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Handheld isothermal amplification and electrochemical detection of DNA in resource-limited settings

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ABSTRACT

This paper demonstrates a new method for electrochemical detection of specific sequences of DNA present in trace amounts in serum or blood. This method is designed for use at the point-of-care (particularly in resource-limited settings). By combining recombinase polymerase amplification (RPA)— an isothermal alternative to the polymerase chain reaction — with an electroactive mediator, this electrochemical methodology enables accurate detection of DNA in the field using a low-cost, portable electrochemical analyzer (specifically designed for this type of analysis). This handheld device has four attributes: (1) It uses disposable, paper-based strips that incorporate screen-printed carbon electrodes; (2) It accomplishes thermoregulation with ± 0.1 °C temperature accuracy; (3) It enables electrochemical detection using a variety of pulse sequences, including square-wave and cyclic voltammetry, and coulometry; (4) It is operationally simple to use. Detection of genomic DNA from *Mycobacterium smegmatis* (a surrogate for *M. tuberculosis*—the main cause of tuberculosis), and from *M. tuberculosis* itself down to ~0.040 ng/μL provides a proof-of-concept for the applicability of this method of screening for disease using molecular diagnostics. With minor modifications to the reagents, this method will also enable field monitoring of food and water quality.

Introduction

Analysis of nucleic acids is an increasingly widely used approach to a range of biomedical analyses including pre-symptomatic detection of infectious diseases [1], examination of perinatal genetics and detection of inherited genetic disorders [2], and speciation of pathogens in samples of food and water [3]. These analyses typically require sophisticated procedures and expensive equipment, operated and maintained by trained personnel. They are confined to centralized laboratories, and are generally of limited usefulness at the point-of-care (PoC), at remote sites, and in resource-limited settings. If made inexpensive and portable, however, analyses of nucleic acids could be particularly useful where central laboratories are inaccessible.

The objective of this work was to simplify two of the most challenging steps required for electrochemical detection at the PoC: i) amplification of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequences of interest and ii) detection of nucleic acids. In particular, i) we show that coupling isothermal amplification of DNA with electrochemical readout enables accurate and rapid detection of DNA, and ii) we demonstrate an affordable, handheld device that uses disposable, paper-based test strips and screen-printed electrodes (SPEs) that can detect DNA after amplification.

Analysis of nucleic acids requires amplification because the DNA in many biological samples (e.g., in pre-symptomatic patients) is present only at such low concentrations that it cannot be detected directly [4]. Polymerase chain reaction (PCR) [5] is the most common approach for amplification of DNA, but it requires precise control of the sequence of thermal steps it uses [6], at a high degree of temperature stability. This characteristic has so far made PCR unsuitable for use in low-cost, portable detectors. However, an open-source, lab-based thermocycler is available for $649 (OpenPCR™) and a $130 (cost of goods) portable detector was recently reported [7].

Abbreviations: CV, Cyclic voltammetry; DNA, deoxyribonucleic acid; dsDNA, double-stranded DNA; HDA, helicase dependent amplification; LAMP, loop-mediated isothermal amplification; NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction; PID, proportional-integral-derivative; RPA, recombinase polymerase amplification; SPE, screen-printed electrodes; SWV, square-wave voltammetry; TB, tuberculosis; uMED, universal mobile electrochemical detector; uMED^{NA}, universal mobile electrochemical detector for nucleic acids

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Isothermal alternatives to PCR eliminate thermocycling and therefore simplify the required instrumentation [8]. Albeit, isothermal methods are less established than PCR, and use expensive, and often proprietary reagents. We have chosen to use recombinase polymerase amplification (RPA) because it operates satisfactorily over a convenient temperature range (39–45 °C), is insensitive to temperature variations of ± 1 °C, and is rapid (10–15 min) [9]. These characteristics make RPA an attractive candidate for applications that target resource-limited environments [10].

Amplification of the initially low concentration of target DNA produces a high concentration of amplicon (i.e., replicated DNA) that can be quantified with conventional analytical techniques at the end of the reaction (end-point measurement). To enable accurate quantification during the amplification (real-time measurement), the most common methods of detection use fluorimetry and rely on the indirect measurement of fluorescent molecules that interact with DNA [11]. Sequence-non-specific fluorescent molecules (e.g., SYBR Green I) [12] intercalate with double-stranded DNA (dsDNA) without any dependence on the amplicon sequence. Upon binding to the minor groove of any dsDNA sequence, the intercalators produce an increase in fluorescence; this increase rises as the reaction progresses and more dsDNA is synthesized. The kinetics of this reaction depend on the initial amount of target DNA. Although accurate, fluorimetric methods still need development to be rugged, affordable in resource-limited operations, and portable.

Although less developed as a method for DNA-based applications than other methods, detection of nucleic acids based on electrochemistry [13] offers an attractive alternative to optical methods because the required instrumentation can be implemented easily with small components that require little energy [14]. Electrochemical methods are also independent of background light, and insensitive to color in the sample, although they are sensitive to electrochemically active samples. The electrochemical approach may directly measure the native electrochemistry of DNA, or (in a process conceptually similar to the optical alternative) use molecules that interact with dsDNA. We have used “electrophores” that are detectable by cyclic voltammetry (CV) and square-wave voltammetry (SWV) when they are complexed with DNA [15].

Electrophores can interact specifically or non-specifically with DNA, with and without amplification of the target sequence [16]. Transition metal complexes (e.g. osmium or ruthenium complexes) and organic dyes (e.g. methylene blue) are two types of non-specific electroactive mediators for DNA detection [17]. Deféver et al. employed Os $[(bpy)_2phen]^{2+}$ to detect cDNA non-specifically after amplification using PCR [18]. They also used methylene blue as an electroactive mediator for real-timePCR of *Chlamydia trachomatis* [18]. Ahmed et al. used $[Ru(NH_3)_6]^{3+}$ for real-time detection using benchtop LAMP of genomic DNA from *Staphylococcus aureus* in solution [19]. In all of these reports, however, experiments were performed in bulk solutions, with a benchtop thermocycler for thermal control, and a benchtop electrochemical analyzer for detection. Cunningham et al. [20] and others [21] have used electrochemical paper-based devices for sequence-specific detection of pure DNA without amplification, using aptamers or other ssDNA probes, as recently reviewed [22]. The only integrated platform for DNA amplification combined with electrochemical detection was developed by Atlas Genetics Ltd. (Trowbridge, UK), and uses ultra-fast PCR and ferrocene-labelled, sequence-specific probes for *C. trachomatis* and *Neisseria gonorrhoeae*. [23] This instrument, however, is not yet commercially available, and has been developed in a benchtop format that is incompatible with PoC use.

This paper describes the first (to our knowledge), fully integrated, PoC device that combines isothermal DNA amplification with electrochemical detection on paper. Nucleic amplification on paper has been shown using methods other than electrochemistry [24]. We implemented the RPA reaction (Fig. SI 1) with $[Ru(NH_3)_6]^{3+}$ as an electroactive mediator for the electrochemical detection of DNA (Fig. 1a), and demonstrated electrochemical detection coupled with RPA on the same device. Fig. SI 2 shows the redox couple for $[Ru(NH_3)_6]^{3+}$. When the ruthenium complex binds to dsDNA, the amount of free $[Ru(NH_3)_6]^{3+}$ drops in proportion to the amount of DNA present in the initial sample; this drop produces a decrease in the cathodic current that can be measured voltametrically. We chose electrochemical detection (specifically SWV) because of it is rapid, sensitive and compatible with inexpensive electrochemical analyzers, and thus for PoC use [25]. Here we have used a handheld, electrochemical unit that builds on the universal mobile electrochemical detector (uMED), that we described in a previous publication [26]. Electrochemistry is particularly attractive for samples in which elaborate purification is required, because it is insensitive to color or particulates in the sample, and because it allows easy electronic transfer of data generated by the assay, or derived from it.

To allow tests to be performed at the PoC or point-of-use, we developed disposable, paper-based test strips that mate with an advanced version of the uMED (which we will call from here on, “*uMED*^{NA}”, NA stands for “nucleic acid”). This uMED^{NA} provides components and algorithms to control the amplification of DNA, and to perform electrochemical detection, both automatically (Fig. 1b, Figs. SI 3 and SI 4). The use of paper-based test strips enabled us to reduce the sample volume, relative to the bulk reaction, and therefore to reduce both the cost of the reagents and the volumes of blood required. By implementing closed-loop circuitry and software for thermoregulation, we enabled the uMED^{NA} to set and stabilize the temperature of the RPA reaction on the paper-based test strips to ± 0.1 °C. The uMED^{NA} provides full capabilities for electrochemical detection using a variety of pulse sequences (particularly, here CV and SWV), on-screen readout, and transfer of the acquired data to “the cloud” by communicating through audio, USB, or Bluetooth. We, and others, have recently shown examples of readers with cloud connectivity for data acquisition of PoC test results [26,27]. Yang et al. and Steinhubl et al. have thoroughly reviewed this emerging field of mobile health (mHealth) technologies [28].

We demonstrate the performance of our system for detection of *Mycobacterium smegmatis* as a simulant for tuberculosis (TB). TB is the third most lethal infectious disease globally, in estimated number of deaths after diarrheal diseases and HIV/AIDS. It is caused by a single agent, *Mycobacterium tuberculosis* [29]. Present assays available for TB PoC diagnosis commonly use nucleic acid or immunological analytical methods [30]. The key challenges to developing an integrated system are to simplify the instrumentation and procedures needed for performing the complicated and operationally difficult (and dangerous) steps required for the collection, processing and presentation of real samples to the analytical device [31]. These steps include obtaining sputum from the patient, concentrating *M. tuberculosis* cells, and lysis of these cells; purification and extraction of DNA; and amplification and detection of DNA. In this paper, we have focused on the last two steps of the process, as performed in bench-top real-time instruments, but acknowledge that the first steps are operationally the most difficult. Sputum, the main clinical sample used for TB analysis, is a particularly difficult matrix to obtain and manipulate. The GeneExpert (Cepheid Inc.) is endorsed by the WHO for TB detection and demonstrates excellent performance [32]. It must, however, still be used in a laboratory environment and is too expensive for most applications in the developing world and at the PoC. Here we use *M. smegmatis* as a surrogate strain for *M. tuberculosis*, although the benchtop RPA assay that we developed can detect 19 members of genus *Mycobacterium*. We have, however, also carried out experiments using samples spiked with authentic DNA from *M. tuberculosis* that demonstrates that the electrochemical method also works with this DNA.

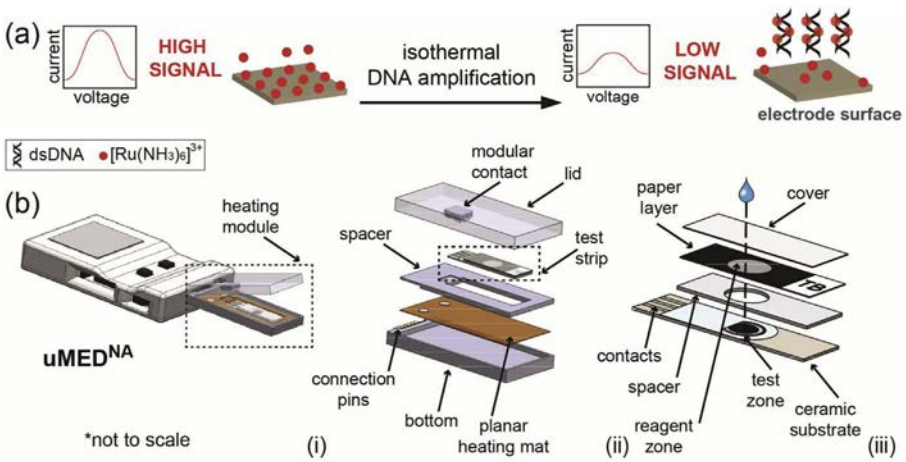

Fig. 1. (a) Schematic representation of the electrochemical RPA approach. (b) Schematic representation of the portable device for DNA amplification and detection: (i) uMED^{NA} connected to the heating module. (ii) Exploded view of module, and contacts to test strip. (iii) Exploded view of the disposable, paper-based test strip. The dotted line represents the flow of liquid through the disposable strip.

Materials and methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma Aldrich (St. Louis, USA) and were of molecular biology grade. All solutions were prepared in sterile glassware or certified nuclease-free plasticware. All pipette tips were sterile aerosol filter tips.

Methods

We describe all the methods required to reproduce our work in the supplementary information section.

Results and discussion

Design and validation of the RPA assay

After identifying a 213-bp region common to both *M. tuberculosis* and *M. smegmatis*, we designed appropriate primers for the RPA assay to amplify this sequence (Table SI 1). To determine the efficacy of the RPA assay, we performed the assay in bulk solution with a benchtop thermocycler and confirmed the efficiency of the amplification process with gel electrophoresis (Agilent TapeStation™). Fig. 2a summarizes the electrophoresis results. The 213-bp band confirms that the forward primer (positions 69–103 of 16S rRNA *M. smegmatis* gene, GenBank X52922.1) and the reverse primer (positions 248–282) successfully amplified the target DNA sequence. We also confirmed that our assay amplified the same target sequence in *Mycobacterium tuberculosis* (Strain H37Rv, GenBank AL123456).

We also tested the real-time efficiency using a fluorescent DNA intercalating dye. Fig. 2b shows real-time RPA results for four different concentrations of genomic DNA on a benchtop fluorescence thermocycler. The threshold of detection (TOD) was at 22 ± 2.1 AU of fluorescence. This value was calculated as 3σ above the average of three negative control experiments. The minimum concentration of genomic DNA detected above the TOD was 0.1 pg/μL.

Validation of the electrochemical probe

To characterize the electrochemical behavior of $[Ru(NH_3)_6]^{3+}$, we first recorded cyclic voltammograms between 0.2 and −0.5 V (*vs.* silver quasi-reference electrode) at different concentrations of the probe in Tris-Acetate buffer (40 mM, pH 7.4). For each measurement, we placed a 50-μL droplet of the sample on a bare, non-porous, ceramic substrate supporting SPEs. Fig. 3 shows CVs and SWVs comparing measurements performed by the uMED^{NA} and a benchtop analyzer for different

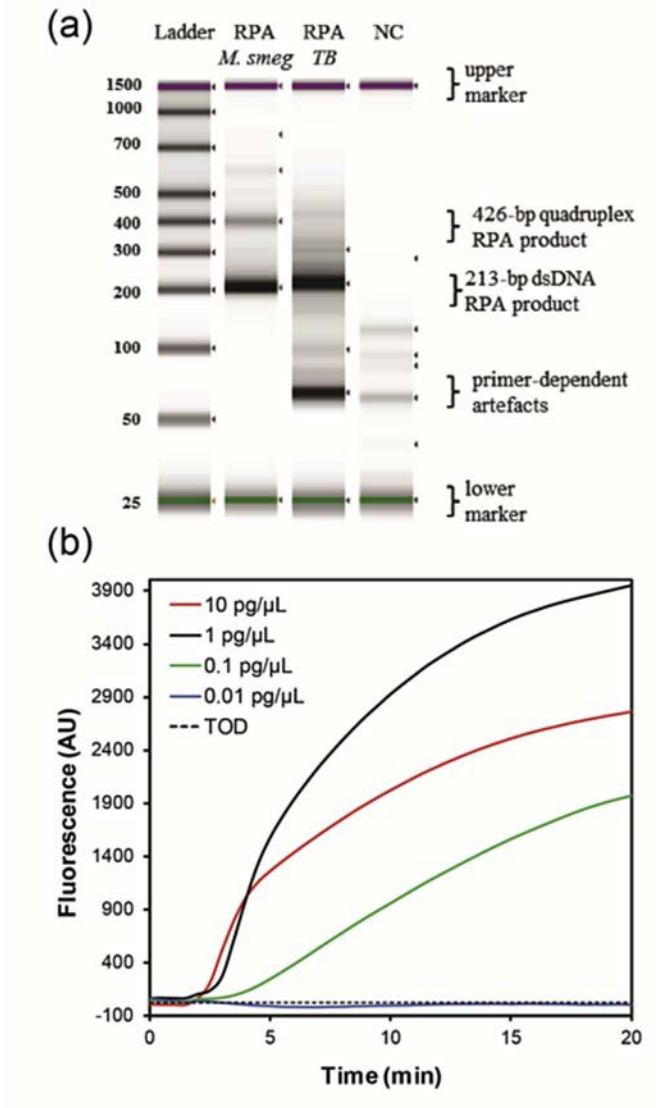

Fig. 2. (a) Gel electrophoresis results (Agilent TapeStation™) for the RPA reaction. The ~210-bp bands in the RPA reactions confirm efficient amplification of the reaction product. NC is the negative control with primers, and no DNA. (b) Real-time fluorescence results for RPA assays at variable concentrations of genomic target DNA (10, 1, 0.1 and 0.01 pg/μL, n = 3). The threshold of detection (TOD) was determined as 3σ above the arithmetic mean of three negative control experiments. The minimum amount of genomic DNA detected above the TOD per reaction was 0.1 pg/μL.

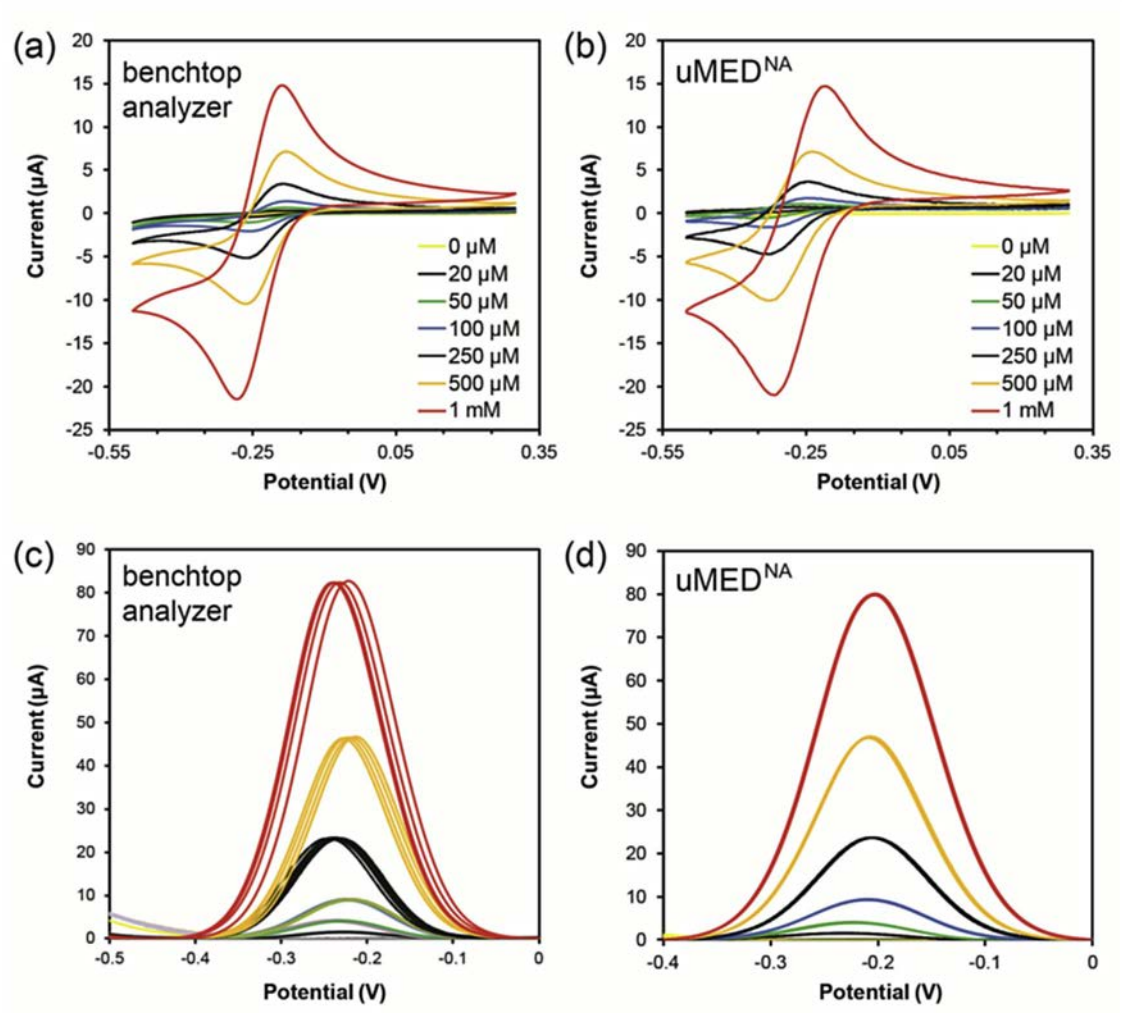

Fig. 3. Electroanalytical results comparing the performance of the uMED^{NA} and a benchtop analyzer over a range of concentrations of $[Ru(NH_3)_6]^{3+}$ in Tris-acetate buffer (25 μL, 40 mM, pH 7.4) using a ceramic substrate with screen-printed carbon electrodes and a Ag/Cl quasi-reference electrode. (a) and (b), Cyclic voltammograms (100 mV/s scan rate). (c) and (d), Square-wave voltammograms (n = 4, 50 μL, 15 Hz frequency, 50 mV amplitude, 50 mV/s scan rate).

concentrations of $[Ru(NH_3)_6]^{3+}$, and establishes the equivalence between measurements performed with each. Using Fig. 3a as an example, at 1 mM of $[Ru(NH_3)_6]^{3+}$we observed two electrochemical processes: (1) a cathodic peak at −0.279 V, and (2) an anodic peak at −0.173 V. The difference between peak potentials (E_{pa}-E_{pc} = 70 mV) and the ratio between peak currents (i_{pa}/i_{pc} = -0.7) indicate the redox reaction taking place is quasi-reversible process. The intensity of the peak current (either anodic or cathodic in CV) was proportional to the concentration of $[Ru(NH_3)_6]^{3+}$. Calibration curves were linear in the range of concentrations tested (R^2 > 0.99). Fig. SI 5 shows these calibration curves for i_{pa} and i_{pc}. Reproducibility of repeat measurements showed an excellent coefficient of variance for both SWV and CV (e.g. for 1 mM $[Ru(NH_3)_6]^{3+}$, 0.43% for SWV, 0.26% for i_{pa} and 0.98% for i_{pc}). Although CV could be used as the basis for analysis, SWV is more sensitive, and we have thus chosen this technique for recording the current due to the electron transfer processes involving $[Ru(NH_3)_6]^{3+}$.

DNA detection by RPA with electrochemical readout on a portable device

To detect DNA with our approach, we first prepared a paper-based test strip that included the necessary reagents (*i.e.*, RPA reagents, and $[Ru(NH_3)_6]^{3+}$), on the ceramic substrate with overlaying layers of tape and paper. We then performed the analysis by: i) adding a 25-μL sample that contained *Mycobacterium* DNA to the test zone, ii) affixing a cover layer to prevent evaporation, iii) inserting the test strip into the heating module and closing the lid, and iv) connecting the module to the uMED^{NA} to perform the analysis. The uMED^{NA} executed the following sequence automatically: i) it performed SWV to measure the background concentration of the redox probe at $t = t_0$, ii) it controlled the RPA reaction by incubating the test strip at 39 °C for 20 min, and iii) it performed SWV again to measure the reduction in the unbound concentration of the redox probe. We used t_0 = 5 min because the reaction mastermix took this time to reach 39 °C.

We programmed the uMED^{NA} using a desktop computer to send all raw data to a custom application written in MATLAB (Mathworks) and to perform all relevant signal analysis (e.g., baseline corrections) automatically. The uMED detects and displays the measured peak current to the user. For an initial peak current I_0 at $t = t_0$ and a final peak current I_t at time t, we define the signal $s(t) = [1 - I_t/I_0]$ x 100% as the % decrease of peak current *vs*. time. This type of quantification has been previously used for detecting DNA, for example, by Won et al. [33] in real-time electrochemical PCR, and by Ahmed et al. [19] in real-time LAMP. We studied the electrochemical behavior of the redox probe, $[Ru(NH_3)_6]^{3+}$ in the RPA reaction medium. Fig. SI 7 shows CVs of the reaction mastermix in the absence and presence of $[Ru(NH_3)_6]^{3+}$ (1 mM). We observed significant double layer capacitance in the CV for the mastermix. The CV for $[Ru(NH_3)_6]^{3+}$ showed: i) a cathodic peak at −0.038 V (−32.2 μA), and ii) an anodic peak at 0.023 V (29.0 μA, with

a difference in peak potentials very close to the 59 mV of a fully reversible one-electron process). In order to decrease the contribution of this capacitive current, and to enhance the Faradaic component in a measurement, proportional to the concentration of the redox probe, we used square wave voltammetry.

We observed two electrochemical processes that occurred during the RPA reaction when implemented with $[Ru(NH_3)_6]^{3+}$. We hypothesize that the process with a peak current of −0.06 V is diffusion-controlled, while the process with a peak current of −0.15 V is surface-confined. The first one is caused by electron transfer involving free $[Ru(NH_3)_6]^{3+}$ that diffuses in solution to the electrode. The surface-confined process is due to $[Ru(NH_3)_6]^{3+}$ interacting with DNA and proteins that adsorb on the electrode. At the start of the RPA reaction, the amount of DNA present was negligible; this low concentration allowed the electroactive molecules to diffuse onto surface of the electrode and to yield a high reduction signal. As the reaction progressed, more dsDNA was synthesized, complexed with $[Ru(NH_3)_6]^{3+}$, and the concentration of free electroactive molecules decreased, which caused a drop in the diffusion-controlled current. Fig. SI 8 shows the results of our confirmatory studies of the electrochemical RPA reaction at different scan rates. Ferapontova et al. suggest that an adsorptive process shows a linear relationship for current I against the square root of frequency $V^{1/2}$; a surface-confined process is linear for I against V [34]. We confirmed that this is the case for our results.

We used the decrease in the peak current at −0.06 V as the basis for quantification of the initial amount of DNA present. We expected this decrease because of binding of the redox probe to dsDNA by hydrogen-bonding and electrostatic interactions (intercalative stacking) [35]. These interactions decrease the number of probe molecules available for reduction at the surface of the electrode. Fig. SI 9 show the results of our tests for the same potential range for $[Ru(NH_3)_6]^{3+}$ only (Fig. SI 9a), DNA only (Fig. SI 9b), and $[Ru(NH_3)_6]^{3+}$ with DNA (Fig. SI 9c). We performed experiments in n = 7 replicates, containing the components of the full reaction, prepared independently.

Detection of M. smegmatis and M. tuberculosis on paper on a portable device

We performed the reaction on paper-based test strips with different initial concentrations of genomic target DNA present at the start of the reaction. We collected SWVs at the start, and at the end of the reaction. Fig. 4 shows a plot of the signal *s(t)* at 0, 1, 35 and 140 ng per sample (corresponding to a concentration of 0, 0.04 ng/μL, 1.4 ng/μL, and 5.6 ng/μL) of *M. smegmatis* DNA with 250 μM of $[Ru(NH_3)_6]^{3+}$. We observed the two peaks at −0.06 V and −0.15 V (as seen in Fig SI. 9c). We used the peak at −0.06 V to calculate the % decrease of peak current, as described in the previous section. Fig. 5a shows the SWV for an RPA reaction with 140 ng of genomic DNA from *M. smegmatis*. We calculated the signal *s(t)* at −0.06 V to be 65.40%. We also collected an aliquot of the same RPA reaction of the paper-based test strip, and confirmed efficient amplification by gel electrophoresis (Fig. 5b).

In summary, our objective was to simplify the procedures and instrumentation necessary to detect nucleic acids. Our approach combined isothermal amplification (RPA) with electrochemical readout of redox-active ($[Ru(NH_3)_6]^{3+}$), and enabled the entire process to be performed automatically by a low-cost (~$30 for components and materials) mHealth device, such as the uMED^{NA}. The disposable test strips that we developed enable pre-storage of all reagents needed for RPA, as well as the redox probe on the paper strip, and yield sensitive and accurate measurements of DNA in low concentrations. Reagents for nucleic-acid-amplification can be stored at 4 °C for up to eight months without significant loss in activity [36]. The use of disposable, paper electrodes also simplifies safe disposal of potentially harmful or pathogenic samples by incineration, and, through reduction of the volume of reagents consumed, decreases the cost of consumable components. The analytical sensitivity of the Cepheid Xpert MTB/RIF assay is at 131 CFU/mL of clinical sputum [37]. This limit of detection

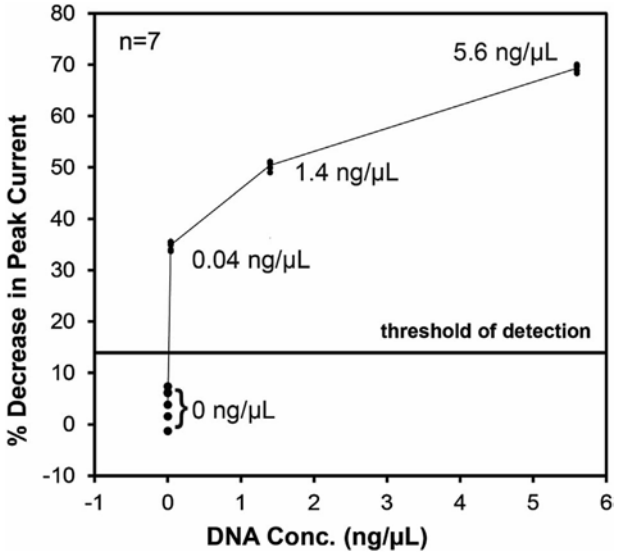

Fig. 4. Plot of signal *s(t)*, the % decrease in the peak current at −0.06 V, from square-wave voltammograms collected on the uMED^{NA} portable device using paper-based test strips at t = 0 and t = 20 min of the RPA reaction (25 μL, 15 Hz frequency, 50 mV amplitude, and 50 mV/s scan rate). Four different initial amounts of genomic DNA from *M.smegmatis* with 250 μM $[Ru(NH_3)_6]^{3+}$were tested and results for 0, 1, 35 and 140 ng are shown in (a), (b), (c) and (d) respectively. These amounts correspond to concentrations of 0, 0.040 ng/μL, 1.4 ng/μL, and 5.6 ng/μL. The threshold of detection was determined as 3σ above the arithmetic mean of seven negative control experiments.

approximately equates to 0.460 ng/μL, using the published size of the genome for TB [38]. We have estimated our LOD at 0.040 ng/μL to equate approximately to 11 CFU/mL of *M. tuberculosis*. This estimation assumes that there is one copy of the genome per CFU.

In the future, the RPA assay can be modified by using electroactive sequence-specific probes to increase the sensitivity, and the addition of lysis agents (e.g. guanidinium thiocyanate) will enable the use of our test-strips for samples of whole blood (as we previously showed for the detection of *E. coli* by LAMP on a "paper machine" [39]). Alternatively, DNA may be detected by chemiluminescence during the RPA reaction, which can be up to x10^2 more sensitive than electrochemical detection [40].

Conclusions

Existing methods of nucleic acid detection require expensive instrumentation and trained personnel to perform complex, multi-step procedures. Isothermal amplification and electrochemical detection of DNA are growing fields, but they have yet to be combined in a way that is applicable to an affordable, portable, web-connectable detection suitable for resource-limited settings (most efforts continue to rely on the use of expensive and difficultly portable benchtop equipment).

Our approach to the RPA assay is promising for the detection of DNA in the field, and we can detect down to 0.040 ng/μL of genomic DNA per sample with our current assay. Precision will need to be further optimized to detect smaller amounts of DNA reliably, but the current range compares well with the benchtop instrument (Cepheid). By altering the RPA reagents on the paper-based test strip (e.g., by choosing different primers to target a different nucleotide sequence), we believe that the assay can be modified for a wide range of applications, including detection of viruses and viral nucleic acids, water monitoring and food testing.

Acknowledgements

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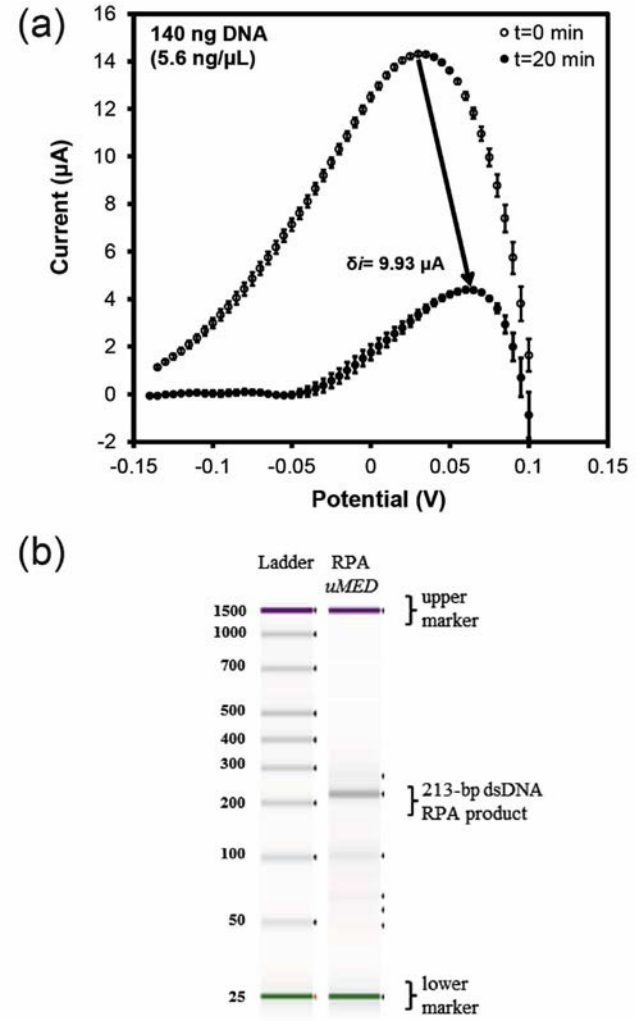

Fig. 5. (a) Square-wave voltammograms collected on the uMED^{NA} portable device using paper-based test strips at t = 0 and t = 20 min of the RPA reaction (25 μL, 15 Hz frequency, 50 mV amplitude, and 50 mV/s scan rate). (b) Gel electrophoresis results (Agilent TapeStation™) for the same RPA reaction on the uMED^{NA} portable device for 140 ng of *M. smegmatis* DNA in the sample (corresponding to a concentration of 5.6 ng/μL).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.11.025.

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