

Sensitive and selective detection of DNA fragments associated with *Ganoderma boninense* pathogen by DNA-nanoparticle conjugate hybridisation

Ekta Rani,^a Siti Akhtar Mohshim,^{b,c} Nor Hidayat Yusof,^d Muhammad Zamharir Ahmad,^b
Royston Goodacre,^{a,e} Shahrul Ainliah Alang Ahmad,^{c,d} Lu Shin Wong^{a*}

^a Manchester Institute of Biotechnology and School of Chemistry, University of Manchester,
131 Princess Street, Manchester M1 7DN, United Kingdom

^b Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and
Development Institute, 43400 Serdang, Selangor, Malaysia

^c Institute of Advanced Technology, Universiti Putra Malaysia, 43400, UPM Serdang,
Selangor, Malaysia

^d Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang,
Selangor, Malaysia

^e Department of Biochemistry, Institute of Integrative Biology, University of Liverpool,
Crown Street, Liverpool L69 7ZB, United Kingdom

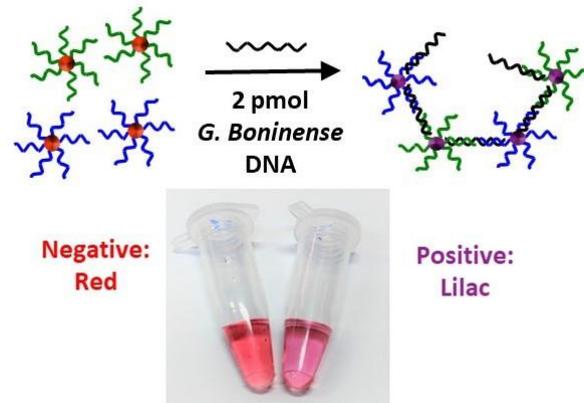
* email: l.s.wong@manchester.ac.uk, tel: +44-161-3068939

Abstract

A colorimetric assay for the detection of DNA fragments associated with the oil palm pathogen *Ganoderma boninense* is reported, which is based on the aggregation of DNA-nanoparticle conjugated in the presence of complementary DNA from the pathogen. Here, various designs of DNA-nanoparticle conjugates were evaluated, and it was found that the best design gave a visually observable colour change with as little as 2 pmol of double-stranded DNA analyte even in the presence of a large excess of a mixture of non-complementary DNA. The assay was also able to differentiate analyte sequences with three or more single nucleotide

mismatches. Overall, this label-free system is rapid, sensitive, selective, simple in design and easy to carry out. It does not require specialist equipment or specialist training for the interpretation of the results and therefore has the potential to be deployed of agricultural diagnostics in the field.

Graphical Abstract



Development of a colorimetric assay based on DNA-nanoparticle conjugates for the oil palm pathogen *Ganoderma boninense*

Introduction

The oil palm *Elaeis genesis* is the most important cash crop in Malaysia, which generated an annual export income of RM 65 billion (~ US\$ 16 billion) in 2016.¹ However, the yearly harvest is substantially reduced by basal stem rot (BSR) disease, caused by infection with fungal pathogen *Ganoderma boninense*,²⁻⁵ and results in economic losses of between RM 225 million to RM 1.5 billion annually in Malaysia⁶. The disease causes a reduction in crop yields and eventual death of the palms, necessitating frequent felling and replanting. Since there is no effective treatment for BSR once it manifests, management of the disease revolves around the isolation of infected plants (e.g. by trenching, ploughing or harrowing the soil around the palm).² As with any transmissible disease, early detection of infection is key to this management strategy. However, such detection is currently difficult since *Ganoderma* is a soil-borne organism that is transmitted through the roots of young plants, and the disease is symptomless at the early stage of infection. Thus, there is a need for quantitative, sensitive and selective detection methods. Furthermore, in order for any diagnostic system to be applicable for use in the field, it must also ideally be portable, affordable, have low power consumption, be tolerant to a wide range of environmental conditions, and not dependent on specialist equipment or training.

Various physical and chemical methods based on volatile molecule “electronic-nose” sensors,⁷ ultrasonic density measurements,⁸ spectral imaging,⁹⁻¹¹ and microwave radar backscattering¹² have been investigated in attempts to detect *G. boninense* in the field. However, these detection methods possess practical disadvantages in regards to complexity, cost and the need for specialist equipment and training to arrive at an unequivocal positive identification. From a molecular biological perspective, the earliest molecular attempts to identify *G. boninense* species relied on antibody-based immunoassays.¹³⁻¹⁵ However, due to the cross-reactivity of the antibodies, false positive results caused by other fungi such as *G.*

miniacinctum and *G. zonatum*, as well as *Trichoderma* and *Botrytis* species, were also observed.¹⁴ Further, their application in the routine analysis is limited by their complex protocols, labile reagents with high cost, relatively low sensitivity and the requirement of highly skilled personnel. Electrochemical methods for the detection of quinoline (a secondary metabolite produced by the palms under *G. boninense* attack),¹⁶ and DNA fragments associated with this organism^{17,18} have also been reported. The latter in particular has been shown to give high sensitivity (~ 1 fM of target DNA) and selectivity towards *G. boninense*. However, these methods rely on electrodes that are somewhat complex to fabricate and still rely on trained personnel for data analysis and interpretation.

In contrast, colorimetric assays that employ gold nanoparticles (AuNP) conjugated to DNA¹⁹⁻²¹ have shown great potential in diagnostics for human diseases²²⁻²³. Here, the presence of an analyte DNA strand bearing a sequence complementary to the DNA on the AuNP results in hybridisation of the DNA and concomitant aggregation of the nanoparticles. This aggregation, in turn, gives rise to an increase in the particles' surface plasmon resonance and thus UV-Vis absorption spectrum (i.e. an increase in λ_{\max} of the spectrum), which translates into a visually observable colour change (typically red to lilac). This analytical approach therefore offers many of the attributes that are desirable in a diagnostic system and would be applicable to field use, being portable, easy to carry out (only simple manipulations and without specialist equipment) and interpret.

Herein, we report a methodology for the application of DNA-AuNP conjugates towards the detection and quantification of DNA sequences related to *G. boninense*, in the context of single- and double-stranded DNA analytes, as well as in the presence of a mixture of non-complementary DNA. Furthermore, these procedures have been carried out independently in the UK (Manchester) and Malaysia (UPM) to demonstrate their reproducibility.

1. Experimental Section

1.1. Materials and Equipment

All chemical reagents were purchased from Sigma Aldrich (Haverhill, UK), Merck (Feltham, UK) or ThermoFisher Scientific (Altrincham, UK) and used as supplied. All reagents were of analytical grade. 20 nm AuNP colloid at a concentration of 1 optical density (OD) unit @ 525 nm in 0.1 mg mL^{-1} sodium citrate was purchased from Alfa Aesar (Heysham, UK). Deionised water (resistivity $\sim 18.2 \text{ M}\Omega$) was used for all the experiments.

The single-stranded (ss) DNA, double-stranded (ds) DNA of the analyte sequences and various DNA probes (with a 5' S-S C6 disulfide linker or 3' S-S C3 disulfide linker) were purchased from Sigma Aldrich. These were all supplied after purification by high-performance liquid chromatography and in lyophilised form. Deoxyribonucleic acid sodium salt from calf thymus (referred henceforth as "CT") was supplied by Sigma Aldrich.

1.2. Preparation of DNA probes-AuNP conjugates

DNA-AuNP conjugates were prepared as previously described²⁴ with few modifications. The disulfide modified oligonucleotides ($100 \text{ }\mu\text{M}$, $40 \text{ }\mu\text{L}$) were mixed with TCEP (20 mM , $40 \text{ }\mu\text{L}$) for 2 h at room temperature (RT) to prepare $50 \text{ }\mu\text{M}$ of DNA solutions with reduced thiols. This solution ($80 \text{ }\mu\text{L}$) was then mixed with 1 mL of citrate-stabilised AuNP colloid, and the mixture was incubated at RT for 12 h. $24 \text{ }\mu\text{L}$ of 1 M NaCl was added to this mixture followed by sonication for 10 s. This process of addition of NaCl and sonication (10 s) was repeated four times at 1 h intervals (i.e. 5 times in total) until a final concentration of 0.1 M NaCl was achieved. The final colloidal solution allowed to stand at RT for 24 h, then centrifuged at $19,000 \text{ g}$ for 30 min. The supernatant was decanted, and then

the pellet of AuNP resuspended in 5 mM phosphate buffered saline (PBS, at pH 7.4) containing 0.01 % v/v Tween 20. This process of centrifugation, decanting and resuspension then repeated three times, and final pellet of AuNP was re-suspended in 100 μ L 10 mM PBS buffer. The OD was then measured by UV-vis spectroscopy at 525 nm. These DNA-AuNP conjugates were typically stored at 4 °C (fridge) and used within a week of preparation. Prior to use, the OD of each colloid was adjusted to 0.7 by the addition of the relevant volume of 10 mM PBS.

1.3. Hybridisation assay

Lyophilised analyte ssDNA and dsDNA were reconstituted in water to make up two stock solutions of either 5 or 1 μ M (stock solutions A and B, respectively). For analyte concentrations of 20 nmol, stock solution A was used, while solution B was used for all other experiments.

1.3.1. For the detection of ssDNA

40 μ L of each DNA-AuNP conjugate was mixed with 10 μ L of hybridisation buffer (10 mM phosphate buffer, pH 7.0 with 0.3 M NaCl). The analyte ssDNA stock solutions were then added in volumes sufficient to make up the desired analyte concentration in a final assay volume of 200 μ L. To these mixtures, 40 μ L of NaCl solution of 5 M was added to achieve a final concentration in the assay mixture of 1 M. Finally; water was added to make up the final assay volume to 200 μ L. This colloidal solution was heated to 95 °C for 2 min, followed by gradual cooling (approximate rate of cooling was 5 °C/min) to RT (25 – 30 °C) over approximately 15 min.

1.3.2. For the detection of dsDNA

To make the working analyte solutions, the dsDNA stock solutions (at volumes to make up the desired analyte concentration in 200 μ L) were mixed with water to a volume of 70 μ L. These mixtures were heated at 95 °C for 2 min, and to this heated solution, a mixture

of DNA-AuNP conjugates (40 μL each), 10 μL of hybridisation buffer (see sub-sub-section 2.3.1) was added. To these mixtures, 40 μL of NaCl solution of either 5, 10 or 15 M was added to prepare the assay to achieve a final concentration in the assay mixture of 1, 2 or 3 M, respectively, to achieve a final volume of 200 μL . This assay was again heated at 95 $^{\circ}\text{C}$ for 2 min, followed by gradual cooling to RT over approximately 30 min.

1.3.3. For the detection of dsDNA in a mixture of calf-thymus DNA

To make the working analyte solutions, the dsDNA stock solutions (at volumes to make up the desired analyte concentration in 200 μL) were mixed with water to a volume of 68 μL . 2 μL of calf-thymus DNA solution (100 mg/77 μL in water) was added. Rest of the procedure was followed as noted in sub-sub-section 2.3.2.

2. Results and Discussion

2.1. Preparation and stability of DNA-AuNP conjugates

For the species-selective detection of *G. boninense*, DNA sequences from the internal transcribed spacer 1 (ITS1) region of its 18S ribosomal gene were chosen as targets.^{5,25} Two short DNA sequences were selected from the ITS1 genes bearing the NCBI Genbank accession numbers EU701010 and BD082757 (termed T1 and T2 respectively, Table 1), and pairs of DNA-AuNP conjugates bearing complementary probe DNA sequences to each target were prepared. Two general assay configurations were investigated for each target. In the “head-to-tail” (HTT)¹⁹ configuration, two probes in series were employed, with each complementary probe sequence attached at 5'-end to the AuNP via a 12 base GC repeat spacer and an alkylthiol linker (Figure 1a). Here, GC spacer is used to increase the spacing between AuNPs conjugated to two probes. In the “tail-to-tail” (TTT) format²¹ one probe sequence was attached at the 5'-end and the other via the 3'-end and the DNA sequences were attached directly to the AuNPs via alkylthiol linkers only (Figure 1b).

To improve the likelihood of identifying probes that would give reliable results, several pairs of DNA-AuNP conjugate probes were prepared for both target sequences. Thus, for target sequence T1, one probe pair in the head-to-tail configuration (termed P1a^{HTT} and P1b^{HTT}) and two pairs in tail-to-tail (P1a^{TTT} and P1b^{TTT}, P1c^{TTT} and P1d^{TTT}) were prepared (Table 1). For sequence T2, one pair of each conjugate was prepared for each configuration (P2a^{HTT} and P2b^{HTT}, P2a^{TTT} and P2b^{TTT}; Table 1).

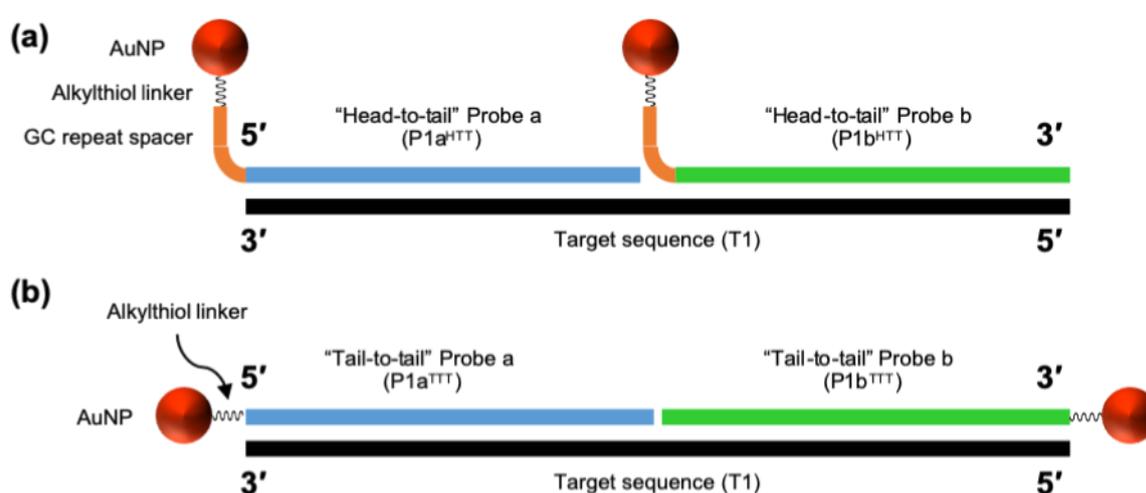


Figure 1. Schematic diagrams illustrating the (a) head-to-tail (HTT) and (b) tail-to-tail (TTT) binding configurations of the DNA-AuNP conjugate probes to the complementary target single-stranded DNA. For simplicity, only one example is shown for each pair of probes (P1a^{HTT} and P1b^{HTT} in (a), or P1a^{TTT} and P1b^{TTT} in (b)), which are complementary to the T1 target sequence.

Table 1. List of DNA sequences used in this study. All the sequences are listed in the 5' to 3' direction. The colour coding is used for clarity and is consistent with that in Figure 1. The mismatches are underlined for emphasis.

Ref.	Description	Sequence (annotated for clarity)
T1	35-mer sequence corresponding to fragment from NCBI Genebank accession number EU701010	TTGGCTCTCGCATCGATGAAGAAGAACGCAGCAGG
M1	T1 with single a mismatch	TTGGCTCTCGCATCGAT <u>CA</u> AAGAAGAACGCAGCAGG
P1a^{HTT} P1b^{HTT}	DNA probes complementary to T1 in a head-to-tail configuration	Alkylthiol linker Poly-GC Binding sequence (Thiol-C6)GGCCGCGGGCGGCCTGCTGCGTTCTTCTTCAT (Thiol-C6)GGCCGCGGGCGGCATGCGAGAGCCAA
P1a^{TTT} P1b^{TTT} P1c^{TTT} P1d^{TTT}	DNA probes complementary to T1 in a tail-to-tail configuration	(Thiol-C6)CCTGCTGCGTTCTTCTTCAT CGATGCGAGAGCCAA(Thiol-C3) (Thiol-C6)CCTGCTGCGTTCTTC TTCATCGATGCGAGAGCCAA(Thiol-C3)
T2	35-mer sequence corresponding to fragment from NCBI Genebank accession number BD082757	TTGGCTCTCGCATCGATGAAGAACGCAGCGAAATG
M2 M2a M2b M2c M2d M2e M2f	T2 with various mismatch(es)	TTGGCTCTCGCATCGATG <u>G</u> GAGAACGCAGCGAAATG TTGGCTCTCGCATCGATG <u>GG</u> AACGCAGCGAAATG TTGGCTCTCGCATCGAT <u>AGGAG</u> ACGCAGCGAAATG TTGGCTCTC <u>A</u> CATCGATG <u>G</u> GAGAACGC <u>GG</u> GCGAAATG TTG <u>A</u> CTCTC <u>A</u> CATCGATG <u>G</u> GAGAACGC <u>GG</u> GCG <u>G</u> ATG TTGGCT <u>A</u> TCGCATCGATGAAGAACGCAGC <u>C</u> AAATG TTGGC <u>CA</u> TCGCATCGATGAAGAACGCAGC <u>C</u> AAATG
P2a^{HTT} P2b^{HTT}	DNA probes complementary to T2 in a head-to-tail configuration	Alkylthiol linker Poly-GC Binding sequence (Thiol-C6)GGCCGCGGGCGGCATTTTCGCTGCGTTC (Thiol-C6)CGGGCGGCGCCGTTTCATCGATGCGAGAGCCAA
P2a^{TTT} P2b^{TTT}	DNA probes for tail-to-tail configuration	(Thiol-C6)CATTTTCGCTGCGTTC TTCATCGATGCGAGAGCCAA(Thiol-C3)
NC	Non-complementary DNA	GGAAGGCCAGCTACAACCCAGCTAGTCAAGGTAAC

Following their preparation, the DNA-AuNP conjugate bearing the P1a^{HTT} probe exhibited the expected deep red colour and a corresponding UV-Vis spectrum with a λ_{\max} at 525 nm (Figure 2a), similar to λ_{\max} for unconjugated AuNP. However, for P1b^{HTT}, a higher λ_{\max} (533 nm) and a broad tail towards the higher wavelengths was observed, which suggests the presence of aggregated AuNPs. DNA-AuNP colloids bearing the P2a^{HTT} and P2b^{HTT} probes also showed similar results (Figure 2b), with P2b^{HTT} showing a broad tail along with λ_{\max} at 533 nm. Both pairs of probes were thus unsuitable for further investigation.

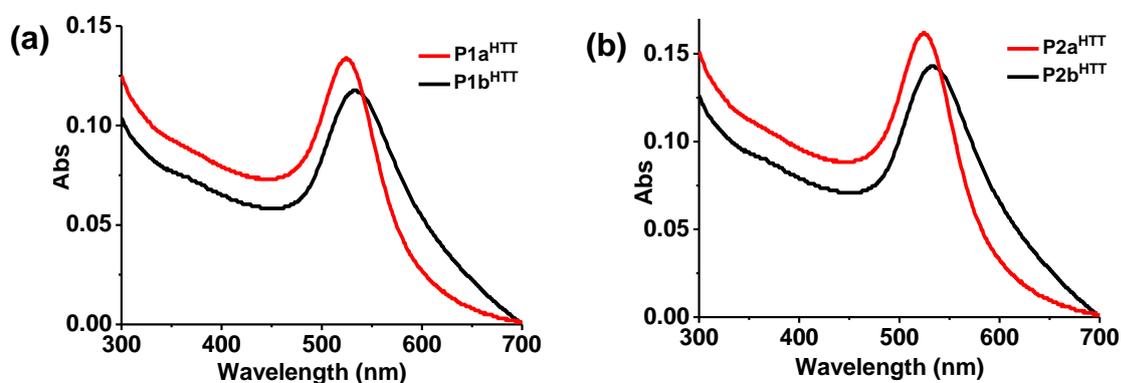


Figure 2. UV-Vis absorption spectra of conjugates prepared using probes (a) P1a^{HTT} and P1b^{HTT}, (b) P2a^{HTT} and P2b^{HTT}.

Probes in the tail-to-tail configuration were then prepared, starting with the P1a^{TTT} and P1b^{TTT} pair. Post DNA conjugation, expected red colour and a λ_{\max} of 525 nm was observed for colloid bearing the P1a^{TTT} probe (Figure 3a). However, despite several efforts to produce the conjugates of P1b^{TTT} under various conditions, stable colloids could not be produced. The AuNPs precipitated during the NaCl addition step, resulting in only minimal amounts of suspended conjugates, as evidenced by the minimal UV-Vis absorbance. An alternative design was then attempted using the same sequence that was complementary to the T1 target sequence, but now differently divided into each probe (referenced as P1c^{TTT} and

P1d^{TTT}; Table 1). This revised design led to the successful preparation of the DNA-AuNP conjugates with the expected optical properties (Figure 3b). Based on these results, probes P2a^{TTT} and P2b^{TTT} were designed for T2 (Table 1), and the prepared conjugates were found to show expected optical properties (Figure 3c).

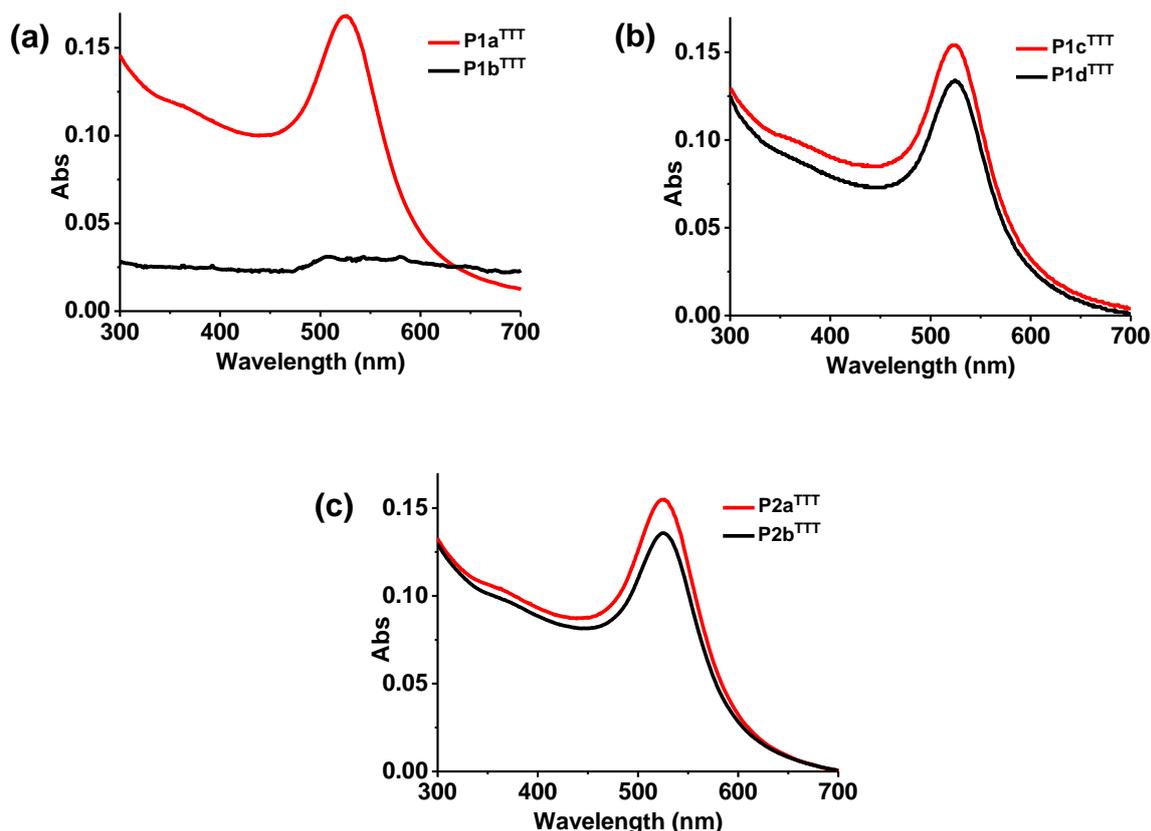


Figure 3. UV-Vis absorption spectra of conjugates prepared using probes (a) P1a^{TTT} and P1b^{TTT}, (b) P1c^{TTT} and P1d^{TTT} and (c) P2a^{TTT} and P2b^{TTT}.

These results suggest that to achieve stable conjugates, a minimum of 20 bases are required when DNA is conjugated to AuNP at 3' position of DNA, though the reason for this observation is unclear. The P1b^{TTT} probe has been previously used successfully in other sensor platforms,¹⁸ but the current result nevertheless shows that the cross-compatibility of DNA probes between different applications cannot be assumed. Thus, the probe pair

P1c^{TTT}/P1d^{TTT} complementary to T1 and P2a^{TTT}/P2b^{TTT} complementary to T2 were taken for further evaluation.

2.2. Hybridisation against single-stranded DNA

In preliminary optimisation experiments, the hybridisation assays were carried out with ssDNA sequences as the analyte, which was added to a mixture consisting of each pair of complementary DNA-AuNP probes. Three types of analytes were tested (Table 1): (i) the target DNA strand consisting of 35 bases fully complementary to their respective probes (i.e. either T1 or T2); (ii) DNA sequences containing a single base mismatch referred to as M1 and M2, but are otherwise identical to T1 or T2, respectively; (iii) “non-complementary” DNA consisting of a randomly generated sequence of identical length and similar GC content to T1 and T2 (termed NC). In all cases, a mixture of equivalent concentrations of DNA-AuNP conjugates were prepared (to an optical density, OD, of 0.7 @ 525 nm of each probe prior to mixing), to which the ssDNA analyte was added to give a final analyte concentration of 1 μ M (corresponding to 20 nmol of analyte in 200 μ L of the total assay mixture). In the negative control, only water was added as the analyte (henceforth referred to as “C” in all figures).

For the hybridisation experiments with the tail-to-tail configuration using probes P1c^{TTT} and P1d^{TTT} against T1, it was found that a hybridisation mixture with 1 M NaCl gave good hybridisation after 30 min. This result was evidenced by a visually observable colour change from a deep red to lilac, and was corroborated by the UV-Vis spectrum that exhibited a λ_{max} increase from 525 to 550 nm, a decrease in the absorbance at their respective λ_{max} (A_{max}) from 0.6 to 0.1 (an approximately 80 % reduction), and a broadening of the peak (Figure 4a & b). In contrast, a negative control experiment where only water was added, no significant change in colour or the UV-Vis spectrum was observed. Similarly, the NC

sequence gave no significant change also, which confirms that probe binding is sequence specific. In the case of the mismatched sequence M1, a result similar to T1 was observed, which indicates this probe pair was able to bind and cannot differentiate this single mismatch from the fully complementary sequence (i.e. this is a false positive result).

A similar experiment was performed for P2a^{TTT} and P2b^{TTT} probes, with T2 or M2. Both these analyte sequences gave colour and spectroscopic changes corresponding to a positive result similar to the above (Figure 4c & d). As before, negative controls consisting of the NC sequence or water only gave no change.

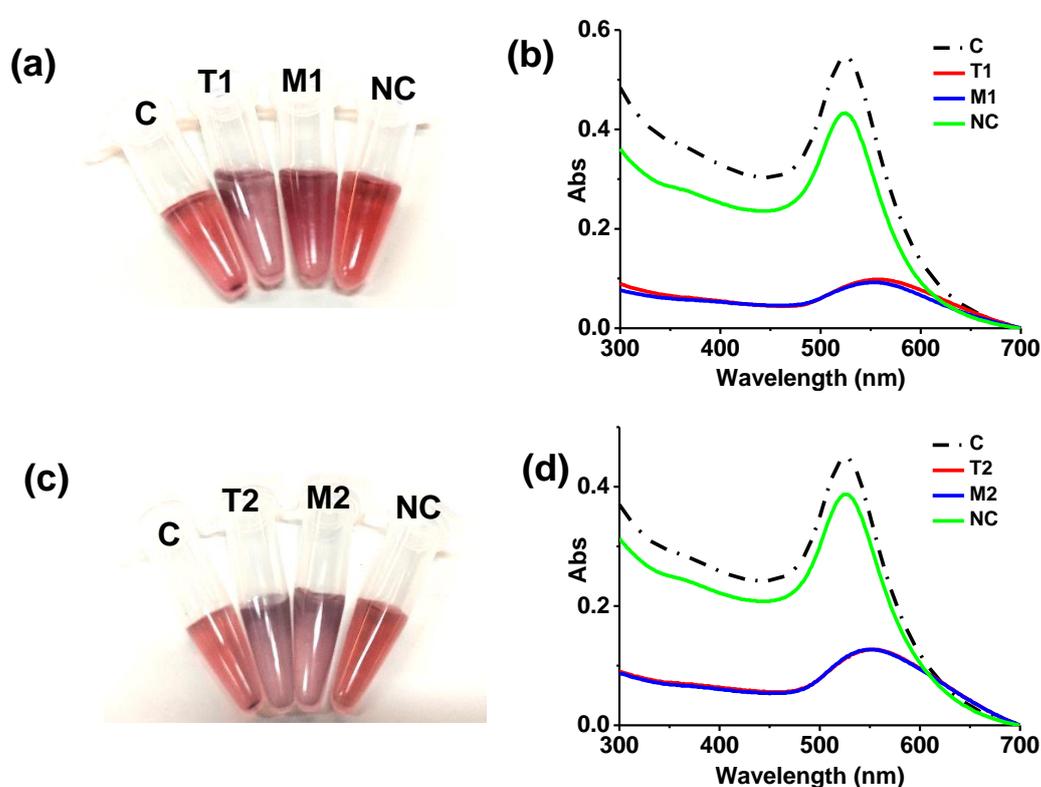


Figure 4. Photographs and UV-Vis spectra of the assay mixtures containing the DNA-AuNP conjugates and analyte for: (a & b) mixtures using P1c^{TTT} and P1d^{TTT} probes; and (c & d) mixtures using P2a^{TTT} and P2b^{TTT} probes. The photographs show each assay carried out at a 200 μ L total volume.

2.3. Response time of hybridisation assay

The rapidity of the assay was then investigated. Here, using probes P2a^{TTT} and P2b^{TTT} against T2, it was found that a change in λ_{\max} from 525 to 548 nm, and decrease in the A_{\max} from 0.7 to 0.2, occurred after 15 min (Figure 5 c), with essentially no further changes even after 45 min (Figure 5). This result thus shows that the 30 min incubation time previously used was, in fact, unnecessary, and thus all subsequent assays were analysed after 15 min.

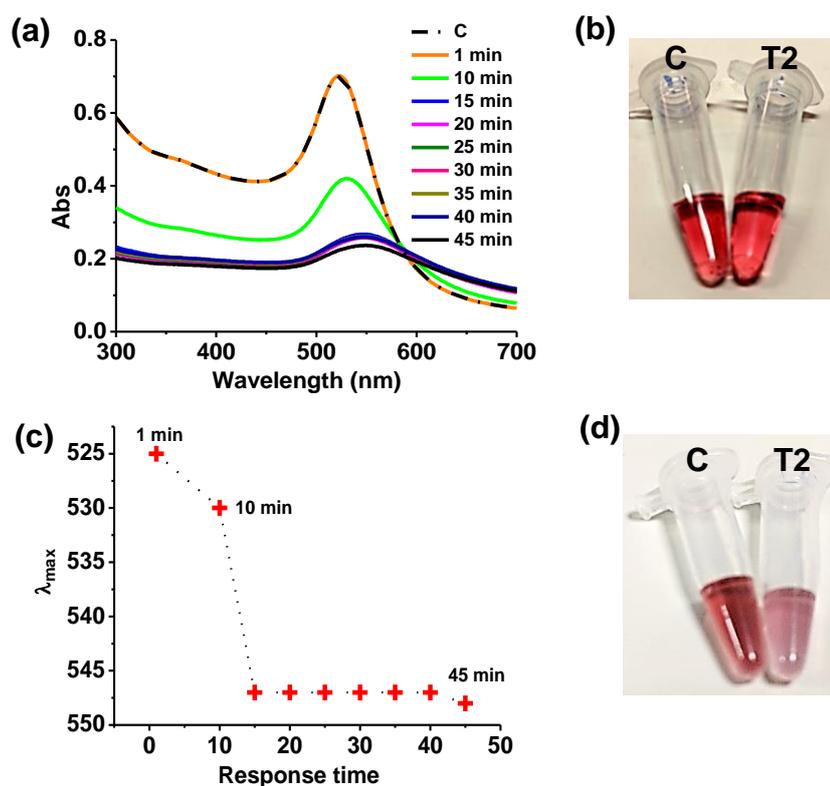


Figure 5. a) UV-Vis spectra of the assay mixtures containing the DNA-AuNP conjugates and target DNA analyte for P2a^{TTT} and P2b^{TTT} probes and c) change in λ_{\max} vs. response time. The photographs show C and T2 DNA analyte (20 nmol) assay post 1 min (b) and 15 min (d) cooling.

2.4. Assay sensitivity against single-stranded DNA analyte

Based on the above results, DNA analyte T2 and DNA-AuNP conjugates prepared using probes P2a^{TTT} and P2b^{TTT} were taken for further study. A series of assays were carried out by adding between 0.1 and 10 pmol of T2 to the conjugates to test its sensitivity. These results were compared with a positive control with 20 nmol and negative control with water as the analyte. It was found that a visually observable colour change was produced with as little as 0.2 pmol of T2 (Figure 6a), although the UV-Vis analysis showed that the λ_{\max} shift for this amount of analyte was smaller than for the higher amounts (10 nm vs 25 nm, Figure 6b). The UV-Vis spectrum corresponding to 0.1 pmol of T2 did show an ~ 50 % reduction in A_{\max} without an accompanying change of λ_{\max} , but this change could not be readily observed visually. Nevertheless, the visual transition overall is very sharp, spanning just one order of magnitude in the pmol – fmol range (i.e. 1.0 pmol was clearly positive, but 0.1 pmol was clearly negative).

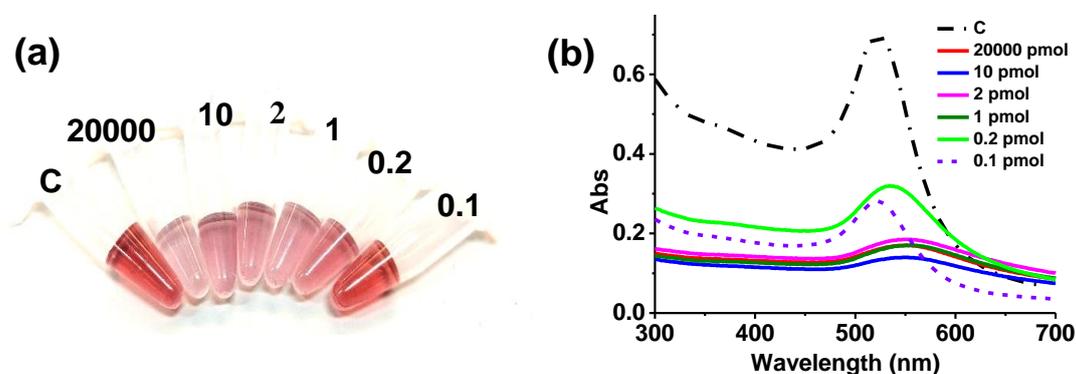


Figure 6. (a) Photograph and (b) UV-Vis spectra of the assay mixtures containing the DNA-AuNP conjugates P2a^{TTT} and P2b^{TTT} and varying amounts of T2. The photograph shows each assay carried out at a 200 μ L total volume, with the labels indicating the amount of T2 DNA added in pmol.

2.5. Effect of mismatch on the hybridisation efficiency of DNA analyte

As the results with the single-base mismatch M2 sequence also gave a positive result, the tolerance of the assay (with probes P2a^{TTT}/P2b^{TTT}) against other mismatched sequences was investigated. For this purpose, the assays were carried out with a series of analyte sequences bearing mismatches at multiple locations, referenced M2a to M2f (Table 1).

It was found that analyte sequence M2a, bearing three mismatches in series at the junction where the two probe sequences meet, also gave a false positive. This result was evidenced by a visually observable colour change and a UV-Vis λ_{\max} shift from 525 to 545 nm and a drop in A_{\max} similar to M2 (Figure 7). The sequence with five mismatches in series (M2b) gave only a minimal change in colour, which was confirmed spectroscopically by a small λ_{\max} increase to 531 nm and A_{\max} reduction of only 15 %. Since this change was not visually distinguishable from the negative controls (C in Figure 7 and NC in Figure 4d), it was judged to be a “negative” result.

In contrast to M2a, if three mismatches were individually distributed throughout the analyte sequence (M2c), this gave a “negative” visual appearance that was similar to M2b (Figure 7a). This observation was consistent with the UV-Vis spectrum showing a small λ_{\max} shift to 533 nm, although there was a significant decrease in the A_{\max} by 40 % (Figure 7b and d). The sequence M2d with five distributed mismatches was however clearly negative, with a visual and spectral result that was similar to M2b.

The inability of these DNA-AuNP conjugates to differentiate either one or three mismatches in series at the meeting point of the two probes (M2 and M2a) can be rationalised by the binding stability of the remaining complementary base pairs. It is known that DNA duplexes with more than ~ 12 complementary base pairs typically exhibit melting temperatures (T_m) well above 37 °C.²⁶ Since even three mismatches would allow both probes to bind with ≥ 14 serial base pairings, it would be expected that stable DNA hybridisation can

still be achieved, which translates to a positive result. In comparison, M2c with three distributed mismatches gave a negative result. However, it is notable that five mismatches in series (M2b) gave a negative result, even though it would still theoretically give > 12 base pairings in series. This result suggests that ≥ 14 serial base pairings are required in these conjugates, which could be due to the presence of AuNP hindering efficient hybridisation.

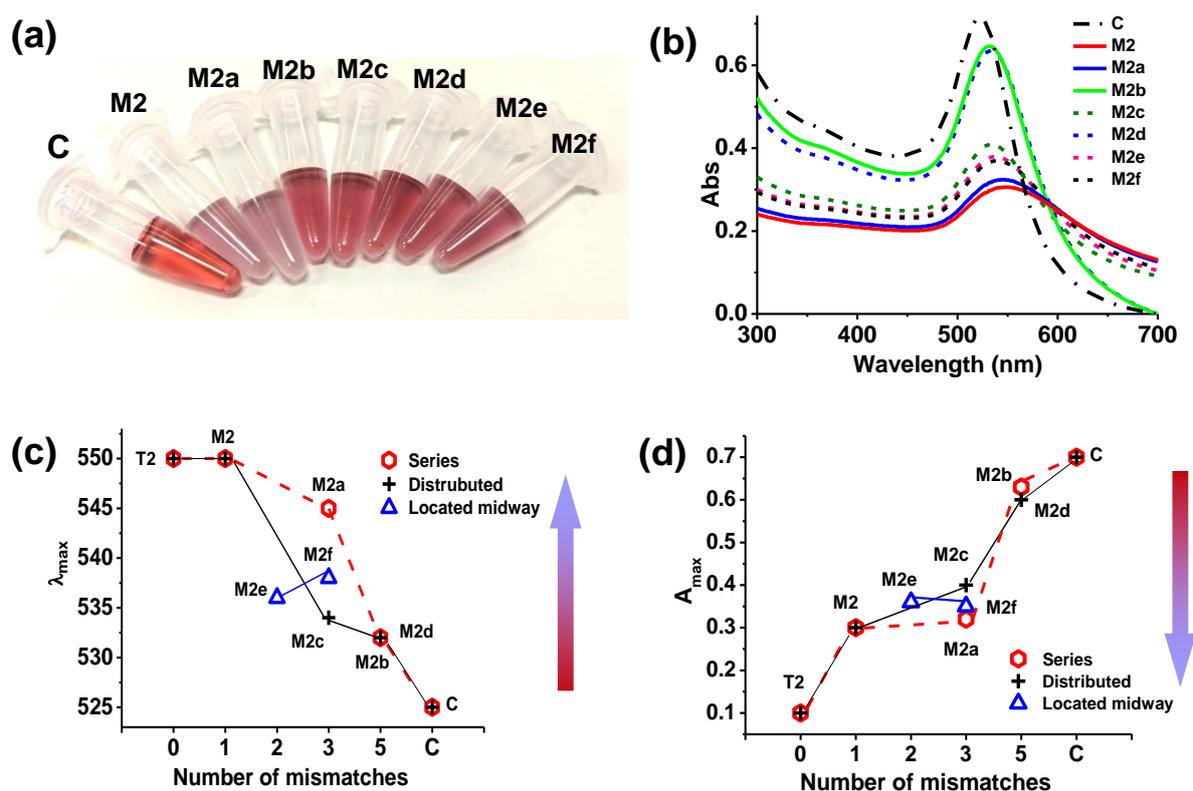


Figure 7. (a) Photograph and (b) UV-Vis spectra of the assay mixtures containing the DNA-AuNP conjugates P2a^{TTT} and P2b^{TTT} and analyte sequences M2a to M2f, as well as M2 for comparison. Graphs of UV-Vis λ_{\max} (c) or A_{\max} (d) against the number of base pair mismatches in the analyte sequences (the inset arrows show the visually observed colour change, and “C” indicates the control reaction where water is added as the analyte). The photograph shows each assay carried out at a 200 μ L total volume, and the amount of mismatched DNA was kept at 20 nmol for consistency with the results observed for M2 (in Section 3.2).

In the case of mismatches located midway within the sequence of each probe (M2e and M2f), these gave an ambiguous result that was mid-way between the positive and negative controls. An intermediate colour and intensity change were visually observed, consistent with the UV-Vis spectra that gave a λ_{max} shift to 538 and 536 nm (for M2e and M2f, respectively) and an A_{max} decrease of $\sim 48\%$ for both (Figure 7d). Thus, though these mismatches could not be differentiated by eye, they could still be identified spectroscopically.

2.6. Assays against double-stranded DNA analytes

As a step towards biologically relevant diagnostics, the assay was next tested against dsDNA fragments. Here, the experiments consisted of dsDNA with the T2 sequence (referred henceforth as T2') and nanoparticle conjugates bearing probes P2a^{TTT} and P2b^{TTT}. The assay procedure was also modified such that the analyte was heated to 95 °C to induce the denaturation of the dsDNA²⁷ and enable subsequent probe hybridisation upon cooling. It was however observed that in contrast to ssDNA (sub-section 3.3), a longer waiting period was needed for the assay to develop (30 vs. 15 min with ssDNA).

A range of analyte amounts was then tested from 0.2 pmol to 20 nmol (in a 200 μL final assay volume). In order to optimise the sensitivity, the concentration of NaCl in the assay mixtures were also varied (from 1 to 3 M)²⁸. It was found that a visually observable positive result could be achieved at 10, 1 and 1 pmol of T2', at all NaCl concentrations that were tested (Figure 8a, c & e). These observations were supported by UV-Vis spectra showing both the expected increases in λ_{max} and decreases in A_{max} , typically by 20–25 nm and 40–50 %, respectively; depending on the amount of analyte (Figure 8b, d & f). However, even the best results obtained here were still 5-fold less sensitive than the equivalent

experiment with ssDNA. This result is likely due to competitive rebinding of the analyte ssDNA to its complementary strand upon cooling.

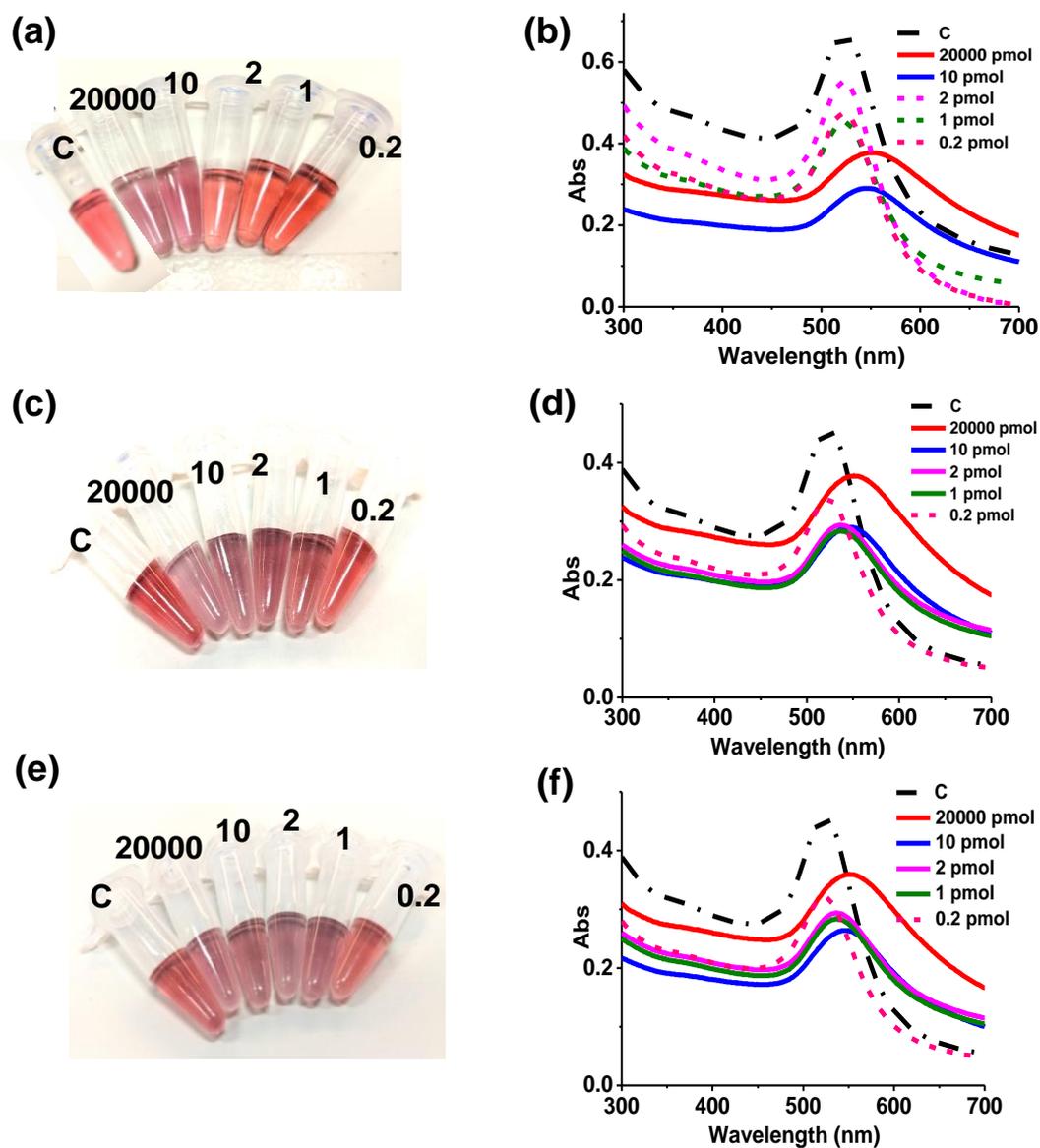


Figure 8. Photographs and UV-Vis spectra of the assay mixtures containing the DNA-AuNP conjugates P2a^{TTT} and P2b^{TTT} and varying amounts of T2', with (a & b) 1, (c & d) 2 M and (e & f) 3 M NaCl. The photographs show each assay carried out at a 200 μ L total volume, with the labels indicating the amount of T2' DNA added in pmol.

2.7. Selectivity in the presence of non-complementary DNA

In order to demonstrate the selectivity of the assay in the presence of a large amount of non-complementary genomic DNA that would be present in any potential biologically derived sample, the optimised dsDNA assay was then carried out in the presence of CT, as a model mixture of non-complementary dsDNA with a variety of lengths. Here, varying amounts of T2' dsDNA from 0.2 pmol to 20 nmol were mixed with 2.6 mg of CT and tested.

Similar to the isolated dsDNA, quantities of T2' as low as 2 pmol gave a visually observable result, even in the presence of a considerable amount of CT (Figure 9a). The UV-Vis spectra also showed the corresponding λ_{\max} increase from 525 to 540 nm, though the A_{\max} reduction, in this case, was more modest at 24 % (Figure 9b). Similar results were obtained regardless of the NaCl concentration used (between 1 and 3 M).

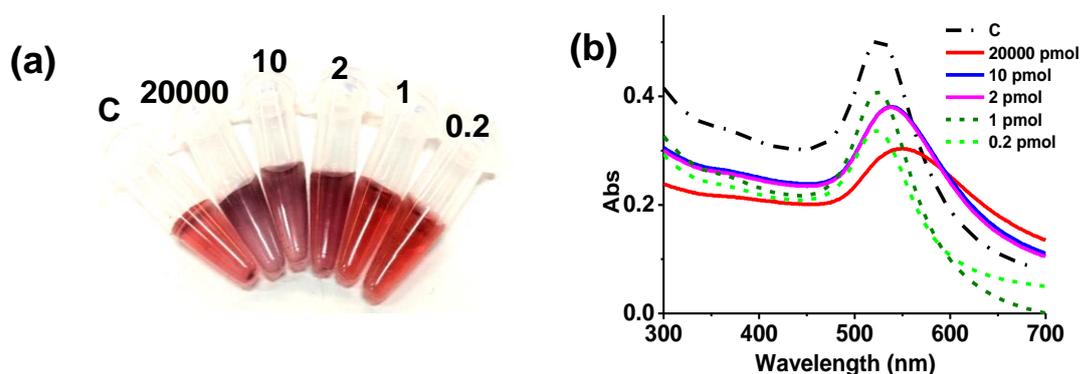


Figure 9. (a) Photograph and (b) UV-Vis spectra of the assay mixtures containing the DNA-AuNP conjugates P2a^{TTT} and P2b^{TTT} and varying concentrations of T2' and in the presence of CT with 1 M NaCl. The photograph shows each assay carried out at a 200 μ L total volume, with the labels indicating the amount of T2' DNA added in pmol.

3. Conclusion

In conclusion, we have demonstrated a label-free, sensitive and selective colorimetric DNA detection method that combines distance-dependent optical properties of AuNP with the hybridisation of complementary DNA for the detection of DNA fragments associated with *Ganoderma boninense* pathogen. A detailed survey was performed involving a range of analyte sequences and two assay configurations to detect single and double-stranded DNA. The target DNA can be easily be visually identified down to a concentration of 0.2 pmol ssDNA or 2 pmol dsDNA, respectively. The sensitivity limit was maintained for the dsDNA even in the presence of a large excess of non-complementary DNA. This protocol is simple to implement and is relatively rapid (within 30 min), without the need for sophisticated equipment and specialist training. Indeed, only the mixing of several solutions at room temperature and heating to a single temperature is required.

These results represent the first steps towards the development of a nanoparticle-based assay for practical application in tropical agriculture. To this end, there is significant scope further improvement of the assay performance. The reported results were carried out at a 200 μ L scale for the purposes of UV-Vis measurements. However, in a field application, scaling down the assay volumes by order of magnitude can be envisioned, and would still be sufficient for a visually observable result, while reducing the amount of analyte needed to the fmol range. The implementation of strand displacement strategies will also be able to achieve clearer differentiation between sequences bearing mismatches.²⁹⁻³¹

Conflicts of interest

There are no conflicts to declare.

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References

1. B. Nambiappan, A. Ismail, N. Hashim, N. Ismail, D. N. Shahari, N. A. N. Idris, N. Omar, K. M. Salleh, N. A. M. Hassan, and A. Kushairi, *J. Oil Palm Res.*, 2018, **30**, 13-25.
2. Y. W. Ho, and A. Nawaw, *Pertanika*, 1985, **8**, 425-428.
3. R. R. M. Paterson, *Crop Prot.*, 2007, **26**, 1369-1376.
4. C. Utomo, and F. Niepold, *J. Phytopathol.*, 2000, **148**, 507-514.
5. R. Hushiarian, N. A. Yusof, and S. W. Dutse, *SpringerPlus*, 2013, **2**, 555-566.
6. B. G. Ommelna, A N. Jennifer, and K. P. Chong, *J. Sustain. Sci. Manag.*, 2012, **7**, 186-192.
7. M. A. Markom, A. Y. Md Shakaff, A. H. Adom, M. N. Ahmad, W. Hidayat, A.H. Abdullah, and N. A. Fikri, *Comput. Electron. Agr.*, 2009, **66**, 140-146.
8. M. M. K. Najmie, K. Khalid, A. A Sidek, and M. A. Jusoh, *Meas. Sci. Rev.*, 2011, **11**, 160-164.
9. C. C. Lelong, J-M. Roger, S. Brégand, F. Dubertret, M. Lanore, N. Sitorus, D. Raharjo, and J-P. Caliman, *Sensors*, 2010, **10**, 734-747.
10. H. Santoso, T. Gunawan, R. H. Jatmiko, W. Darnosarkoro, and B. Minasny, *Pre. Cis Agric.*, 2011, **12**, 233-248.
11. P. Ahmadi, F. M. Muharam, K. Ahmad, S. Mansor, and I. A. Seman, *Plant Dis.*, 2017, **101**, 1009-1016.
12. I. C. Hashim, A. R. M. Shariff, S. K. Bejo, F. M. Muharam, and K. Ahmad, *IOP Conf. Ser.: Earth Environ. Sci.*, 2018, **169**, 012066-6.
13. T. W. Darmono, and A. Suharyanto, *Menara Perkebunan*, 1995, **63**, 15-22.
14. S. Shamala, D. Chris, O. Sioban, and A. Idris, *Malays. J. Microbiol.*, 2006, **2**, 30-34.
15. A. Z. Madihah, A. S. Idris, and A. R. Rafidah, *Afr. J. Biotechnol.*, 2014, **13**, 3455-3463.
16. F. S. Akanbi, N. A. Yusof, J. Abdullah, Y. Sulaiman, and R. Hushiarian, *Sensors*, 2017, **17**, 1538-14.

17. S. W. Dutse, N.A. Yusof, H. Ahmad, M. Z. Hussein, Z. Zainal, R. Hushiarian, and R. Hajian, *Anal. Lett.*, 2014, **47**, 819-832.
18. R. Hushiarian, Nor A. Yusof, A. H. Abdullah, S. A. A. Ahmad, and S. W. Dutse, *Anal. Chem. Res.*, 2015, **6**, 17-25.
19. R. Elghanian, J. J. Storhoff, R. C. Mucic, R. L. Letsinger, and C. A. Mirki, *Science*, 1997, **277**, 1078-1081.
20. J. J Storhoff, A. D. Lucas, V. Garimella, Y. P. Bao, and U. R. Müller, *Nat. Biotechnol.*, 2004, **22**, 883-7.
21. F. Li, H. Zhang, B. Dever, X. Li, and X. C. Le, *Bioconjugate Chem.*, 2013, **24**, 1790-1797.
22. K. Liang, S. Zhai, Z. Zhang, X. Fu, J. Shao, Z. Lin, B. Qiu, and G. N. Chen, *Analyst*, 2014, **139**, 4330-4334.
23. C. Chen, M. Luo, T. Ye, N. Li, X. Ji, and Z. He, *Analyst*, 2015, **140**, 4515-4520.
24. E. Rani, S. A. Mohshim, M. Z. Ahmad, R. Goodacre, S. A. A. Ahmad, and L. S. Wong, *Polymers*, 2019, **11**, 561-12.
25. A. Sakamoto, T. Mikawa, M. Murase, A. Tanaka, and R. Kuran, Patent: JP 2001314199-A 1, 2001.
26. D. de Bruin, N. Bossert, A. Aartsmarus, and D. Bouwmeester, *J. Nanobiotechnology*, 2018, **16**, 37-8.
27. X. Wang, H. J. Lim, and A. Son, *Environ. Health Toxicol.*, 2014, **29**, e2014007-8.
28. K. Sato, K. Hosokawa, and M. Maeda, *J. Am. Chem. Soc.*, 2003, **125**, 8102-8103.
29. X. Wang, M. Zou, H. Huang, Y. Ren, L. Li, X. Yang, and N. Li, *Biosens. Bioelectron.*, 2013, **41**, 569-575.
30. D. Yao, T. Song, X. Sun, S. Xiao, F. Huang, and H. Liang, *J. Am. Chem. Soc.*, 2015, **137**, 14107-14113.
31. T. Song, S. Xiao, D. Yao, F. Huang, M. Hu, and H. Liang, *Adv. Mater.*, 2014, **26**, 6181-5.