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Short communication

Nanoparticle based DNA biosensor for tuberculosis detection using thermophilic helicase-dependent isothermal amplification

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ABSTRACT

The present study describes the development of a DNA based biosensor to detect *Mycobacterium tuberculosis* using thermophilic helicase-dependent isothermal amplification (tHDA) and dextrin coated gold nanoparticles (AuNPs) as electrochemical reporter. The biosensor is composed of gold nanoparticles (AuNPs) and amine-terminated magnetic particles (MPs) each functionalized with a different DNA probe that specifically hybridize with opposite ends of a fragment within the *IS6110* gene, which is *M. tuberculosis* complex (MTC) specific. After hybridization, the formed complex (MP-target-AuNP) is magnetically separated from the solution and the AuNPs are electrochemically detected on a screen printed carbon electrode (SPCE) chip. The obtained detection limit is 0.01 ng/μl of isothermally amplified target (105 bp). This biosensor system can be potentially implemented in peripheral laboratories with the use of a portable, handheld potentiostat.

1. Introduction

Tuberculosis (TB) is the world's second deadliest infectious disease (1.7 million people die annually) and it has been identified as a leading cause of death among HIV-positive patients (WHO, 2009). The standard test for TB diagnosis is smear sputum microscopy, which is unable to identify half of the positive TB infections. Smear-negative TB disease is highly common in HIV co-infected patients (Perkins et al., 2006). Among the novel detection methodologies are the nucleic acid amplification tests (NAATs). These have been extensively explored for the rapid detection of *Mycobacterium tuberculosis*. NAATs have high specificity and sensitivity, and can provide same day results (detection time: from 2 to 8 h on processed specimens). However, NAATs require highly specialized personnel, expensive equipment, and are used only in proficient laboratories that can afford reference reagents to monitor the assay performance. Therefore, they are suitable for reference and peripheral laboratory implementation, but difficult to use in resource-constrained settings (Palomino, 2005).

Isothermal amplification techniques have been recently developed as an alternative to polymerase chain reaction (PCR) for target DNA amplification and detection without the use of a thermocycler (Gill and Ghaemi, 2008). Thermophilic helicase-dependent isothermal amplification (tHDA) utilizes a thermostable UvrD helicase to unwind the double stranded DNA (dsDNA) and generate single stranded templates that are used for further polymerase amplification (Lixin et al., 2005). The dsDNA separation and amplification are performed at the same temperature (60–65 °C) which makes this technique suitable for development of point-of-care microbial detection systems, since a thermocycler is not required for DNA denaturation (95 °C) and amplification (Jeong et al., 2009). tHDA protocols have been developed for the detection of several pathogens including: *Helicobacter pylori* (Gill et al., 2008), *Clostridium difficile* (Chow et al., 2008), *Staphylococcus aureus* (Goldmeyer et al., 2008), *Neisseria gonorrhoeae* (Lixin et al., 2005). tHDA has also been used in microfluidic chips with integrated sample preparation and amplification to detect *Escherichia coli* (Mahalanabis et al., 2010) and for the multiplex detection of *S. aureus* and *N. gonorrhoeae* using a microarray onchip-amplification approach (Andresen et al., 2009).

Recently, nanomaterials have been introduced to enhance molecular detection performance. Nanoparticles have been widely used over the last decade in the development of new diagnostic devices, especially quantum dots (QDs) and gold nanoparticles (AuNPs), (Azzazy et al., 2006). Sensitivity enhancement has been achieved with the use of nanoparticles as tags or electrochemical labels. One of the most explored applications of AuNPs in clinical diagnostics is the use of AuNP probes (AuNP-ssDNA probe) for the detection of DNA targets. The unique tunable physicochemical properties of AuNPs, plus their good biological compatibility, conducting capability, and high surface-to-volume ratio make them ideal candidates for electronic signal transduction of biological

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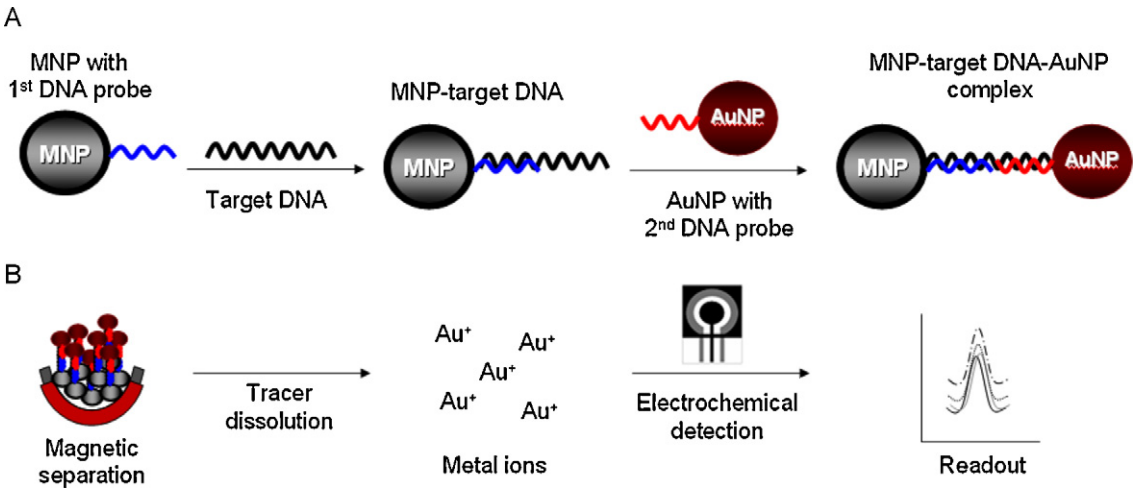

Fig. 1. Schematic of the target DNA detection system: (A) formation of complex target sandwich (MP-target DNA-AuNP). (B) Magnetic separation, metallic tracer (Au^{3+}) dissolution and electrochemical detection.

recognition events in DNA-based electrochemical biosensing platforms (Guo and Wang, 2007).

Gold nanoparticles are commonly synthesized using the Turkevich method (Turkevich et al., 1951). Recently, a green alkaline based AuNPs synthesis procedure was developed in our laboratory using a linear oligosaccharide (dextrin) as a coating agent. The bio-compatible AuNPs were successfully functionalized with DNA probes using a citrate ligand exchange methodology (Anderson et al., 2010). Here we describe the development of a portable, nanoparticle, DNA-based biosensor to detect *M. tuberculosis* using dextrin coated AuNPs as electrochemical labels and tHDA for the DNA target amplification.

2. Materials and methods

2.1. Nanoparticle synthesis and characterization

The gold nanoparticles were synthesized following an alkaline based methodology recently developed in our laboratory using dextrin as coating agent (Anderson et al., 2010). A 50 mL solution of 2.5 g/L dextrin and 2 mM gold chloride ($HAuCl_4$) was adjusted to pH 9 using 10% sodium carbonate and incubated at 50 °C with agitation in the dark for 8 h. After the reaction was completed, the solution changed from pallid yellow to dark red. The formed AuNPs (average size: 12.5 nm) were characterized using UV–vis spectrophotometry (UV–VIS–NIR; Shimadzu) and transmission electron microscopy (TEM) (JEOL 100CX). The amine coated magnetic particles (average size: 1 μm) used in this study were commercially available (Sigma–Aldrich Cat No. I7643-5ML).

2.2. tHDA primers and hybridization probes

The tHDA primers were designed to specifically amplify a fragment of the *IS6110* gene which is tuberculosis (TB) complex specific (forward primer: 5′ GAG CGT AGG CGT CGG TGA CAA AGG 3′ and reverse primer: 5′ GCT TCG GAC CAC CAG CAC CTA ACC 3′ GenBank: AJ242908.1). Specific fragments within the sequence (130–500 bp) have been widely used for PCR protocols targeting *M. tuberculosis* complex (MTC) species (Dalovisio et al., 1996; Fernandez et al., 2009). The tHDA primers were designed considering the optimal conditions for isothermal amplification (IsoAmp II Universal tHDA kit, biohelix). The obtained tHDA product (amplicon) was used as a template for the hybridization assay. Two different DNA probes (capture probe-MP: 5′-ss-AAA AAA AAA AAA GAG CGT AGG CGT CGG TGA 3′ and reporter probe-AuNP: GTG CTG GTG GTC CGA AGC AAA AAA AAA AAA-ss-3′) were also designed to specifically hybridize with the fragment generated by the tHDA reaction. All oligonucleotides were evaluated for specificity using the primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers and probes were designed using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and purchased from IDT (Integrated DNA Technologies).

2.3. Nanoparticle functionalization

The dextrin coated AuNPs (~12.5 nm in diameter) synthesized using the alkaline procedure were functionalized with a thiolated probe (TB 3′thiol) following a common methodology applicable for citrate coated AuNPs ligand exchange (Anderson et al., 2010; Hill and Mirkin, 2006). Briefly, the thiolated DNA probe (5 nmol) was reduced with DTT and purified using a Sephadex column (Nap-5, GE Healthcare). One milliliter of the dextrin coated AuNPs (AU = 2 at λ520 nm) was mixed with the purified probe solution. The dextrin molecules coating the surface of the nanoparticles were exchanged for the thiolated DNA probe over a series of salting steps. After ligand exchange the particles were stored under refrigeration until use.

The amine coated MPs (~1 μm in diameter) were functionalized with a secondary thiolated DNA probe (TB 5′thiol) using sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate (sulfo-SMCC) as cross linker (Zhang et al., 2009). Briefly, the amine coated MNPs (10 mg) were conjugated with sulfo-SMCC and incubated with the DTT reduced and purified thiolated DNA probe (10 nmol). After functionalization, sulfo-NHS acetate was used to block the unreacted linker groups on the MP surface. After passivation, the particles were washed and stored under refrigeration until use.

2.4. Helicase dependent isothermal amplification (tHDA)

As a proof-of-concept, a synthetic target ssDNA containing the same oligonucleotide sequence of the *IS6110* fragment (190 bp) was used (IDT Technologies). In order to evaluate the primers designed for the isothermal amplification, a commercially available kit for isothermal amplification was used (IsoAmp II Universal tHDA kit, biohelix). The amplification was conducted following the tHDA conditions for one-step 50 μl tHDA reaction (1× annealing buffer II, 4 mM $MgSO_4$, 40 mM NaCl, 3.5 μl dNPT solution, 1 ng synthetic target, 75 nM primer, 3.5 μl enzyme mix). The reaction was covered

tHDA Parameter	TB tHDA (*IS6110* fragment)	Optimal
Amplicon size	105 pb	80-120 bp
Amplicon Tm	74°C	68-75°C
Amplicon GC%	63%*	40%
Primer size	24 b	24-33 bases
Primer Tm	71°C	60-74°C
Primer GC%	62%*	35-60%

*The specific region selected within the I*S6110* has high CG content.

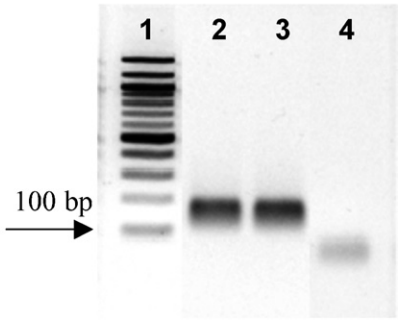

Fig. 2. tHDA parameters obtained for the specific isothermal amplification of a *IS6110* fragment and agarose gel electrophoresis of tHDA products using individual reactants—lane 1: 100 bp ladder. Lanes 2 and 3: tHDA amplification fragment using 5 ng of synthetic target. Lane 4: blank.

with mineral oil (50 μl) to avoid evaporation. The isothermal amplification reaction was also optimized using individual reactants: 1× thermo pol II buffer, 4 mM $MgSO_4$, 3 mM dATP, 200 μM dNPTs, 20 U Bst DNA polymerase large fragment, 100 ng thermostable helicase, 1 ng synthetic target DNA, 75 nM forward primer (105-F), 75 nM reverse primer (105-F). Both enzymes were purchased from New England Biolabs. The total reaction volume was 50 μl and same volume of mineral oil was added to avoid evaporation (Lixin et al., 2005; Vincent et al., 2004). Both reactions were incubated in a regular heating block for 90 min at 65 °C (Eppendorf Thermomixer R). After the reaction, the product was purified using a silica membrane column (Miniellute, Qiagen) to eliminate the remaining primers. The purified product was serially diluted for the hybridization assay.

2.5. Hybridization assay and electrochemical detection

The product obtained from the tHDA (10–0.01 ng/μL) was denaturated at 95 °C for 10 min and allowed to hybridize with the capture DNA probe (TB 3′thiol) on the MPs (0.8 mg) for 45 min at 44.5 °C with continuous rotation to form a MP-target DNA complex. After magnetic separation and several washing steps to eliminate the unreacted target, AuNPs (40 μL) labeled with the reporter probe (TB 5′thiol) were added and incubated for 2 h at 44.5 °C with continuous rotation to form a hybridized sandwich complex consisting of MP-target DNA-AuNP (Zhang et al., 2010). The sandwich complex was pulled and separated with a magnet. After several washing steps to eliminate the non-hybridized AuNPs, the complex was resuspended in 50 μL of water and transferred to a SPCE electrode (Gwent Electronic Materials, Ltd.) (Fig. 1). The SPCE is composed of working (carbon) and reference (silver/silver chloride) electrodes. The solution was allowed to dry onto the carbon electrode for 30 min, and then 50 μL of 1 M HCl solution was added to dissolve the AuNPs and generate Au^{3+} ions. A constant 1.25 V was applied to the electrode for 2 min to oxidize the gold ions (potentiostat/galvanostat 263A and PowerSuite software, Princeton Applied Research). Differential pulse voltammetry (DPV) was performed from 1.25 V to 0.0 V (with a step potential of 10 mV, modulation amplitude of 50 mV, and scan rate of 33.5 mV/s) to generate the voltammogram produced by the reduced gold ions on the SPCE (Pumera et al., 2005).

3. Results and discussion

3.1. Nanoparticle synthesis, characterization and functionalization

The dextrin coated AuNPs were characterized using UV–vis spectra and TEM. Absorbance peaks were obtained at 520 nm which is characteristic of the AuNPs production. The average particle diameter (12.4 nm) the TEM images (supplementary data). We have demonstrated the functionalization capabilities of the dextrin AuNPs with DNA probes in a previous paper (Anderson et al., 2010) using fluorescence labeling to confirm DNA probe attachment. In this study, two different particle sizes were evaluated for ligand exchange capability (12.4 nm and 10.4 nm). Both

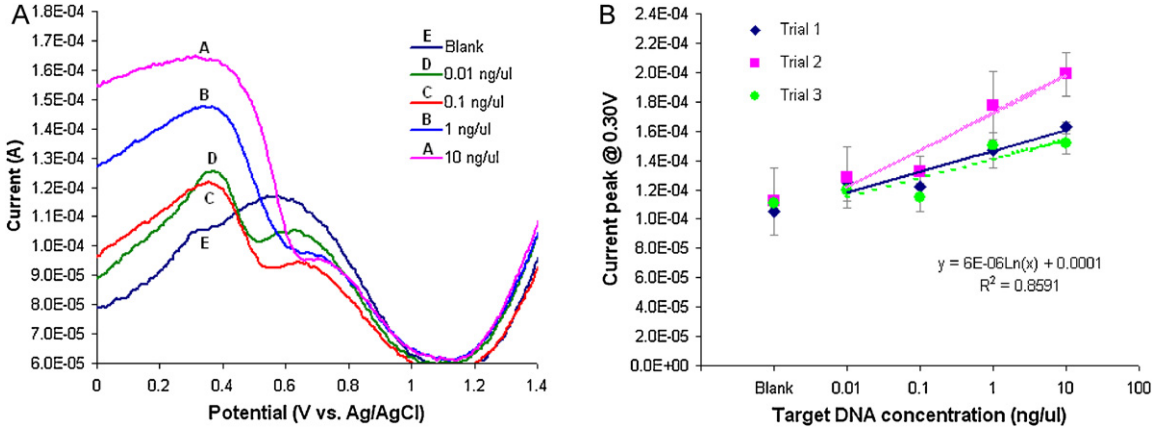

Fig. 3. (A) Mean DPV response of sandwich complex (MNPs-target DNA-AuNPs) after hybridization for different DNA target concentrations on SPCE. Each concentration was run in triplicates. The gold reduction peak was observed between 0.30 and 0.35 V DPV scan from +1.25 V to 0 V, step potential at 10 mV, modulation amplitude at 50 mV, and scan rate at 50 mV/S. (B) Logarithmic correlation between the target DNA concentration (ng/μl) and the gold reduction peak for three different trials. Each concentration was run in triplicates. The displayed equation correspond to trial 1, which is the same trial represented in the DPV response plot (A).

particles where successfully functionalized using a thiol-DNA-6-FAM oligonucleotides following the same capping ligand exchange technique used to functionalize citrate-generated AuNPs with DNA probes (Hill and Mirkin, 2006). The novel dextrin AuNPs have the same functionalization efficiency as the citrate coated AuNPs using the same ligand exchange procedure that is commonly used for oligonucleotide AuNP surface coating (Anderson et al., 2010). Therefore, the dextrin coated AuNPs can be used as an alternative to citrate AuNPs for DNA applications. The study presented in this paper is the first application of dextrin coated AuNP into a biosensor platform.

3.2. Helicase dependent isothermal amplification (tHDA)

Successful amplification was obtained using the designed tHDA primers, synthetic target DNA and the tHDA kit following the conditions for one-step reaction (90 min of incubation at 65 °C) (IsoAmp II Universal tHDA kit from biohelix). After the primer evaluation with the commercially available kit, the isothermal amplification reaction was also optimized using individual reactants. Fig. 2 shows the parameters used for the tHDA protocol design for the isothermal specific amplification of a *IS6110* fragment and successful amplification products (bands) using individual reactants from agarose gel electrophoresis. This is the first report that successfully uses the helicase isothermal amplification technique (tHDA) instead of PCR for a tuberculosis detection application in a DNA based biosensor platform.

3.3. Hybridization assay and electrochemical detection of AuNPs

Direct oxidation of AuNPs onto the carbon electrode surface was optimized at 1.25 V for 2 min obtaining a reduction peak of gold ions between 0.30 and 0.35 V. Fig. 3 shows the DPV response obtained with different DNA concentrations after hybridization, sandwich complex formation (MP-target-AuNP), magnetic separation and AuNPs dissolution. After the gold was dissolved and oxidized by the acidic solution, the gold ions were reduced between 0.30 and 0.35 V on the SPCE (Fig. 3A). Three separate trials were run using four different DNA concentrations (0.01–10 ng/μl) plus a blank with no target DNA. Each sample was run in triplicates. A linear log-correlation was observed between the target DNA concentration and the gold reduction peak (Fig. 3B). The presence or absence of the target was identified with a detection limit of 0.01 ng/μl. Variability in the peak height was observed in different trials between the two lowest concentrations detected (0.1 and 0.01 ng/μl). This variability may be the result of inter particle distances. At low concentrations, the particles are more dispersed and have more accessible surface area to interact with the acidic solution producing slightly higher reduction peaks. Therefore, the semi-quantitative detection limit obtained using synthetic targets was 0.1 ng/μl.

These results represent the first application of dextrin AuNPs as electrochemical labels and the use of tHDA amplification products as DNA targets. In order to evaluate the biosensor functionality, citrate AuNPs were used during initial runs with PCR products as targets for the hybridization reaction (data not shown). No difference was observed when using dextrin AuNPs in place of citrate AuNPs as electrochemical labels. This was expected since there is no remaining capping agent, dextrin or citrate, on the surface of the AuNP after the oligonucleotide functionalization procedure. The particle's coating molecules are exchanged for thiolated DNA probes and the coating material is liberated to the suspension liquid. After functionalization, the particles are centrifuged and washed to eliminate the supernatant with unreacted materials. Since the elemental gold that is reduced in the electrochemical detection is the same for citrate and dextrin coated AuNPs, there is no effect of the coating material in the detection system.

4. Conclusion

The present study describes the development and optimization of a nanoparticle DNA-based biosensor to detect a tuberculosis specific DNA fragment using the electrochemical detection of gold nanoparticles. In order to make the platform more suitable for resource-constrained setting diagnostics, an isothermal amplification reaction was optimized in lieu of PCR which requires a thermocycler. The isothermal helicase dependent amplification was successfully optimized using individual reactants and a regular heating block, which can be replaced with a water bath. The detection time with the proposed platform, including amplification and hybridization is 6 h; without considering sample preparation (DNA extraction). The main advantages of the proposed platform are that the detection system is not expensive, it can be portable and there is no need of a thermocycler due to the isothermal amplification.

The platform uses dextrin coated AuNPs and, to the best of our knowledge, this is the first study that reports the application of these alkaline synthesized particles as electrochemical labels. No difference was observed when using dextrin or citrate AuNPs as electrochemical labels in the detection system; nevertheless, the main advantage of using dextrin coated AuNPs is the synthesis. The particles are produced under alkaline conditions (pH 9) and can be synthesized at room temperature. This opens the possibility for future explorations of bio-molecule (DNA probes) functionalization during synthesis. The ligand exchange method traditionally used to functionalize citrate AuNPs with DNA probes requires 72 h post-synthesis (Hill and Mirkin, 2006).

Due to the main focus of the present study (proof-of-concept) synthetic targets were used to develop and optimize the detection system. Further studies of sensitivity and specificity using bacterial DNA and biological matrices (e.g. sputum) are necessary in order to address the main issues and constraints of sample preparation (DNA extraction); and optimize the platform using real samples. The designed platform can potentially be implemented in peripheral laboratories for the detection of TB using a handheld potentiostat and a portable PC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.055.

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