31] H_2O (18 mL) was added into 0.5 mL $HAuCl_4$ (0.01 M), and then 0.5 mL of 0.01 M sodium citrate was added and stirred at room temperature. Then, 0.5 mL freshly prepared $NaBH_4$ (0.1 M) was added. When the solution color changed from colorless to orange, stirring was stopped, and the solution was left undisturbed for 2 h. The stock AuNPs (5 nm) concentration was about 65 nM based on its absorbance at 520 nm (extinction coefficient: 8.6×10^6 $M^{-1}cm^{-1}$).[32]

LAMP reaction

The LAMP reactions were performed in a final volume of 25 μL containing 1×isothermal amplification buffer, dNTPs (0.8 mM), Mg^{2+}(4.0 mM), FIP (1.6 μM), BIP (0.2 μM), *Bst* 2.0 DNA polymerase (0.32 units μL^{-1}), 1×SYBR Green I and a specified amount of single-stranded M13mp18 DNA. BSA (final 0.01%), PEG and PVP (final 0.01%), Br^- and A_{15} (final 1 μM), GSH (final 0.1 mM), AuNPs (final 5.2 nM), lysozyme (final 0.01%), glucose oxidase (final 0.01%), catalase (final 0.01%), deoxyribonuclease (final 0.01%) and pyruvate dehydrogenase (final 0.01%) were used during the LAMP. These ligands were first pre-incubated with the AuNPs for 1 h before the other LAMP reagents were added. Then the mixture was incubated at 65 ℃ for 1 h and heated at 80 ℃ for 10 min to terminate the reaction. The reaction kinetics were recorded on a CFX96 real-time thermocycler (Bio-Rad).

Electrophoresis

LAMP products (15 μL), 30% glycerol (15μL) and loading dye (2 μL) were mixed thoroughly and then electrophoresed in 2.0% agarose gel at 120 V for 60 min using 1×TBE buffer. The gel was documented with a ChemiDoc™ MP imaging system (Bio-Rad). Zeta potential analysis was performed on a ZetaSizer (Malvern).

Results and Discussion

The single-stranded M13mp18 DNA was used as the template and four specific primers were designed to carry out our LAMP reaction (Figure 1). In the following explorations, we utilized the most common 13 nm citrate-capped AuNPs (Figure 2A), although 5 nm AuNPs were also used for comparison.

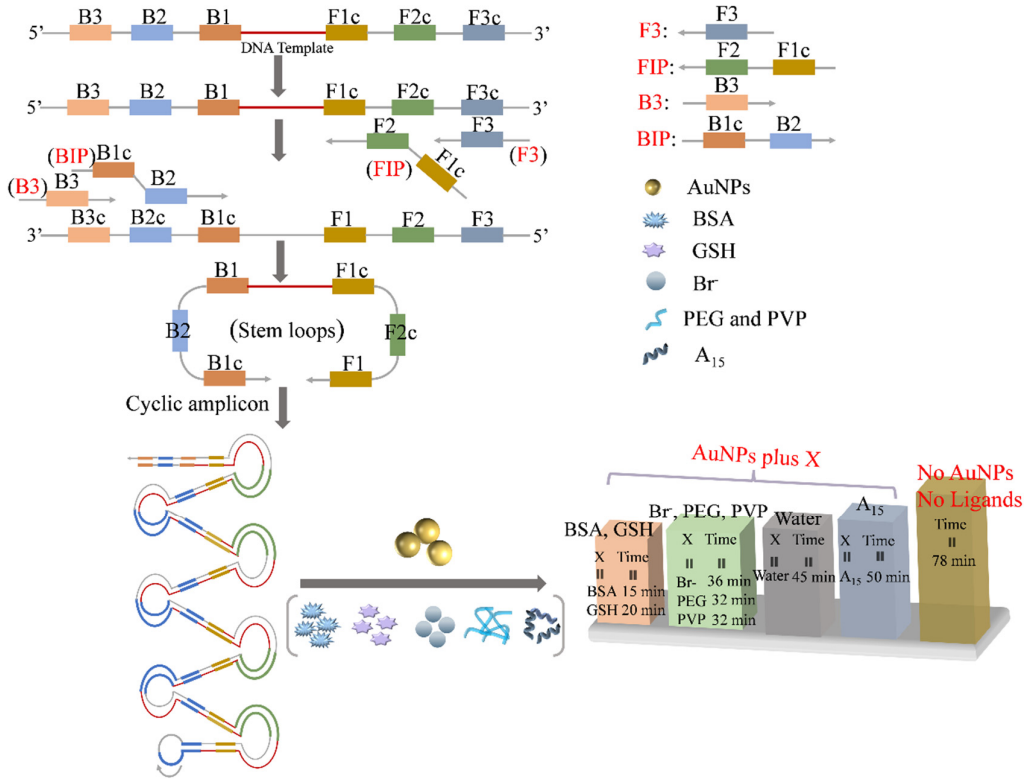


Figure 1. Scheme of AuNP-assisted LAMP (AuNPs-LAMP). A total of four primers were used.

Citrate-capped AuNPs accelerated LAMP

Since LAMP efficiency is strongly affected by Mg^{2+} and dNTPs, we first optimized their concentrations (Figure S2). The optimal concentrations of Mg^{2+} (4.0 mM) and dNTPs (0.8 mM) were used in the following tests. We then studied the effect of AuNP concentration and monitored the amplification process via real-time fluorescence in a qPCR instrument. As double-stranded DNA products were produced, SYBR Green I fluorescence was enhanced. These obtained amplification curves demonstrate that the AuNPs accelerated LAMP

significantly (Figure 2B). The time when the fluorescence emerged gradually decreased from 81 min to 47 min as the AuNPs increased from 0 to 5.2 nM (Figure 2C). Furthermore, the LAMP products in the presence of different AuNP concentrations were also electrophoresed (Figure 2D), and the typical LAMP pattern confirmed a successful amplification. Noticeably, a gradual ascent in the DNA yield was observed as more AuNPs were added, consistent with the real-time fluorescence result. Hence, the AuNP concentration was fixed at 5.2 nM in the subsequent studies. The same effect was also observed with 5 nm AuNPs (Figure S1).

To confirm the effect of AuNPs, we then centrifuged the 13 nm AuNPs and separately tested the supernatant and the redispersed precipitate. The redispersed AuNPs were still able to accelerate the LAMP, whereas the supernatant showed little effect (Figure S3). Thus, the AuNPs were indeed responsible for the acceleration of LAMP.

We then followed the LAMP reaction without and with the AuNPs at different temperatures and analyzed the products by electrophoresis. A large number of amplification products were produced at 50 ℃ in the presence of the AuNPs, whereas 55 ℃ was required in the absence of AuNPs (Figure 2E and 2F). All results illustrated that the AuNPs expedited the LAMP.

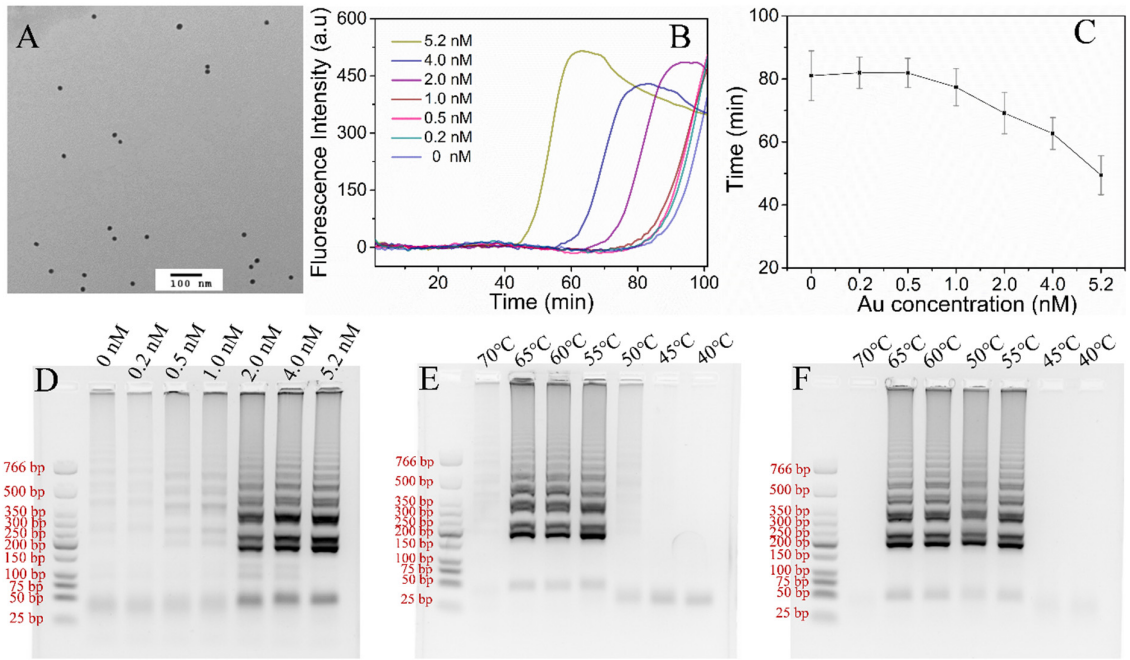


Figure 2. (A) A TEM micrograph of the 13 nm citrate-capped AuNPs used in this work. (B) Real-time amplification curves of LAMP with the AuNPs at different concentrations. (C) The time of emergence of

fluorescence signal in the LAMP with different AuNP concentrations. (D) Electrophoresis micrograph of the LAMP products at different AuNPs concentrations. Electrophoresis analysis of the (E) original LAMP and (F) AuNPs-assisted LAMP at different temperatures.

Effect of mixing order

Since LAMP is a complex reaction system containing not only the template DNA and four primers, but also a polymerase, dNTPs, and Mg^{2+}. AuNPs can strongly adsorb DNA and they might also adsorb the polymerase.[33] Such adsorption can be so strong that the initially adsorbed molecules onto AuNPs might hardly be displaced.[34] Therefore, we reason that the order of mixing might affect the outcome of the LAMP reaction. Most previous works simply mixed AuNPs with all LAMP reagents.[35-36] We here systematically varied the order of mixing. We respectively mixed the AuNPs with one of the reagents and then added the rest reagents. Interestingly, all the samples showed a similar extent of acceleration (fluorescence signal emerged in about 20 min) except for mixing the AuNPs with the primers first, which emerged the signal in 45 min (Figure 3A and 3B). Nevertheless, this was still faster than the sample without AuNPs (78 min). The electrophoresis results of these samples (Figure 3C) also supported the observation. The four primers were DNA oligonucleotides added at a high concentration (a total of 1.8 μM) that can strongly adsorb to AuNPs (5.2 nM). Our group recently showed that once a DNA oligonucleotide is adsorbed on AuNPs, it can hardly be displaced by adding its complementary strand or aptamer targets.[34,37] Thus, the excess primers can cover the gold surface if they were pre-incubated, which might explain the effect of the primers.

While the template DNA was also single-stranded, it was very long and added in a low concentration (0.02 pM) and they could not saturate the AuNP surface. In addition, the adsorption of very long DNA is known to be disfavored compared to the adsorption of oligonucleotides on AuNPs.[38-39] Our LAMP reaction only amplified a small portion of the entire 14450-nt template DNA sequence. All these factors may explain the lack of a strong effect by mixing the template DNA and AuNPs first.

It needs to be noted that the color of the AuNPs changed from red to purple upon mixing

with Mg^{2+} and with the polymerase (Figure 3D). The free AuNPs displayed a characteristic absorption peak at 520 nm, which disappeared as Mg^{2+} or polymerase was added, indicating the aggregation of the AuNPs. We also performed UV-vis spectroscopy and measured the zeta-potentials to test these reagents' impact on the AuNPs (Figure S4). All the AuNPs remained negatively charged regardless of the added reagents, and the Mg^{2+} sample had the surface charge closest to zero. Mg^{2+} can induce AuNP aggregation by screening their surface negative charges. In addition, polymerase as a large protein can crosslink the AuNPs. Nevertheless, these two aggregated samples still showed a similar acceleration effect on LAMP. Therefore, the AuNPs can exert their function even in an aggregated state. In fact, after LAMP, all the AuNP containing samples were aggregated, regardless of the order of addition (Figure 3D, bottom panel).

From this mixing order experiment, it appears that the surface of the AuNPs was indeed important. When covered by a high concentration of DNA primers, the function of the AuNPs was compromised. The other molecules might have less adsorption affinity or concentration to fully cover the surface, and their related concentrations are listed in Table S1.

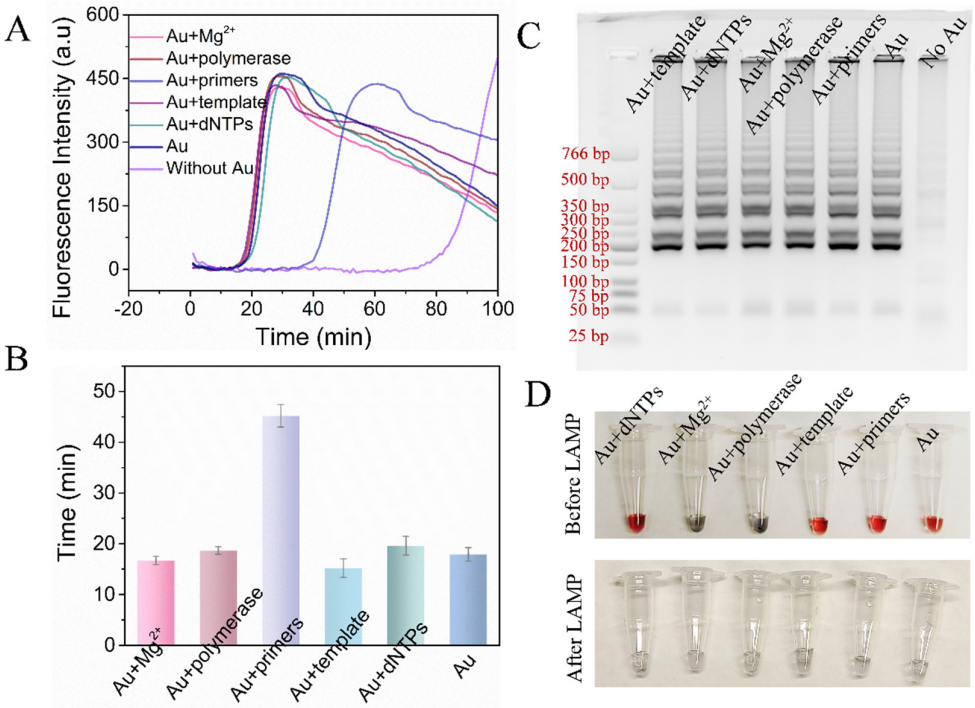


Figure 3. (A) Real-time amplification plots of AuNP-assisted LAMP with different orders of mixing. (B) The corresponding time of LAMP signal appearance with different orders of mixing AuNPs. (C)

Electrophoresis image of AuNP-assisted LAMP with different orders of mixing. (D) Digital photographs of the samples before and after the LAMP reaction.

Modulation of LAMP using different surface ligands

In the above experiments, the acceleration effect of the AuNPs decreased when they were mixed with the primers first. Therefore, we then turned our attention to a few other common surface ligands, which might provide deeper insights into AuNP-LAMP acceleration. Five groups of surface ligands were tested: polymers, inorganic ions, peptides, proteins, and single-strand DNA. These ligands were first incubated with the AuNPs and then added to the LAMP reaction.

PEG and PVP are two common polymers, and they had a weak effect on LAMP acceleration in the absence of AuNPs (accelerated for ~5 to 10 min, Figure 4A, 4C and 4D). PEG was reported to increase the activity of polymerase, which could also be helpful for the isothermal nucleic acid amplification.[40-41] When the AuNPs were added to PEG and PVP, similarly boosted LAMP amplifications were obtained, and they showed ~10 min acceleration compared to using the AuNPs alone (Figure 4D). Therefore, the effect of these polymers and AuNPs appeared to be additive. The concentrations of PEG and PVP were only 0.01% in our work. Thus, they might serve as blocking or surface-capping agents instead of crowding agents for AuNPs.[42] These weakly adsorbing polymers might not strongly affect the interaction between AuNPs and other components in the LAMP system.

We also pre-incubated the AuNPs with Br^-. We studied Br^- since it has a similar affinity to some nucleobases such as adenine and cytosine on gold and may affect the adsorption of other molecules.[43] Br^- accelerated LAMP without and with AuNPs by around 5 to 10 min (Figure 4C). Free Br^- might reduce unwanted DNA secondary structures to enhance the LAMP.[44] After introducing AuNPs, Br^- can absorb onto AuNPs and take some surface sites to prevent primer adsorption to further speed up the LAMP.

We then examined a protein, BSA. The effect of BSA on LAMP and AuNP-LAMP was similar, where both samples showed very fast reaction (below 20 min). Thus, in this case, the effect of the AuNPs was not obvious and the system was dominated by BSA. From the

photographs in Figure 4E and 4F, only the BSA samples retained the same red color after the LAMP, whereas the other samples all turned purple, demonstrating that BSA worked to protect the colloidal stability of the AuNPs during LAMP. BSA was reported to serve as a sacrificial protein to alleviate the inhibition of polymerase to enhance LAMP amplification.[45-46]

GSH, a thiol containing tripeptide, could also accelerate LAMP. Incubation GSH with the AuNPs further reduced the amplification time. Since GSH is a peptide, it might play a similar role as BSA to accelerate the LAMP. We reason that grafting a dense layer of GSH on the AuNPs can produce a protein-like surface. GSH carries a positive and two negative charges at neutral pH. Given the zwitterionic nature of GSH, it might accelerate LAMP by antifouling and decreasing sequence bias and reducing the secondary structure of the nucleic acids (similar to the effect of betaine, a zwitterionic acid amino analog).[47-48] The GSH layer can impede primers absorption as well.

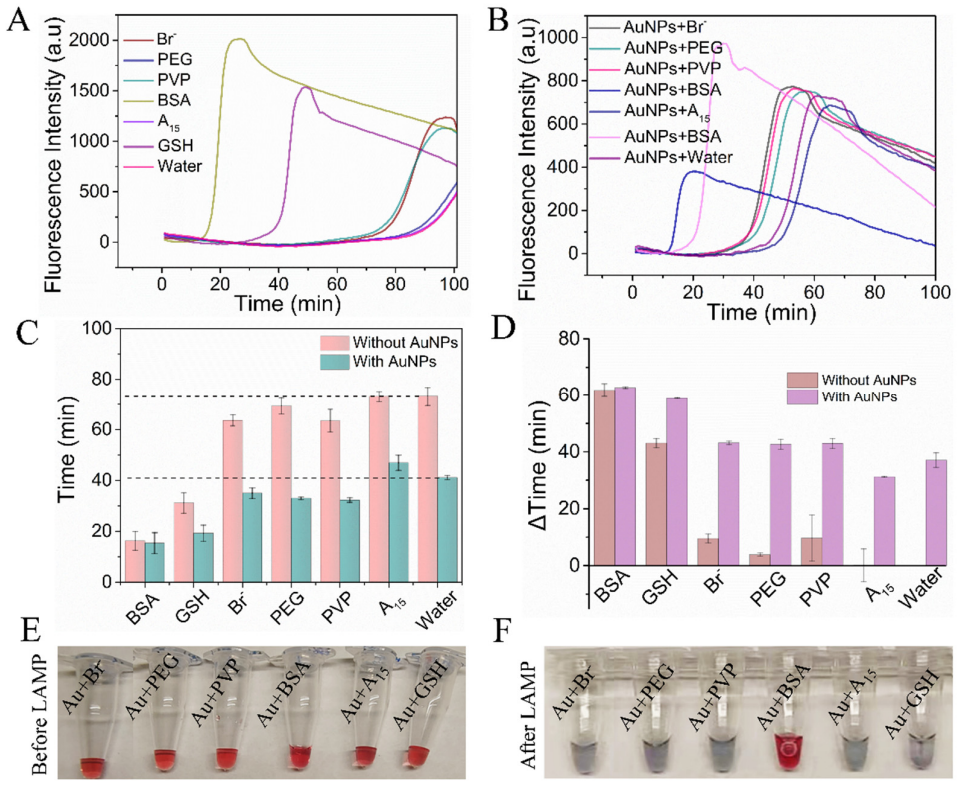


Figure 4. Real-time amplification plots of LAMP with different surface ligands (A) in the absence of the AuNPs and (B) in the presence of the AuNPs. (C) The LAMP time in the presence of various surface

ligands in the absence or presence of the AuNPs. (D) The difference in amplification time (Δtime) of AuNP-free and ligand free samples with surface ligand and AuNP-ligand added samples. Digital photography of AuNPs mixed with different surface ligands (E) before and (F) after the LAMP.

Adding free A_{15} DNA showed a negligible effect on LAMP. We chose to test A_{15} DNA due to the strong inhibition effect when the primers were first mixed with the AuNPs. Poly-A DNA has the highest affinity to AuNPs in all the DNA sequences. A_{15} in the concentration range from 0.1 μM to 1 μM delayed the LAMP (Figure S5), aligning with the result of the primers. Therefore, when the AuNPs were capped by DNA, LAMP was inhibited, but when the AuNPs were first adsorbed with GSH, LAMP can be enhanced. Thus, the nature of the surface ligand is critical for the function of the AuNPs. We also tested these surface ligands on the colloidal stability of the AuNPs using UV-vis spectrometry (Figure S6), no AuNPs aggregation was found. Capping of the AuNPs by GSH and BSA was also confirmed by zeta-potential measurement (Figure S7).

The acceleration effects are summarized in Figure 4D, where a higher bar indicates more acceleration. Although BSA-AuNPs had the largest time drop, the effect of AuNPs was not obvious since free BSA had a similar acceleration effect. Since the main focus of this work is to understand the effect of AuNPs, GSH was chosen to be our experimental model in the following explorations.

Mechanistic discussion

Among the AuNP-free samples tested in Figure 4, the most significant acceleration was achieved in the presence of BSA followed by GSH. They are amino acid-based polymers. When the AuNPs were added, further acceleration was achieved for all the samples, except for A_{15}. Based on these data, we propose that the effect of the AuNPs was to provide a structural scaffold to create a favorable surface environment for the LAMP reaction. When this environment is similar to a protein surface, the acceleration is the most favorite. This effect can be achieved by BSA alone. GSH-capped AuNPs can nicely mimic a protein surface and thus it also showed good acceleration. On the other hand, when the AuNPs were capped

by DNA, such a surface property is less favorable. Other molecules such as Br^-, PVP and PEG, they can help decrease the adsorption of DNA and thus they worked better than DNA but not as good as the protein or peptide.

To confirm our hypothesis, we then tested a few other proteins and a significant acceleration was also found (Figure S8). They exhibited even better performance on LAMP in the absence of the AuNPs, supporting that the acceleration effect can be realized by these proteins alone. A mechanism diagram is displayed in Figure 5, where a protein-like surface is emphasized. Since so many different proteins work similarly, the effect did not require a specific protein. It could be that the protein-like surface can prevent nonspecific adsorption of reagents (polymerase and template DNA) to the tube surface.

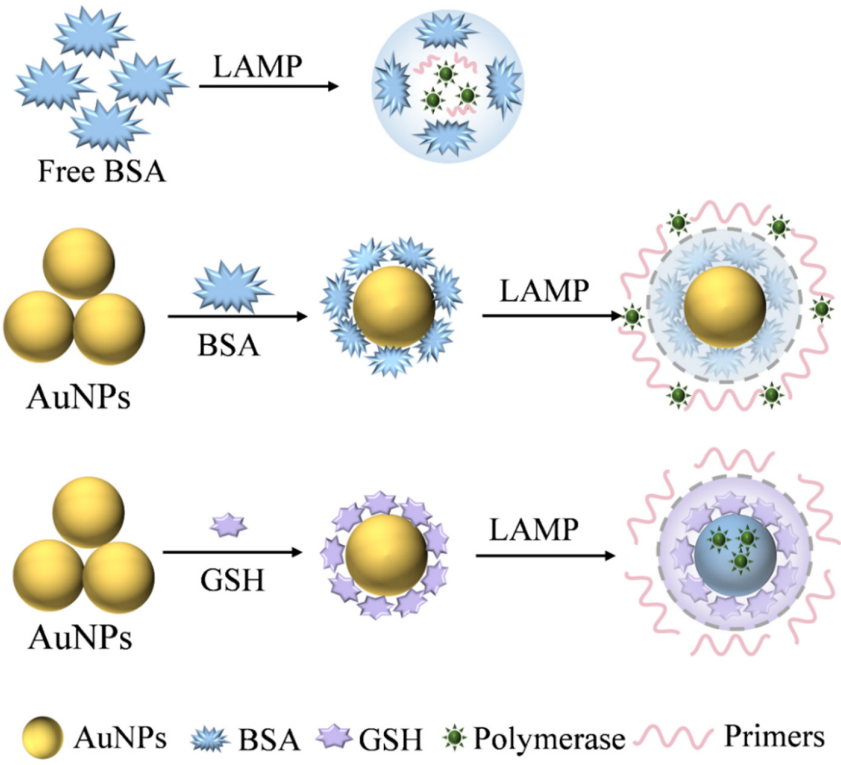


Figure 5. The mechanism diagrams showing the effect of free BSA protein, BSA-capped AuNPs and GSH-AuNPs during the LAMP amplification.

To test the generality of our observation, we further explored the effect of graphene oxide (GO) on LAMP. Faster LAMP amplification was also observed and the fluorescence signal

emerged in about 30 min when the final GO concentration was 0.01 mg/mL. When both GO and GSH were present, the time was shortened to about 15 min (Figure S9). Thus, the effect of nanomaterials and GSH was general and can also be achieved with GO.

GSH-capped AuNPs

Since GSH enhanced the effect of AuNPs, we further studied GSH in detail. First, we varied the concentration of GSH (Figure 6A, B). The speed of AuNP-LAMP accelerated when the GSH concentration was increased from 0 to 2 mM, after which, its effect saturated. Furthermore, we also varied the concentration of the AuNPs (5.2 nM, 0.52 nM and 0.052 nM) in the presence of 0.1 mM GSH (Figure S10). A higher concentration of AuNPs was more helpful to boost the LAMP. The shortest reaction time of about 15 min was achieved using the GSH-capped AuNPs.

We also studied the time of pre-incubation of GSH with AuNPs (Figures 6C, D). GSH was pre-incubated with AuNPs for up to 120 min respectively. A long pre-incubation time was more helpful to enhance the AuNP-LAMP reaction attributable to more absorption of GSH on AuNPs. This experiment indicated that the GSH-AuNPs conjugate played a more important role than free GSH. According to these results, 0.1 mM GSH and a pre-incubation time of 1 h were chosen in the following detection experiments.

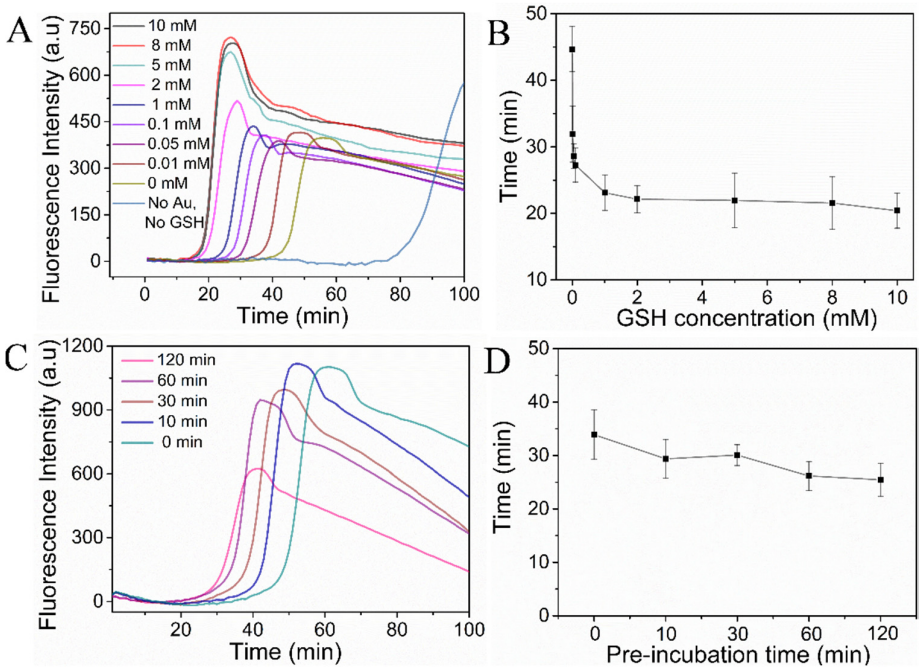


Figure 6. Real-time amplification plots of LAMP with GSH-capped AuNPs with (A) different concentrations of GSH, and (C) different pre-incubation times. The LAMP time at (B) different concentrations of GSH, and (D) different pre-incubation times.

Highly sensitive detection of DNA

Finally, we tested the analytical performance of GSH-AuNP assisted LAMP. We varied the concentration of the DNA template for the LAMP in the presence of GSH (0.1 mM) without and with the AuNPs. In the presence of AuNPs, the time increased from 41.9 min to 99.1 min when the DNA template was decreased from 1.26×10^{7} to 1.26×10^{2} copies μL^{-1} and no fluorescence was detected when the template concentration reached 1.26×10^{1} copies μL^{-1}, demonstrating that the limit of detection of this system was around 1.26×10^{2} copies μL^{-1} (Figure 7A). Moreover, the linearity of time and template concentration is displayed in Figure 7C with a correlation coefficient of 0.972 (1.26×10^{7} to 1.26×10^{3} copies μL^{-1}). When the AuNPs were absent, the corresponding time increased from 44.6 to 96.5 at the template concentrations ranging from 1.26×10^{7} to 1.26×10^{3} copies μL^{-1} with a correlation coefficient of 0.983 (Figure 7B and 7C). No fluorescence were observed as the template concentration was below 1.26×10^{3} copies μL^{-1}. These results illustrated that the GSH-capped AuNPs were able to offer a higher sensitivity compared with free GSH (Figure 7D).

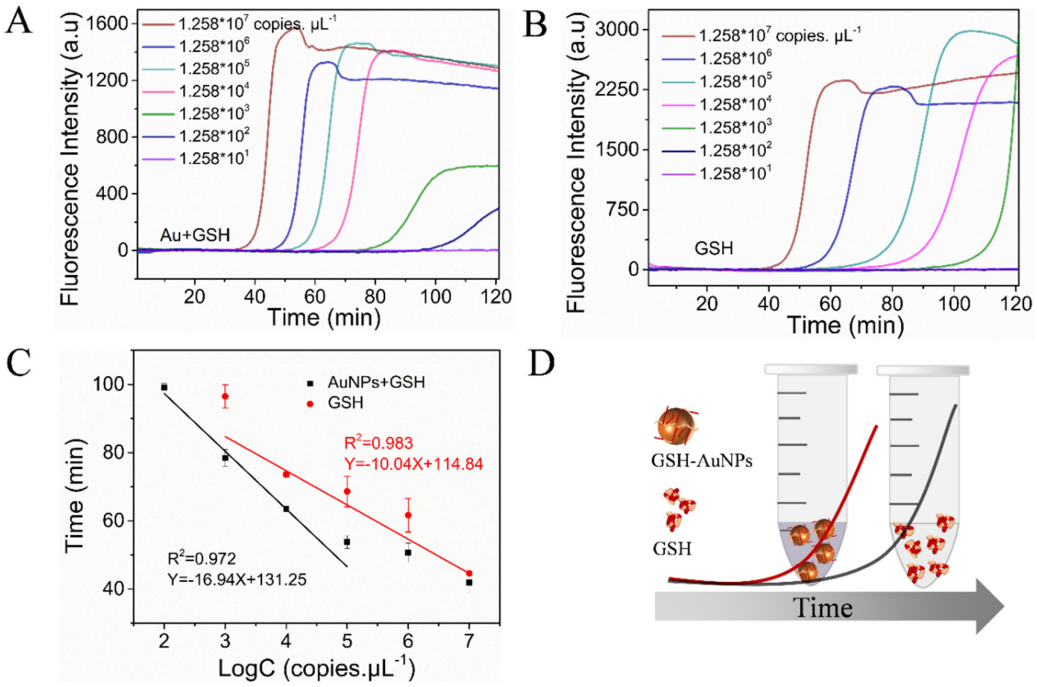


Figure 7. Real-time LAMP amplification plots of (A) GSH capped-AuNPs, and (B) AuNP-free GSH involved LAMP with different concentrations of template DNA. (C) The linear correlation between template DNA concentration and LAMP time. (D) Schematic of GSH-capped AuNPs and free GSH in the LAMP reaction.

Conclusions

In conclusion, we systematically studied the effect of surface ligands on AuNP-LAMP and the potential of GSH-capped AuNPs for LAMP-based DNA sensing. Directly adding citrate-capped AuNPs showed acceleration, which can be further enhanced by using certain surface ligands. Such an improvement was affected by surface interaction with AuNPs. Once the surface is protein-like, a good acceleration was achieved. Free protein molecules can achieve a similar acceleration effect. Thus, a limitation of this AuNP system is that its effect can be dominated by proteins. AuNPs likely only played a template role. On the other hand, when AuNPs were capped by DNA oligonucleotides, the acceleration effect was lower. Using GSH, we obtained higher sensitivity compared to that of free GSH. This work not only gives a picture of the surface ligand-capped AuNPs for LAMP amplification but also provides a

deeper understanding of the role of AuNPs during the LAMP.

Acknowledgments

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Conflicts of interest

There are no conflicts to declare.

Supporting Information

AuNPs (5nm and 13nm)-assisted LAMP (Figure S1); Electrophoresis images (Figure S2 and S3); UV-vis and Zeta potentials characterizations (Figure S4, S6 and S7); Additional real-time amplification plots of LAMP (Figure S5); Real-time amplification plots of enzymes on LAMP (Figure S8); Real-time amplification plots of GO on LAMP (Figure S9); Real-time amplification plots of varied AuNPs concentration on LAMP in the presence of GSH (Figure S10); The table of components concentrations of AuNPs-LAMP (Table S1).

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