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COMMUNICATION

Naked-eye visualization of nucleic acid amplicons using hierarchical nanoassembly

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This study reports the development of a rapid visualization method for DNA amplicons. Oligonucleotide-coated gold nanoparticles hierarchically assemble on DNA networks to form globular nanostructures, which precipitate into a distinct visible red pellet. This aims to overcome challenges associated with nanoparticle aggregation and dye-based colorimetric detection in LAMP assays.

Loop-mediated isothermal amplification (LAMP) assays have gained growing interest over the last few years due to their ability to achieve high sensitivity and specificity while offering rapid detection and portability.[1-4] Requiring only a heating block, LAMP reactions can be visualized with the naked eye using a variety of methods. These include the use of pH sensitive metal indicating dyes such as hydroxynaphthol blue or fluorescent intercalating dyes such as,[5] SYBR green I[6] that react with the LAMP amplification products to produce a change in color, which is detected visually. However, there have been reports of the inability to reproduce color changes at certain concentrations of reagents used in LAMP reactions.[7] Other dyes used for colorimetric detection include PicoGreen, propidium iodide, and ethidium bromide.[8] However, these methods depend on the use of certain concentrations of the dye to produce a desirable color change, which oftentimes can cause inhibition of the LAMP amplification reaction without extensive optimization.[9] Another significant disadvantage of these methods is the difficulty in distinguishing subtle color changes, especially in the field with varying natural light.[10,11] Color perception by different individuals is also different and has to account for users with color blindness, which most times makes it necessary to use standard color quantification methods for secondary validation.[12]

A limited number of studies have explored the use of gold nanoparticles (AuNPs) due to their optical properties.[13] The change in pH and/or ionic strength following the LAMP reaction leads to the aggregation of the AuNPs, resulting in a color change from red (single AuNPs) to purple (aggregated AuNPs).[14-18] However, the aggregation can occur due to other changes in the reaction medium that potentially alters its pH or ionic strength irrespective of the presence or absence of amplified DNA. This could give rise to higher false negatives and decrease the sensitivity of the assay.[19] In addition, the colour change from red to purple is often hard to perceive in small volumes such as 25 µL, which is usually the sample size of LAMP assays, and may require longer than the stipulated time for visible changes to be detected. Such problems question the applicability of most colorimetric tests for onsite detection and creates a need to develop alternative visual identification methods that are fast and easier to interpret.

In this study, AuNPs functionalized with non-complementary oligonucleotides (AuNP-oligos) are used to detect nucleic acid amplicons through their rapid and non-specific assembly. A subsequent precipitation results in a distinctive red pellet visible to the naked eye (Fig. 1). The fungus, *Bretziella fagacearum*, that has a significant ecological importance,[20] was used as a model organism to conduct the LAMP assay and generate amplicons. More information on the oligonucleotide sequences used is provided in the supplementary information.

LAMP reactions for pure *B. fagacearum* DNA extracts were conducted to yield DNA amplicons that served as samples for the development of the new visualization method. Serial dilutions of the amplicons were used to evaluate the limit of detection (LOD). Fig. 2 shows a typical bright ladder-like pattern of DNA copies obtained from a LAMP reaction in well 1, which confirms the presence of the amplicons. It should be noted that with each subsequent well the bands grow fainter as they represent serial dilutions of the amplicons. Fluorescence detection of the amplicons and their dilutions were also done

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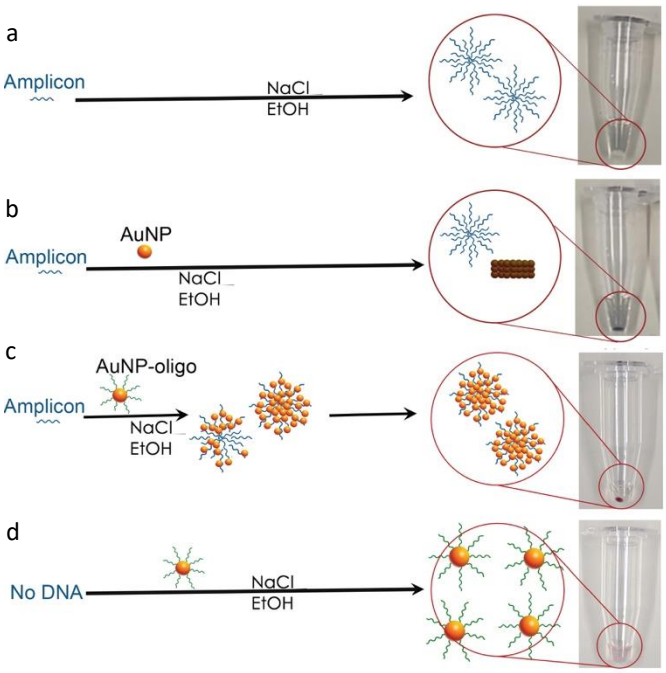

Fig. 1 Naked-eye visualization of DNA amplicons using nanoassembly. From top to bottom: a) DNA amplicons form globular networks under the effect of salts and ethanol, b) uncoated gold nanoparticles (AuNPs) aggregate under the same conditions, c) AuNPs coated with oligonucleotides (AuNP-oligos) bind non-specifically to the amplified DNA causing assembly of single nanoparticles with the DNA amplicons to form globular nanostructures, which precipitate into visible red pellet, d) In the absence of the DNA amplicons, the AuNP-oligos remain suspended in solution as single nanoparticles.

using a Qubit fluorometer to confirm amplification and to quantify the DNA concentrations.

The visualization test begins with the addition of the non-complementary AuNP-oligos to the LAMP reaction products, which gives it a uniform pink color. This was followed by the addition of salt and ethanol to induce precipitation of the DNA copies,[21,22] which eventually settled down as red pellets upon centrifugation. The high salt concentration causes DNA molecules to become insoluble in alcohol, causing them to precipitate.[21,22] The AuNP-oligos, in this case, are used as a coloring agent for the amplicons, owing to their natural red color, where they assemble closely to form a red pellet. This makes the amplicons distinctly visible to the naked eye while the rest of the solution remains clear. The solutions of samples that did not contain the DNA amplicons, remained uniformly pink with no detectable change. The time taken to conduct this test was less than ten minutes.

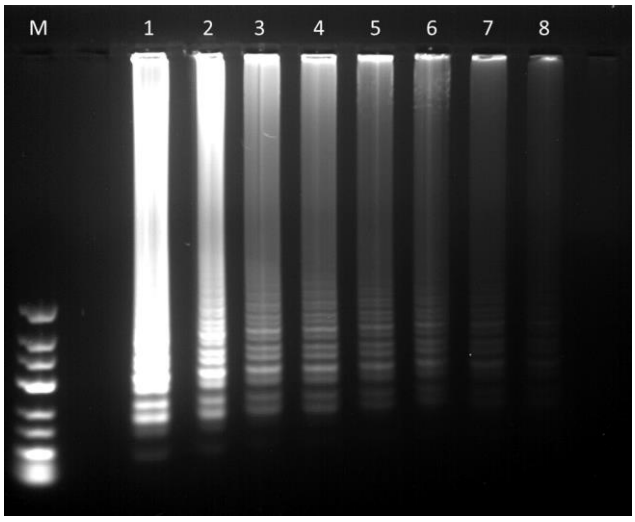

Fig. 2 Agarose gel image of LAMP products. Well M serves as a marker. Well 1 contains *B.fagacearum.* LAMP amplicons and wells 2-8 are 2x, 4x, 6x, 8x, 10x, 20x and 40x dilutions of the amplicons in well 1.

It is important to note that when AuNPs aggregate due to Van der Waals interactions, they usually form dark blue or gray precipitates in solution.[23] The formation of a red pellet in the presence of the amplicons indicates that AuNP-oligos did not aggregate but assembled in an organized fashion through DNA clustering. Transmission electron microscopy (TEM) imaging was conducted to confirm the binding of the AuNP-oligos to the DNA amplicons. Fig. 3 shows single AuNP-oligos (around 10 nm) inside and around the DNA amplicon network, forming globular nanostructures of hydrodynamic diameter around 300-500 nm. Here the oligonucleotide sequences serve two purposes. One is facilitating the non-specific assembly of the AuNPs with the DNA amplicons and two is stabilizing the AuNPs to prevent aggregation.

The precipitation and agglomeration of these nanostructures result in the red pellet observed at the bottom of the centrifuge tube. The red color suggests that most nanoparticles still maintain an interparticle distance higher than 20 nm. An interparticle distance below 20 nm would have resulted in a change of color from red to purple due to localized surface plasmon resonance effect as we have previously reported.[23,24] Maintaining the red color and generating a visible red pellet is important to distinguish between AuNP-oligos-

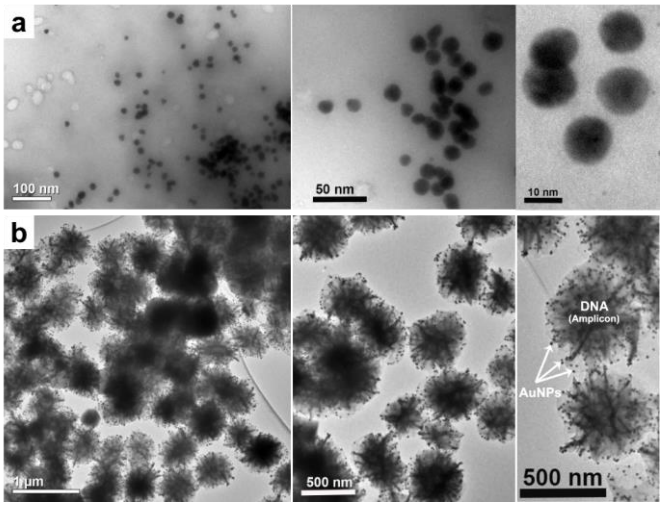

Fig. 3 Transmission electron microscopy (TEM) images of AuNPs-oligos DNA conjugates (a) before and (b) after nanoparticle assembly following the addition of DNA amplicons. In the presence of the amplicons, the AuNP-oligos conjugates (10 nm) assemble into globular nanostructures of 300-700 nm.

amplicon assembly and other non-specific aggregation of AuNPs.

To verify the nature of this interaction, a series of control tests were conducted. First, LAMP products were incubated with the prepared AuNP-oligos for five minutes at 65°C (LAMP reaction temperature), followed by amplicon precipitation using salt and ethanol. Tests at room temperature and at 95°C (DNA melting temperature) were also conducted. The results showed no difference in AuNP-oligos-amplicon interactions at different temperatures. This confirms that the binding of the DNA amplicons with the oligonucleotides on the surface of the AuNPs is non-specific. This is because higher temperatures are required to melt the double stranded LAMP products before specific hybridization, which was not the case at RT or 65°C. Such non-specific interaction is likely due to a combination of factors including low stringency conditions created by high concentration of salts for non-complementary hybridization.[25] Furthermore, the positive sodium ions from the salts interact with the negative charges of the DNA amplicons' phosphate backbones, creating a salt bridge.[26] At this point, the DNA molecules likely form an expanded network with the bound AuNP-oligos. When ethanol was added, the DNA with bound AuNPs started clustering into globular nanostructures due to low solubility in ethanol, which then precipitated as a distinctive and visible red pellet at the bottom of the centrifuge tube. Tests with a different target amplicon obtained from conducting LAMP assay on *Listeria ivanovii* gene,[27] a foodborne bacterial pathogen, yielded similar results using the same AuNP-oligos (results provided in Supplementary Fig. S1). Further, tests with AuNPs functionalized with complementary oligonucleotide sequences (provided in supplementary information) that are specific to the target fungal gene (*B. fagacearum*) also yielded a similar result (Fig. 4). This means that under these conditions synthesized AuNP-oligos can act as labels for any amplicons obtained from a DNA amplification reaction irrespective of complementarity and is not selective for the source of amplicons. Therefore, as long as the designed LAMP assay primers are highly specific to the target template sequence, the final reaction with AuNP-oligos would serve as a rapid non-specific visual confirmation. Furthermore, the non-specific binding of AuNP-oligos with the amplicons for naked-eye detection eliminates the need to maintain high temperatures for hybridization, making it more feasible for field testing. Triplicates were maintained throughout.

Tests were also conducted with samples containing AuNPs synthesized without any oligonucleotides. Samples without the addition of NaCl and ethanol were also used as controls (Fig. 1 and Supplementary Fig. S2). The addition of AuNPs without the oligonucleotides caused the nanoparticles to aggregate in the presence of the salt and settle down as a dark grey aggregate irrespective of whether the sample was positive or negative. This showed that the oligonucleotide sequences on the AuNPs were necessary for their stability and attachment to the DNA amplicons for a successful distinction between positive and negative samples. Moreover, without the addition of the salt and ethanol, both positive and negative samples remained pink in colour for samples with AuNP-oligos and amplicons. This showed that the addition of NaCl and ethanol was necessary to cause the nanoassembly of the DNA amplicons and AuNP-oligos, which led to the appearance of the red pellet in positive samples. Therefore, the AuNP-oligos, and adequate concentrations of salt and ethanol were essential ingredients for a visual detection of LAMP amplicons at the end of the amplification reaction. Results from this test are shown in Supplementary Fig. S2.

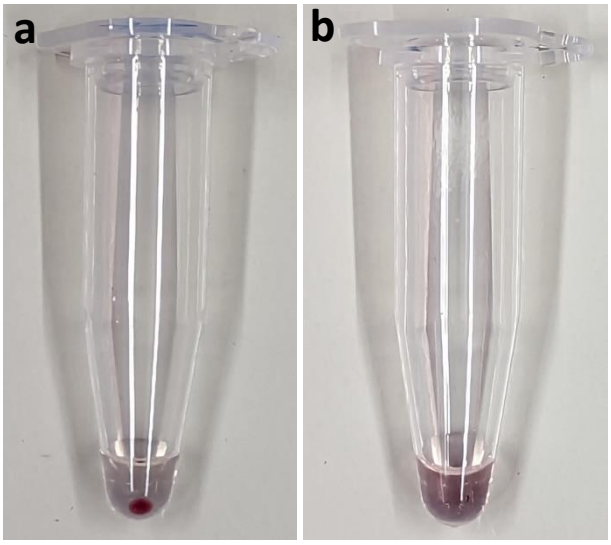

Fig. 4 Visualization of *Bretziella fagacearum (B. fag)* DNA amplicons by nanoassembly. a) AuNPs were coated with oligonucleotide probes that are specific to *B.fag,* then mixed with the *B.fag* DNA amplicon to cause nanoassembly and precipitation into a visible red pellet. The control sample (b) was obtained by replacing the *B.fag* DNA amplicon with nuclease-free water.

To determine the minimum concentration of amplicons that is required to induce the formation of the red pellet, serial dilutions of the LAMP products were also tested. The concentrations of the amplicons were determined using a Qubit fluorometer. Given that the sample volume and concentration of the AuNP-oligos in solution was too low for UV-visible-based quantification using the nanodrop, AuNP-oligo assembly with the DNA amplicons was evaluated using ImageJ software. The change in the gray value between the free nanoparticles in solution (supernatant) and the nanoparticles assembled into a red pellet was quantified. The process is illustrated in Supplementary Fig. S3. This approximates the ratio of nanoparticle assembly caused by the DNA amplicons. The results depicted in Fig. 5 show that the lowest concentration of the amplicons that yielded a visible red pellet in the assay was found to be around 45 µg/mL, which was calculated to be 1.87 x 10^{11} copies/µl using an online tool (https://www.technologynetworks.com/tn/tools/copynumber calculator). It is important to note that this concentration is up to 20 times lower than the DNA amplicon concentrations usually produced by nucleic acid amplification during LAMP assays, confirming that the proposed process can be a powerful visualization method for LAMP assays.

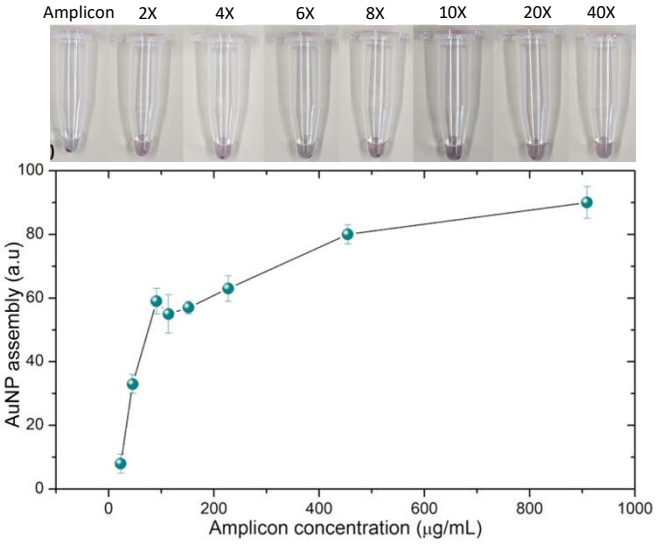

Fig. 5 Serial dilutions of the DNA amplicons and corresponding changes caused by AuNP-oligo assembly. Measurements were done in triplicates.

The nanoassembly of AuNP-oligos and DNA amplicons into a distinct structure visible to the naked eye is demonstrated to be a simple and efficient method for rapid visualization of LAMP reaction products. The new approach overcomes the limitation faced by current visual colorimetric detection methods, including nuanced colour change, and concentration-dependent reaction of dyes. Unlike other studies that explored the use of nanoparticles for LAMP assay detection, the approach reported here relies on a novel method of applying AuNP-oligos, where visual detection is defined by the behaviour of the AuNP-oligos-amplicon complex rather than colour changes. While the binding of the oligonucleotides to the DNA amplicon is non-specific, the nanoassembly and subsequent formation of a red pellet is highly dependent on the presence of DNA amplicons in the sample. Furthermore, the nanoassembly approach allows visualization of amplicons at concentrations down to 1.87 x 10^{11} copies/µl (45 µg/mL), which is up 20 times lower than the average amplicon concentration obtained from LAMP-based nucleic acid amplification.

Author Contributions

A.A. directed the research. V.T.N collected and prepared the samples, conducted the LAMP experiments, and developed the rapid visualization test for red oak samples. V.T.N. analyzed the data and wrote the manuscript with input from all the authors.

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Conflicts of interest

There are no conflicts to declare.

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