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13. SUPPLEMENTARY NOTES

14. ABSTRACT: There is a need for a point-of-care device for rapidly screening and monitoring of cardiomyopathy conditions and progression to decrease incidence, death occurrences, and healthcare costs. The overall goal 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, demonstrated how to construct an impedimetric multi-array biosensor platform based on platinum wires functionalized with aptamers, and progressively navigated the platform all the way beginning from construction to optimization and demonstration 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 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 then 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. 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
Impedimetric Biosensors, Aptamer, Cardiac Biomarkers, Self-assembled monolayer, Brain natriuretic peptide, Troponin T, Cardiomyopathy, Heart Failure, Point of care.

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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 the patient at home or at the patient bedside, in ambulance use by paramedics, including during clinical visits as a 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. suffer from some form of cardiovascular diseases (CVDs) in 2016, and the costs burden (both direct and indirect) of cardiovascular diseases exceed $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 increase from $318 billion to $749 billion and indirect costs (attributable to lost productivity) for all fatal and nonfatal CVDs are estimated to increase from $237 billion in 2015 to $368 billion in 2035 [3, 4]. Further, CVDs and stroke accounted for 14% of total US health expenditures in 2014 to 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 the result of CVDs being clinically silent with non-specific symptoms 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 for a point-of-care (POC) device 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 disease. The current diagnostic techniques for CVDs are entirely dependent on the use of expensive non-invasive imaging techniques, use of invasive methods, or dependent on the timely and accurate interpretation of the physical symptoms experienced by patients. Unfortunately, current protocols dictate that medical professionals treat any people 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.

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 by 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 and described 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 task) 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 (**On going).**

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 (**On-going**).

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 apt for likely reducing biofouling. Cysteamine (C), glutaraldehyde (G), and Streptavidin/NeutrAvidin (V) self-assembled monolayers (SAM) are first formed on the VAP wires. 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.

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

concentrations, we used an arbitrary set of concentrations and times for the SAM functionalization. This was achieved by surface functionalizing the platinum electrodes with cysteamine (10 mg/mL), glutaraldehyde (25% w/v), Neutravidin (1 mg/mL), and aptamer (1480 µg/mL) added in succession. Therefore, in order to determine the optimal concentrations and times of each functionalization layer, we bound each layer to the electrode surface at varying concentrations for varying times and then bound the subsequent layer at a set concentration and time. The first SAM combination we focused on was that described as PCGNA. 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**). In order to assess the optimal concentration and binding time, each binding step was further analyzed separately using the following equation after execution of EIS,

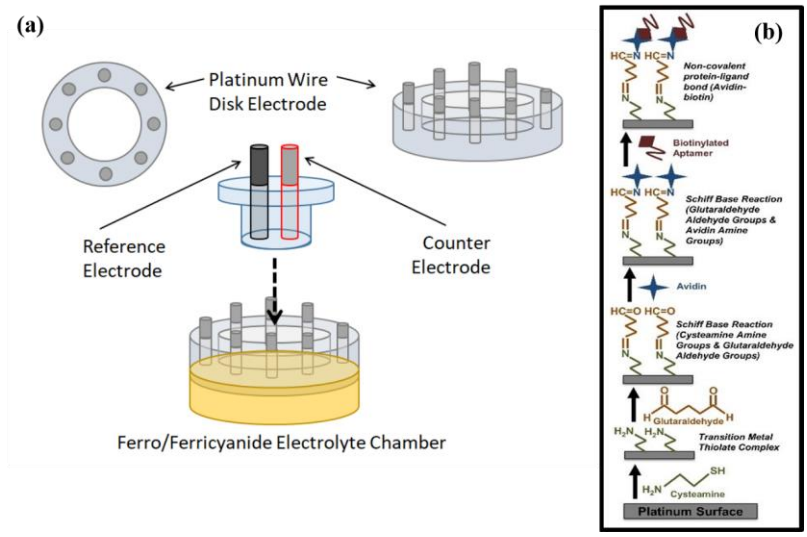

Figure 1: (a). Schematic of the electrochemical cell setup demonstrating the electrochemical cell setup and how the platinum disk working electrodes, silver reference electrode, and platinum counter electrode fit into the Ferro/Ferricyanide electrolyte chamber; (b). Functionalization schematic showing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

$$\%\Delta R_{ct}\ \text{(binding layer)} = \frac{R_{ct}\ \text{(binding layer)} - R_{ct}\ \text{(optimization layer)}}{R_{ct}\ \text{(optimization layer)}} \times 100$$

wherein the optimization layer is the SAM component that was being optimized, and was therefore being prepared at various concentrations and incubation times. The binding layer was the SAM component that follows the optimization layer (in the case of PCGNA, we were optimizing C, then the binding layer would be G), and that layer was bound at a single concentration and incubation time. Therefore, as a higher $\%\Delta R_{ct}$ indicated greater binding of the binding layer, the incubation time and concentration of the optimization layer that resulted in the highest $\%\Delta R_{ct}$ with the binding layer was considered the optimal incubation time and concentration for that particular SAM component. We determined that the **PCGNA** combination representative of being the most complex grouping, was the best for both antigen detection and coverage of the underlying SAM layers (**Figure 2**). We also determined that the optimal binding times and concentrations for each layer were **20 mg/mL cysteamine for 30 minutes, 5% w/v glutaraldehyde for 15 minutes, 1 mg/mL Neutravidin for 30 minutes, and 1480 µg/mL aptamer for 30 minutes** (**Figure 2 a-d**). Therefore, it was concluded that future studies will now proceed with this particular optimized SAM, as it was found to be sufficiently robust, reproducible, sensitive, and highly correlated to the target concentration range during the antigen detection steps for both, brain natriuretic peptide

(BNP) and troponin T (TnT), the two accepted biomarkers known for assessing prevalence and severity of cardiovascular disease conditions.

Subtask 1.2. Determine whether the functional layers of the SAM can be removed without compromising the biosensor performance (**Completed, February 2020**): Once the optimal incubation times and concentrations for every functional layer for all combinations were derived, we then proceeded to test the concentrations of BNP and TnT antigen against each SAM combination (**Figure 3)**. For BNP (B = BNP aptamer, **Figure 3 a, c**), while the slope was relatively high for PCB, PGB, and PCGB, the error bars demonstrate the lack of precision those particular SAM combinations have between each concentrations. The SAM combination studied and executed that exhibited a high correlation value, and reasonable sensitivity and precision is PCGNB. For TnT (T = TnT aptamer, **Figure 3b, d**), we perceived poor precision across all the SAM combinations save for PCGNT-TnT and PCGST-TnT, although the correlation coefficients for PCT-TnT, PGT-TnT, PCST-TnT, and PGNT-TnT were all relatively high. Therefore, once again, we observed that Avidin conferred a degree of stability to the biosensor, especially in regards to precision. Both PCGNT-TnT and PCGST-TnT exhibit good correlation and reasonable sensitivity and precision for sensing and detecting TnT.

We also determined that despite the complexity of the SAM combinations, for PCGNT-TnT (especially in comparison to other SAM combinations tested), we were able to maintain reproducibility between all various functionalization steps. Therefore, although the SAM complexity does lead itself to more variability and more risk of error due to the

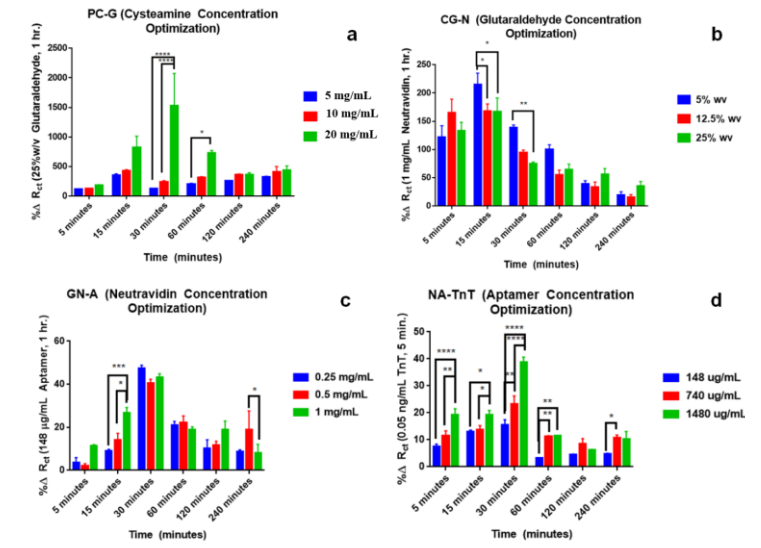

Figure 2: PCGNA concentration & time optimization. The figure depicts the optimization of concentration and binding time for the functionalization layers (a) Cysteamine, (b) Glutaraldehyde, (c) Neutravidin, and (d) Aptamer. n =3 for all bars, error bars stand for ± SEM, and significance levels were calculated via two way ANOVA with Tukey's multiple comparison post-hoc testing.

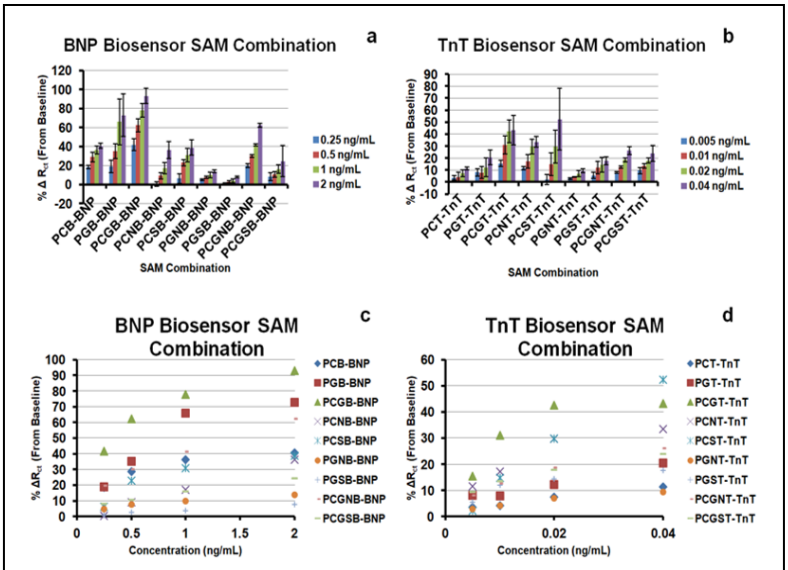

Figure 3: Antigen detection for SAM combinations. The figure depicts antigen detection for all 9 SAM combinations in bar and scatterplot format for BNP (a and c respectively) and TnT (b and d respectively). Corresponding calibration curves and correlations were. n =3 for all bars, error bars stand for ± SEM.

multiple steps and incubations required to build up a more complex SAM, the error can be marginalized by performing incubation steps with caution (and can probably be trivial if the process was automated rather than manually performed by hand). In addition, we determined that in our situation, the more complex SAM system guaranteed better coverage of the underlying linkage groups, thus preventing any biosensing interference that could be generated by the antigen interacting with the underlying SAM layers. We also quickly realized that elimination of any one of these layer leads to the instability of the SAM and results in irreproducible biosensor performance and therefore was not pursued further.

Subtask1.3. Optimize antigen incubation time to enhance sensitivity, precision, and linearity of the calibration curves (**Completed, April 2020**): Based on the findings of **subtask 1.1** and **1.2**, we used the optimal functionalization layer optimization to determine which antigen incubation time would be most favorable. BNP-aptamer biosensors were thus successively treated with 1 µl of 0.25 ng/mL, 0.5 ng/mL, 1.0 ng/mL, and 2.0 ng/mL BNP, and TnT-aptamer biosensors were successively treated with 1 µl of 0.005 ng/mL, 0.01 ng/mL, 0.02 ng/mL, and 0.04 ng/mL TnT. Three antigen incubation times were then tested – 1 minute, 2.5 minutes, and 5 minutes – at room temperature, and EIS measurements were correspondingly then taken after each antigen incubation for obtaining the calibration curves. While all previous experiments focused on the functional layers itself, this experiment focused on the antigen detection capability as a function of time for both BNP and TnT (**Figure 4**). We clearly observed a reduction in sensitivity as the incubation time decreased for both BNP and TnT, which was expected as the reduced time would likely provide less or even insufficient time for antigen binding. We also perceived a reduction in the correlation with increase in time for BNP (**Figure 4a**), which was probably the result of the

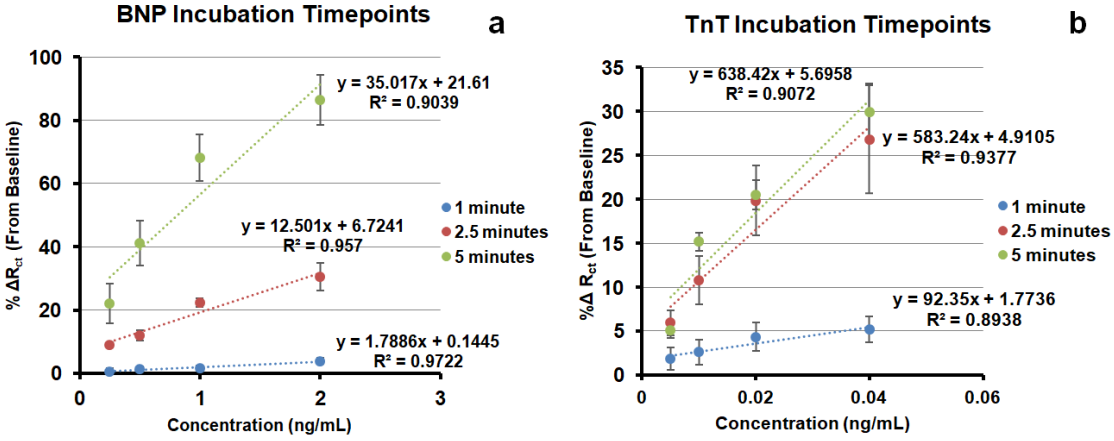

Figure 4: Antigen incubation time for BNP and TnT biosensors Various timepoints (1 minute, 2.5 minute, and 5 minute) for antigen incubation tested on (a) BNP biosensors and (b) TnT biosensors. Error bars represent ±SEM for n = 3, and calibration curve equations and corresponding correlation coefficients are located by each line for each timepoint.

biosensor saturating and therefore, not demonstrating a linear response throughout the concentration range.

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. Calculate single-frequency for each cardiac biomarker that exhibits excellent antigen detection and retest biosensors at that single frequency (**on going**): The optimal single-frequency was calculated by comparing the Bode plots for the optimal functionalization combination

(PCGNB/T) antigen detection and determining the frequency at which the percent change in absolute impedance, known as $Z_{modulus}$ ($\%\Delta Z_{mod}$) between the various concentrations correlated best with the concentrations themselves.

One of the major aspects of using EIS as the transducer is the rapidity and fast response time of the assay. However, in order to make the assay even faster, we compared the resultant Bode plots from the BNP and TnT antigen assays and calculated the single frequency at which both antigen detection assays demonstrated the highest correlation across the entire concentration range (**Figure 5** and **Table 1**). A Bode plot is another interpretation of an EIS spectra, just like a Nyquist plot, but instead of depicting the impedance as real and imaginary Cartesian coordinates based off a parametric frequency response, a Bode plot alternatively gives the overall absolute impedance, separating the real and imaginary components as a function of frequency. Based off the Bode plots, the highest change in Z_{mod} across all the concentrations of BNP and TnT occur between 100 Hz and 10 Hz (**Figure 5 a-d**).

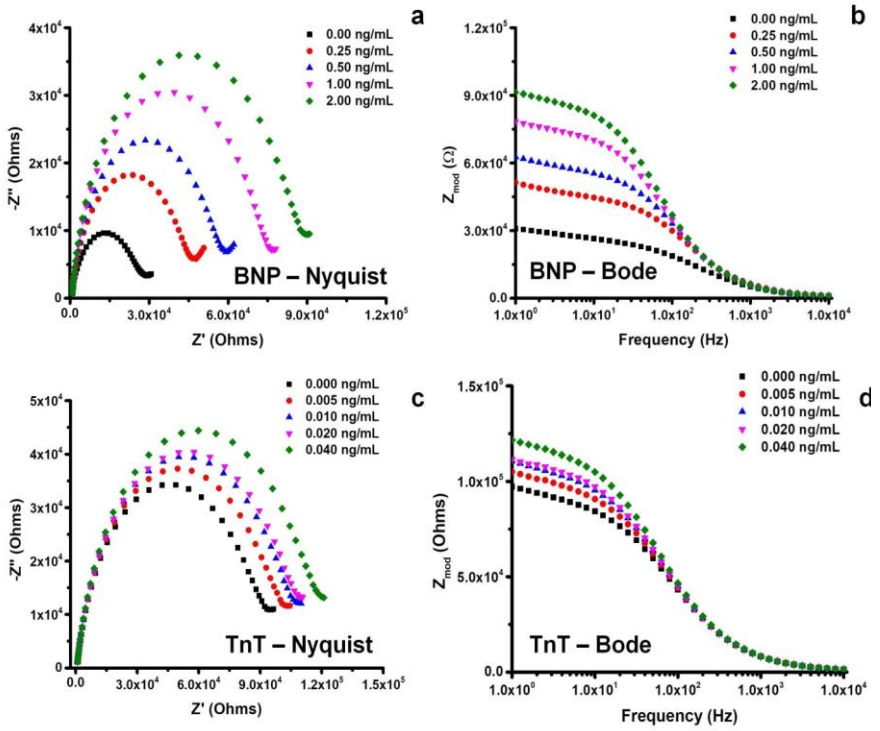

Figure 5: Nyquist and Bode plots of BNP and TnT antigen curves Nyquist (a, c) and Bode (b, d) plot representations of EIS spectra for the baseline and four successive concentrations for BNP (a, b) and TnT (c, d) antigen detection.

Beyond 10 Hz, we actually see a slight drop in the change in Z_{mod}, although that change had to be examined numerically rather than graphically. Therefore, we calculated the calibration curves across those frequencies and determined the frequency at which the

Table 1. Single frequency calibration curve correlation coefficients. Table demonstrating the values of the calibration curve correlation coefficients between frequencies 100 Hz to 10 Hz across the given concentration ranges.

Frequency (Hz)	**Correlation (R^2) $\%\Delta Z_{mod}$: Concentration**	
	BNP (0.25ng/mL-2.0ng/mL	**TnT (0.005 ng/mL – 0.04 ng/mL)**
100.00	0.953	0.937
79.41	0.955	0.985
63.10	0.958	0.986
50.01	0.959	0.967
40.01	0.959	0.987
31.58	0.961	0.989
24.99	0.961	0.998
20.00	0.962	0.999
15.79	0.960	0.995
12.50	0.957	0.993
10.00	0.959	0.990

calibration curve correlation coefficient was the highest (**Table 1**). At the frequencies between 100 Hz and 10 Hz, the changes in Z_{mod} across all the concentrations were nearly similar (similar slopes), so we examined the correlation coefficient to assess differences between frequencies. Based off the calculations, we determined that the optimal single frequency for rapid (15-30s) testing for antigen detection was **f = 20 Hz**, which demonstrated the highest correlation coefficient (**Table 1**).

Subtask 2.2 Develop an electrochemical technique to regenerate aptamers without impacting biosensor performance to create a reusable biosensor (**on-going**): 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 of regeneration was to use the electrochemistry principles itself to manipulate the aptamer into unfolding and refolding into the current sensing configuration. Thus BNP biosensors (without the antigen) were prepared 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. With the applied voltage, especially at higher magnitudes of voltage, we observed that the biosensor layers themselves were being stripped off, even with a voltage application lasting for 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, were perceived to be too strong and removed the biosensor layers themselves. Thus far, the results obtained in this study remain inconclusive, and further experiments are underway to optimize the methodology of aptamer regeneration. These include exploring even smaller

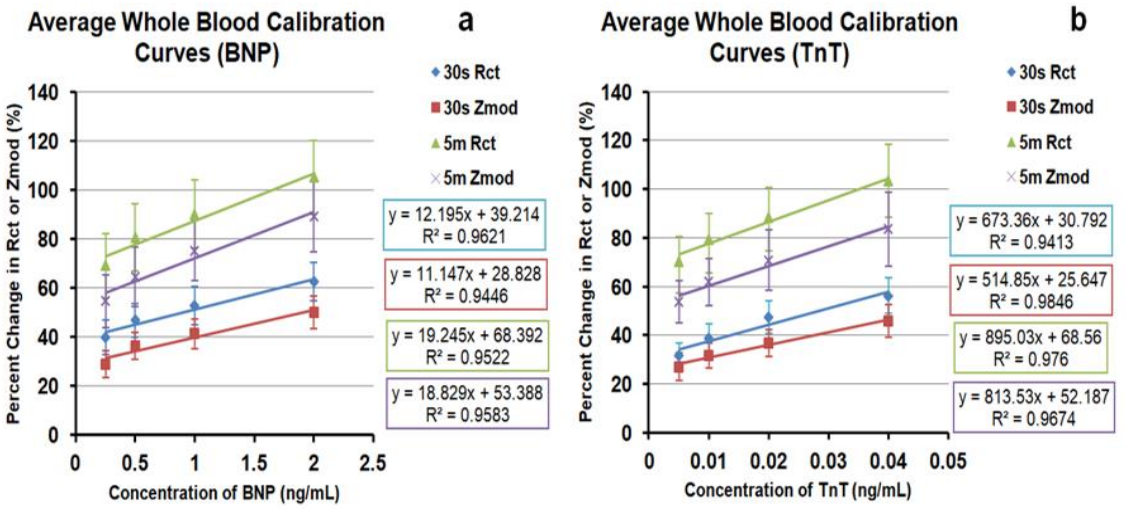

Figure 6: **Average whole blood calibration curves**. Average calibration curves for (a) BNP biosensors and (b) TnT biosensors tested via four different methodologies - 30s Z_{mod} (20 Hz), 30s R_{ct}, 5m Z_{mod} (20 Hz), and 5m R_{ct}. Percent change represents the percent change of the signal (R_{ct} or Z_{mod}) between the biosensor baseline (0.00 ng/mL) value and the respective concentration.

magnitude of pulsed voltages and currents of shorter time. These studies planned to be executed.

Subtask 2.3 Test the biosensors against clinically obtained whole blood samples to evaluate the effectiveness as a potential ex-situ CVD screening device (**on-going**): In order to determine the antigen detection capabilities of the biosensor and the resistance of the biosensor to interference and bio-fouling, various biological samples were tested. Concentrations of 0.2 ng/mL, 0.6 ng/mL, 1.0 ng/mL, and 2.0 ng/mL BNP, and 0.005 ng/mL, 0.01 ng/mL, 0.02 ng/mL, and 0.04 ng/mL of

TnT were prepared using the following solutions – (1) PBS (the buffer that has been utilized for all the testing thus far), (2) Dulbecco's Eagle Medium (DMEM) containing 10% Fetal Bovine Serum and (3) five separate rat whole blood samples (R3, R36, R37, R38, R39) obtained from Sprague Dawley Rats that were sacrificed after 4-16 weeks following a **post-biodegradable magnesium alloy implantation study that was executed as part of a another separate project (Rat Gluteal Muscle Biocompatibility Study: IACUC # IS00013709). It is important to mention here that we did not perform any in-vivo experiments to date within this project. Rather, we received leftover rat blood samples from a different project (Rat Gluteal Muscle Biocompatibility Study: IACUC # IS00013709) which was subsequently used in the experiment. Because we used leftover rat blood samples from an alternate and separate project instead of obtaining and collecting the blood ourselves, it was deemed not necessary to gain permission from ACURA.** Desired concentrations were prepared in 25 µl volumes of blood samples which were stored at 4°C until testing. Each concentration was tested on a different batch of electrodes (instead of testing all of the successive concentrations on only one set of electrodes) to avoid increased interference from any biological substances present in the samples. Two concentration incubation times (30 seconds and 5 minutes) and two testing methods (single-frequency vs. frequency-range EIS) were employed for determining the antigen calibration curves.

These screening experiments were conducted with various antigen concentrations and mainly we selected and prepared the calibration curve for the whole blood samples for comparison and our findings for each method is shown in **Figure 6.**

In order to avoid and mitigate the bio-fouling/interference from the other proteins and biological factors present in the biological samples, especially for human serum and the rat whole blood samples we developed a novel corrective approach for testing the biosensor.

To perform the novel corrective approach for each sample, three sets of electrodes were prepared. These include, one set of functionalization from Platinum to Neutravidin bound with biotin, called electrode B_B, and two other sets with platinum to Neutravidin bound with respective sensing agents, respectively (herein referred to as electrode B_S). The rat whole blood samples, received from another project as excess, (Rat Gluteal Muscle Biocompatibility Study: IACUC # IS00013709), tested using the B_B and B_S methods were used to obtain a corrected value (B_Φ) that provides a value obviating the interference due to biofouling and any other possible nonspecific adsorption of biomolecules. The developed novel corrective bio-sensing method was verified using rat blood samples utilizing known concentrations of BNP and TnT. The B_Φ corrected values of BNP and TnT were very similar or identical to that of the known concentration as shown in **Figure 7** and therefore, show the validity of the proposed

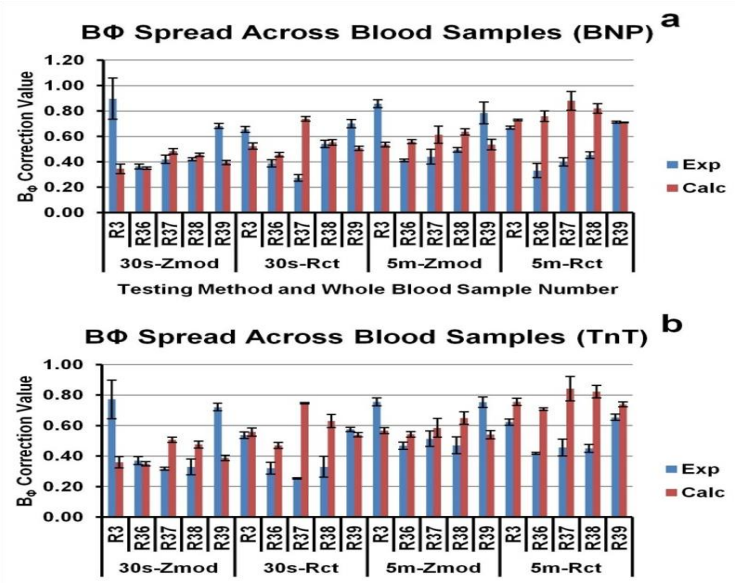

Figure 7. B_Φ correction value comparisons

Figure 7: Spread of B_Φ values across whole blood samples for BNP (a), and TnT (b) per each testing method, error bars representing ±SEM, n = 3

correction method. The robustness of this novel bio-sensor correction method will also be tested using human blood and serum samples in the remaining time of the project. The results will be reported in the subsequent reports to follow.

Key Research Outcomes/Accomplishments

- Designed and developed a simplistic upright vertically aligned platinum wire-based aptasensor, a multi-array impedimetric biosensor that can detect biomarkers indicative of myocyte stress (BNP) and myocyte injury (TnT).
- Optimized the ideal parameters such as concentration and incubation time for self-assembled monolayers (SAM) of the biosensor.
- The aptamer based biosensor shows reproducible sensitivity, accuracy and selectivity for BNP and TnT biomarkers.
- Further experiments are needed to optimize the biosensor regeneration methods.
- Proof of concept for an impedimetric aptasensor design has been demonstrated using rat whole blood samples implementing a novel developed calibration and corrective approach.

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

The project has provided opportunity for the 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 first year of this project thus far, efforts were directed at achieving the planned project goals of fabricating the various SAM combinations, preparing the electrodes and performing detailed and systematic testing as summarized above. The results have therefore, not yet been presented in various 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 first year 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. 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.

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. Successful outcome of this project will yield biosensor for 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 sensing 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 studies will also pave the way for other disease detections studies (e.g. Traumatic Brain Injury), as the platform developed in this grant is very versatile and can serve 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 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 a 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 the 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 1st phase (12 months) of the project focused on the formation of self-assembled monolayers (SAM) of the biosensor. 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. However, 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 July 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 June 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 have 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. In regards to the rat blood usage, we did not perform any rat cultures or rat experiments. We used the rat blood from left over blood of another project as outlined above.

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.

Nothing to report. Manuscripts and presentations 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.

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. The optimal SAM combination was then used to develop biosensors to test in rat whole blood samples to create a unique calibration curve model. We tested rat whole blood samples 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 following the approval of the no-cost extension.

Inventions, patent applications, and/or licenses

Nothing to report at present. Invention related to the novel calibration and detection method will be filed very soon. We anticipate reporting the filing of this invention disclosure and conversion

of the disclosure to a provisional patent application in the remaining time of this project before the completion of the project goals outlined above.

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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