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Electrochemical-Nucleic Acid Detection with Enhanced Specificity and Sensitivity

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Abstract

Development of a rapid and label-free Electrochemical Impedance Spectroscopy (EIS) biosensor for nucleic acid detection based on sensitivity and specifies was proposed to fill the technology gap between rapid and portable detection of DNA sequences. Current biosensors have inherent limitations to sensitivity and specificity due to the monovalent character of the detection method. These limitations were significantly improved upon by constructing cooperative probes, called Tentacle Probes™ (TP), which utilize divalent binding to achieve both high sensitivity as well as extremely high specificity.

Immobilization of Tentacle Probe onto gold disk electrode produced statistical significance, with $p<3.0*10^{-13}$. Tentacle Probe with EIS detection was able to detect a difference between the B. AnthracisgyrA gene and B. Cereus gyrA Single Nucleotide Polymorphism sequence at a lower limit of detection of 20nM. Furthermore, B. Cereus gyrA is a common soil bacterium that causes false positives for B. AnthracisgyrA. This method of detection is current a model system for DNA detection; other genes could be detected with this system. With the potential to be transformative by providing a platform technology for both DNA and protein detection with high sensitivity this system offers extremely low rates of false positives. There is a high capacity for the technology to be multiplexed for high-throughput applications or quick diagnostic tests for medical purposes. Thus, the biomarker development will also have the potential to be assessed through noninvasive methods.

Keywords: Tentacle probe; Cyclic voltammagram; Amperometric; Electrochemical impedance spectroscopy; Redox probe/electron mediator

Abbreviations: TP: Tentacle Probe; CV: Cyclic Voltammagram; Amp-It: Amperometric I-T; EIS: Electrochemical Impedance Spectroscopy; RP: Redox Probe/Electron Mediator

Introduction

The point-of-care electrochemical impedance based technique offers what existing techniques such as PCR and ELISA do not currently have, rapid analytical results, sensitive biosensors, high portability, and low cost [1,2]. A nucleic acid biosensor would need to exemplify all these characteristics. For example, 4.6 million deaths annual occur from Bacterial Infectious Disease (BID). In addition, the majority of the deaths that occur, approximately 95%, happen due to improper diagnosis and treatment [3]. This is because BIDs are easy to misdiagnose, many reported cases occur in rural communities of developing countries that do not have access to proper health facilities with their own laboratories [4]. Moreover, the current State Of The Art (SOTA) method of nucleic acid detection requires cell cultures and gene amplification through Polymerase Chain Reaction (PCR) and Enzyme-Linked Immunosorbent Assays (ELISA); while these methods do provide sufficient results, they require SOTA laboratories and are time-consuming processes [5].

To increase the specificity without comprising the sensitivity we proposed a modified version of a typical Molecular Beacon (MB) called the Tentacle Probe (TP). An MB is a nucleotide hybridization probe that detects the very specific nucleic acids. Furthermore, a MB is a hairpin shaped structure that will detect target nucleic acid sequences. When detection occurs the quencher group moves from the fluorophore group and fluorescence can occur as a result of binding to the target nucleic acid sequence. The TP is formed in a similar fashion however is able to bind more effectively to target nucleic acid sequence [6]. There are several methods and devices that exist to detect nucleic acid sequences. The most common method used clinically is culture isolation of the genes to determine the microbial identification and epidemiology [3]. However, currently culture based nucleic acid detection is a lengthy process with a further lengthened period upon which treatment is administered [7]. Due to these technical limitations the World Health Organization has attempted to address this problem. To counteract the formation of devices that are too complex to use in developing countries, novel methods of detection are considered. Current devices that are introduced to the market must meet certain requirements such as, limited resource setting, and minimal laboratory support. The requirements as stated by the world health organization are collectively referred to by the acronym ASSURED. ASSURED stands for the capabilities that new biosensors should have when introduced to developing countries; specifically, (i) Affordable, (ii) Specific, (iii) Sensitive, (iv) User-friendly, (v) Rapid, (vi) Equipment-free, and (vii) deliverable to those who need it [7,8].

To fulfill the WHO requirements this study combined the strengths offered by the TP and electrochemical detection called electrochemical impedance spectroscopy (EIS). The electrochemical technique EIS is used as a potential method of detection that offers DNA hybridization sensing up to lower limit of detection 10 nM concentration [9].

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Nucleic Acid electrochemical sensors convert the hybridization event of congruent base pairing that occurs on the electrode surface to a useful electrical signal [10,11]. The electrochemical biosensor is a technology that addresses some of the criteria imposed by the WHO and the ASSURED requirements. EIS detection is a method that is affordable, sensitive, and rapid. In addition, the operation of the device is comparable to a glucose meter; it has high potential to become user-friendly with further development. Finally, the technology is equipment-free and deliverable to developing countries.

In the world of Molecular Biology, detection of nucleic acid sequences is done through the use of PCR and (MB). However, PCR/MB technology can experience false positive readings between 9% and 57% and ultimately do not meet the criteria set by the WHO [12]. TP is able to outperform the MB due to its increased specificity and sensitivity. A more detailed explanation can be found in Satterfield et al.[6], but briefly it is described as a hairpin structure that contains a fluorophore and quencher group in close proximity. The hairpin structure opens when target sequence is present, and thus, the fluorophore group moves away from the quencher and fluorescents occurs. This was precisely the goal of the TP, eliminating false positive detection without sacrificing the specificity and sensitivity, making the method of detection more rapid. Satterfield successful detected wild-type (WT) target sequence with a lower limit of detection (LLD) of 15.4 nM with PCR and TP technologies. This was significantly better than PCR/MB, however, the technology of PCR and TP does not meet all of the requirements put forth by the WHO. Electrochemical point-of-care biosensor detection offers greater advantages over other optical systems, such as MB/TP, and PCR, all of which exhibit fluorescent or chemiluminescent labels [13,14]. Electrochemical detection does indeed off the strong specificity, rapid results, and good sensitivity, however, the process is not equipment-free, user-friendly, or deliverable to developing countries.

Detection concerning nucleic acids in systems is challenging. As with many systems there is a tradeoff between sensitivity and specificity. This is true with other nucleic acid methods of detection such as PCR and ELISA techniques. Many of these systems involve several steps and specific antibody tagged fluorescent labels. Additionally, the number of binding events and the capture kinetics of the target biomarkers limit the detection. There are currently novel methods of electrical response of nucleic acids that perform the detection at the nano scale such as nano needle biosensors that perform detection in real time such as EIS [15]. Attempts have been made in order to address the concerns of increasing specificity and sensitivity through the use of label free biosensors. The method of detection we propose is a novel method of detection that strictly addresses the concerns of the WHO ASSURED program.

The Tentacle Probes is a cooperative rt-PCR probe developed by Caplan and collaborators, achieves exquisite specificity by decoupling the binding of the probe to the analyte DNA sequence from the detection event in which the fluorescent part of the probe binds to analyte DNA. Tentacle Probes combine a traditional Molecular Beacon (rt-PCR probe with a quencher on the 3' end, fluorophore on the 5' end, and self-hybridizing sequences at both ends so that the probe forms a stem-loop structure when not bound to analyte DNA) with an additional linear piece of DNA (called the Capture region) connected by a poly (ethylene glycol) linker. Caplan and co-workers have demonstrated that Tentacle Probes achieve their exquisite specificity by making the detection region binding independent of the solution concentration of the analyte DNA. The capture region of the TP binds to the analyte DNA first, and then the detection region of the probe and the part of the analyte DNA to which the detection region will bind are held in proximity to each other with known concentration (the molarity of one molecule in the volume in which that strand of DNA can move=1/(4/3 π L^3 * 6.022x10^23) where L is the length of the detection region in decimeters) [6].

Literature has shown that there is often a tradeoff between specificity and sensitivity. The EIS-TP system has promise as a revolutionary nucleic acid detection device. The EIS offers rapid, affordable, equipment-free, and user-friendly point-of-care detection while the TP can make detection extremely specific and sensitive. Furthermore, this work explored the impedance-based electrochemical technique (EIS) in conjunction with TP; TP was immobilized onto the GDE. The following experiments were performed to find the optimal functioning temperature of the TP while on the GDE and the optimal frequency to perform LLD.

Materials and Methods

Chemical and reagents

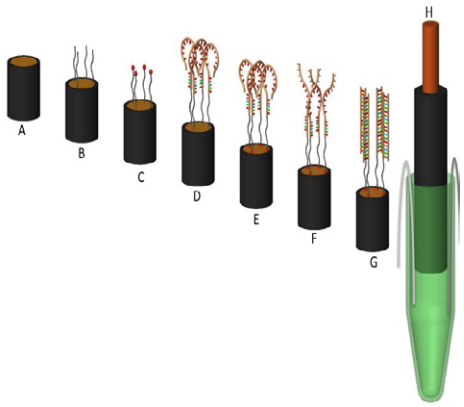
Unless otherwise stated, all chemical reagents were purchased from Sigma or Sigma-Aldrich (St Louis, MO, USA). 130 mM NaCl, 10 mM phosphate buffer (PBS), and 3 mM KCL tablets were purchased from Calbiochem (Gibbstown, NJ, USA), pH electrode storage solution was purchased from Thermo Electron Corporation (Beverly, MA, USA), Potassium hexacyanoferrate (III) was purchased from EMD Chemicals (Billerica, MA, USA), N-Hydroxysulfosuccinimide Sodium Salt (NHS) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). HEPES free acid 99% was purchased from Acros Organics, Thermo Fisher Scientific (Waltham, MA, USA). TP and target sequences were synthesized and purified through dual high performance liquid chromatography by Biosearch Technologies (Novato, CA, USA). TP was synthesized through the addition of a capture region to the hairpin using polyethylene glycol sequence (Table 1) [6]. Table 1 shows the B. AnthracisgyrA WT and B. Cereus gyrASNP gene. In addition, B. Cereus gyrA is a common soil bacterium that causes false positives for B. AnthracisgyrA. The TP, WT, and SNP were suspended in HEPES buffer (pH of 7.0) with .18 M NaCl. Solutions were rapidly frozen using liquid nitrogen and stored in 193.15 K fridge. The redox probe reagent that was used is potassium hexacyanoferrate (III) dissolved in 10 mM phosphate buffer saline.

Electrodes maintenance

The electrodes used in this experiment were gold disk electrodes (GDE), Ag/AgCl reference electrodes, and platinum electrodes, and were all purchased from CH Instruments (Austin, TX, USA). For reference, the reference electrodes were stored in a 3 M KCl solution between datasets, and the counter electrode stored in a pH electrode storage solution. Polishing pads were used to clean the gold disk working electrodes. The Buehler felt pads were covered with deionized

Name	Sequence
TP	GAT TAA AAT GTC CAG TGT ACC AG - PEG 9mer - CF560 - ccc TGG CGG AAA AGC TAA TAT AGT AA ccgccaggga -BHQ1
WT	ATT ATT ACT TTA CTA TAT TAG CTT TTC CGC CAT CTA AAA TTC TAT TTT CTG GTA CAC TGG ACA TTT TAA TCA ATG TAT TC
SNP_{det}	ATT ATT ACT TTA CTA TAT TAT CTT TTC CGC CAT CTA AAA TTC TAT TTT CTG GTA CAC TGG ACA TTT TAA TCA ATG TAT TC

Table 1: The table shows the TP and target sequences of the WT and SNP.



Scheme 1: This illustration shows the immobilization chemistry process to covalently bind TP onto the end of the 16-MHDA SAM of the GDE. A: Bare Au disk Electrode. B: 1 mM 16-MHDA Linker. C: EDC/NHS coupling. D: 100 nM TP Immobilization. E: 1% Ethanolamine Blocking. F: Activated TP. G: TP biding to WT. H: Au Electrode setup using Eppendorf tube. Once immobilized a solution that contains the WT would show binding occur and result in a current and impedance change in real time as seen on a Nyquist graph.

water (DI) and Al_2O_3 particles of 1µg grit. Each GDE was polished on these pads using 120 figure-eight like rotations. After the GDE's were polished they were then sonicated in DI to remove any excess contaminants/alumina.

Immobilization technique

The method of preparing the gold disk electrode is precisely explained in Belle et al. and it is illustrated in Scheme 1, but is briefly described here. The above details the cleaning of the electrode and it is necessary for the electrode to be essentially bare so the self-assembly attachment can occur. The self-assembling monolayer 16-MHDA is incubated (1 mM) on the GDE surface for one hour at room temperature (RT). The 16-MHDA was suspended in ethanol and served as the link between the gold surface and 100 nM of TP. After one hour the electrode is rinsed with DI and incubated with NHS/EDC to prepare the carboxylate MHDA groups for TP attachment. 10 µL was placed directly on the surface of the gold disk and is incubated on the now enhanced GDE surface for one hour. Lastly, 1% ethanolamine in DI is incubated on the surface of the GDE for 30 min. This prevented non-specific binding on any unbound 16-MHDA. GDE's were stored at 277.15 K until WT and SNP experiments were performed. In short, immobilization of the TP occurred via 1 mM 16-mercaptohexadecanoic acid (16-MHDA) in ethanol, 10 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide in PBS (EDC for short), 80 mM (in PBS) NHS, and 1% (in DI) Ethanolamine.

Hot plate and electrochemical cell

The electrochemical cell that was a part of the experiment was a 2 mm GDE, Ag/AgCl reference electrode, and platinum electrode. The electrochemical cell was established using a 1.5 mL eppendorf tube into which the GDE was placed, bending the Ag/AgCl electrode (d=0.5mm) and Pt electrode (d=0.5 mm) wires into the Eppendorf tube. Alligator clips connected the cells to the 660C CH Instruments Electrochemical Analyzer. The electrochemical cell was placed into a VWR hot plate catalog number 12365-476 with a wells attachment and an IR Thermometer by EXTECH which measures temperature was used to set the temperature to perform the experiments. However before the immobilization of TP, cyclic voltammetry sweeping from +1 to -1 V 6 times and was performed on all bare GDEs using 100 microliters of sample redox probe. This allowed us to find the DC offset of the electrochemical cell or also the average oxidation and reduction peaks (formal potential).

Electrochemical sensing techniques

Electrochemical impedance based spectroscopy uses an AC voltage signal, which used a 5 mV sine wave of varying frequencies ranging from 100 kHz to 1 Hz using the formal potential. In short, the solution was stimulated with a voltage while the current was measured and impedance calculated in real time. The formal potential is used in every EIS step thereafter. From the measured current the impedance and phase are calculated and plotted as Nyquist graphs as seen in Figure 1. For all experiments the initial values are set, such that, Initial E=Calculated formal potential for that corresponding GDE from CV, High Frequency=100 kHz, Low Frequency=1 Hz, and amplitude=5 mV. After the GDE is prepared the following experiments can be performed.

According to Satterfield et al. [6] there is distinct differentiation of WT and SNP at 500 nM at 333.15 K. To perform this experiment 500 nM of targets SNP and WT was used. The relationship between the redox probe and samples is 1:1 ratio. Thus, the samples were pipetted into the tube with a mixture of 180 µL of HEPES and 180 µL of ferricyanide (100 mM) redox probe and 2 µL of 500 nM WT stock were placed into an eppendorf tube. The procedure is identical when using SNP. 222 µL of HEPES and 225 µL of ferricyanide (100 mM) redox probe and 3 µL of 500 nM SNP stock was placed into an eppendorf tube. The solutions were then placed into the hot plate; there was a 5-minute wait time between samples, thus allowing each sample to reach the desired temperature. The temperature was checked with a heat gun before the sample was run.

After the optimal temperature and frequency was established for the EIS/TP conjugated method a concentration gradient at constant temperature was performed. After the GDE is prepared, the WT and SNP in HEPES diluted through a serial gradient consisting of 10 solutions ranging from 0-500 nM is run from lowest to highest

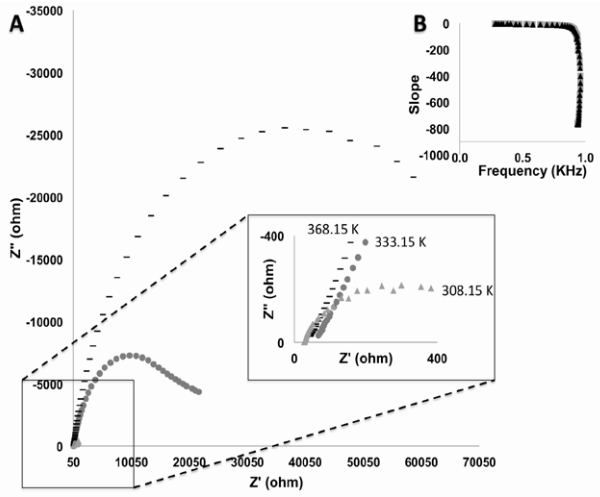


Figure 1: This is a representation of a Nyquist curve based off impedance readings of TP immobilized on GDEs at three temperature values of 368.15K, 333.15K, and 308.15K. (B) This graph shows the relationship of a logarithmic fit slope at the optimal frequency of 175.8.

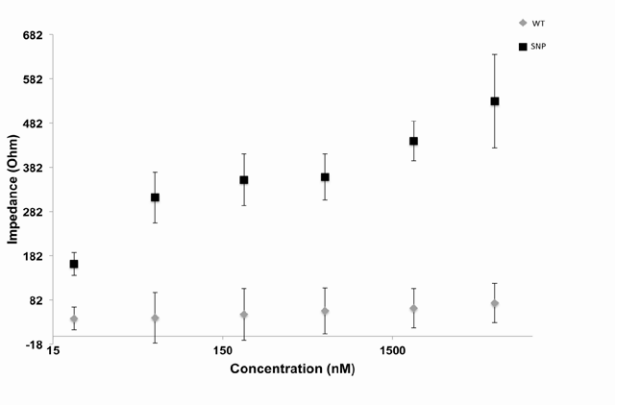


Figure 2: Overlay curve that plots normalized impedance as a function of WT and SNP concentration at the optimal binding frequency of 175.8 Hz and optimal binding temperature of 333.15 K in purified solution. The black curve, denoted by squares, is the SNP and the WT is the gray curve, denoted by diamonds.

concentration. The LLD for nucleic acid biosensor was performed. The amount included in each solution is illustrated above. Analyses were performed using a two way ANOVA with an alpha value of 0.05 and confidence interval of 95%. Additionally, taking the absolute value of delta change from the initial concentration to following concentration values data in Figure 2 was normalized.

Results and Discussion

Affordability, specificity, and rapid detection addressed

The protocol described in Scheme 1 is necessary for the proper covalent immobilization of TP so the interactions with WT are guaranteed during the EIS sweep. While the current is measured, from the EIS frequency sweep, Nyquist plots are generated that compare the real and imaginary impedance. This datum is collected in real time due to the magnitude and phase changing from the input to the output signal at the frequency range. To properly provide a solution for the WHO concept for new developing biosensors and answer the criteria of ASSURED [7], electrochemical DNA biosensors must be affordable. The electrochemical EIS system is a point-of-care, label free, low cost, highly sensitive method of biosensor detection [1,16]. Additionally, the development of the TP-EIS system will produce a novel method of sensitive and specific DNA detection whereas if separate it would falter in one of the two aspects.

The results provide by Figures 1A and 1B address the issue of sensitivity and rapid detection. Figure 1A shows a Nyquist curve for the varying temperature of 368.15 K, 333.15 K, and 308.15 K for a concentration of 100 nM TP immobilized onto the GDE. The light gray curve, denoted by triangles, indicates a temperature of 373.15 K with purified WT solutions made using only HEPES and the redox probe. The darker gray, denoted by circles, curve indicates a temperature of 308.15 K with purified WT solutions made using only HEPES and the redox probe. The black curve, denoted by rectangles, indicates a temperature of 333.15 K with purified WT solutions made using only HEPES and the redox probe. Changing the temperature of the solution provided varying impedance values. The solution comprised of WT, HEPES, and redox probe at the highest temperature tested (368.15 K) generated the lowest impedance. Concurrently the solutions at the lowest temperature (308.15 K) showed only moderate impedance and the greatest impedance resulted from a temperature of 333.15 K.

Figure 1A overlays the Nyquist curves of the constant concentration and varying temperatures. It is evident from these Nyquist curves that the 333.15 K yields a greater responsivity then at 308.15 K and 368.15 K. The cooperativity of the TP is predicated through the use of mathematical modeling. The cooperativity modes are derived from collision theory, where the probability of the first binding event is proportional to the product of the reagent's concentrations. In addition, an increase in temperature will increase the kinetics of the TP and WT binding Satterfield et al. [6] Scatterfield found that 333.15 K was the optimal performance temperature. There was a significant difference between the WT and SNP in detection that there is a measurable difference. To summarize, the initial concentration of three electrodes of TP were constant as was the solution containing WT. The TP and WT designed by Satterfield will negatively affect the bioactivity of TP, WT and SNP when exposed to prolonged exposure of temperatures nearing 368.15 K. At this temperature there was hardly any TP still immobilized to detect the WT. TP was designed to have a measurable WT to SNP detection difference at temperature of 333.15. The increase in temperature would increase the kinetics of the TP and WT binding [16,17]. Concluding, that the temperature is an important factor to consider when using TP, the optimal temperature is correlated to the strongest impedance in such a way that the TP exhibits sensitivity to the WT at certain temperatures. Moreover, this addresses the specificity of the WHO guidelines by developing a DNA biosensor that does not sacrifice specificity for sensitivity, all the while being rapid, affordable. While the technique is not yet portable, the potential design would match characteristics to blood glucose sensors and thus in fact be portable and user-friendly.

The data was placed into an EXCEL template after the concentrations were run. This allowed the spreadsheet to analyze the frequency verses the impedance at each frequency at the three temperature values. This presented the opportunity to look at all the frequencies, which allowed calculations of linear and log fit slopes and R-squares (degree of fit). The ideal frequency was determined by picking the best R-square (>0.95) and slope and plotting the impedance at that specific frequency (Figure 1B). Furthermore, Figure 1B illustrates the challenges that were presented with selecting the optimal frequency. It is evident that the relationship is logarithmic and the reproducibility (R^2) is an important factor. Figure 1B shows an n=3 of a sample of WT and after several immobilization, 175.8 Hz was chosen as it consistently displayed an R^2>0.95. Essentially, it offers confirmation to the issue that is presented when the temperature of the solution and GDE is changed from 308.15 K to 368.15 K. The impedance signal is negatively affected. However, at the approximate 333.15 K temperature there was greatest differentiation between the WT and SNP as indicative of individual R^2 and slope values (differentiation as great as 95%). Comparatively slopes were consistent around the concentration of 500 nM, from which the LLD of 20 nM of WT and SNP was tested and determined.

Sensitivity, user and equipment availability addressed

Figure 2 illustrates data collected at increasing concentrations (0-6000 nM) these tests were performed at the evaluated optimal frequency of 175.8 Hz and optimal binding temperature for WT and SNP of 333.15 K, as the TP designed by Satterfield et al. [6] was optimized for detection at 333.15 K. The black curve, denoted by squares, is the SNP curve and the gray curve, denoted with diamonds is the WT curve. With N=4 for both WT and SNP a two-way ANOVA showed statistically significant difference, $p<3.3*10^{-13}$, between the WT and SNP at a LLD of 20 nM. In addition, Figure 2 displays the sensor response to the increasing WT and SNP concentrations. The difference

between the WT and SNP indicates that the conjugated TP was able to differentiate between B. AnthracisgyrA WT gene and B. Cereus gyrA SNP gene. The EIS/TP conjugation as this provided evidence that EIS and TP together offer solutions to weakness in both techniques. EIS/TP is able to enhance specificity without sacrificing sensitivity. From a clinical standpoint, nucleic acid detection majorly uses cell culture, quick time PCR, and enzyme-linked immunoassays. These methods do provide sufficient results, they prove to be time-consuming and require SOTA laboratories, which most developing countries do not have. In addition, just PCR technology can experience false positive readings between 9 and 57% [11] and ultimately not meet the criteria of the WHO of being specific and sensitive. Electrochemical detection offers greater sensitivity of target at low concentration [18-23]. The combination of immobilizing the TP to the surface GDE has provided evidence of achieving the specifications of the WHO ASSURED criteria and stands as an important method of future point-of-care technology.

The results from this research show great promise in providing evidence to the sensitivity of the EIS/TP detection setup. The EIS/TP combination shows promise as a point-of-care technology. In an ideal case, a hand-held meter could be used that would heat the sample to the optimal temperature and perform EIS at the optimal temperature for the TP and WT. Assuming that the sample can be prepped quickly, testing takes less than 90 sec to gather data. The applications of such a biosensor are enormous and it meets the equipment free and user-friendly criteria imposed by the WHO. There is still a considerable amount of work required to take the research data components such as EIS and TP technology and fabricate a real time sensor that would fit the WHO criteria, - the results show only the immobilization onto a GDE sensor and displays data in terms of impedance - however, this data still offers the possibility of integration. If the design challenges could be overcome this device would become a SOTA device, potentially mirroring commercial glucose meters.

Conclusion

The bench top data presented is but one new method of nucleic acid detection. The resulting immobilization with EIS provided additional specificity and sensitivity to the existing method of detection. The quantified LLD being 20 nM in a purified solution is far more specific and sensitive than some current biosensors and PCR technologies. The resulting false positives that come from the SOTA detection of commercial devices are eliminated with the TP-EIS system. The ability to detect nucleic acid sequences at this LLD the combination of TP-EIS offers to become the next step in biosensor DNA detection.

Future work will include efforts to before detection in complex solution of B. AnthracisgyrA gene and B. Cereus gyrA. Furthermore, implementation of nanoparticles to develop a technique for simultaneous biomarker detection

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