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Development Toward a Triple-Marker Biosensor for Diagnosing Cardiovascular Disease

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ABSTRACT: Cardiovascular disease (CVD) is the leading cause of death in the United States and is responsible for 30% of all deaths globally.[1] The diagnosis and management of CVD requires monitoring of multiple biomarkers, which comprehensively represents the state of the disease. However, many assays for cardiac biomarkers today are complicated and laborious to perform. Rapid and sensitive biosensors capable of giving accurate measurements of vital cardiac biomarkers without complex procedures are thus in high demand. In the work presented below, rapid, label-free biosensor prototypes for three Food and Drug Administration–approved biomarkers are reported: B-type natriuretic peptide (BNP), cardiac troponin I (cTnI), and C-reactive protein (CRP). The sensors were prepared by immobilizing each biomarker's antibody onto gold working electrodes with platinum counter and silver/silver chloride reference electrodes. The sensors were tested using electrochemical impedance spectroscopy (EIS), a femto-molar sensitive technique capable of label-free, multi-marker detection if a biomarker's optimal frequency (OF) can be identified. The OFs of BNP, cTnI, and CRP were found to be 1.74, 37.56, and 253.9 Hz, respectively. The performance of the BNP biosensor was also evaluated in blood and achieved clinically relevant detection limits of 100 pg/mL.

KEY WORDS: cardiovascular disease, electrochemical impedance spectroscopy, optimal frequency, biomarkers, multi-marker biosensor, imaginary impedance

ABBREVIATIONS: BNP, B-type natriuretic peptide; **CRP**, C-reactive protein; **cTnI**, cardiac troponin I; **CVD**, cardiovascular disease; **DI**, deionized water; **EIS**, electrochemical impedance spectroscopy; **OF**, optimal frequency; **POC**, point of care

I. INTRODUCTION

Cardiovascular disease (CVD) is a class of heart conditions associated with the functions of the heart and blood vessels. In 2008, approximately 17.3 million people died from CVDs, which accounts for 30% of global deaths.[1] Today, CVD continues to be the number-one dominating cause of death globally, with the most prevalent types of CVD being coronary heart disease, responsible for 7.3 million deaths, and stroke, responsible for 6.2 million deaths.[1] Other CVDs include cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis and pulmonary embolism.[1] It is estimated that, by 2030, approximately 23.6 million people will die from CVDs.[1] Therefore, efforts in prevention, detection, and treatment can help CVD patients and individuals who are at risk. Current methods of CVD detection involve elaborate laboratory tests for the quantification of CVD biomarker concentration. With the limitations of the current diagnostic techniques and the growing epidemic of CVDs, the design of a point-of-care (POC) sensor capable of detecting and monitoring multiple biomarkers in a rapid and accurate platform is both beneficial and critical for the diagnosis, prognosis, and management of the disease.

A. Current Methods of Diagnosis

The typical technique for assessment of cardiovascular condition is a blood test in the hospital setting to provide information on certain selected biomarkers for the diagnosis of patients. Other diagnostic methods include electrocardiograms (ECGs), ECG stress tests, nuclear stress tests, computerized tomography (CT) scans, echocardiograms (Echo), magnetic resonance angiograms (MRAs), angiography/arteriography, cardiac catheterization, and lipid

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profile blood tests.[2] However, all these techniques require the analysis and interpretation of results through blood work and laboratory means, which produces lag time and delays to the treatment of patients. Such a delay can cause potential problems and complications depending on the time sensitivity of the patient's condition. In addition, many of the techniques mentioned are very expensive, which may prevent or discourage patient and insurance company compliance.

B. Biomarkers for CVD

The diagnostic techniques for CVD which utilize blood tests, assess for biomarker levels. A biomarker may be defined as a molecular indicator of biological, pathogenic, or pharmacological processes and responses and is measured and evaluated for therapeutic intervention.[3] There are thousands of proteins in blood with only a few identified and clinically accepted CVD biomarkers; therefore, the accuracy of detection methods is crucial and can be greatly increased with sensitive and precise measurements of biomarkers. The measurement of biomarkers allows for a comprehensive evaluation of a patient's condition, prognosis, and course of treatment. The three clinical biomarkers for CVD employed in the present study are B-type natriuretic peptide (BNP), cardiac troponin I (cTnI), and C-reactive protein (CRP).

1. BNP and Current Assays

Natriuretic peptides are used extensively in clinical applications and as a gold standard for biomarkers in detecting heart failure.[3] Of the natriuretic peptides, BNP is one of the commonly used biomarkers and possesses predictive value for CVD. The detection limit for BNP is 100 pg/mL. Below the limit is typically considered to be in the normal range and the higher the value of BNP above the limit, the more likely and severe it is for heart failure. BNP is released by myocytes in the atria and ventricles when the myocardium stretches in response to increased filling pressure, arterial pressure, or cardiac dilation.[4] Therefore, BNP is a powerful biomarker for risk classification in atrial and ventricular distension; detection of heart failure, acute coronary syndromes, and stable coronary heart disease; and providing stratification information.[5]

There are many different types of BNP assays, beginning with radio immunoassay (RIA), a competitive immunoassay method with the use of radio-labeled tracers.[6] RIAs suffer from lack of specificity due to interference from the multiple related peptides in plasma samples and is not label-free.[6] Therefore, noncompetitive immunoradiometric assay (IRMA), a two-site sandwich immunometric measurement method, was developed and demonstrated an increase in BNP specificity.[6] However, another technique, micromosaic immunoassay (μMIA), possesses the ability to screen for multiple CVD biomarkers such as BNP, cTnI, and CRP simultaneously.[7] Such an approach shows a reduction in both assay time and required sample volume and provides evidence for the potential to detect markers in human samples. Although μMIA displays the potential of a point-of-care device for the detection of multiple markers, it has an eight-step process, which may restrict its use for speed and simplicity, along with limitations in the use of fluorescence labeling.

2. cTnI and Current Assays

The myofibril thin filament in control of heart muscle contraction and relaxation is regulated by the cardiac troponin complex.[3] The complex is composed of the troponin C (cTnC), cTnI, and troponin T (cTnT) subunits.[8] Troponins are typically undetectable in healthy patients and the reference range varies between assays. After the emergence of chest pains, serum cTnI levels begins to increase in 3 to 6 hours, peak in 12 to 16 hours, and remain elevated for approximately 5 to 6 days.[3] Studies by Horwich et al. and Ilva et al. have suggested that cTnI may be a useful biomarker for diagnosis and prognosis assessment of acute myocardial syndromes, and have shown the correlation between elevation in cTnI and mortality.[9,10] Therefore, identifying cTnI levels has become a standard practice for early rule-outs, risk prognosis, and assessment of outcome in patients with acute myocardial syndromes.

Multiple techniques exist for the detection of cTnI levels. Recently, high-sensitivity cTnI assays have been achieved using chemiluminescence. The

objective of such advances in cTnI analysis is to increase the speed and sensitivity of assays. A recent example is the Architect STAT Troponin-I assay (Abbott Diagnostics), which uses three murine monoclonal antibodies.[11] High-sensitivity cTnI assays are particularly useful for early detection and quick analysis due to their increased sensitivity.[12] The technique is very precise and has the ability to provide prognostic information in stable patients. While the assay has many advantages, there is wide variability in assay characteristics between manufacturers, such as the limit of detection. Also, biological variability in the low detectable levels is frequently observed in normal individuals over time and fluctuating levels of cTnI can lead to false-positive interpretations.[12] Due to these variabilities, there is an absence of standardization for current high-sensitivity cTnI assays.

3. CRP and Current Assays

CRP is a biomarker associated with atherosclerosis, a chronic blood vessel inflammation disease.[13] It is released during the immune response of inflammation, and its elevated concentrations indicate increasing CVD risk. Increased CRP levels have also been found to correlate with a higher risk of cardiovascular death, myocardial infarction, or stroke in patients with stable coronary artery disease.[13] The Centers for Disease Control (CDC) and the American Heart Association (AHA) identified a level of < 1 mg/L to be low risk, 1–3 mg/L to be average risk, and > 3 mg/L to be high-risk for cardiovascular events.[13] While CRP levels below 3 mg/L are considered normal, the baseline CRP concentration varies among individuals; therefore, they should be identified for each patient for the determination of appropriate cut-off values. With the use of baseline and current CPR concentration levels, the risk of CVD can be assessed and action can be taken toward prevention or maintenance of patients with CVD.

CRP detection has been performed in a variety of different techniques by various research groups. The typical assays are immunonephelometric or immunoturbidimetric, using a single polyclonal antibody.[14] However, some techniques lack sensitivity to assess and predict risk of coronary diseases and their detection range is not very low. Also, they all require highly skilled professionals and expensive laboratory equipment. Immunoradiometric assays and ELISA tests provide higher sensitivity, lower detection limits, and more accurate and precise measurements; however, they are technically demanding and time consuming.[14] A recent study investigated electrochemical techniques including EIS and cyclic voltammetry (CV) and demonstrated the immobilization of CRP and anti-CRP on the surface of a gold electrode.[14] Such an approach shows the potential of electrochemical techniques, particularly EIS, for the development of a point-of-care immunosensor that would be more efficient, affordable, and convenient.

C. Electrical Impedance Spectroscopy

Several limitations can be seen in current techniques of detection for the three biomarkers of CVD. However, the potential of electrical impedance spectroscopy (EIS) has been demonstrated. Impedance spectroscopy is used to analyze the electrical resistance of a system through assessment of the surface reactions and alterations of bulk properties, study the binding on a transducer surface, and characterize changes in surface properties in immobilization of biological molecules.[15] Impedance is determined through application of a potential while the current response is measured, resulting in a complex impedance value due to current differences in amplitude and phase shift.[15] A specific range of frequencies allows for characterization of surfaces, layers, or membranes and assessment of diffusion activity.[15] The impedance of a system in EIS is based on ohmic resistance, capacitance, constant phase element, and Warburg impedance.[15]

To assess a biological molecule, an electrochemical cell is created where current flows through the working electrode, the molecule, the solution, and the counter electrode in order to measure overall system impedance.[15] EIS utilizes a closed-field technique in which the binding of biological molecules to the antibodies on the surface produces a larger signal compared to binding further away from the surface. From these measurements, a frequency detection range can be determined and overall system impedance can be related to the concentration of the

biological molecule.[15] In addition, EIS has been utilized in a frequency-tuning experiment, providing evidence of its ability to multiplex multiple biomarkers onto a single sensor surface. By multiplexing biomarkers, a more robust detection technique can be used for CVD and user error is minimized by eliminating the need to label the targets.[16] Therefore, EIS has potential to be a rapid, label-free, and ultrasensitive technique to analyze biomarkers.

EIS has been shown to be an effective technique for detecting biomarkers in the cardiovascular system.[17] It can characterize a biomarker much more quickly compared to current assays for BNP, cTnI, and CRP. The ability of EIS to quickly detect only one of these biomarkers is a major advantage. Lin et al. used EIS techniques to detect both LDL and HDL levels at the same time.[17] With the success of detecting two different biomarkers, it should be possible to detect the three biomarkers discussed in the present paper. In order to obtain concurrent detection, each biomarker must be characterized individually.

In the study reported here, detection and characterization of BNP, cTnI, and CRP were performed using the impedimetric sensor technique employing EIS. The impedance values obtained allowed for concentration changes of the biomarkers to be analyzed. To study a biomarker through EIS, the optimal detection frequency of binding was determined. The use of a molecular recognition element (MRE) specific to the target biomarker was employed to better separate frequencies and avoid optimal frequency overlap from the multiple biomarkers.[18] The work reported shows the development toward a rapid, label-free EIS sensor for BNP, cTnI, and CRP and future advancement of a POC device for CVD risk assessment. In the process, the relationship between optimal frequency and molecular weight was explored as well. Furthermore, BNP, the smallest biomarker, was selected for the investigation of EIS response in blood.

II. MATERIALS AND METHODS

A. Electrochemical Sensor Fabrication

BNP and anti-BNP were purchased from Bachem and Hytest, respectively; cTnI and anti-cTnI were purchased from HyTest; and CRP and anti-CRP were purchased from R&D Systems. New Zealand white rabbit blood was purchased from Bioreclamation, and Phosphate-buffered saline (PBS) was purchased from VWR International. All other chemical and reagents in the present study were purchased from Sigma-Aldrich unless noted otherwise. The gold disk working electrodes (GDEs) with a gold surface thickness of approximately 2.5 mm, silver/silver chloride (Ag/AgCl) reference electrodes, and platinum wire counter electrodes were purchased from CH Instruments. All electrochemical measurements were performed on the a CHI660C Electrochemical Analyzer from CH Instruments at room temperature.

We present an adaptation (Fig. 1) of a previous study by Lin et al.[17] The surfaces of the GDEs were polished on Buehler felt pads using 3.0-, 1.0-, and 0.05-μm-grit alumina oxide slurry in deionized (DI) water in approximately 120 figure-eight motions. The GDEs were then sonicated for 15 min in DI water. Afterwards, cyclic voltammetry (CV) was performed from −1.0 to +1.0 V in a 100-mM potassium ferricyanide solution made in PBS with a pH of 7.4 to find the formal potential. This was followed by EIS using the formal potential determined from our experiment and a sine wave sweep from 1 to 100 kHz of a 5-mV AC. From these experiments, we determined the impedance and surface topography of the bare GDEs. The GDEs were then rinsed in DI water to begin the immobilization process. On each GDE, 1-mM solution of 16-mercaptohexadecanoic acid (16-MHDA) with ethanol was incubated for 1 h in order to form a self-assembly monolayer (SAM), followed by an impedance measurement for each GDE's formal potential to enforce quality control. The GDEs were then incubated with a solution of 40-mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 20-mM sulfo-derivative of N-hydroxysuccinimide (NHS) for 1 h in order to activate the carboxylate groups at the tail of the 16-MHDA. Afterwards, the GDEs were washed with DI water and followed by the immobilization of 100 μL of a solution containing 50-μg/mL antibodies (BNP, cTnI, or CRP) prepared in PBS with a pH of 7.4 for 1 h. The GDEs were then washed with PBS, and 1% ethanolamine in DI water was used to block the remaining reactive sites for 30 min. Lastly, the GDEs were rinsed with PBS and stored at 4ºC until needed.

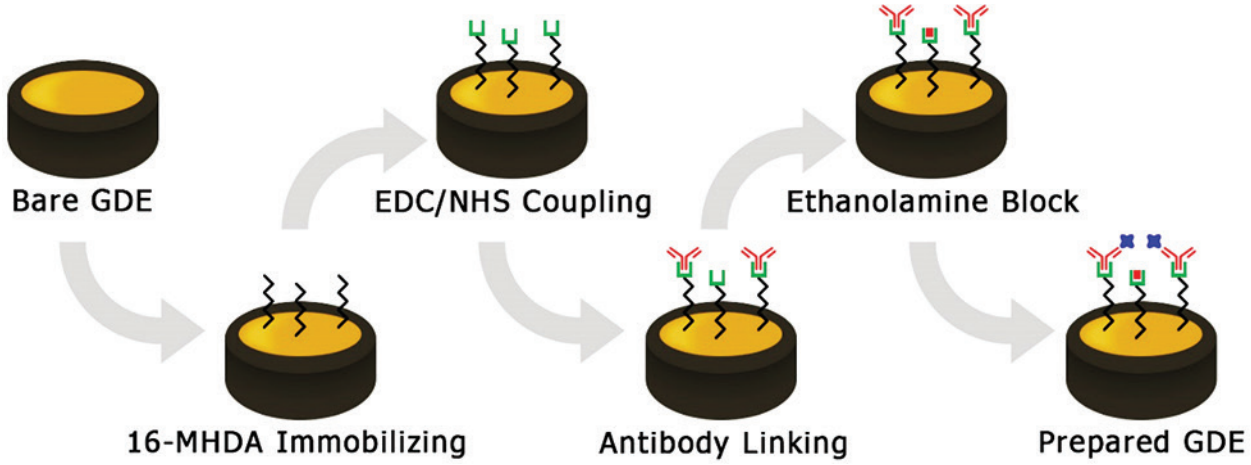

FIG. 1: Schematic of sensor fabrication

B. Electrochemical Characterization

The purified BNP, cTnI, and CRP samples were prepared by dilution in PBS. Then 50 µL of each sample was mixed with 50 µL of 100-mM potassium ferricyanide to create a 1:1 ratio and 100-µL volume of each sample at a specific concentration gradient. The sample gradients were 0, 10, 100, 1000, and 10,000 pg/mL for BNP; 1e-5, 1e-4, 1e-3, 0.01, 0.1, 1, 10, 30, and 300 ng/mL for cTnI; and 0, 0.1, 0.5, 0.75, 1.5, 3, 5, and 10 µg/mL for CRP. For the BNP in blood, rabbit blood was diluted to 10% by combining it with the purified BNP concentrations and 100-mM potassium ferricyanide. Each sample's impedance at the formal potential of each GDE was measured by EIS from 1 to 100 kHz. The resulting impedances were then related to the concentrations of the biomarkers and used to calculate the slope, representing sensitivity, and the RSQ, representing specificity. Finally, by plotting the slope and RSQ against the frequency, the optimal frequency at which the biomarker is best detected could be determined.

C. Optimal Frequency Determination Using Imaginary Impedance

The four output parameters of EIS are real, imaginary, and complex impedance and phase angle. Impedance is concentration-dependent because when targets enter the area where the immobilized MREs are, MRE-target complexes are formed, blocking electron flow and changing the impedance.[17] Earlier studies utilized complex impedance for determination of optimal frequencies.[19,20] In contrast, the present study used imaginary impedance, which peaks at a specific frequency location and forms a parabolic curve. Slope and RSQ are determined by correlating the imaginary impedance to the concentration of the biomarker. Then the optimal frequency is found by plotting the slope and RSQ and locating the frequency at which the slope curve peaks; it is often correlated with a high RSQ as well. Following the estimation of optimal frequency, a calibration curve is generated by correlating the imaginary impedance to the biomarker concentrations at the OF.

III. RESULTS

A. Electrochemical Characterization of Purified BNP, cTnI, and CRP

The three biomarkers, BNP, cTnI, and CRP, were all electrochemically analyzed via EIS to study their imaginary impedance. By correlating the impedance and the concentration of a biomarker, the slope and RSQ are determined. The optimal frequency of each marker is then discovered by plotting the slope and RSQ together and determining where the two curves have the best trade-off. As shown in Fig. 2, the OFs of BNP, cTnI, and CRP were found to be 1.74, 37.56, and 253.9 Hz. After identifying the OF, a calibration curve was established at the respec-

tive frequency for each biomarker and, as shown in Fig. 3, the markers had a linear relationship between concentration and imaginary impedance. These observations suggest that at each biomarker's optimal frequency, concentrations of BNP, cTnI, and CRP can be established for diagnostic use.

B. Electrochemical Characterization of BNP Marker in 10% Blood

In order for the technique described here to be useful in the detection of biomarker levels in POC testing, the device must be able to detect these markers in blood. The sensor's ability to detect BNP was tested in a 10% blood solution. As shown in Fig. 4(a), the optimal frequency in 10% blood was found to be 69.75 Hz. This frequency is much higher than the 1.74 Hz for BNP in the nonblood solution. However, based on the RSQ, the frequency at which the RSQ is the highest is still 1.74 Hz, indicated by the small increase in the RSQ curve near 1.0 Hz. The calibration curve of BNP at 1.74 Hz in 10% blood was then produced. A clear difference was observed in the slopes between purified BNP at 1.74 Hz [Fig. 3(a)] and 10% blood BNP at 1.74 Hz [Fig. 4(b)] and was most likely due to interference from other molecules in blood. Such interference affects detection levels and is a major issue with respect to application of the technique.

IV. DISCUSSION

A. Purpose of Electrochemical Characterization

Through the use of EIS to study imaginary impedance, the CVD biomarkers BNP, cTnI, and CRP were successfully characterized. Each CVD biomarker possesses a unique optimal frequency, indicating that the sensor can be used to identify the three markers without signal overlap. In addition, the unique OFs suggest the potential of a multi-marker

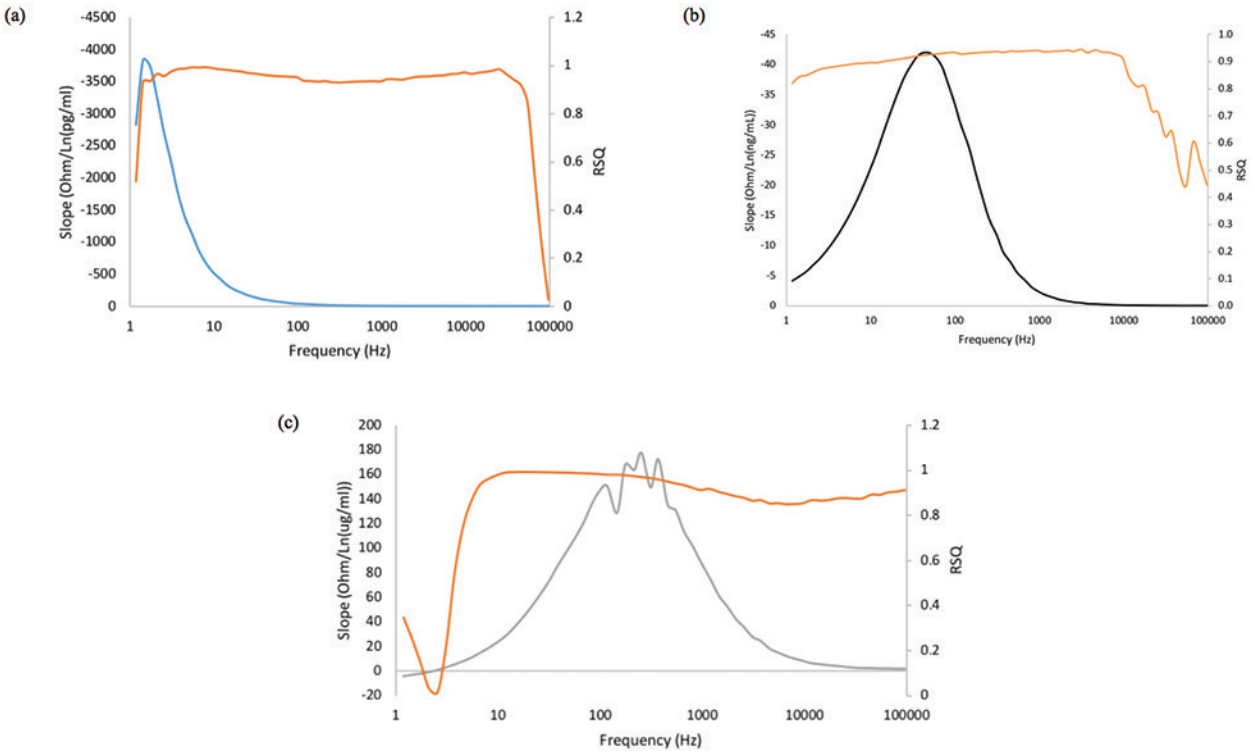

FIG. 2: (a) BNP (blue lower line), (b) cTnI (black), and (c) CRP (grey) were electrochemically analyzed. The slope and RSQ (orange or lighter) were overlaid after correlating the impedance values with the sample concentrations. The optimal frequencies of BNP, cTnI, and CRP are 1.74, 37.56, and 253.9 Hz, where the slope and RSQ have the best trade-off.

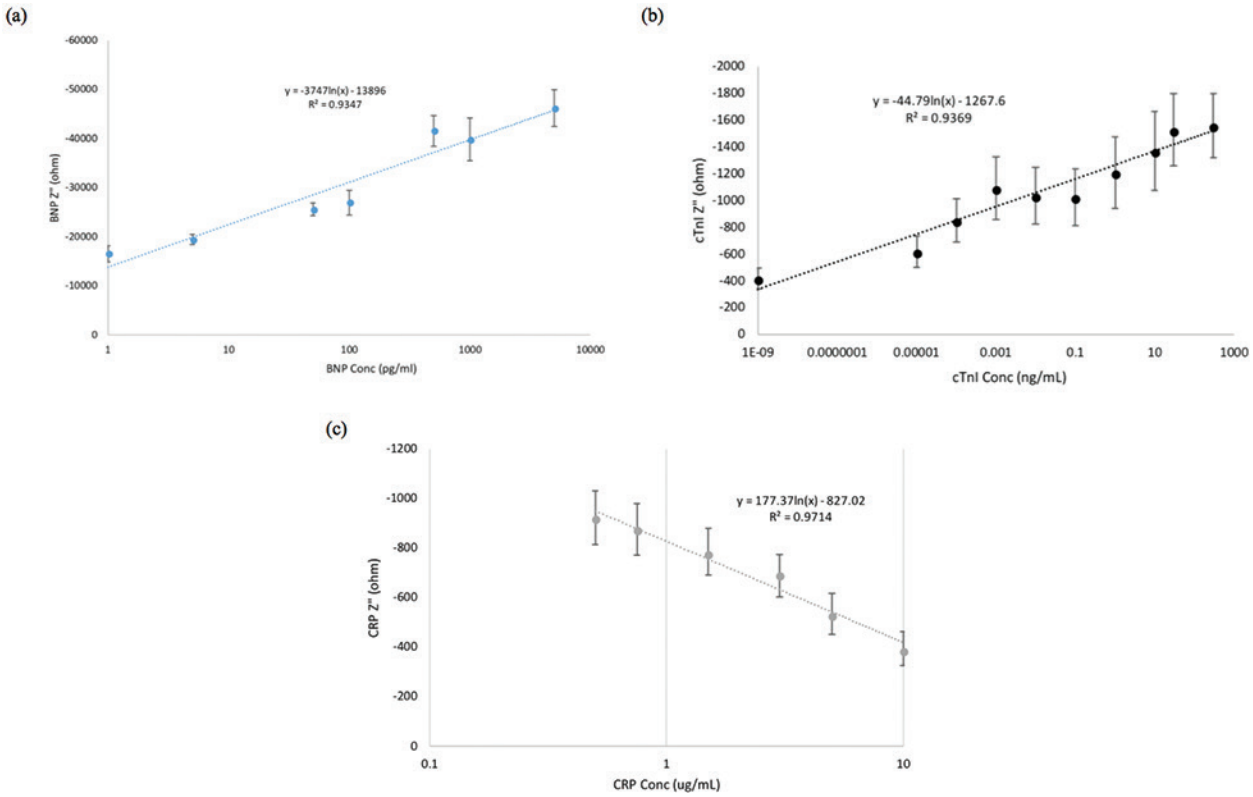

FIG. 3: Calibration curves of the (a) BNP sensor at 1.74 Hz, (b) cTnI sensor at 37.56 Hz, and (c) CRP sensor at 253.9 H

detection sensor by co-immobilizing the three biomarkers on a single sensor. A calibration curve was established for the markers as well, and the linear relationships reveal the sensor's ability to detect the levels of BNP, CRP, and cTnI in a solution. With the use of EIS techniques, the sensor has the potential to provide quick and effective information about each of the three biomarkers for use in a clinical setting.

B. Effect of Blood in Detection of BNP levels

In order for the sensor developed in our laboratory to be effective in detecting levels of BNP in a clinical setting, it must be able to measure a biomarker's level in blood. The sensor's performance was evaluated in a 10% rabbit blood solution for its ability to electrochemically characterize BNP. The 10% blood solution resulted in a higher optimal frequency of 69.75 Hz for BNP than the purified solution of 1.74 Hz. However, it can be argued that the OFs were the same for both conditions. In Fig. 4(a), a shift in the position of the slope curve can be seen but, based on the curve of the RSQ, the frequency at which the RSQ is the highest is still 1.74 Hz, indicated by the small increase at the beginning of the curve near 1.0 Hz. These results were most likely due to interfering molecules in the blood. Blood possesses many molecules with the ability to interfere with electrochemical analysis. In order to reduce such interference, additional experiments would need to be performed to evaluate the method of sensor preparation for the avoidance of molecule interference. Although the 10% blood solution presented interference in the data, the sensor was still able to meet clinically relevant detection limits of 100 pg/mL.

C. Potential Relationship between Molecular Weight and OF

The molecular weight of BNP is typically in the range of 3–4 kDa,[6] the molecular weight of cTnI is approximately 24 kDa,[21] and the literature indicates the mo-

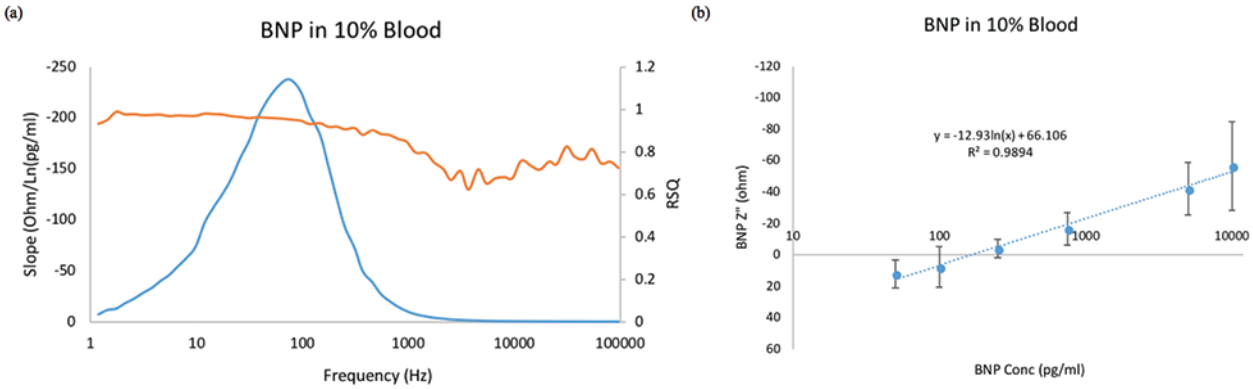

FIG. 4: (a) BNP in 10% blood was electrochemically analyzed. The slope (blue lower curve) and RSQ (orange upper curve) were overlaid and used to determine the optimal frequency at 69.75 Hz. (b) Calibration curve of the BNP sensor in 10% blood at 1.74 H

lecular weight of CRP to be approximately 25 kDa.[22] However, according to the Mayo Clinic, CRP generally exists as a pentameter, resulting in a combined molecular weight of 105 kDa.[23] Of the three biomarkers tested, BNP was detected at the lowest frequency of 1.74 Hz, followed by cTnI at 37.56 Hz. CRP was detected at the highest frequency of 253.9 Hz. This suggests a potential correlation between molecular weight and optimal frequency, since variously sized targets bind to their corresponding MREs and form complexes in varying sizes. This connection is also supported by other studies.[17,24]

D. Limitations of Each Biomarker

Although BNP, cTnI, and CRP have shown predictive value in CVD, each one has limitations. For BNP, conditions such as abnormal kidney function and ischemia may result in increased levels; therefore, the utilization of BNP alone may yield inaccurate diagnostic results.[25,26] For this reason, BNP concentrations are typically measured with NT-proBNP, a BNP precursor. NT-proBNP is produced by myocytes in response to elevated pressure or myocardial stretching and cleaves into the biologically active BNP.4 The combination of the two peptides is a powerful biomarker for CVD and allows risk stratification strategies, prognostic information, and detection of conditions such as heart failure, acute coronary syndromes, and stable coronary artery disease.[3]

The use of cTnI levels is a standard practice for early rule-outs, stratification of risks, and assessment of outcome in patients with acute myocardial syndromes. However, multiple challenges exist, such as the lack of standardization depending on the group of patients, presence of post-translational modification of cTnI in serum cTnI, and variation in commercially available troponin assays.[27] The lack of standardization and the variability in troponin assays inhibit comparisons not only between assays but between hospitals and medical systems for conventional assays.

The limit of detection for CRP is 3 mg/L; levels below the cut-off are generally deemed normal. However, since CRP is a proinflammatory biomarker, its baseline concentrations vary among patients. Therefore, it is necessary for patients to identify their baseline CRP levels, before determining the appropriate cut-off concentrations and to use this as an assessment tool for when elevation occurs. Also, CRP blood tests only assess large-scale inflammation, whereas high-sensitivity CRP (hs-CRP) blood tests are used for detecting vascular health.[28]

E. Benefits of EIS

When EIS is compared to currently used assays for BNP, cTNI, and CRP, EIS shows potential as a rapid, label-free, ultrasensitive, and multi-marker technique for the evaluation of biomarkers. The closed-field technique of EIS, where biological molecules binding to antibodies on the surface develop a larger

signal, determines a frequency detection range and relates system impedance to concentration of biological molecules. The unique optimal frequency of each biomarker and the use of MRE to better separate the frequencies indicate the potential for a multimarker sensor that identifies each biomarker without signal overlap. Also, the ability of EIS to multiplex multiple biomarkers onto a single sensor surface allows stronger detection, eliminating the need for target labeling as well, which minimizes user error. The benefits of EIS suggest that it would be more accurate than other techniques and lead to cost reduction.

V. CONCLUSION

Our study detected the presence of BNP, cTnI, and CRP in purified solutions and BNP in 10% blood solution in a sensitive and label-free manner. The process was completed through the immobilization of antibodies onto gold disk electrodes and the acquisition of EIS measurements. In the purified solutions, the resulting optimal binding frequencies for BNP, cTnI, and CRP were found to be 1.74, 37.56, and 253.9 Hz. In particular, the BNP sensor was evaluated in blood and achieved clinically relevant detection limits of 100 pg/mL. The unique OFs of each biomarker suggest the possible integration of the three onto a single sensor without signal overlap. The signal-analyzing approach demonstrates the potential of EIS as a rapid, label-free, ultrasensitive, multi-marker biosensor for the detection and monitoring of biomarkers indicative of cardiovascular disease.

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