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

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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 human whole blood and serum samples to create a unique calibration curve model. We also developed a novel corrective approach to in effect "erase" the impact of biofouling and a patent application was filed. Additionally, we 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. A standalone IRB for utilizing the biosensor platform for testing and validation of clinically relevant BNP levels in blinded human whole blood and serum was submitted and approved. Following this approval, two different methods of functionalization of the electrodes using washing method of fully functionalized electrode (Method 1) and complete functionalization of each electrode (Method 2) were used to obtain calibration curves for detecting five different clinically relevant concentrations of BNP in human blood and human serum samples. To further validate the efficacy of this novel biosensing platform technology, the innovative 2-electrode corrective approach will be used to test human blood (n = 20) and human 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

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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 (Completed and results were described in previous report submitted on June 11, 2021).

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 (The standalone IRB for this project is now officially approved and the biosensor testing of clinically relevant human whole blood and serum samples will be carried out in the upcoming months)

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.

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

Subtask 2.3 Test the biosensors against clinically obtained whole blood samples to evaluate the effectiveness as a potential ex-situ CVD screening device:

Prior to testing the human whole blood and serum samples containing clinically relevant BNP levels, it is important to prepare the calibration curve for the human serum and whole blood samples. In order to determine the complete antigen detection capabilities of the biosensor and the resistance of the biosensor to any interference from non-specific adsorption of myriad proteins, various biological samples of serum and whole blood samples from unknown patients were tested. Concentrations of 0.1 ng/mL, 0.3 ng/mL, 0.6 ng/mL, and 0.9 ng/mL and 1.2 ng/mL BNP were respectively prepared in the following (1), human serum and (2) human whole blood sample solutions. The calibration curves were prepared following two different methods described below.

Method 1: This method involves the testing of all the concentrations on the same batch of electrodes after successive addition of each concentration and washing steps. The detailed procedures followed are given below.

i) **Electrode functionalization**: Vertically aligned Pt electrodes were treated with each functionalized layer. Electrodes were first treated with 1 µL of 20 mg/mL cysteamine prepared in de-ionized water for 30 min at room temperature, followed by 1µL of 25% glutaraldehyde in de-ionized water for 15 min at room temperature for thiolation and carboxylation of the Pt-surface. The surface was then treated with 1.5 µL of 1 mg/mL Neutravidin prepared in 10 mM phosphate buffer solution (PBS) for 30 min at room temperature, followed by incubation with 1480 µg/mL biotinylated BNP aptamer for 30 min at room temperature. During functionalization, the electrodes were washed with de-ionized water for 30 sec and air dried before adding each subsequent layer. The functionalized electrodes were then stored in electrolyte solution at room temperature overnight.

ii**) Antigen testing**: Five concentrations of BNP were prepared by adding known amounts of BNP to human serum and human whole blood samples that originally contain very little or no BNP. Each concentration selected falls within the clinical range representing low to high risk for cardiovascular disease (CVD). The BNP-aptamer biosensors were thus successively treated with 1 µL of 0.1 ng/mL, 0.3 ng/mL, 0.6 ng/mL, and 0.9 and 1.2 ng/mL concentrations, respectively in order to develop a calibration curve for future biosensor testing. Each concentration was added after 30 sec of washing and air-drying steps. EIS measurements were then taken after each antigen incubation to obtain the calibration curves. All the antigen binding steps were conducted at room temperature to examine the rapid detection capability of the biosensor. The biosensor testing was performed for the above antigen concentrations using electrochemical impedance spectroscopy (Rct across 10,000 Hz – 1 Hz) following 5 min incubation.

Fig. 1(a) shows the calibration curve obtained for detecting BNP in human serum and Fig. 1 (b) shows the corresponding calibration curve obtained for detecting BNP in human whole blood.

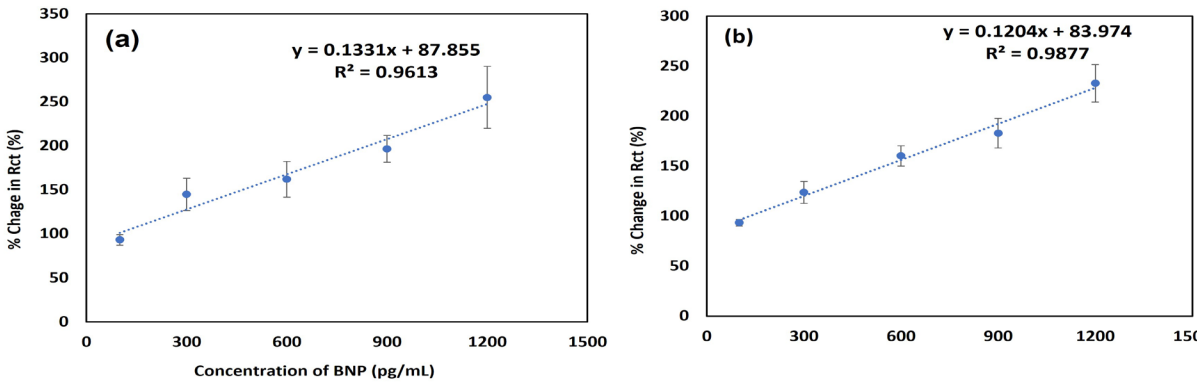

Fig.1(a) Calibration curved prepared for detecting BNP in human serum and (b) human whole blood samples (n=3 electrodes) using Method 1. The calibration curves depict the average percent change in charge-transfer resistance as a function of concentration.

Method 2: In Method 2 each concentration was tested on a different batch of freshly prepared electrodes (instead of using the same set of electrodes for testing successive concentrations of the antigen) for achieving better accuracy and also avoid increased interference from any biological substances present in the human serum and whole blood samples. The detailed procedures involved in this method is provided below.

i) Electrode functionalization: The vertically aligned Pt electrodes were treated with each functionalized layer. Electrodes were first treated with 1 µL of 20 mg/mL cysteamine prepared in de-ionized water for 30 min at room temperature, followed by 1µL of 25% glutaraldehyde in de-ionized water for 15 min at room temperature for thiolation and carboxylation of the Pt-surface. The surface was then treated with 1.5 µL of 1 mg/mL Neutravidin prepared in 10 mM phosphate buffer solution (PBS) for 30 min at room temperature, followed by incubation with 1480 µg/mL biotinylated BNP aptamer for 30 min at room temperature. During functionalization, the electrodes were washed with de-ionized water for 30 sec and then air dried before adding each subsequent layer. The functionalized electrodes were stored in electrolyte solution at room temperature for overnight.

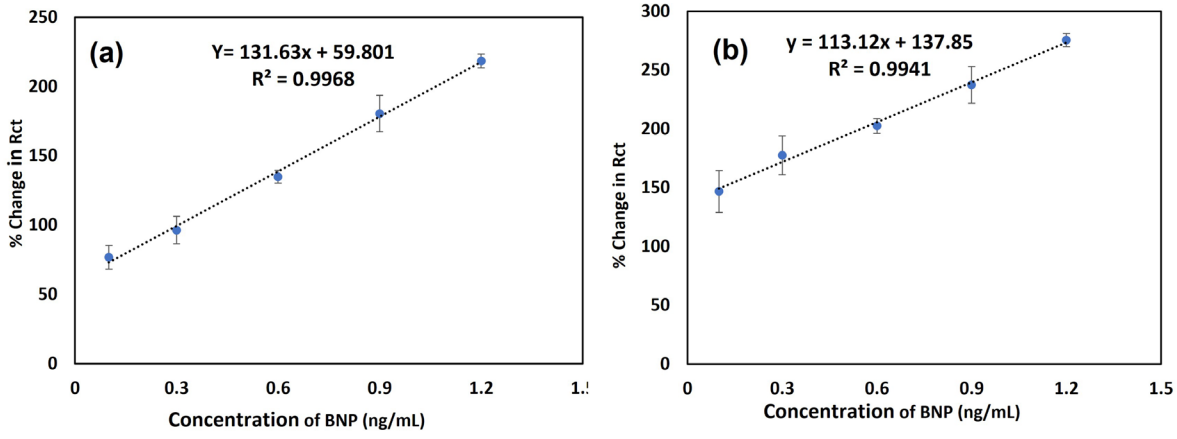

Fig.2 (a) calibration curved prepared for detecting BNP in human serum and (b) human whole blood samples (n=3 electrodes) by Method 2. The calibration curves depict average the percent change in charge-transfer resistance as a function of concentration.

ii)Antigen testing: Each concentration of BNP was prepared by adding known amounts of BNP to human serum and human whole blood samples that originally contained little or no BNP. Each concentration was then added to a different batch of fully functionalized electrodes and EIS measurements were then taken after antigen incubation to obtain the calibration curves. All the antigen binding steps were conducted at room temperature for 5 minutes to examine the rapid detection capability of the fully functionalized cardiosense biosensor. The biosensor testing was performed for the antigen concentrations using electrochemical impedance spectroscopy (Rct across 10,000 Hz – 1 Hz). Fig. 2 (a) & (b) shows the calibration curves obtained using method 2 for detecting BNP in human serum and human whole blood samples, respectively.

Key Research Outcomes/Accomplishments

- Calibration curves for human whole blood and human serum were generated using the electrodes prepared by Method 1. This method involves the following steps as shown schematically in Fig. 3.

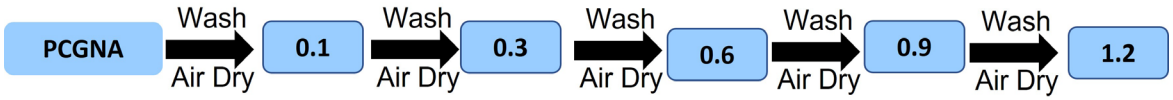

Fig. 3. Schematic representation of the procedure used for obtaining the calibration curves utilizing a single set of fully functionalized electrodes by Method 1. PCGNA above represents fully functionalized electrodes of vertically aligned platinum containing BNP Aptamer. The concentrations tested were: 0.1, 0.3, 0.6, 0.9 and 1.2 ng/mL of BNP.

- The calibration curves for detecting BNP in human serum and human whole blood samples were generated using five different sets of fully functionalized electrodes prepared following Method 2. This method involves executing the sequence of steps as shown schematically in Fig. 4.

- The calibration curves were obtained using human serum and human whole blood samples following the above two methods before testing the unknown BNP amounts in blinded human whole blood and human serum samples for determining the clinically relevant unknown concentrations of BNP.

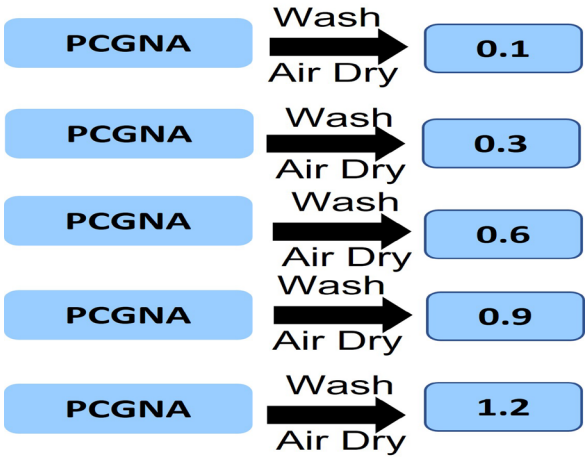

Fig.4. Schematic representation of the calibration curves prepared by Method 2. Each PCGNA electrode above represents a fully functionalized electrodes of vertically aligned platinum to BNP Aptamer , Concentration tested were 0.1, 0.3,0.6,0.9 and 1.2 ng/mL BNP

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 to the Materials Science & Technology Symposium to be held in October, 2022. The results have, however, not yet been presented in any 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 following testing and detection of the BNP amounts in blinded human serum and human whole blood samples. 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 for detecting clinically relevant BNP concentrations using blinded human blood (n =20) and human serum samples (n =20).
- Test the developed novel calibration and correction method to avoid cross selectivity and any interference due to biofouling as well as any other non-specific biofactors related adsorption.
- Verify the concentration of the measured BNP in human blood and human serum with the enzyme-linked immunosorbent assay (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, a 1-year no-cost extension was originally requested which was approved in 2020. However, we were unable to perform any blood-related experiments since the originally approved IRB that was obtained by our collaborator was considered not acceptable for executing and testing of the biosensor on human blood samples. A separate, standalone IRB specifically for the present project associated with the original PI, Dr. Moni K. Datta had to be submitted to the University of Pittsburgh, human research protection office (HRPO) before initiating any of the planned blood related experiments. A second no-cost extension was therefore requested which was approved in 2021. The separate standalone IRB in the name of Dr. Moni K. Datta was also approved in 2022. However, Dr. Moni K. Datta left the University of Pittsburgh in March of 2022. Hence, a change in PI had to be requested followed by a change in PI on the approved IRB. This was completed in April 2022. Due to the above changes, 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 June 2020. We now have an approved standalone IRB in the name of the current PI, Dr. Prashant N. Kumta and we also have the project transferred to the current PI. Hence, we are in a good position now to proceed and complete all the remaining specific aims and tasks before the end of the no-cost extension period of November 2022.

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 submitted an invention disclosure to the University of Pittsburgh, which was accepted, and a provisional patent application was filed in October 2021. The details are given below.

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. Prashant N. Kumta, Mitali S. Patil, Abhijit Roy, Moni Kanchan Datta, Sangeetha Kunjukunju, U.S. Provisional Patent Application No. 63/254,284 , Filing Date: October 11, 2021.

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, accuracy, ease of detection, 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 the planning stages 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 (until March 10, 2022)	9
Prashant N. Kumta	Co PD/PI (until March 10, 2022) and now the PI since March 10, 2022	1
Abhijit Roy	Assistant Professor until August 31, 2021	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 changes in the support of the PI and key personnel as outlined in the table above.

Dr. Moni Kanchan Datta, who was the original PI on this grant resigned his position and left the university on March 10, 2022. He was replaced by the Co-PI, Dr. Prashant N. Kumta who is now the official PI of the project.
Dr. Abhijit Roy, who was the Co-investigator on this grant resigned his position and left the university on August 31, 2021.

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:

1] Z. Guo, L. Murphy, V. Stein, W.A. Johnston, S. Alcala-Perez, K. Alexandrov, Engineered PQQ-Glucose dehydrogenase as a universal biosensor platform, J. Am. Chem. Soc. 138 (2016) 10108–10111.
[2] I.O. K'Owino, O.A. Sadik, Impedance spectroscopy: a powerful tool for rapid biomolecular screening and cell culture monitoring, Electroanalysis 17 (23) (2005) 2101–2113.
[3] E.J. Benjamin, P. Muntner, A. Alonso, M.S. Bittencourt, C.W. Callaway, A.P. Carson, A.M. Chamberlain, A.R. Chang, S. Cheng, S.R. Das, F.N. Delling, L. Djousse, M.S.V. Elkind, J.F. Ferguson, M. Fornage, L.C. Jordan, S.S. Khan, B.M. Kissela, K.L. Knutson, T.W. Kwan, D.T. Lackland, T.T. Lewis, J.H. Lichtman, C.T. Longenecker, M.S. Loop, P.L. Lutsey, S.S. Martin, K. Matsushita, A.E. Moran, M.E. Mussolino, M. O'Flaherty, A. Pandey, A.M. Perak, W.D. Rosamond, G.A. Roth, U.K.A. Sampson, G.M. Satou, E.B. Schroeder, S.H. Shah, N.L. Spartano, A. Stokes, D.L. Tirschwell, C.W. Tsao, M.P. Turakhia, L.B. VanWagner, J.T. Wilkins, S.S. Wong, S.S. Virani, E. Amer Heart Assoc Council, C. Prevention Stat, and S. Stroke Stat, Heart Disease and Stroke Statistics-2019 Update A Report From the American Heart Association. Circulation, 2019. 139(10): p. E56-E528.
[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.
[5] T. Dahiya , S. Yadav, N. Yadav, A. Mann, M. Sharma, J.S. Rana . Monitoring of BNP cardiac biomarker with major emphasis on biosensing methods: A review. Sensors International. 2(2021) 100103.