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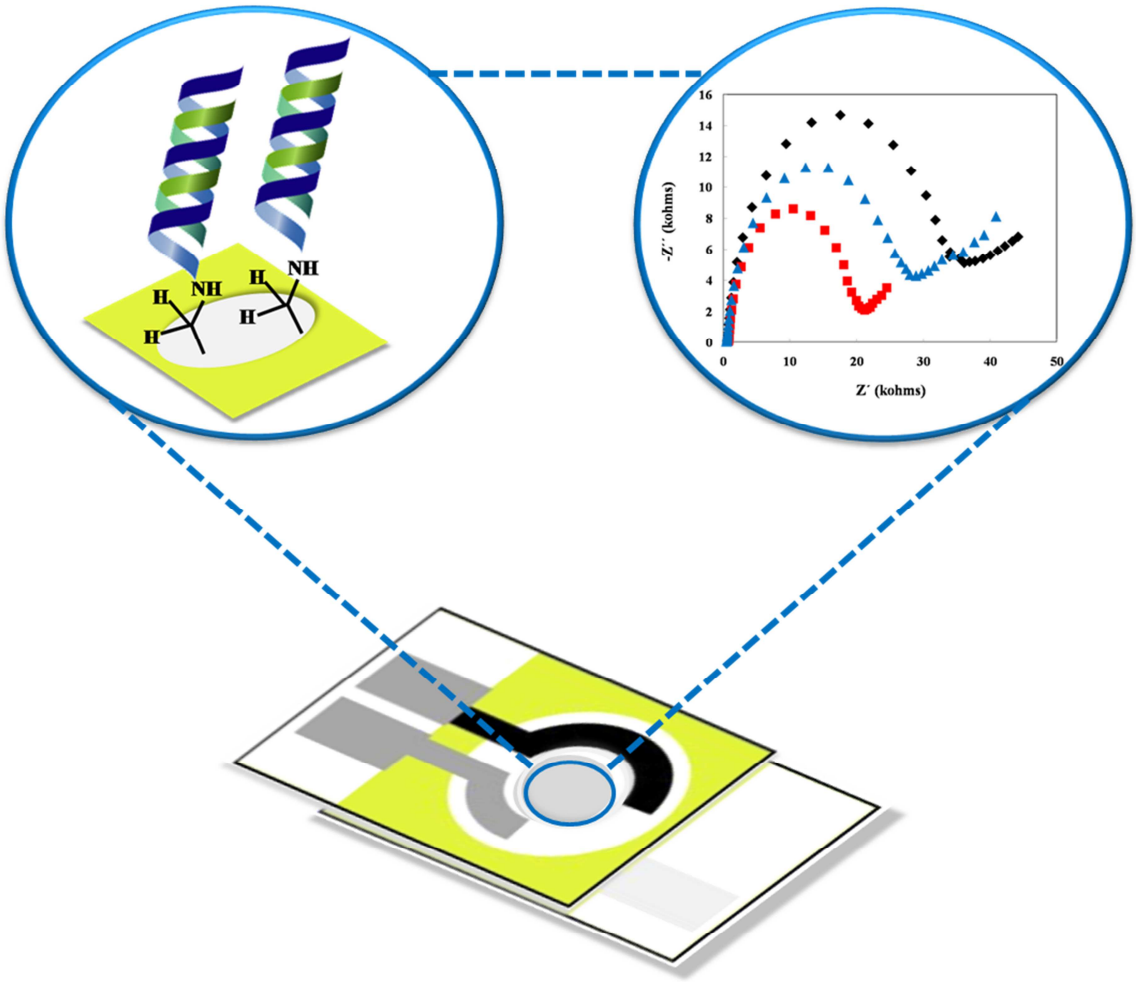

Electrochemical impedance-based DNA sensor using pyrrolidinyl peptide nucleic acids for tuberculosis detection

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ABSTRACT

A label-free electrochemical DNA sensor based on pyrrolidinyl peptide nucleic acid (acpcPNA)-immobilized on a paper-based analytical device (PAD) was developed. Unlike previous PNA-based electrochemical PAD (ePAD) sensors where the capture element was placed directly on the electrode, acpcPNA was covalently immobilized onto partially oxidized cellulose paper allowing regeneration by simple PAD replacement. As an example application, a sensor probe was designed for *Mycobacterium tuberculosis* (MTB) detection. The ePAD DNA sensor was used to determine a synthetic 15-base oligonucleotide of MTB by measuring the fractional change in the charge transfer resistance (R_{ct}) obtained from electrochemical impedance spectroscopy (EIS). The R_{ct} of $[Fe(CN)_6]^{3-,4-}$ before and after hybridization with the target DNA could be clearly distinguished. Cyclic voltammetry (CV) was used to verify the EIS results, and showed an increase in peak potential splitting in a similar stepwise manner for each immobilization step. Under optimal conditions, a linear calibration curve in the range of 2-200 nM and the limit of detection 1.24 nM were measured. The acpcPNA probe exhibited very high selectivity for complementary oligonucleotides over single-base-mismatch, two-base-mismatch and non-complementary DNA targets due to the conformationally constrained structure of the acpcPNA. Moreover, the ePAD DNA sensor platform was successfully applied to detect PCR-amplified MTB DNA extracted from clinical samples. The proposed paper-based electrochemical DNA sensor has potential to be an alternative device for low-cost, simple, label-free, sensitive and selective DNA sensor.

Keywords: Paper-based electrochemical DNA biosensor, acpcPNA, Electrochemical impedance spectroscopy, *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) has long been a crucial global health problem and is still one of the top 10 causes of death worldwide. In 2015, the World Health Organization (WHO) reported that 10.4 million people were infected with TB and 1.8 million people died from this disease. In humans, TB is caused by mycobacteria, *M. tuberculosis* (MTB). Standard TB diagnostic methods rely on the identification of MTB and mainly employ sputum smear microscopy, culture of bacilli and/or polymerase chain reaction (PCR) [1-6]. Although these methods are sensitive, they have limitations. Sputum smear tests require trained personnel and use of a microscope to identify MTB. Bacillus culture and PCR are complicated and time-consuming methods. The requirement of highly trained personnel, sophisticated instrumentation and multiple detection steps limits the feasibility of applying these methods for routine diagnosis of TB in developing countries.

Detecting specific DNA sequences has attracted increasing attention for disease diagnosis. Many detection methods for point of care assays, including fluorescent [7], electrochemiluminescence [8], enzymatic [9], surface plasmon resonance [10] and electrochemical [11-13], have been proposed. Among the existing approaches, electrochemical DNA sensors have become an interesting option as they offer simplicity, portability and low-cost. Generally, electrochemical DNA sensors rely on oligonucleotide probes immobilized on an electrode surface. After exposure to a sample containing the specific complementary target sequence, the electrochemical response is monitored upon DNA hybridization by either direct or indirect methods. Although direct detection based on guanine (G) and/or adenine (A) oxidation allows a

simple and reagentless process, the oxidation signal depends on the number of electroactive bases as well as their distance from the electrode, impacting sensitivity and detection limit. On the other hand, indirect methods require additional electroactive labels or indicators, including redox couples such as methylene blue [14, 15], daunomycin [16] or anthraquinone [11, 17, 18], metal ion complexes with osmium or ruthenium [19-21], or nanoparticles [22] to generate sensitive electrochemical signal. However, strategies involving labels are time-consuming and complicate the detection process.

A label-free assay would be preferable for reducing time and complexity in DNA diagnostics [23, 24]. Electrochemical impedance spectroscopy (EIS) is a particularly useful detection mode for DNA diagnostics because it does not require labels or indicators [25-28]. The resistance to charge transfer (R_{ct}) at the electrode surface is a sensitive indicator for monitoring the change of interfacial surface properties following DNA hybridization. Label-free detection based on EIS is therefore an ideal platform for developing sensitive, low-cost detection when combined with portable instrumentation for point-of-care applications.

In the field of electrochemical DNA sensors, one of the key components to achieve specific DNA detection is the probe. Vilaivan's group proposed a new conformationally constrained pyrrolidinyl PNA system which possesses an α,β-peptide backbone deriving from D-proline/2-aminocyclopentanecarboxylic acid (known as acpcPNA) [29, 30]. Compared to the original aegPNA [31], acpcPNA exhibits a stronger affinity and higher sequence specificity binding to DNA and RNA. It also provides the characteristic selectivity of antiparallel/parallel

binding to the target and a low tendency for self-hybridization. Moreover, it can be modified at nucleobase or backbone to incorporate diverse functionalities. These properties allow acpcPNA to be widely used as a probe for biological applications [11, 18, 32, 33].

The combination of PNA probe and label-free detection based on EIS has been reported [34-36]. However, the devices are expensive and require the complicated steps to fabricate. Therefore, it is not ideally suited for point-of-care diagnostics. Paper-based analytical devices (PADs) have continuously received significant interest as a point-of-care sensor. Because PADs are simple, inexpensive, portable and disposable [37-42], they can act as a preliminary screening tool for diagnostics in developed and developing countries alike. Additionally, paper is typically made of cellulose which is compatible for biological samples and can be chemically modified with a variety of functional groups [40]. The emerging of acpcPNA probe and PADs has been recently reported by our research network [11, 43-45] and provides advanced possibilities of alternative point-of-care sensor. In 2015, Jirakittiwut et al. [44] proposed a fabrication of paper-based DNA sensor by covalently modifying cellulose paper with acpcPNA probe. The presence of DNA was determined by the visual color of cationic colorimetric dyes bound to DNA by electrostatic interaction. This new colorimetric DNA assay concept offers a low-cost fabrication with great specificity way for DNA detection. In addition, a paper-based device for multiplex detection of DNA oligonucleotides based on electrostatic interaction between acpcPNA and silver nanoparticles leading to distinct color change in the absence and presence of DNA was developed [45]. Colorimetric detection; however, is limited by the need for low background surfaces, a limited dynamic range, low sensitivity, and variability in environmental illumination.

Electrochemical sensing offers a quantitative alternative detection system for the DNA assay. Electrochemical detection can also improve the performance of paper-based DNA sensor in terms of sensitivity and selectivity while providing lower detection limits.

Here, we report a label-free electrochemical PAD (ePAD) for DNA detection using acpcPNA as a probe and electrochemical impedance spectroscopy (EIS) as the detection motif. Unlike previous electrochemical PNA sensors where the probe was attached to the electrode and paper used as a transport mechanism, the acpcPNA probe was covalently immobilized onto cellulose paper. The quantification of DNA was performed by monitoring the change in the charge transfer resistance using EIS before and after the acpcPNA-DNA hybrid is formed. The proposed concept was used for the detection of an MTB oligonucleotide and successfully applied with PCR-amplified DNA from a clinical sample. This simple-to-fabricate label-free electrochemical paper-based DNA sensor has the potential to be developed as an alternative diagnostic device for simple, low-cost, sensitive and selective DNA and RNA detection.

2. Materials and methods

2.1 Chemicals and materials

Analytical grade reagents, including LiCl from Wako Pure Chemicals Industry (Japan), $NaIO_4$ from CARLO ERBA reagents (Italy), 2,4-dinitrophenylhydrazine (2,4-DNP) from H&W (USA), $NaCNBH_3$ from Acros Organics (USA), NaCl, KH_2PO_4, Na_2HPO_4 and KCl from Merck (Germany), potassium ferricyanide and potassium ferrocyanide from Sigma-Aldrich (Singapore),

were used without further purification. The 18 M Ωcm^{-1} resistance water was obtained from a Millipore Milli-Q water system. Dimethylformamide and acetonitrile were products of Merck. Carbon graphene and silver/silver chloride ink were obtained from Gwent group (United Kingdom). The screen-printed block was made by Chaiyaboon Co. Ltd. (Thailand). Synthetic oligonucleotides were obtained from Pacific Science (Thailand). The sequences of the oligonucleotides used in this study are shown in Table 1.

Table 1. List of oligonucleotides

Oligonucleotide	Sequence (5'-3')
Complementary DNA	5'-ATAACGTGTTTCTTG-3'
Single-base-mismatch	5'-ATAACGTCTTTCTTG-3'
Two-base-mismatch	5'-ATAACGTCTCTCTTG-3'
Non-complementary DNA	5'-CACTTGCCTACACCA-3'

The forward (5'-CCTGCGAGCGTAGGCGTCGG-3') and reverse DNA primers (5'-CTCGTCCAGCGCCGCTTCGG-3') were used for the preparation of the PCR samples. The clinical sample of MTB was obtained from Asst. Prof. Chaniya Leepiyasakulchai, Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University and was collected using university human subjects board approved procedures (MUIRB2016/041.2803).

2.2 Synthesis of acpcPNA

The acpcPNA probe was designed to be complementary to the synthetic oligonucleotide MTB targets with a sequence of CAAGAAACACGTTAT (written in the N→C direction). The

acpcPNA probe was synthesized by solid-phase peptide synthesis using Fmoc chemistry as previously described [29]. L-lysinamide was included at the C-terminus to provide a handle for subsequent immobilization via the ε-amino group and the N-terminus was end-capped by acetylation. The progress of the reaction was monitored by MALDI-TOF-MS analysis on a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The acpcPNA on the solid support was treated with 1:1 (v/v) aqueous ammonia:dioxane in a sealed tube at 60 ºC overnight to remove the nucleobase protecting groups. The PNA was cleaved from the solid support with trifluoroacetic acid (TFA) and purified by reverse-phase HPLC (C18 column, 0.1% (v/v) TFA in H_2O-MeOH gradient). The identity of the acpcPNA was verified by MALDI-TOF MS analysis, and the purity was confirmed to be >90% by reverse-phase HPLC.

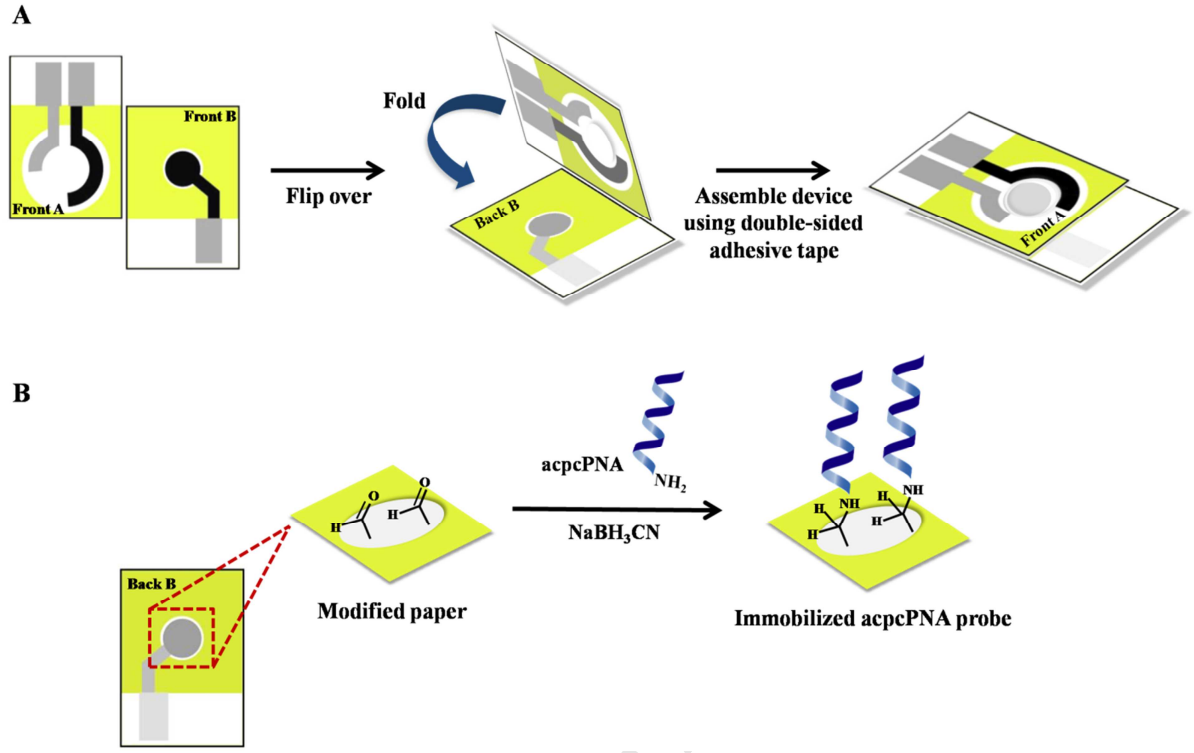
2.3 Fabrication of 3D paper-based electrochemical DNA sensor

A 3D ePAD DNA sensor was fabricated using the wax-printing technique [46]. The patterned paper was designed using Adobe Illustrator based on the origami concept [47] and printed onto the filter paper (Whatman No.1) using a wax printer (Xerox Color Qube 8570, Japan). The printed paper was subsequently melted on a hot plate (175 ºC, 50 s) to generate a hydrophobic barrier. For electrode fabrication, three-electrode systems were prepared using an in-house screen-printing method. Carbon graphene ink was used as a working electrode (WE) and counter electrode (CE). Reference electrode (RE) and conductive pad were screen printed using silver/silver chloride ink. The design and operation of the paper-based electrochemical DNA

sensor was shown in Scheme 1. As shown in Scheme 1A, the sensor consists of two layers: layer A and layer B. Layer A contains CE and RE while layer B contain WE (4 mm i.d.). The sample reservoir is punched in layer A to allow a solution connection directly from the top to the bottom layer when the 3D DNA sensor was fully assembled by folding layer A over layer B. The design of the paper-based DNA sensor was shown in Fig. S1.

2.4 Immobilization of acpcPNA

Covalent immobilization of acpcPNA on the paper WE was carried out according to previous reports [48-50] with slight modification. The immobilization process is shown in Scheme 1B. First, 2.10 M of LiCl in $NaIO_4$ (0.04 M) was spotted onto the back of WE. The reaction was allowed to proceed in the dark at room temperature for 15 min to generate the aldehyde groups on the surface of the cellulose paper [51]. Next, the WE was washed with Milli-Q water to remove the inorganic salts and allowed to dry at room temperature. For covalent immobilization of the acpcPNA, a solution of acpcPNA (2 µM) containing $NaBH_3CN$ (1 mg/mL) in DMF was prepared. 3 µLs of acpcPNA solution was dropped onto the aldehyde modified paper and allowed to react for 12 hr in the dark and humid condition. Afterward, the acpcPNA-immobilized paper was washed for 30 min with Milli-Q water:acetronitrile (1:1) and allowed to dry at room temperature. Finally, the 3D DNA sensor was stored at 4ºC prior to use.



Scheme 1. (A) Design and operation of 3D electrochemical paper-based DNA sensor. (B) The process of acpcPNA covalent immobilization.

2.5 Hybridization of DNA

The solution of MTB DNA oligonucleotide target was dropped onto the acpcPNA-immobilized WE and incubated for 15 min at room temperature. Then, the non-hybridized sequence was removed from the WE by washing with 0.1 M PBS (pH 7.4). The same procedure was applied for the selectivity test with single-base mismatch, two-base mismatch and non-complementary targets.

198

199 *2.6 PCR-amplified MTB analysis in real sample*

200 The extracted DNA from clinical sample was amplified by standard PCR [52]. The
201 amplification reaction was performed using *Thermus aquaticus* (Taq) polymerase and the
202 following condition: 1 µM each of AmpliTaq DNA polymerase, nucleotides, 10X reaction buffer
203 and primer were mixed together. Subsequently, 5 µL of the extracted DNA was added. The
204 sample was denatured at 94 ºC for 5 min and then 25 cycles of amplification were performed.
205 The cycle consisted of denaturation at 94 ºC for 2 min, annealing at 68 ºC for 2 min and
206 extension at 72 °C for 2 min. The PCR product was visualized by electrophoresis in a 2% agarose
207 gel containing ethidium bromide under UV light.

208

209 *2.7 Electrochemical measurement*

210 For electrochemical measurements, the EIS was used throughout the experiment using a
211 PGSTAT30 potentiostat (Metrohm Siam Company Ltd., Switzerland) and CV was performed on
212 a CHI1232A electrochemical analyzer (CH Instruments, Inc., USA). All electrochemical
213 experiments were carried out in 5 mM $[Fe(CN)_6]^{3-/4-}$. After the immobilization and hybridization
214 process, 100 µL of $[Fe(CN)_6]^{3-/4-}$ was added onto the modified working electrode. EIS was recorded
215 in the frequency range of 0.01 Hz – 100 kHz with the potential at 0.1 V and A.C amplitude at
216 5mM. The R_{ct} after the hybridization was measured and compared with the R_{ct} in the absence of
217 the DNA target.

218

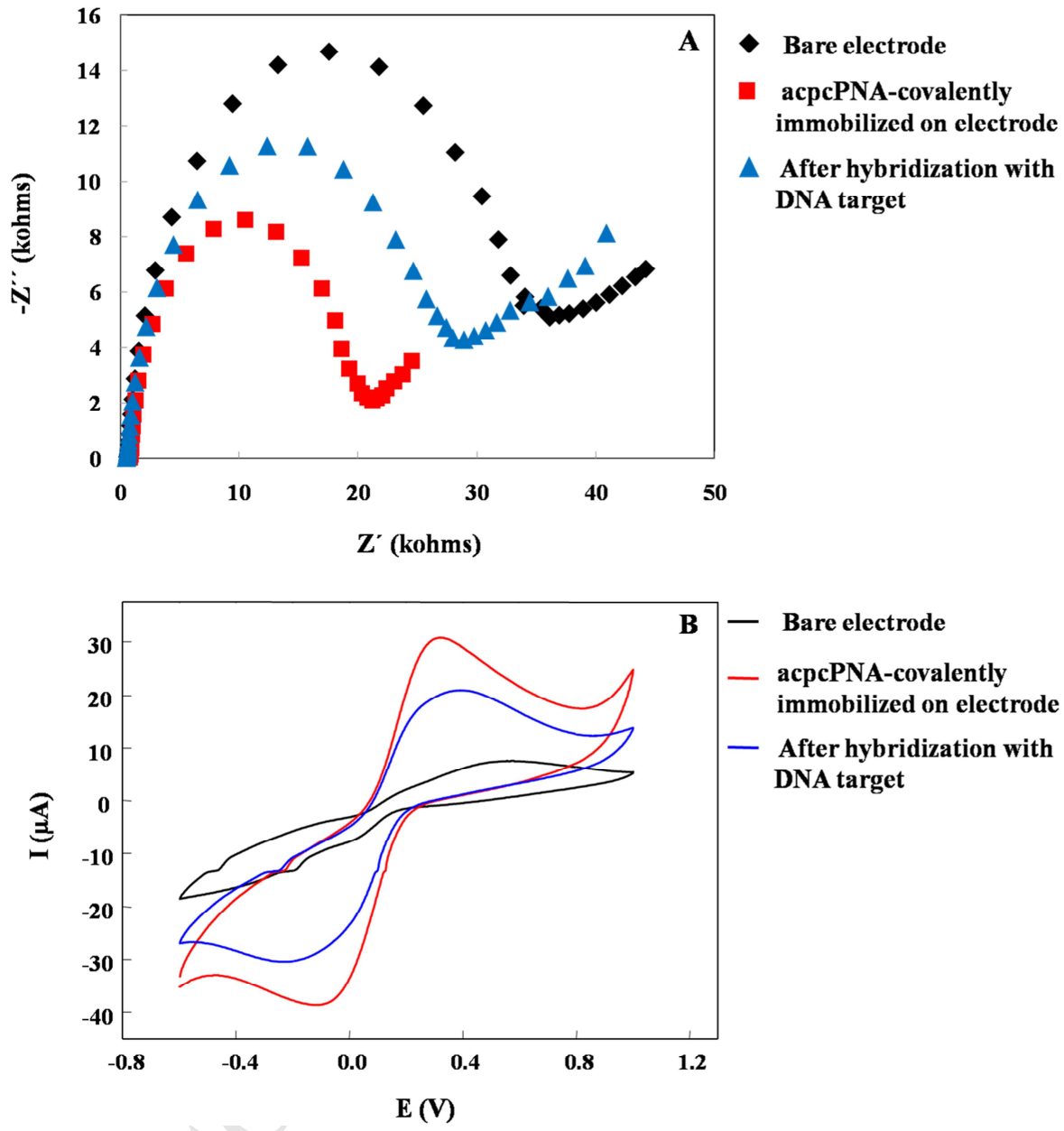
3. Results and Discussion

3.1 Characterization of acpcPNA - covalently immobilized electrode

In order to confirm the success of covalent immobilization of acpcPNA, the change of R_{ct} in EIS was monitored in a stepwise electrode modification using 5 mM $[Fe(CN)_6]^{3-,4-}$. The semicircle of the Nyquist curve represents the electron transfer resistance and the R_{ct} derived from a diameter of semicircle portion of the Nyquist plots can indicate the efficiency of electron transfer from electrode surface to the redox couple solution. First, the electron transfer resistance of bare electrode and modified paper electrode without the immobilization of acpcPNA probe was measured (Fig. S2). The bare and modified paper electrodes provided Nyquist curves giving R_{ct} values of 34.00 (±0.08) kΩ and 34.16 (±1.25) kΩ, respectively, indicating that the electron transfer of electrode surface is not affected by the paper modification process. Fig. 1A shows the Nyquist plots obtained from bare electrode and acpcPNA-covalently immobilized on the electrode before and after hybridization with the DNA target. It can be seen that the bare electrode has the largest semicircle with an R_{ct} of 34.00 (±0.08) kΩ. After the acpcPNA was immobilized on electrode, the R_{ct} value (18.95 (±0.35) kΩ) decreased, indicating that the acpcPNA modified electrode provided a lower charge transfer resistance and higher electron transfer to the electrode. We believe the increased electron transfer was due to the electroactivity of guanine (G) and adenine (A) bases in the acpcPNA probe sequence as has been suggested previously [53-55]. When the acpcPNA modified electrode was hybridized with the DNA target, the R_{ct} value

239 increases to 27.05 (±0.15) kΩ due to the increase in electrostatic repulsion between the negatively
240 charged DNA and redox probe.

241 CV was also used to further verify electrode modification. As shown in Fig. 1B, cyclic
242 voltammogram shows a significant shift in the peak potential and the increasing of anodic peak
243 current after acpcPNA immobilization. The ΔE_p of bare electrode was 0.67 (±0.01) V, while the
244 ΔE_p of acpcPNA modified electrode was 0.35 (±0.02) V. Also, the current response of bare
245 electrode (7.13 (±0.26) µA) was lower than acpcPNA-modified electrode (28.89 (±0.95) µA). This
246 result suggests that G and A bases of acpcPNA probe have ability to accelerate the electron
247 transfer process. Upon the hybridization of DNA target, the ΔE_p slightly shifted to 0.44 (±0.01) V,
248 while the anodic peak current (19.94 (±0.76) µA) decreased, which implied that the electron
249 transfer is low due to the electrostatic repulsion phenomenon of PNA-DNA duplexes and redox
250 couple. According to the characterization evidences, the change of R_{ct} in EIS, ΔE_p and peak
251 current in CV clearly indicate successful covalent immobilization of acpcPNA on the electrode.



252

253 **Fig.1** (A) Nyquist plot and (B) cyclic voltammogram of bare electrode, acpcPNA-covalently

254 immobilized on electrode before and after hybridization with an equimolar concentration of

255 target DNA in 5 mM $[Fe(CN)_6]^{3-/4-}$.

256

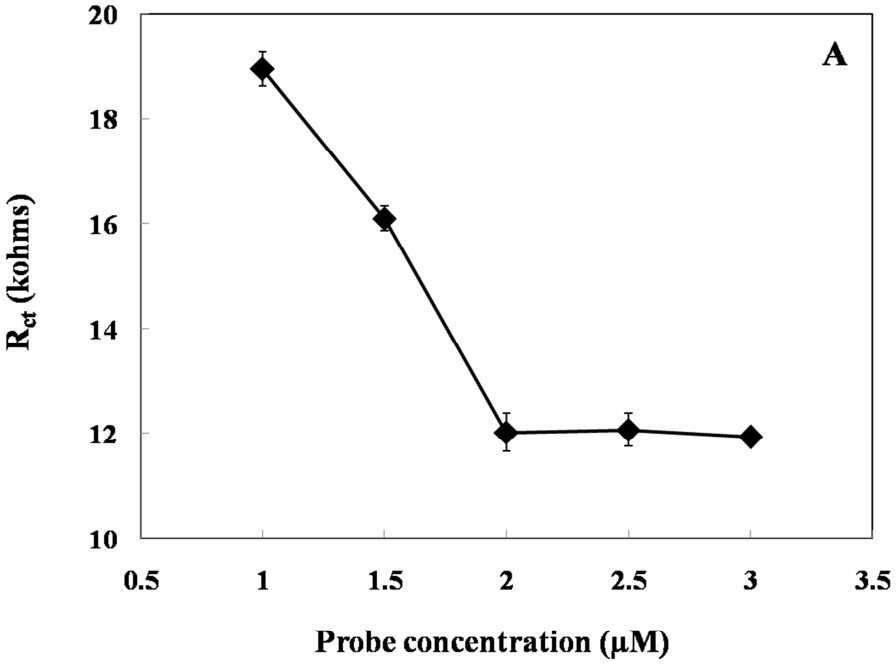
257

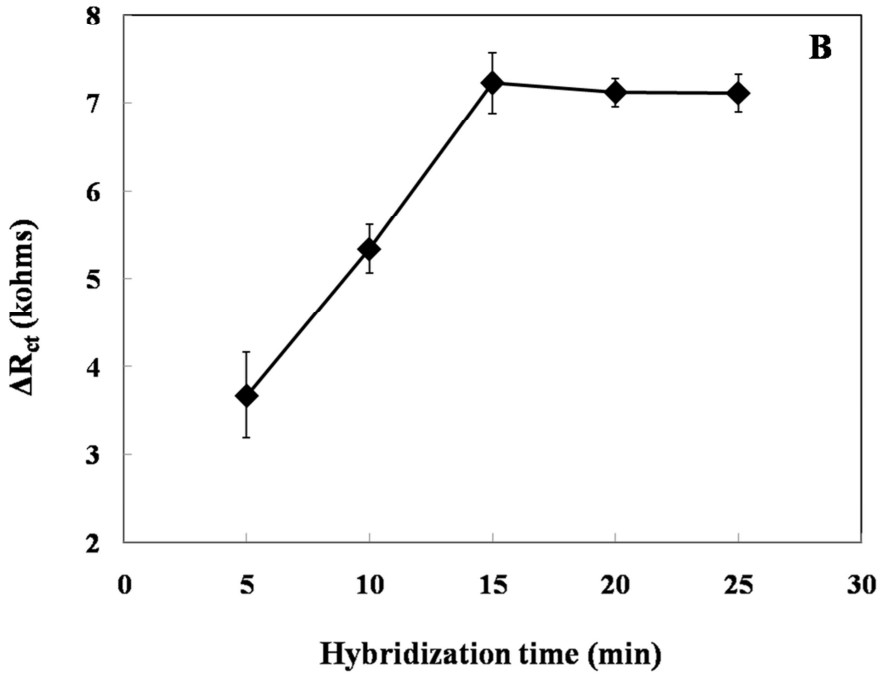
3.2 acpcPNA probe concentration

The effect of acpcPNA probe concentration on R_{ct} obtained from EIS was investigated as shown in Fig. 2A. As the PNA concentration increased, R_{ct} for acpcPNA functionalized electrode decreased until a plateau was reached at a concentration of 2 µM. The excess the PNA concentration will increase the PNA surface density, which hindered accessibility of redox molecules to reach the working electrode surface, resulting in a constant R_{ct}. Therefore, a 2 µM of the acpcPNA probe concentration was selected for further studies.

3.3 Hybridization time

The influence of DNA hybridization time on electrode impedance was studied next. The DNA target was incubated with the 2 µM acpcPNA-modified electrode for different periods of time before washing with PBS (0.01 M, pH 7.4). In Fig. 2B, ΔR_{ct} obtained after hybridization of DNA target increases with the increasing the hybridization time, and the largest ΔR_{ct} was obtained at 15 min. This result indicated that increasing hybridization time provided higher efficiency of the binding between the acpcPNA probe and the DNA target, leading to an increase in charge-transfer resistance. The advantage of short time required for hybridization was proposed. While the hybridization of others DNA assay using EIS requires more than 30 min incubation, our method requires only 15 min to provide highest efficiency of the binding between the immobilized acpcPNA probe and the DNA target. Accordingly, the hybridization time at 15 min was selected as the optimal condition.


A
20
18
16
14
12
10
R_{ct} (kohms)
0.5
1
1.5
2
2.5
3
3.5
Probe concentration (μM)


B
8
7
6
5
4
3
2
ΔR_{ct} (kohms)
0
5
10
15
20
25
30
Hybridization time (min)

278

Fig. 2 The effect of (A) acpcPNA probe concentration and (B) hybridization time of 1 μM DNA target on the R_{ct} value using a 2 μM acpcPNA probe concentration. The error bars represent one standard deviation (SD) obtained from three independent measurements (n=3).

3.4 Analytical performance

The analytical performance of 3D paper-based electrochemical DNA sensor for MTB determination was evaluated. To assess the detection limit and linear range, ΔR_{ct} as a function of DNA target concentration was determined (Fig. 3). The calibration curve (inset Fig. 3), which was plotted as a function of logarithmic DNA concentration and ΔR_{ct}, provided a linear relationship from 2 nM to 200 nM with a correlation coefficient of 0.997. The limit of detection (LOD, S/N=3) and limit of quantitation (LOQ, S/N=10) were found to be 1.24 nM and 3.69 nM, respectively. The wide linear range and low detection limit suggest that our proposed method is useful for MTB DNA detection after amplification.

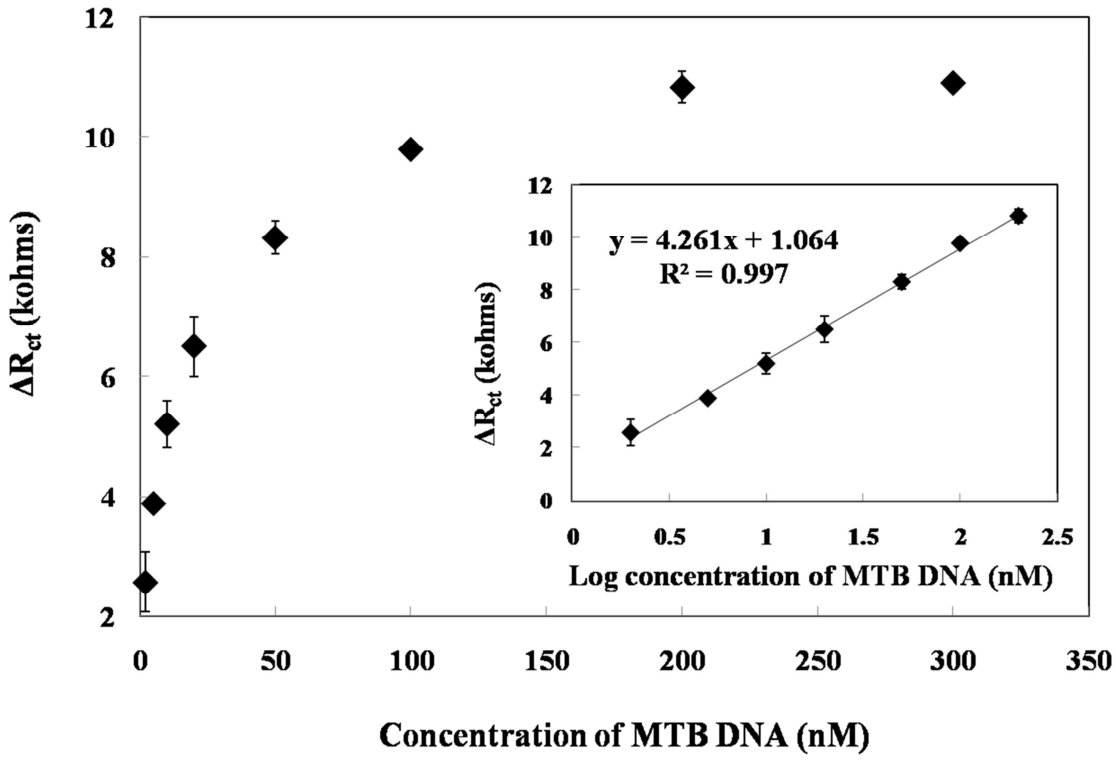


Fig. 3 The change of R_{ct} (ΔR_{ct}) versus DNA target concentration and calibration curve between ΔR_{ct} and log DNA target concentration (inset) for MTB detection in 5 mM $[Fe(CN)_6]^{3-/4-}$, frequency 0.01 Hz-100 kHz, potential 0.1 V. The error bars represent one standard deviation (SD) obtained from three independent measurements (n=3).

3.5 Selectivity

In order to evaluate the selectivity of the sensor, the R_{ct} obtained from MTB DNA target was compared to single-base mismatch (DNA_{m1}), two-base mismatch (DNA_{m2}) and non-complementary DNA (DNA_{nc}) using a 2 µM acpcPNA probe concentration. In the presence of DNA_{com}, the R_{ct} increased dramatically; whereas the R_{ct} did not change for DNA_{m1}, DNA_{m2} and DNA_{nc} as shown in Fig. 4. These results indicated that there was no significant interference by

the mismatched and non-complementary DNA targets. Thereby, the proposed DNA sensor provided high selectivity for MTB detection, which re-emphasizes the usefulness of acpcPNA probes.

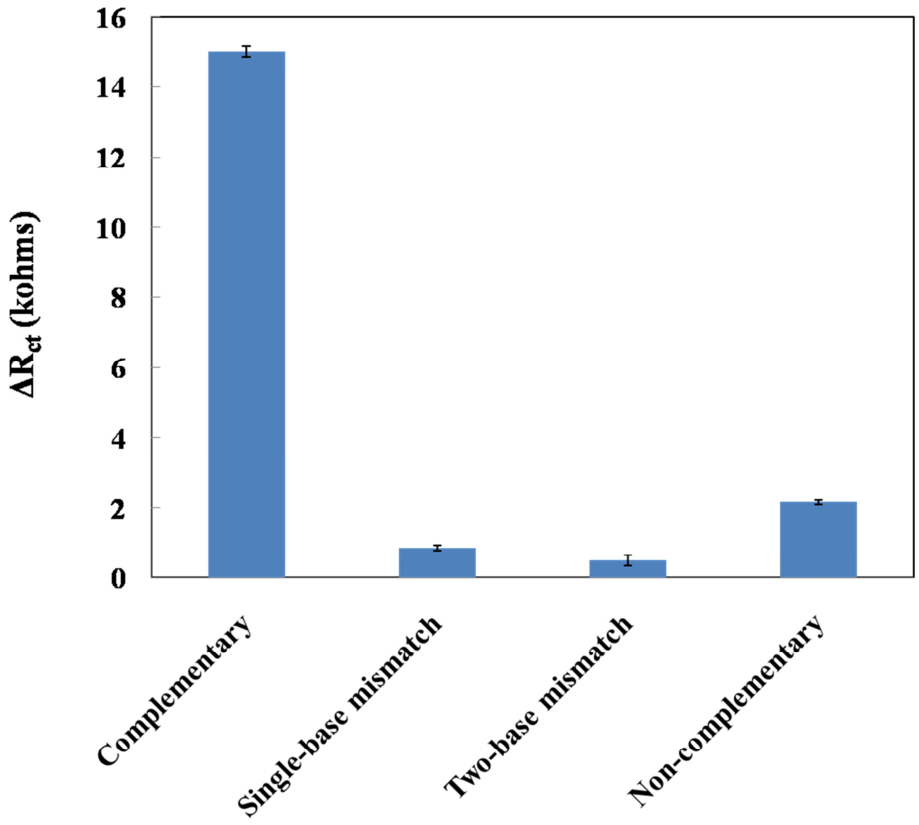


Fig. 4 The ΔR_{ct} in electrochemical impedance spectroscopy of MTB detection after hybridization with 1 µM of DNA_{com}, DNA_{m1}, DNA_{m2} and DNA_{nc} using a 2 µM acpcPNA probe concentration. The error bars represent one standard deviation (SD) obtained from three independent measurements (n=3).

3.6 Stability and reproducibility

To investigate the stability of the proposed label-free DNA biosensor, the sensor was stored at 4 ºC in dry environment over one month before testing the R_{ct} response before and after hybridization with 20 nM DNA target. The R_{ct} response was collected on a weekly basis. It was observed that the change of R_{ct} decreased to 96.2% and 94.1% of the initial response for acpcPNA-covalently immobilized on electrode before and after hybridization with DNA target, respectively (Fig. S4). The electrode-to-electrode reproducibility of the DNA biosensor was also examined as shown in Figure S3. The relative standard deviations (RSDs) of five electrodes were tested in the concentration range of 2-200 nM. The %RSDs was determined to be between 0.58% and 3.46%. These results indicate that the proposed DNA biosensor offers acceptable stability and reproducibility.

3.7 Real sample analysis

To evaluate the efficiency of the proposed method, the developed label-free DNA biosensor was used to detect MTB in PCR-amplified DNA from a blood sample. It was observed that the R_{ct} was increased in the presence of positive sample of MTB (Fig. S5), which confirmed the occurrence of hybridization in the proposed system. To increase the sensitivity, the signal could be improved by performing the hybridization twice onto the same electrode to accumulate more analyte bound to the immobilized probe. It was found that the R_{ct} increased with the increasing of amount of PCR positive sample as shown in Figure 5. Therefore, it clearly shows

that this label-free DNA biosensor platform is applicable for determining MTB in an extracted and amplified DNA from clinical samples.

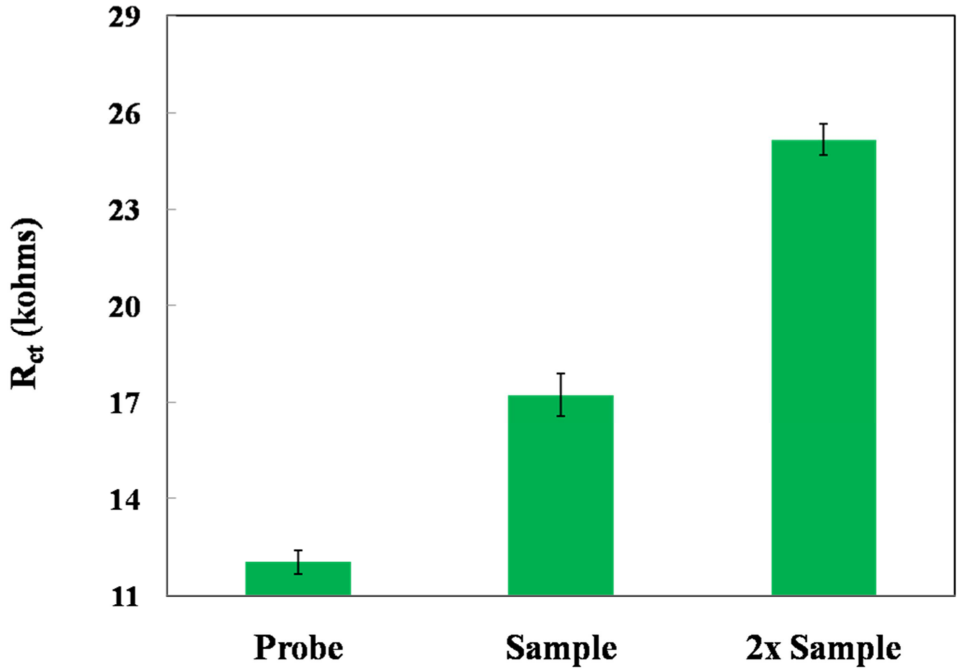


Fig 5. R_{ct} response of the label-free DNA biosensor in the presence of PCR-amplified MTB positive sample from clinical sample. The error bars represent one standard deviation (SD) obtained from three independent measurements (n=3).

4. Conclusions

A new label-free electrochemical DNA biosensor platform using acpcPNA as a probe was successfully developed for MTB detection by measuring changes in resistance to charge transfer. Unlike previous methods that required direct electrode modification, the acpcPNA probe was covalently immobilized onto partially oxidized cellulose paper by reductive alkylation. The

346 change of surface resistance in the presence of target DNA monitored by EIS was used to
347 achieve the quantitative measurement. The label-free system offers the advantage over the more
348 widely used electroactive indicators or labels since it eliminates the complicated and time-
349 consuming steps for labeling or adding indicators without compromising the performance in
350 terms of detection limit and linearity range. The proposed DNA sensor demonstrated very high
351 selectivity of acpcPNA against single-base mismatch, two-base mismatch and non-
352 complementary DNA targets. This sensing system can be employed to determine the PCR
353 amplification of MTB from clinical samples. As a result, this ePAD DNA sensor is well suited as
354 an alternative platform that provides a simple and cost-effective solution to achieve a rapid
355 screening of MTB and can be readily applied to other DNA targets.

356

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364

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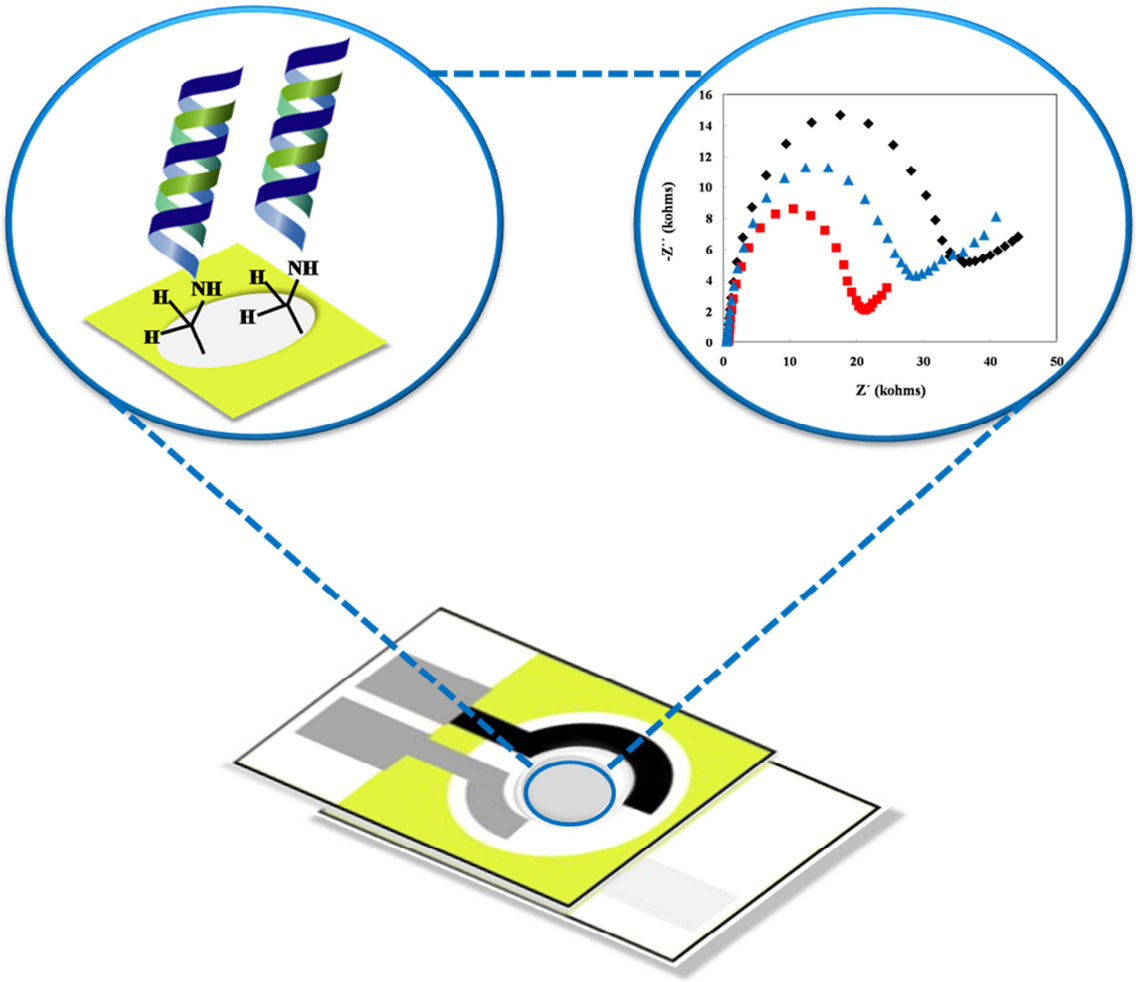
525 **Graphical abstract**



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Highlights

- An electrochemical paper-based PNA detection method is reported.
- The approach uses modified paper as opposed to modified electrodes to enable rapid replacement
- Technique was demonstrated on tuberculosis as a model application.