AWARD NUMBER: W81XWH-19-1-0174
PR182377

TITLE: Novel Aptamer Based Biosensor Platforms for Detection of Cardiomyopathy Conditions

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REPORT DATE: JUNE 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
JUNE 2021	Annual	15 MAY 2020-14 MAY 2021

4. TITLE AND SUBTITLE
Novel Aptamer-Based Biosensor Platforms for Detection of Cardiomyopathy Conditions

5a. CONTRACT NUMBER
W81XWH-19-1-0174

5b. GRANT NUMBER
PR182377

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
4200 Fifth Avenue
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: There is a need for a point-of-care biosensing device for rapid screening and monitoring of cardiomyopathy conditions and progression to lower incidence, death occurrences, and healthcare costs. Overall aim of this project is to create a cardiomyopathy condition screening and monitoring tool to simplify the current biochemical marker testing procedures by developing vertically aligned platinum wire aptamer-based multi-array biosensor for precise, accurate, reliable and rapid measurement of the presence of relevant cardiac marker levels in the human whole blood and serum using electrochemical impedance spectroscopy. This work thus far, has demonstrated how to construct an impedimetric multi-array biosensor platform based on platinum wires functionalized with aptamers, and has successfully navigated the platform all the way beginning from construction to optimization and validation of feasibility in biological samples. Initially, we optimized the optimal platinum wire diameter and surface finish which was necessary to create a biosensor that does not experience saturation within the acceptable clinical ranges of brain natriuretic peptide (BNP) and troponin T (TnT) antigens, the accepted cardiac biomarkers. Following validation, the focus then shifted into assessing the self-assembled monolayer (SAM) approach utilized to tether the BNP and TnT specific aptamers to the electrode surface, determining both the optimal incubation time and concentrations necessary for each layer as well as assessing the necessity of each layer. The best self-assembled-monolayer (SAM) combination that provided reliable, accurate and most sensitive response was determined to be Platinum-Cysteamine-Glutaraldehyde-Neutravidin-Aptamer and this SAM combination showed excellent precision, reasonable sensitivity, and stable insulation of the linker proteins that can easily interfere with the biosensor readings. The optimal SAM combination was also used to develop biosensors to test in rat whole blood samples to create a unique calibration curve model. We also tested rat whole blood samples by using a novel corrective approach developed to in effect "erase" the impact of biofouling. We then tested commercially available screen-printed platinum electrode to detect BNP biomarker using the developed layer by layer (LbL) based SAM fabrication protocol and the results show instability of the SAM layers on these electrodes. Substrate modification was thus needed including design and fabrication of a miniaturized form of the platinum wire multi-array biosensing platform. We further studied storage stability of the BNP aptamer surface functionalized biosensor stored in a vacuum desiccator at 25^{o}C. The results show that surface functionalized electrodes not only detect BNP, but the biosensor response was also very similar to the un-aged biosensor. We also developed an electrochemical surface cleaning method greatly lowering the time for electrode preparation since the entire step of repeated and tedious mechanical polishing of the electrode prior to surface functionalization is eliminated by this cleaning process. To further validate the novel corrective approach, we plan to test human blood and serum samples (n =20) and verify the concentration of the measured BNP in blood and serum against the ELISA derived values (clinically used method/gold standard method) serving as controls.

15. SUBJECT TERMS
Cardiomyopathy, Heart Failure, Point of Care, Impedimetric Biosensors, Cardiac Biomarkers, Aptamer, Self-assembled monolayer, Brain natriuretic peptide, Troponin T

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	17	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

Table of Contents

1. Introduction: There is an increasing demand for sensitive point-of-care (POC) technologies to rapidly monitor the concentrations or activities of biomolecules in biological samples in a cost-effective manner [1]. Electrochemical impedance spectroscopy (EIS) is an ideal measurement approach for POC biosensors as EIS is a highly sensitive, inexpensive, and label-free technique that is amenable to miniaturization, rendering EIS based biosensors highly promising for direct use by patients at home or at the bedside of patients, by paramedics in the ambulance for emergency use, including during clinical visits as a critically useful screening device [2]. According to the American Heart Association and National Health and Nutrition Examination Survey, approximately 121.5 million people in the U.S. suffered from some form of cardiovascular diseases (CVDs) in 2016, and the cost burden (both direct and indirect) of cardiovascular diseases exceeded $351.2 billion [3]. By 2035, 45.1% of the US population is projected to have some form of CVD and between 2015 and 2035, the total direct medical costs of CVD are projected to escalate from $318 billion to $749 billion with the total indirect costs (attributable to lost productivity) for all fatal and nonfatal CVDs estimated to increase from $237 billion in 2015 to $368 billion in 2035 [3, 4]. Further, CVDs and stroke accounted for 14% of the total U.S. health expenditures in 2014 – 2015, more than any major diagnostic group. Unfortunately, the prevalence and costs of cardiovascular diseases are projected to continue to spiral over the years despite CVDs being largely preventable due to the rise in incidences of obesity, hypertension, and diabetes. This high prevalence is due to CVDs being clinically silent with only non-specific symptom evidence until signs of serious complications arise, which has led to a lack of standard methods for CVD diagnosis. Delays in accurate diagnosis and treatment of CVDs are often associated with poor clinical outcomes and increased healthcare costs. Hence, it is imperative that a point-of-care (POC) device be developed for rapidly screening and monitoring of CVD and cardiomyopathy (CM) related heart failure (HF) risks to decrease incidence, deaths, and healthcare costs. Although many CMs are inherited, biochemical markers are a fundamental part of the diagnostic work-up and are useful in the prognostic assessment of the disease. The current diagnostic techniques for CVDs rely entirely on the use of expensive non-invasive imaging techniques, use of invasive methods, or on the timely and accurate interpretation of the physical symptoms experienced by patients. Unfortunately, current protocols dictate medical professionals treating any individual reporting chest pains (one of the most common symptoms of heart attacks) as potential acute myocardial infarction (AMI) patients. Therefore, resources are often constrained leading to situations where people with a milder form of CVDs or other unrelated diseases are also unnecessarily admitted and tested for possible heart attacks. However, in medical facilities with fewer resources, lack of these more sophisticated testing procedures could lead to possible misdiagnosis, thus potentially running the risk of treating patients for an entirely different condition rather than the real disease.

2. Keywords:

Cardiovascular diseases, Biosensor, Impedimetric, Cardiomyopathy, Point-of-Care, Brain natriuretic peptide (BNP) and troponin T (TnT)

3. Accomplishments:

What were the major goals of the project?

Major goals of the project: The overall goal of this project is to simplify, accelerate and improve the biochemical marker testing process. This involves developing vertically aligned platinum wire aptamer-based multi-array biosensor for precise, accurate, reproducible, and rapid detection as well as measurement of the presence of relevant cardiac marker levels in the human whole blood and serum using electrochemical impedance spectroscopy (EIS). To meet the proposed objectives, two specific aims and related subtasks were crafted which are described in the following below:

Specific Aims: **1) Optimization of the self-assembled monolayer (SAM) of the platinum wire multi-array biosensing platforms by assessing the ideal concentrations, incubation times, and combinations of the functional layers and antigen concentrations**.

Major Task 1: Optimize the incubation times and concentrations for all the SAM components, determine the need for each of the SAM components, and accurately isolate the ideal antigen detection time (**completed, 2020**).

The specific steps (**sub tasks**) to achieve **Specific Aim 1** and **Major Task 1** involve the following:

Subtask 1.1 Assess the optimal incubation times and ideal concentrations for each functional layer of the SAM (**completed, October 2019**).

Subtask 1.2 Determine whether the functional layers of the SAM can be removed without compromising the biosensor performance (**completed, February 2020**).

Subtask 1.3 Optimize the antigen incubation time to enhance sensitivity, precision, and linearity of calibration curves (**completed, April 2020)**.

Specific Aim 2: Simplify the optimized biosensor for single-frequency antigen detection, aptamer regeneration and biosensor testing against clinical blood samples derived from patients to assess the specificity, selectively, accuracy, and reusability of the single-frequency aptasensor.

Major Task 2: Fabrication of a multi-array impedimetric aptasensor on a platinum platform for accurate antigen detection, aptamer regeneration and reusability of biosensors for cardiac markers (**on going**).

The specific steps (**sub tasks**) are:

Subtask 2.1 Determine the single frequency for each cardiac biomarker exhibiting excellent antigen detection and retest the biosensors at the exact single frequency **(Completed, December 2020).**

Subtask 2.2 Develop an electrochemical technique to regenerate aptamers without impacting the biosensor performance to create a reusable biosensor (**On going**).

Subtask 2.3 Test the biosensors against clinically obtained whole blood, serum, and plasma samples to evaluate the effectiveness of the biosensors as a potential ex-situ cardiomyopathy screening device (**Once the IRB is approved, we will test sensor performance using clinical samples)**

What was accomplished under these goals?

Significant research results under Specific Aim 1: For precise, accurate, rapid detection, screening and management of vital blood cardiac markers we created Cardiosense, an aptamer-based biosensor with vertically aligned platinum (VAP) electrode wires (**Figure 1a**). Platinum, Pt a noble metal with high electrical conductivity including the desired biocompatibility, as well as oxidation immunity compared to silver, and lower absorptivity than gold is chosen as the apt substrate for likely reducing biofouling. Cysteamine (C), glutaraldehyde (G), and Streptavidin/NeutrAvidin (N) self-assembled monolayers (SAM) are first formed on the VAP wires using the Layer by Layer (LbL) method. SAMs tether the biotin-based aptamer (biological detection element) to Pt maintaining contact between the two elements for transducing to a readable output (**Figure 1b**). The major tasks of this aim were to optimize the incubation times and concentrations for all the SAM components, determine the necessity of each SAM component, and finally assess the ideal antigen detection time. All of the proposed tasks of **specific aim 1** were completed and the results were detailed in the previous report. After completion of those tasks, we

further extended our investigation of the aptamer biosensor to (i) assess the storage stability of the self-assembled monolayers of biosensor over time and (ii) check the feasibility of the developed aptamer-based approach to the commercially available screen-printed platinum-based electrodes.

(i) Assess the storage stability of self-assembled mono layers (SAM) of biosensor at different conditions:

The meticulous self-assembled monolayers derived biosensor electrodes were prepared with cysteamine (10 mg/mL), glutaraldehyde (25% w/v), Neutravidin (1 mg/mL), and aptamer (1480 µg/mL) added in succession. In PCGNA, platinum (P) is thiolated by cysteamine (C) to expose the amine linkage groups. These amine groups bind to the carboxyl groups on one end of glutaraldehyde (G), exposing the carboxyl groups on the other end of glutaraldehyde. These exposed carboxyl groups bind to amine groups in Neutravidin (N), and the biotin group on the biotinylated aptamer (A) binds to the biotin binding sites present in Neutravidin (**Figure. 1b**).

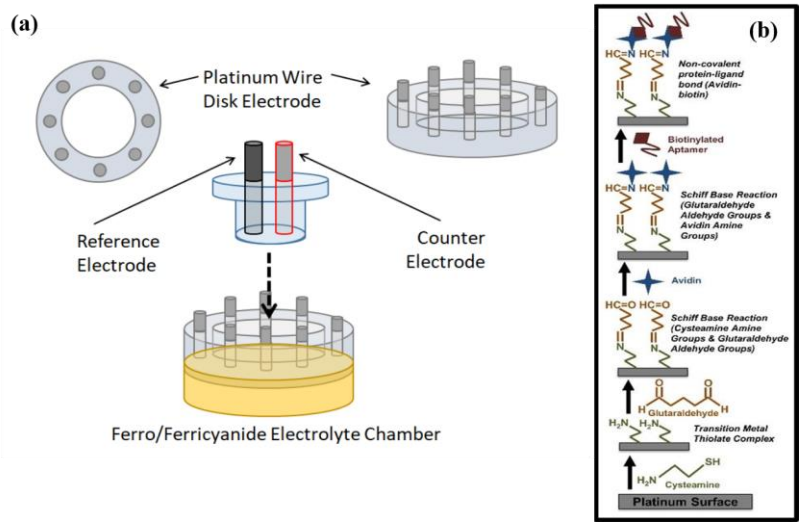

Figure 1. (a). Schematic of the electrochemical cell setup showing the various components and demonstrating the electrochemical cell setup including how the platinum disk working electrodes, silver reference electrode, and platinum counter electrode fit into the Ferro/Ferricyanide electrolyte chamber; (b). Schematic of the functionalization steps showing the accompanying chemical interactions between the functionalization layers.

After the completion of fabrication and optimization of the self-assembled mono layers of biosensors, time depended stability of the SAM layers (**Fig. 1b**) and their influence on biosensor response were investigated. It is important to assess the time dependent stability (or storage stability) of the self-assembled mono layers in terms of the commercial applicability of the developed biosensor. In order to investigate the storage stability of the self-assembled monolayer

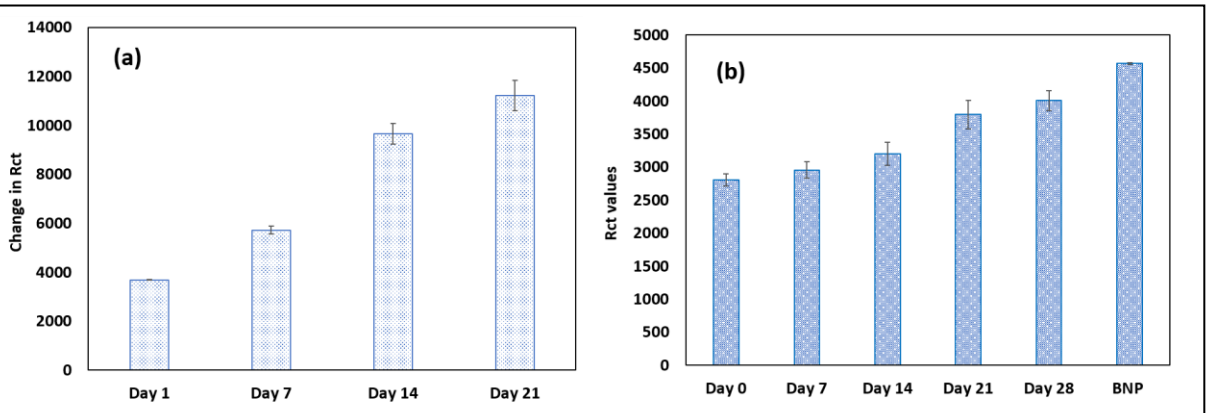

Figure 2. (a). Stability of LbL functionalized electrodes in phosphate buffer over a period of 21 days (n=3); (b) Stability of LbL functionalized electrodes at room temperature over a period of 28 days (n=3).

of biosensor, we prepared the surface functionalization SAM (PCGNA) on electrodes. Two different conditions were used to determine the SAM stability and are described below:

(A). In order to evaluate the stability in liquid medium, the surface functionalized electrodes were immersed into the electrolyte solution (0.5M Potassium Ferro-Ferri cyanide in Phosphate buffer) and EIS testing was performed at different time intervals (**Fig. 2a**).

(B). To evaluate the stability of SAM at room temperature, these functionalized electrodes were stored inside a vacuum desiccator at room temperature (25^{o}C) and EIS testing was performed at different time intervals such as Day 0, 7, 14, 21 and 28 (**Fig. 2b**).

Fig. 2a shows that the R_{ct} values of the electrodes increases with increase in storage time when stored in Phosphate buffer (PBS) electrolyte solution. This is likely due to presence of various inorganic and organic salts in the PBS which may alter the interactions between the various layers in SAM contributing to the increase in R_{ct} change. The electrodes stored at room temperature in a vacuum desiccator also showed increased in R_{ct} values over a period of 28 days. However, the rate of change in R_{ct} value with time was smaller compared to the electrodes stored in PBS.

After 28 days of storage at 25^{o}C, we performed the antigen (Brain natriuretic peptide, BNP) detection capability of the stored electrodes. The results showed that surface functionalized electrodes can detect the BNP and the sensor response was very similar to the un-aged biosensor.

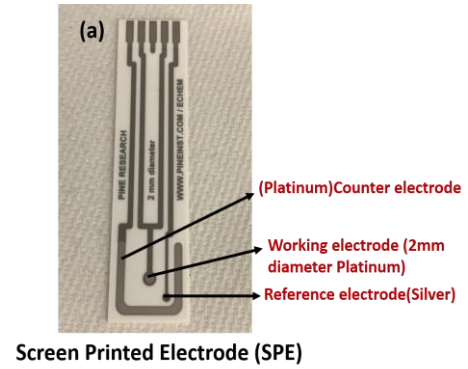

Figure 3. (a). Photographic image of a screen-printed electrode (SPE); (b) Experimental setup showing use of the screen-printed electrode (SPE).

(ii) Feasibility studies on aptamer sensor using commercially available screen-printed electrode platform: In order to develop a point of care (POC) test unit for detection of CVDs biomarkers at home, it is imperative to miniaturize all of the three components of the biosensor, i.e. biosensor electrodes, transducer and display. As first step towards this direction, we planned to replace the platinum wire multi-array biosensing platform with a commercial screen-printed platinum electrode. **Fig. 3a** shows the image of a screen-printed electrode and **Fig. 3b** shows the experimental set up for measurement of sensor response using the commercial screen-printed electrode.

Electrochemical characterization of screen-printed electrodes (SPEs) was conducted by cyclic voltammetry (CV) and EIS measurements. Functionalization and antigen binding assessments were assessed by measuring EIS after each step of functionalization. CV experiments were carried out across a potential range of -0.3V to 0.6V at a scan rate of 100mV/s and EIS experiments were directed across a frequency range of 300,000 Hz – 0.01 Hz with an AC voltage of 10 mV rms and were analyzed using the Z-view (Scribner Associates, Inc.) to determine the charge-transfer resistances.

In the CVs (**Fig.4a**), the current of the working electrode (Pt) was measured across a voltage range at a particular scan rate (mV/s), and the potential was cycled across +0.6V and -0.3V at a scan rate of 100 mV/s to exhibit the oxidation and reduction cycle of the redox couple, $Fe(CN)_6^{3-/4-}$, present in the electrolyte. In the oxidation reaction, the electrons flowed from the electrode surface to the redox couple and exhibited a positive peak current, known as cathodic peak current (IpC). In the reduction reaction, the electrons flowed from the redox couple of the electrode surface, thus exhibiting a negative peak current, known as the anodic peak current (IpA). For EIS (**Fig. 4b**), the opposition of the circuit to an AC current flow of 10 mV rms across a frequency range of 300,000 Hz – 0.01 Hz was measured. As the EIS was measured from an AC current flow (rather than DC current flow), the resulting impedance possessed both frequency dependent phase and magnitude (thus making impedance a vector quality. Resistance, the opposition of a circuit to a DC flow is a scalar quality, possessing only magnitude). The resultant Nyquist plots demonstrated the Cartesian coordinates derived from the parametric coordinates of the frequency response, where the real component of impedance (Z') was plotted on the X-axis and the imaginary component of (-Z") was plotted on the Y-axis.

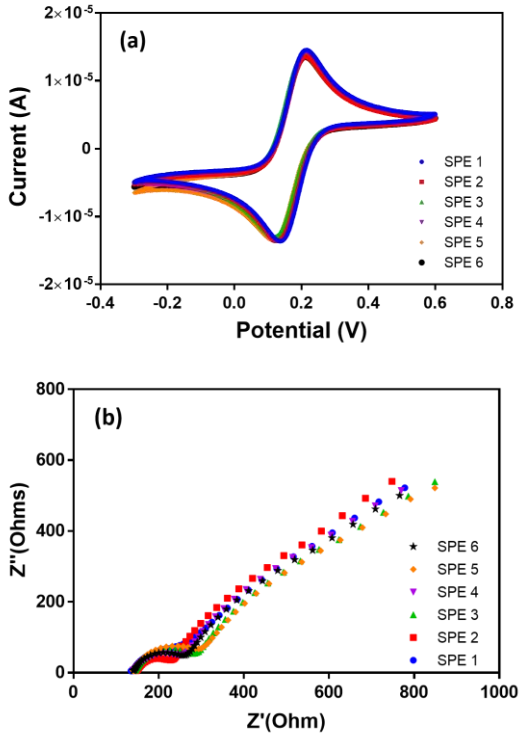

Figure 4. (a) Cyclic voltammogram of SPE; (b) EIS experimental data of SPEs.

In order to prepare the surface functionalization, screen-printed electrodes were treated with subsequent functional layers. Electrodes were first treated with 10 mg/mL of cysteamine prepared in de-ionized water for 30 min at room temperature, followed by 25% glutaraldehyde in water for 15 min at room temperature for thiolation and carboxylation of the surface. The surface was then treated with 1 mg/mL Neutravidin prepared in PBS for 30 min at room temperature, followed by incubation with 148 µg/mL biotinylated BNP s for 30 at room temperature. The charge-transfer resistances of the SPEs obtained at every step of the functionalization were measured and shown

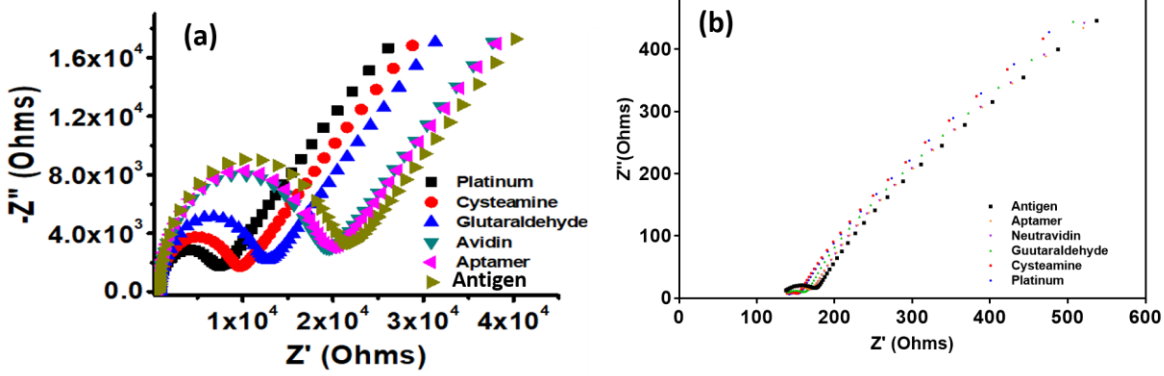

Figure 5. EIS experiments conducted after each step of functionalization for (a) current biosensor developed by this group, and (b) the screen-printed electrode; P: platinum, Cysteamine (C), Glutaraldehyde (G), Neutravidin (N), and Aptamer (A) and BNP antigen (Ag).

in **Fig. 5**. The results demonstrates that the charge transfer resistance increases slightly with addition of each layer of cysteamine (C), glutaraldehyde (G), neutravidin (N), and aptamer (A). These changes in the R_{ct} values with the addition of each subsequent layer are however, much lower when compared to the current platinum wire multi-array biosensing platform. Moreover, the sensor response towards BNP antigen was also very weak and, therefore, these SAM functionalized SPEs cannot be used for reliable detection of BNP. This may be due to the presence of an amorphous carbon and other impurities from use of dispersants and other surfactants in the screen-printed commercial electrodes, which interferes with the adsorption-based SAM formation process. However, the ability of the system to exhibit impedance change with each layer is a testament to the validity of such a platform with carefully assembled electrodes to detect and sense the required biomarkers. We are hence, currently working on the design to miniaturize our existing current platinum wire multi-array biosensing platform for use as a POC sensing unit for home use.

Key research accomplishment under Specific Aim 2: While the previous aim focused on optimizing the biosensor fabrication and reducing the biosensor SAM complexity, this aim (**major task**) was primarily focused on optimization of the biosensor data collection.

Subtask 2.1 This task was completed, and the results were detailed in the previous report.

Subtask 2.2 Develop an electrochemical technique to regenerate aptamers without impacting the biosensor performance to create a reusable biosensor (ongoing): One of the many advantages for use of aptamers is the stability of the aptamer allowing for the biosensor to be reused (regenerated) in such a way that only the aptamer unfolds, releases the antigen, and then refolds back into its original configuration, thus allowing the aptamer and correspondingly, the biosensor to be reused for subsequent measurements. Our strategy for regeneration was to use the electrochemistry principles in itself to manipulate the aptamer into unfolding from inactive and refolding into the current active sensing configuration. Thus, BNP biosensors (without the antigen) were prepared

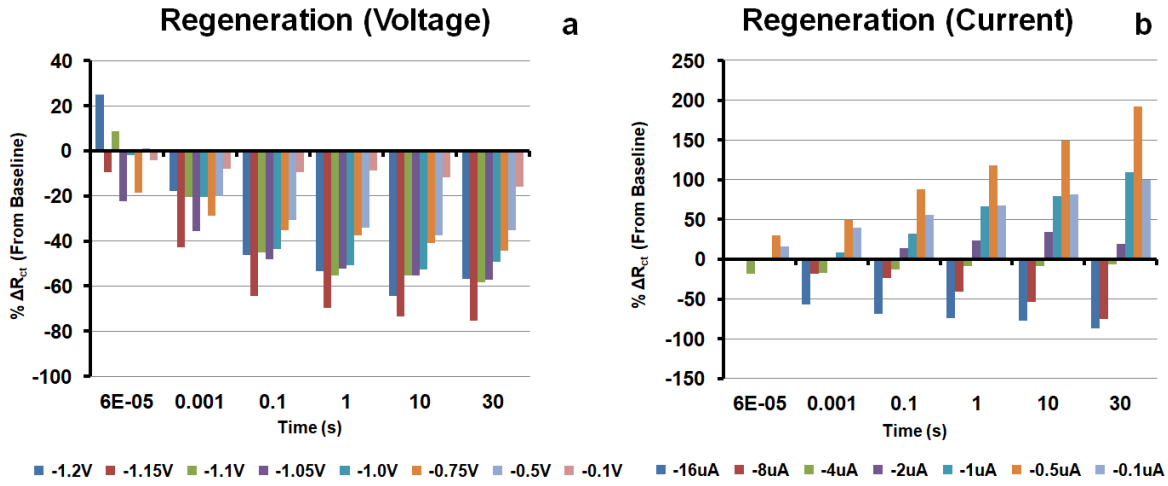

Figure 6. Regeneration strategies: Based off of a BNP biosensor, two regeneration strategies (a) Applied voltage, and (b) applied current, were utilized by applying a certain magnitude of voltage or current over time (ranging from 60 µs – 30 s).

and then exposed to various voltages and currents for a variety of times to assess if the voltages or currents disrupted the intact biosensor system. The results are shown in **Fig. 6**. With the applied voltage, especially at higher magnitudes of voltage, we observed that the biosensor layers are completely stripped off, even with a voltage being applied for a short duration of only 1 ms. With

the applied current, alternatively it was observed that any current above -1.0 µA actually caused an increase in R_{ct}, whereas lower magnitudes were, similar to the voltage, perceived to be too strong and resulted in removal of the biosensor layers themselves. Thus far, the results obtained in this study was inconclusive. As part of the regeneration of biosensor, further experiments were focused on modification of the SAM platform and functionalization which will be reported in the next report as well as the complete elimination of functionalized layers from cysteamine to aptamer with the use of electrochemical cleaning process as described in detail below.

Table 1: Parameters used to clean the electrodes using cyclic voltammetry (CV).

Parameters	**Platinum Electrodes**
Number of segments	200
Initial Potential	-300 mV
Upper Potential	1700 mV
Lower Potential	-300mV
Final Potential	-300 mV
Sweep Rate	500 mV/s
Electrolyte	0.5 M H_2SO_4

Electrochemical cleaning of platinum wire multi-array electrodes of the biosensing platform: A reliable way to surface regeneration and formation of a pristine platinum surface is important for recycling and reusability of the sensor electrodes after each use. One way to achieve this is to remove all the SAM layer by electrochemical cleaning. The process involves repeated application of an electrical potential to cycle the working platinum electrode in a dilute sulfuric acid solution. The specific parameters used for the electrochemical cleaning procedures are given in **Table 1**. Repeated cycling of voltage removes the surface contaminants including the SAM layers and improves the electrochemical performance of the electrodes. In order to investigate the effect of electrochemical cleaning, we used H_2SO_4 cleaning before and after SAM surface functionalization of the electrodes. The results are shown in **Figs. 7**. After electrochemical cleaning of the functionalized electrodes, the cleaned platinum electrodes showed baseline R_{ct} values corresponding to the pristine platinum surface. This results clearly demonstrate that the developed electrochemical cleaning method is equally effective in regenerating the pristine platinum surface and the reproducibility of the method was also verified by repeated measurements (n=3). This step also significantly reduces the time for electrode preparation since the entire step of repeated and tedious mechanical polishing of the electrode prior to surface functionalization is completely eliminated by the electrochemical cleaning process.

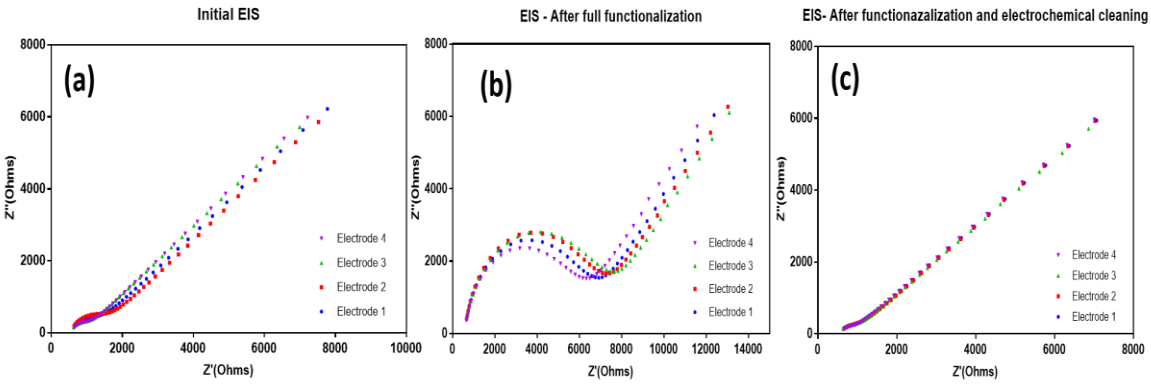

Figure 7. Effect of electrochemical cleaning after immobilization.The top layer of the BNP-antigen prepared in phosphate buffer. (a) Initial EIS (b) After functionalization (c) After functionalization and cleaning.

Subtask 2.3 Test the biosensors against clinically obtained whole blood samples to evaluate the effectiveness as a potential ex-situ CVD screening device: **This step is awaiting the approval of the IRB. Once the IRB is approved, the biosensor testing will be conducted on clinical samples.**

Key Research Outcomes/Accomplishments

- The results showed and affirmed the short term (< 1 month) storage stability of the surface functionalized layer of the BNP sensor under vacuum desiccator at 25^{o}C.
- The commercially available screen-printed Pt electrodes cannot be used to detect BNP biomarker using the developed LbL based SAM fabrication protocol. This necessitates modification of the platform including designing and fabrication of miniaturized form of the platinum wire multi-array biosensing platform.
- Development of an electrochemical technique to regenerate aptamers without impacting biosensor performance to create a reusable biosensor has not been successful yet necessitating modification and optimization of the sensing substrate platform which is currently ongoing. However, a reliable method to regenerate pristine metallic platinum surface has been developed.

What opportunities for training and professional development has the project provided?

The project has provided opportunity for a post-doctoral fellow to work on this project and thereby gain experience in SAM generation, fabrication of electrodes, detection and testing. In mentoring the post-doctoral fellow, the project has provided an excellent avenue for the PI and all of the Co-PI's to gain experience in various aspects of organization, execution and training.

How were the results disseminated to communities of interest?

In past two years of this project thus far, efforts were directed at achieving the planned project goals of fabricating the various SAM combinations, preparing the electrodes, regeneration of the electrodes, performing detailed and systematic testing as summarized above. A part of these results has been submitted for oral presentation in MHSRS 2021 conference (Abstract ID is: MHSRS-21-04108). The results have, however, not yet been presented in various other conferences for rapid release of advances made to the diverse community comprising clinicians, materials scientists, chemical engineers, electrical engineers, electrochemists and solid-state chemists. We, however, anticipate that these successful results achieved in the two years of work as well as advances made in the basic understanding of the synthesis, fabrication, interface stability and reactions, including changes in the microstructure and ensuing electrochemical reactions, and comparison with theory will be published in peer reviewed archival journals very soon as well as presented at various biosensor conferences in the coming months. Significant achievements will also be posted in future on a secure internet website: http://nano.dental.pitt.edu/ and on http://www.engr.pitt.edu/; the university homepage of the PI and Co-PI. The website will serve as a laboratory notebook site and hence, will also act as a medium for exchanging the results and initiating stimulating discussions between various scientific communities.

What do you plan to do during the next reporting period to accomplish the goals?

Goals and objectives for next reporting period:

- Determine the sensitivity and selectivity of the biosensor using human blood and serum samples (n =20).

- Test the developed novel calibration and correction method to avoid cross selectivity and interference due to biofouling and other non-specific biofactors related adsorption.
- Verify the concentration of the measured BNP in blood and serum with the ELISA derived values (clinically used method/gold standard method) serving as the control.
- Improve the regenerative capability of the biosensor with or without the self-assembled monolayers by optimizing and modifying the biosensor platform.
- Finalize the design to miniaturize the sensor platform including sensing element (i.e., platinum electrodes) as well as the reference and counter electrode for home use.

4. Impact:

What was the impact on the development of the principal discipline(s) of the project?

The completion of this study will develop and optimize biosensor for cardiac biomarker, brain natriuretic peptide (BNP), detection in blood for cardiovascular disease (CVD) detection, management and monitoring. Validation of the fully optimized and miniaturized biosensor against clinically relevant whole blood, serum and plasma will greatly influence the specific clinical arenas. Currently, all biomarker detections, including BNP, in clinical and hospital settings use the standard benchtop assays needing costly instrumentation and trained personnel very much lacking the needed portability as well as rapid detection. Successful outcome of this project will yield biosensor for rapid and precise cardiac blood BNP detection, screening and heart failure patient condition management with high precision, accuracy, reproducibility and sensitivity. Furthermore, the studies will pave the way to design a prototype handheld biosensor for use by physicians and nurses in emergency rooms, smaller clinics, technicians and paramedics in ambulances including patients at home. Development of such a biosensing device will also prove to be very much handy especially under conditions of a pandemic wherein patients cannot easily access the services of the clinics and hospitals. The completion of this study will develop and optimize the biosensor for detection of the specific cardiac biomarker, namely, brain natriuretic peptide.

What was the impact on other disciplines?

The proposed research focuses on the development and optimization of biosensors for detection of cardiac markers in blood samples, thus producing a rapid and on-demand biosensing tool for CVD screening and monitoring. The proposed research will also further elucidate exactly how various components of the biosensor interact with one another (especially on a functional group level) and will thus provide new findings for clearly advancing the biosensor functionalization strategies. The platform is very versatile, and the studies will also pave the way for other disease detections studies (e.g. Traumatic Brain Injury), attesting to the versatility of the platform developed in this grant serving as a universal platform for immobilizing any aptamer, antibody, or enzyme, thus allowing for the detection of numerous proteins and markers implicated in various diseases. Therefore, the optimization procedures outlined herein will have universal scientific implications as various biosensor studies can utilize the findings of this proposal to develop more rapid, sensitive, accurate, reproducible, and precise biosensors.

What was the impact on technology transfer?

The project will result in several publications and the results of the studies will form the basis of one or more patent applications. It is possible that these disclosures and patent applications when awarded could lead to technology innovations that could potentially be licensed and even lead to the initiation of startup company ventures. The publications resulting from this work will help disseminate the work and as a result, it is possible that this novel approach can easily form the basis of new revolutionary biosensors for detection of cardiac markers in blood samples, thus

producing a rapid, accurate, sensitive, reproducible and on-demand biosensing tool for CVD screening and monitoring.

What was the impact on society beyond science and technology?

Successful outcome of the experiments outlined in this study will lead to development of a biosensor that can accurately detect cardiac markers in blood with high precision and sensitivity. In addition, the materials and strategies proposed in this study have been expressly selected keeping the concept of miniaturization and portability in mind. As result, the platform studies will allow to develop a prototype handheld device that can be operated not only by a physician or nurse in the emergency room setting, but also by doctors in smaller clinics, technicians and paramedics in ambulances, or potentially even by patients at home. Development of such a tool will also be particularly useful in the event of a pandemic wherein patients cannot easily access and visit clinics and hospitals. Therefore, performing the proposed study successfully will pave the way for development of a handheld point-of-care device for rapid, sensitive, accurate, reproducible and on-demand CVD and cardiomyopathy screening and monitoring. The studies will also open new avenues for early disease/condition detection, personalized medicine, with better understanding and involvement of patients enabling patients to make effective healthcare choices enhancing their decision-making ability ultimately helping to reduce the prevalence, morbidity and mortality of various diseases. Furthermore, the individuals who were trained on this project could eventually become engineers, administrators or choose faculty as well as industry careers and their eventual success could be attributed to the contribution, training and the overall experience gained from working on this project. Hence, the project will have a tremendous impact on improving the society aside from contributing to bounds of science, engineering and academia.

5. Changes/Problems:

Changes in approach and reasons for change:

There were no major changes or modifications to the approaches required to be taken during the formation of self-assembled monolayers (SAM) of the biosensor. We have also tested the short-term stability of the SAM. However, we need to understand the long-term (3-6 months) stability of these SAM also. Thus far, however, we have not been able to develop an electrochemical technique to regenerate aptamers without impacting the biosensor performance to create a reusable biosensor. We plan to achieve this with further optimization and modification of the functionalization of the substrate platform which we plan to do in the coming months and will be reported in the next report. Additionally, we have developed a reproducible and rapid electrochemical process to regenerate the clean and pristine platinum surfaces to reproducibly create and form the SAM layers for biosensor detection. This rapid regeneration step significantly reduces the time needed for electrode fabrication and testing.

Actual or anticipated problems or delays and actions or plans to resolve them:

Due to COVID lockdowns, we were unable to perform any laboratory work from March 2020 to September 2020. Following a lengthy approval process for having a mitigation plan in place, the laboratory has been partially open from September 2020. Consequently, the work was slowed down and the milestone of the project was delayed from the original project plan that was to be executed following inception in September 2020. We anticipate that these COVID lockdowns will hamper the time needed to achieve the milestones of the second phase of the project to some extent. Therefore, we requested a no cost extension of 12 months from the project manager in order to effectively complete the remaining tasks and milestones in a time efficient manner.

Changes that had a significant impact on expenditures:
No changes or alterations on the planned and actual expenditures incurred.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
No human subjects, vertebrate animals, biohazards and or/select agents involved.

Significant changes in use or care of human subjects:
No human subjects involved.

Significant changes in use or care of vertebrate animals:
No vertebrate animals involved.

Significant changes in use of biohazards and/or select agents:
No biohazards were considered in this research.

6. Products:

Publications, conference papers, and presentations:

Journal publications.
Nothing to report. Manuscripts and publications covering the findings and completion of the work to date and what is planned in the remaining time of the project are in planning stage at present. We anticipate completing and submitting these manuscripts before the end of the project.

Books or other non-periodical, one-time publications.
Nothing to report. Manuscripts and other publications covering the findings and completion of the work to date and what is planned in the remaining time of the project are in planning stage at present. We anticipate completing and submitting these manuscripts before the end of the project.

Other publications, conference papers, and presentations.
We have submitted an abstract entitled "Novel Aptamer based Biosensing Platforms for Detection of Cardiovascular Diseases" to the Military Health System Research Symposium (MHSRS) 2021 for oral presentation (Abstract ID is: MHSRS-21-04108) under the research topic- Updates on Military Women's Health. The Military Health System Research Symposium (MHSRS) is the Department of Defense's premier scientific meeting that focuses specifically on the unique medical needs of the Warfighter. This annual educational symposium brings together nearly 3,000 healthcare professionals, researchers, and DoD leaders for four days of critical learning, intensive idea sharing, and relationship building.
We also anticipate completing and submitting manuscripts covering the findings of the work before the end of the project.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
This work thus far, demonstrated how to construct an impedimetric multi-array biosensor platform based on vertically aligned platinum wires functionalized with aptamers, and progressively navigated the system all the way through from construction to optimization to demonstrating feasibility in biological samples. Initially, we focused on creating the multi-array biosensing platform without compromising the reproducibility between electrodes and determining the optimal wire diameter and surface polish (**0.5 mm diameter polished to 5 µm or 1200 grit**) necessary to create a biosensor that does not experience saturation for biomarker detection within

the clinical ranges of TnT and BNP antigens. Following validation, the focus shifted into assessing the SAM layer utilized to tether the BNP and TnT specific aptamers onto the electrode surface, determining both the optimal incubation time and concentrations necessary for each layer as well as assessing the necessity of each layer. We tested in all 9 different SAM combinations, and in the end, we determined that the best combination that provided reliable, accurate and most sensitive response was the **PCGNA** (Platinum-Cysteamine-Glutaraldehyde-Neutravidin-Aptamer) SAM combination, especially as it showed excellent precision, reasonable sensitivity, and stable insulation of the linker proteins that can easily interfere with the biosensor readings. We have also tested the time dependent stability of the SAM layers over a period of one month. The results showed that the SAM layer are stable under vacuum at 25 ^{o}C. Moreover, it is possible to regenerate pristine platinum surface after electrochemical cleaning. However, the developed SAM method was not successful to generate aptamer-based CVD biosensor using commercial screen-printed platinum electrodes. The data however, provided a pathway for miniaturization. Plans are in place to design a miniaturized multi-array biosensing platform from 0.5 mm platinum wire without compromising the sensitivity, selectivity, reproducibility and stability of the electrodes. The optimal SAM combination was also used to develop biosensors to test in rat whole blood samples to create a unique calibration curve model. We tested rat whole blood samples collected from another project as outlined in the earlier report by using a novel corrective approach to essentially "erase" the impact of biofouling and any interference arising from non-specific biomolecular interaction or adsorption. We believe that this novel corrective approach can be extended to test human whole blood and serum samples in a similar fashion eliminating any non-specific adsorption and accurately sensing elevated, median and low levels of BNP and TnT representative of the clinically accepted ranges. These studies will be executed in the remaining time of the project during the no-cost extension phase following the approval of the IRB.

Inventions, patent applications, and/or licenses

We are in the process of submitting an invention disclosure to the University of Pittsburgh.

The title of the invention: Novel two electrode-based correction method to eliminate biofouling from label-free affinity biosensors for detection of biomarkers from animal and human blood, serum, and body fluids.

The main claim of this invention is development of a corrective method which can nullify the influence of any interfering factors present in animal and human blood, serum, biological body fluids or in any natural or synthetic solution that could bind to the label free biosensor (i.e., Non-specific adsorption) and influence its sensitivity, specificity, and reproducibility.

Other Products

Nothing to report at present. Manuscripts covering the findings and completion of the work to date and what is planned in the remaining time of the project are in planning stage at present. We anticipate completing and submitting these manuscripts before the end of the project.

7. Participants & other collaborating organizations

What individuals have worked on the project?

Name	Most Senior Project Role	Nearest person month worked
Moni Kanchan Datta	PD/PI	9
Prashant N. Kumta	Co PD/PI	1
Abhijit Roy	Assistant Professor	1
Mary Keebler		1
Sangeetha KunjuKunju	Post-doctoral fellow	6

Full details of individuals who have worked on the project:

Name	Moni Kanchan Datta (mkd16@pitt.edu)
Project Role	PD/PI
Research Identifier	ORCID ID: Moni Datta (0000-0002-1837-2000)
Nearest Person Month Worked	9
Contribution to project	Principal investigator of the project involved in coordinating, planning and execution of the research. Worked extensively on the synthesis, structural characterization and electrochemical characterization of biosensor and interpretation of the results.
Funding Support	Fully funded from the current project

Name	Prashant N. Kumta (pkumta@pitt.edu)
Project Role	CO-PD/PI
Research Identifier	ORCID ID: prashant kumta (0000-0003-1227-1249)
Nearest Person Month Worked	1
Contribution to project	Co-principal investigator of the project involved in coordinating, planning and execution of the research.
Funding Support	No support from the current project.

Name	Abhijit Roy (abr20@pitt.edu)
Project Role	Co-investigator
Research Identifier	ORCID ID: https://orcid.org/0000-0002-5132-3825
Nearest Person Month Worked	1
Contribution to project	Involved in coordinating, planning and execution of the research. Worked extensively on the synthesis, and characterization of biosensor and interpretation of the results
Funding Support	Partial support from the current project.

Name	Mary Keebler
Project Role	Co-investigator
Research Identifier	ORCID ID
Nearest Person Month Worked	1
Contribution to project	Involved in coordinating, planning and execution of the research.
Funding Support	No support from the current project.

Name	Sangeetha KunjuKunju
Project Role	Post-Doctoral
Research Identifier	ORCID ID: Sangeetha (0000-0003-0338-8269)
Nearest Person Month Worked	6
Contribution to project	Synthesis and characterization of aptasensor.
Funding Support	Supported by the current project.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

There have been no changes in the support of the PI and any key personnel.

What other organizations were involved as partners?
No other organizations have been involved as partners in this project. The project is fully conceived and executed at the University of Pittsburgh.

8. Appendices. Nothing to report.

References:

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[4] N.R.I.N. RTI International. Projections of Cardiovascular Disease Prevalence and Costs: 2015–2035: Technical Report [report prepared for the American Heart Association]. Research Triangle Park.