AWARD NUMBER: W81XWH-19-1-0174

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

PRINCIPAL INVESTIGATOR: Prashant Nagesh Kumta

CONTRACTING ORGANIZATION: University of Pittsburgh, Pittsburgh, PA

REPORT DATE: March 2023

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
March 2023	Final	15May2019-14Nov2022

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

6. AUTHOR(S)
Prashant N. Kumta
Sangeetha Kunjukunju

E-Mail:pkumta@pitt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
4200 Fifth Avenue
Pittsburgh, PA 15260

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: A point-of-care biosensing device for rapid screening and monitoring of cardiomyopathy conditions and progression to lower incidence death occurrences, and healthcare costs is needed. The 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 apt cardiac marker levels in the human whole blood and serum using electrochemical impedance spectroscopy. This work demonstrated construction of an impedimetric multi-array biosensor platform based on platinum wires functionalized with aptamers and successfully navigated the platform beginning from construction to optimization and validation of feasibility in biological samples. Initially, we optimized the optimal platinum wire diameter and surface finish 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 shifted to 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 needed for each layer and assessing the need of each layer. The best self-assembled-monolayer (SAM) combination that provided reliable, accurate and most sensitive response was determined as Platinum-Cysteamine-Glutaraldehyde-Neutravidin-Aptamer and this SAM combination showed excellent precision, reasonable sensitivity, and stable insulation of the linker proteins that 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 fully "erase" biofouling impacts and a patent application was filed. Utilizing the approved standalone IRB, we applied the innovative 2-electrode corrective approach to test human blood (n=15) and human serum samples (n=15) and successfully validated the measured BNP levels in blood and serum against clinical gold standard of ELISA showing excellent agreement of the novel impedimetric biosensor proposed approach.

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**
					USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	18	**19b. TE8EPHONE 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 (WB) 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 human whole blood (WB), serum, and plasma samples to evaluate the effectiveness of the biosensors as a potential ex-situ cardiomyopathy screening device. **The biosensor testing of clinically relevant human WB and serum samples has been completed and are detailed in this report.**

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. 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. 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 WB samples to evaluate the effectiveness as a potential ex-situ CVD screening device:

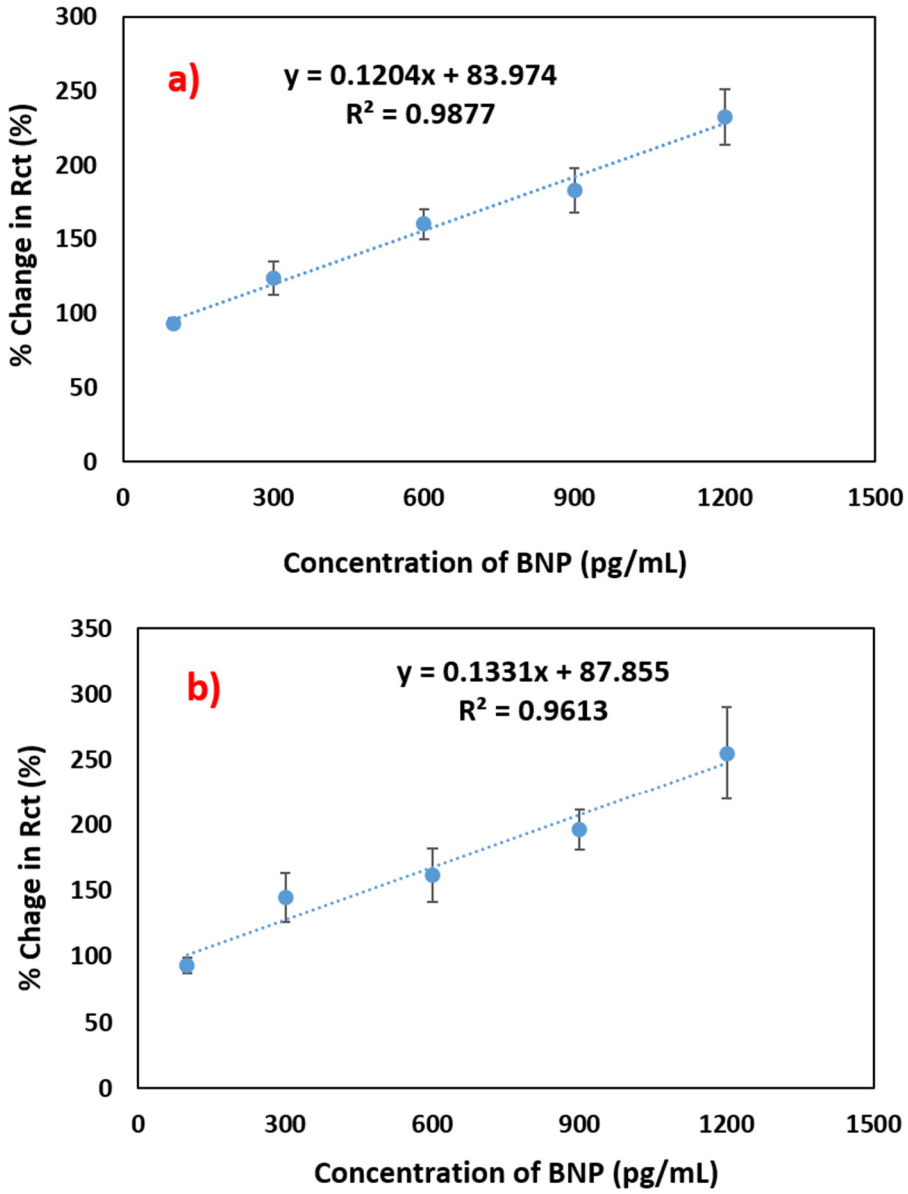

Fig. 1: BNP calibration curve generated from the washing method. 1 electrode is used and sequential concentrations of known BNP in a) human whole blood and b) human serum are tested on the same electrode. Each data point represents an average of three measurements.

Proof of concept for an impedimetric aptasensor design was demonstrated earlier. In this study, the bio sensor response (% changes in charge transfer resistance (R_{ct})) were recorded using known concentration of BNP added to a very low BNP containing human WB and serum samples to generate linear calibration curves (**Figures 1 and 2**). The BNP concentration

range used for the calibration curves was 100-1200 pg/mL. The amount of BNP present in the unknown serum samples (n=15) was measured using an enzyme-linked immunosorbent assay (ELISA) assay, which is currently the clinical gold standard for the determination of BNP concentration. These human serum samples were then used to obtain the sensor response utilizing the developed biosensor and the concentration of the unknown BNP containing samples was determined using the calculated calibration curves. Two separate methods were used to generate the BNP calibration curves, namely, the washing method (**Fig. 1**) and the full functionalization method **(Fig. 2**).

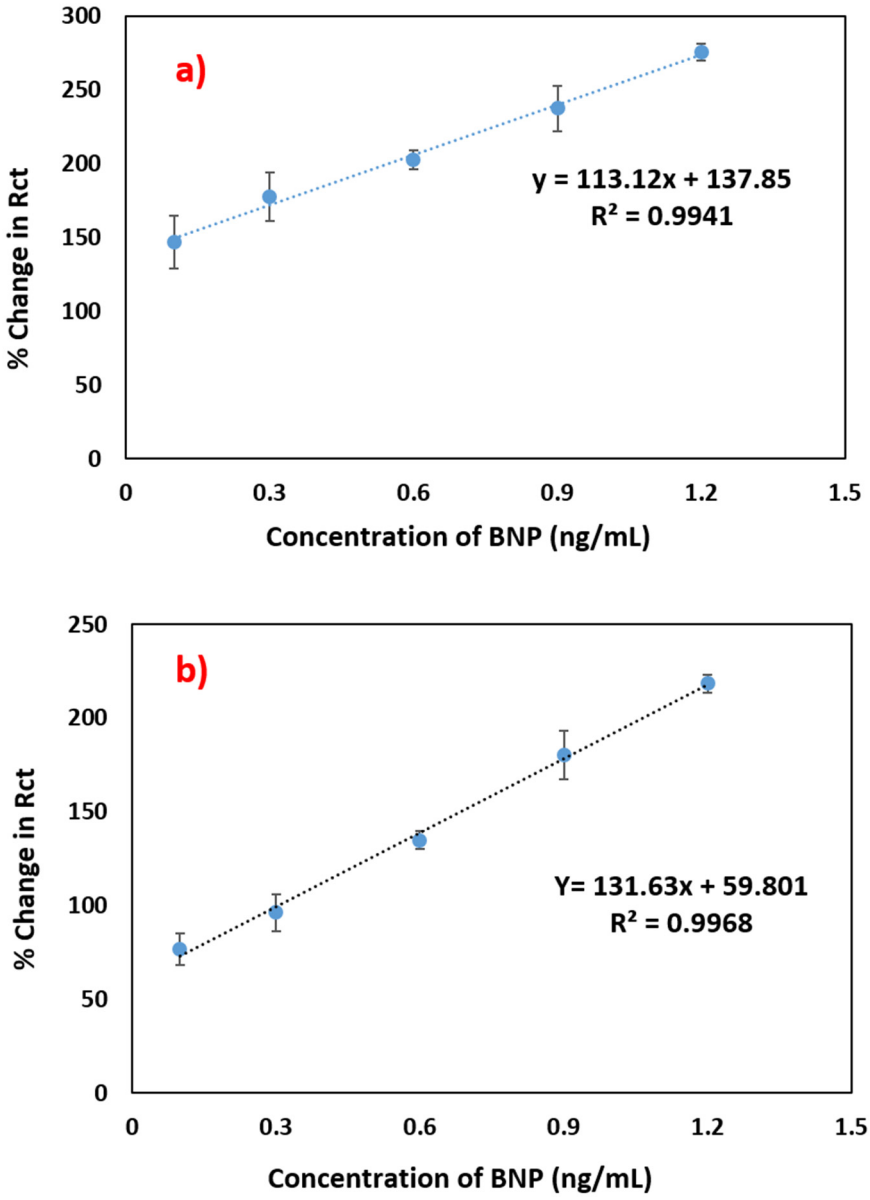

Fig. 2: BNP calibration curve generated from the full functionalization method. An individual electrode is used to test an individual known concentration of BNP (5 electrodes total) in a) human whole blood and b) human serum. Each data point represents an average of three measurements.

The process of washing method utilizes a single electrode to test each of the concentrations of BNP in serum or WB sequentially, with a washing and drying step between each concentration. The full functionalization method on the other hand, uses individual electrodes to test each individual known concentration of BNP in human WB or serum. More in-depth description and details of the two calibration curves methodologies have been reported in the **June 2022 Report (Subtask 2.3)**. **Fig. 1 (a, b)** reveals that the washing method generates larger standard deviations

at the higher concentrations of BNP as compared to the full functionalization method (**Fig. 2 (a,**

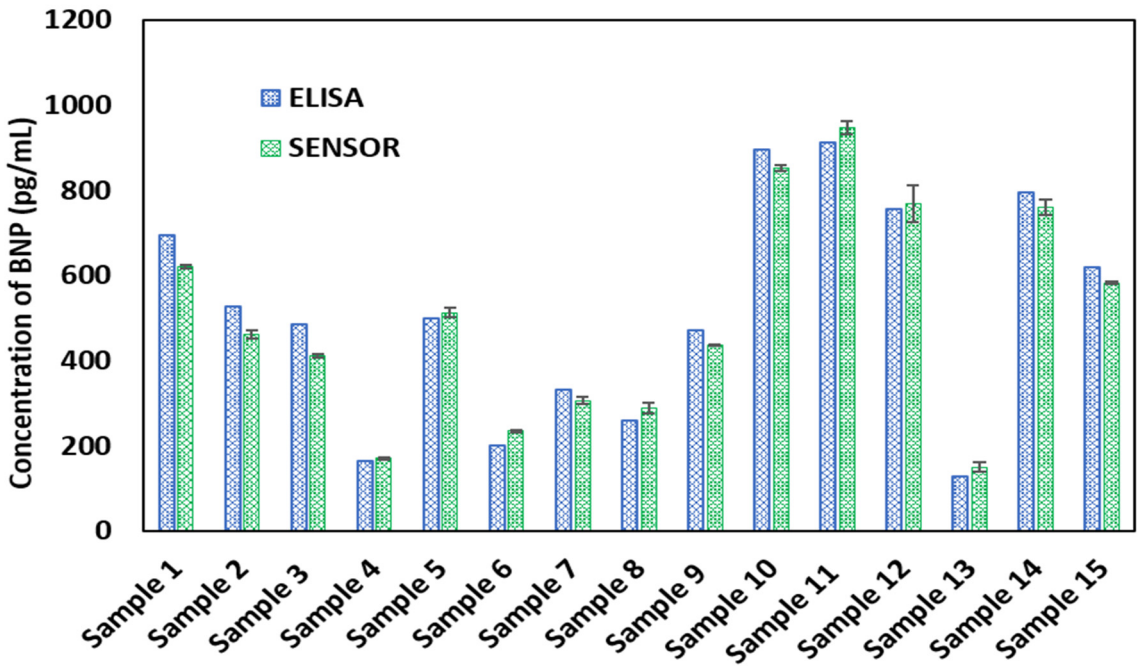

Fig. 3: Concentration of BNP in unknown human serum sample (n=15). Change in R_{ct} values were measured after 5 minutes of incubation time of the human serum samples on the two-electrode biosensing electrode. Each data point represents an average of three measurements.

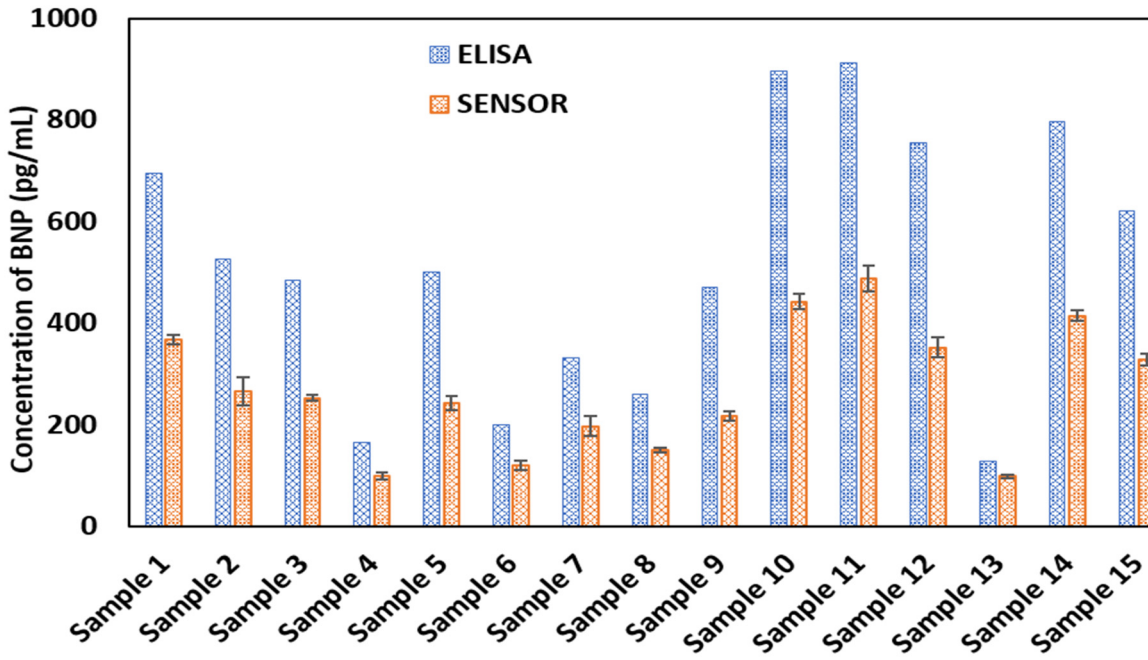

Fig. 4: BNP Concentration in unknown human whole blood samples (n=15). Change in R_{ct} values were measured after 5 minutes of incubation time of the human whole blood samples on the two-electrode biosensing electrodes. Each data point represents an average of three measurements.

b)). We believe the reason for this occurring is due to the washing steps not fully removing all analyte components, thus resulting in oversaturation of the electrodes. Additionally, the full

functionalization method generates calibration curves with R^2 values closer to 1 which thus correlates to a better linear fit. Thus, the full functionalization method calibration curves were used to calculate the unknown BNP concentration in the human serum and WB samples.

A two-electrode correction method was used to negate any unspecific protein adsorptions and consequent biofouling on the biosensor electrodes. The two-electrode approach is based on baseline and biosensing electrodes that are exposed to conditions with and without the detecting analyte, thus enabling proper corrections to be made to completely remove and eliminate the prevalence of any species contributing to biofouling. **Fig. 3** correspondingly shows the results obtained for BNP concentration measured using the two-electrode biosensing approach of 15 unknown serum samples. The results demonstrates that BNP values obtained using the developed biosensor are very similar to the standard ELISA values. The BNP concentration in the WB samples obtained from the same patients were also tested using the two-electrode biosensing approach and the results are shown in **Fig. 4**. Please note that ELISA assay for BNP works only with serum samples. The value of BNP concentration in WB is almost half of the serum as the volume of WB is twice that of the serum. The volume of serum obtained after blood clotting is reduced by ~ 50% in volume due to the removal of various cells and platelets in the form of solid clotting. The BNP values of WB samples obtained using the biosensor follows the same trend obtained for the human serum samples and therefore, confirms the use of this developed impedimetric biosensor for detecting the specific BNP biomarker levels in both human WB and serum samples.

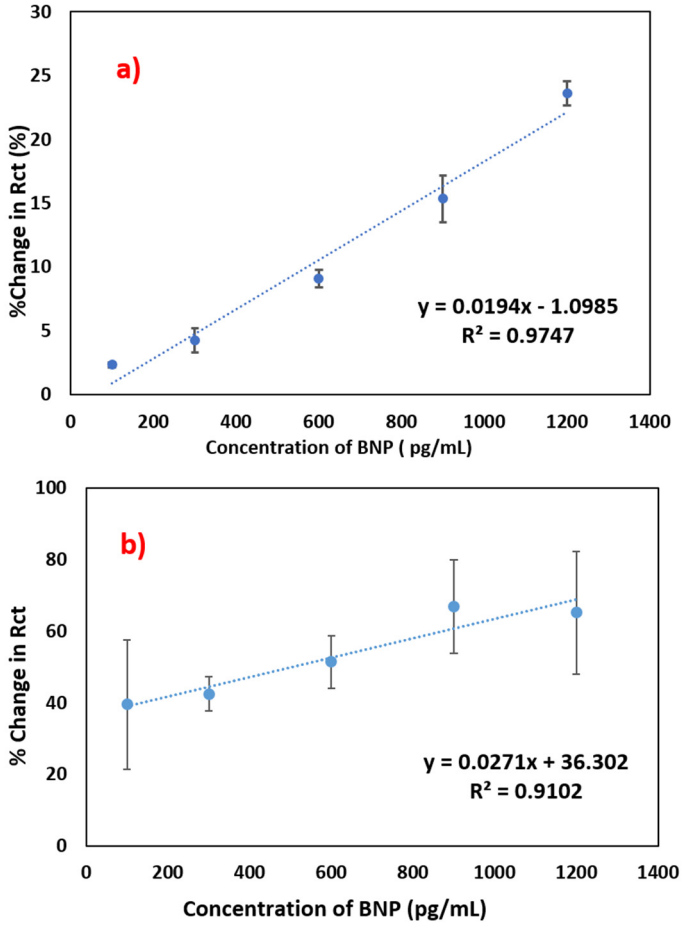

Fig. 5: Stability testing of the biosensor electrode**.** BNP calibration curve generated using the washing method after the self-assembled monolayer coated electrode was (a) freshly made and (b) stored for 7 days in a vacuum environment at 25°C. Each data point represents an average of three measurements.

The tests demonstrate the ability of the biosensor to accurately, and reproducibly detect unknown concentrations of BNP in both human serum and human whole blood without any deficiencies or problems. These results therefore demonstrate and prove the original hypothesis of the proposed Discovery project study and successfully fulfils the primary objective of the proposed study.

However, further validation and optimizations are required using larger sample cohort sizes (n=100) to specify the linearity, sensitivity, analytical specificity, precision, and other performance characteristics of the developed biosensor. This larger cohort of sample detection will be part of the future work that can be planned on a continued submission of a proposal for further determining the accuracy, specificity, and reproducibility of this novel impedimetric biosensor approach for detecting cardiovascular blood-based biomarkers.

In addition to accurately, and reproducibly detecting the unknown concentrations of BNP in both human whole blood (WB) and human blood serum, the time dependent stability of the self-assembled monolayers (SAM) on the electrode is crucial to the development of a miniaturized benchtop biosensor platform. Previously, the functionalized electrodes were stored and tested for change in the charge transfer resistance following storage for 7 days, 14 days and 21 days. The electrodes registered an increase in charge transfer resistance, but the efficacy of the biosensor to detect analyte concentrations was not tested. Hence, a brief short shelf-life stability analysis of the biosensor was conducted by coating an electrode with the SAM layers and storing the electrode in a vacuum environment at 25°C for 7 days. A calibration curve was generated using known BNP concentrations in PBS and shown in **Fig. 5**. The data show that the obtained R^2 accuracy values are slightly lower after storage of the electrode for 7 days (**Fig. 5b**) than the original calibration obtained on the freshly made electrodes with identical washing method shown in **Fig. 5a**. This marginal loss in accuracy is, however, not a deterrent since the trend shows the ability of the functionalized biosensor electrode to still detect the concentrations of the BNP biomarker. The marginal drop in detection accuracy could occur from either presence of contaminants during storage, marginal change in the protein configuration of the SAM layers and more importantly, due to the washing method not removing the concentration of the analyte during the washing of the analyte prior to detection of the subsequent concentration, one of the limitations of the washing method outlined above. The shelf-life stability studies should be conducted using the more accurate full functionalization method outlined above and for longer duration of times. These are part of the long-term studies which will be part of the future work on the subsequent continuation of the studies via submission of a proposal for further determining the shelf-life stability of the biosensor in addition to determining the accuracy, specificity, and reproducibility of this novel impedimetric biosensor approach for detecting cardiovascular blood-based biomarkers as outlined above.

Key Research Outcomes/Accomplishments

- Calibration curves for human whole blood (WB) and serum samples were generated using the washing method and full functionalization method.
    - Full functionalization method generated linear calibration curves with less error.
- The human serum and WB samples were tested with the developed biosensor utilizing the novel two electrode correction approach.
    - The BNP concentrations obtained using the developed biosensor were very similar to ELISA values for both the human WB and serum unknown samples.
    - Clearly demonstrates the effectiveness of the developed biosensor as a potential ex-situ CVD screening device for accurately and reproducibly detecting unknown BNP concentrations in both human WB and human serum blood samples.

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 the past 2.5 years and entire 4 years duration 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 were planned to be presented as an oral presentation to the Materials Science & Technology Symposium held in October, 2022. Unfortunately, due to the personnel engaged in the project testing positive for Covid 19, the presentation had to be withdrawn. The results were therefore not presented and have 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 past 2.5 years of work and the entire 4 years of the comprehensive proof of concept study, 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 including the testing and detection of the BNP amounts in blinded human serum and human WB 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.

4. Impact:

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

The completion of this study has developed and optimized an impedimetric 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 human whole blood (WB) and serum has clearly demonstrated the effectiveness of the developed biosensor as an ex-situ CVD screening device and 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. The successful outcome of this project will yielded a biosensor for rapid, reproducible, accurate, 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 has developed and optimized the biosensor for detection of the specific cardiac biomarker, namely, brain natriuretic peptide.

What was the impact on other disciplines?

The proposed research focused 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 has further elucidated exactly how various components of the biosensor interact with one another (especially on a functional group level) and thus provided new findings for clearly advancing the biosensor functionalization strategies. The platform is very versatile, and the completed studies will also pave the way for other disease detections studies (e.g., Traumatic Brain Injury), including detection of immunosuppressant drug (ISD) level detection in patients who have undergone vascular composite allotransplantation (VCA) attesting to the versatility of the platform developed in this grant serving as a universal platform for immobilizing any aptamer, antibody, or enzyme specific to the biomarker to be detected, thus allowing for the detection of numerous proteins and markers implicated in various diseases. Therefore, the optimization procedures completed 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 completed project will result in several publications and the results of the studies will form the basis of one or more patent applications (provisional patent filed in Oct. 2021, more details below). 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 also 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?

The successful outcome of the experiments outlined in this study have led to the development of an impedimetric biosensor that can accurately detect cardiac markers in human whole blood and serum with high precision, accuracy, reproducibility, 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, the successful performance of the proposed study will now guide the 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 to the approaches required to be taken during the formation of self-assembled monolayers (SAM) of the biosensor. The only minor change made was the reduction in the blood and serum sample size from 20 to 15 due to 5 of the received unknown samples being very close in BNP concentrations. Thus, analysis of these 5 samples would not result in significant variations in the detection levels and would not contribute to any major findings or alteration of the biosensor device capabilities. We have also tested the short-term stability of the SAM. However, we need to understand the long-term stability of these SAM spanning 3, 6 and even 12 months duration. 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. This is primarily because of the method of attachment or functionalization of the sensing monolayers particularly, the attachment of the initial functional layer of Cysteamine (C) which is physically adsorbed to the underlying metallic substrate platform that has a strong affinity to mercaptan groups on Cysteamine. The physical adsorption of the cysteamine layer renders the platform very susceptible to any electrical stimulus of voltage or current that was used to trigger the removal of the analyte following detection to regenerate the electrode enabling reuse. There is a need to therefore modify the substrate platform which is part of the future study in the subsequent continued grant application discussed above. We plan to achieve this with further optimization and modification of the functionalization of the substrate platform which we plan to do as a continuation of the project. Despite the inability to successfully regenerate the biosensor electrode, we have nevertheless, developed a reproducible, accurate, and sensitive 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 and were able to successfully complete all the remaining specific aims and tasks before the end of the no-cost extension period of November 2022. Thus, the project has been successfully completed.

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:
There were no changes in use or care of 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 stages at present. We anticipate completing and submitting these manuscripts as soon as possible in the coming months, and the source of funding will be acknowledged.

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 are in the planning stages at present. We anticipate completing and submitting these manuscripts as soon as possible in the coming months, and the source of funding will be acknowledged.

Other publications, conference papers, and presentations.
We anticipate completing and submitting manuscripts covering the findings of the current work in the upcoming months, and the source of funding will be acknowledged.

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

Technologies or techniques
This work as a whole, 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 by measuring the change in charge transfer resistance. However, the efficacy of the biosensor to detect the biomarker was not tested. In this report, the short-term stability was tested for a period of 7 days and 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 WB samples to create a unique calibration curve model. We tested rat WB 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 have shown that this novel corrective approach can be successfully extended to test human WB and serum samples in a similar fashion eliminating any non-specific adsorption and accurately sensing elevated, median, and low levels of BNP representative of the clinically accepted ranges. These human whole blood and serum studies have been executed in the remaining time of the project during the no-cost extension phase.

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 provisional patent application was converted and a full U.S. Patent Application on October 11, 2022, and a PCT application, International Application Number: PCT/US22/46216 was filed.

The main claim of this invention is development of a novel two-electrode 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.

The United States Patent and Trademark office recorded application on December 7, 2022.

Other Products

Nothing to report at present. Manuscripts covering the findings and completion of the work to date are in progress and in the planning stages at present. We anticipate completing and submitting manuscripts covering the findings of the current work in the upcoming months, and the source of funding will be acknowledged.

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	Co-Investigator	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 n. 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. Special Reporting Requirements:
Nothing to report.

9. 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.