Chapter 7

Enzymatic Detection of Traumatic Brain Injury Related Biomarkers

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Abstract

Electrochemical detection methods have been popular in the medical diagnostics field. Several well-known devices such as the self-monitoring blood glucose meter have relied on electrochemical techniques for their sensitivity, and ability to make direct measurements without optical labels. Currently, no point-of-care or handheld diagnostic tool exists to quantify the severity of a traumatic brain injury (TBI). We have shown that enzymatic detection of norepinephrine (NE), a biomarker which can indicate TBI severity, using impedance-based electrochemical techniques can achieve the required sensitivity, ~100 pg/mL. Furthermore, the first steps have been taken to quantify NE in whole blood solutions and to optimize the technique for a handheld device.

Key words Biosensor, Enzyme, Biomarker, Norepinephrine, Traumatic brain injury, Electrochemical impedance spectroscopy, Impedance time, Point-of-care technology

1 Introduction

Traumatic brain injury (TBI) is the leading cause of injury-related deaths in the USA (>1.7 million), resulting in $60 billion in direct and indirect annual costs [1, 2]. Moreover, 40% of TBI survivors have negative and often permanent long-term outcomes including cognitive, physical and/or sensory deficits post-injury [3]. Currently, TBI is diagnosed in a clinical setting using the Glasgow Coma Scale (GCS), which is a score resulting from the assessment of verbal, eye, and motion responses, and is often accompanied by computerized tomography (CT) imaging in moderate to severe cases [4, 5]. Although TBI awareness has increased with improved recognition of its signs and symptoms, the barrier to progress lies in the minimal advancements in diagnostic and treatment modalities for TBI [6].

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With increasing awareness regarding TBI and its detrimental effects, the need for a point-of-care technology (POCT) to rapidly diagnose TBI and predict the resulting outcomes has become vital.

The catecholamines, including norepinephrine (NE), have been shown to correlate to trauma, such as TBI events [7–9]. Asymptomatic blood concentrations of norepinephrine in blood range from 70 to 1.7 ng/mL [10]. Catecholamine blood concentrations fluctuate and can reach levels of 49 ng/mL or higher, depending on severity to trauma sustained [11]. Several studies have shown that norepinephrine blood concentrations within 1–7 days of injury strongly correlate with severity of the TBI [7, 11]. Hamill, et al., reported that norepinephrine levels were strongly correlated in mild to moderate TBIs and increased four to five times post injury. While the half-life of catecholamines in the blood are on the order of minutes [12], the concentrations continue to fluctuate on the order of days as the injury begins the healing process, thus with a low-cost POCT with a low limit of detection, norepinephrine levels can be quantified as often as necessary. Thus, it is the goal of this work to employ electrochemical methods to enzymatically quantify the TBI biomarker, norepinephrine.

Electrochemistry is the study of electrical effects such as current, and correlating that information to another phenomenon, such as a chemical change [13]. This is often achieved using a three-electrode system: working, counter, and reference electrodes, which can be made of a variety of materials. The counter electrodes provides the stimulating signal while the working electrodes measures the resulting signal with respect to the reference electrode (like a multimeter using two leads to measure current). In the fields of medical devices and biosensors, several very popular modes of measuring physiologically relevant molecules include potentiometric, amperometric, and impedimetric techniques [13]. An example of a potentiometric technique is cyclic voltammetry (CV), which stimulates the solution with a saw-tooth voltage wave, while current is measured (Fig. 1a). The self-monitoring blood glucose meter uses the more sensitive Amp-it technique which applies a DC voltage (typically the oxidation potential informed by the CV) to the solution as current is measured over time. Two impedance-based techniques are used in this work: electrochemical impedance spectroscopy (EIS) and the impedance-time (Z–t) technique. The EIS technique uses an AC voltage signal which changes frequency over time to stimulate the solution and measures the resulting AC current signal, then compares the phase and amplitude differences to generate a Nyquist plot as shown in Fig. 1b [13, 14]. The Z–t technique works very similarly, however the AC voltage signal does not change frequency and yields a complex impedance versus time plot (Fig. 1c). One advantage Z–t has over EIS is that it requires less hardware to generate the stimulating signal while maintaining the same sensitivity, and is therefore more

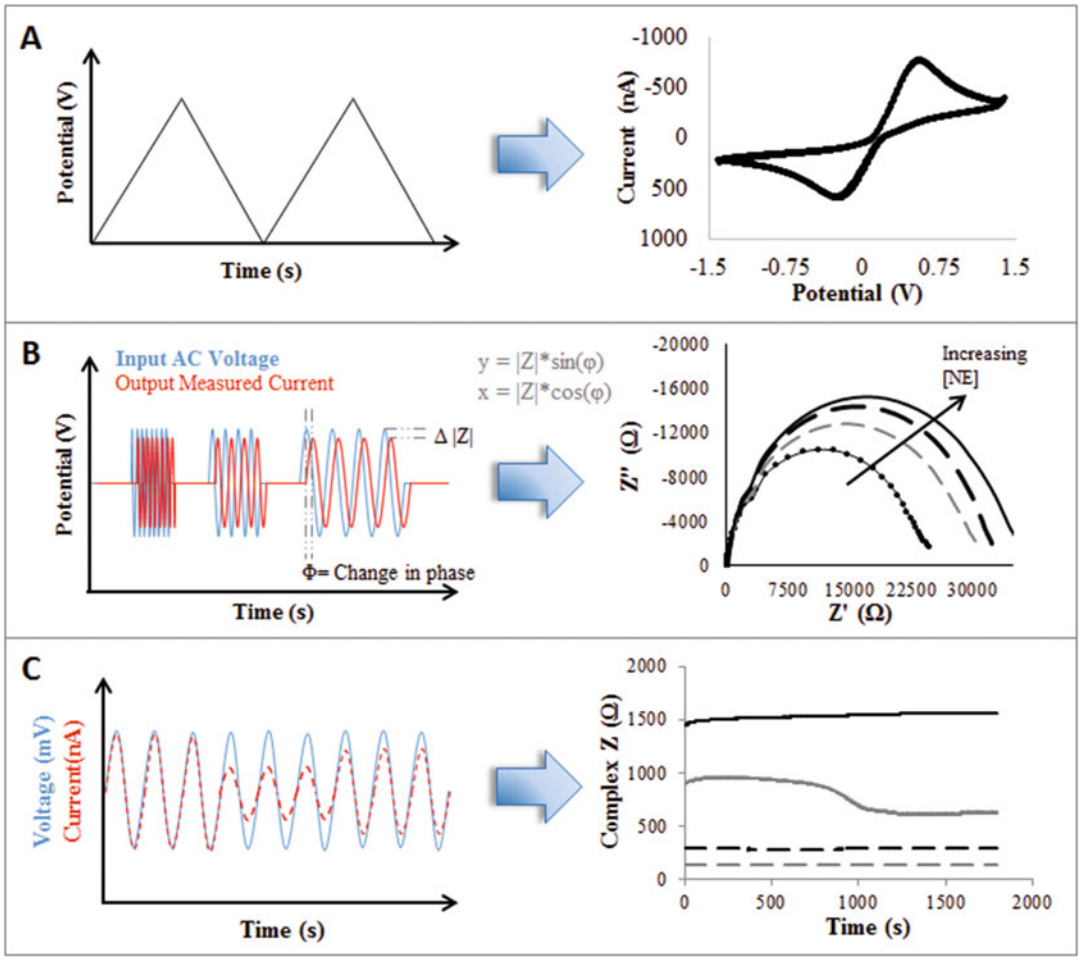

Fig. 1 Panel (**a**) shows the stimulating saw tooth voltage signal which produces the cyclic voltammogram at the right to obtain oxidation and reduction information from. Panel (**b**) depicts the AC voltage stimulating signal in the EIS technique and the gray measured current signal which results in the right Nyquist curve where the *y*-axis is imaginary impedance and *x*-axis is real impedance (showing [NE] = 1, 50, 500, and 5000 pg/mL). Panel (**c**) illustrates the simplified Z–t signal which measures the same thing EIS does, but the stimulating signal does not change frequency, resulting in an amp-it-like graph at the right on a 500 pg/mL NE solution (from the top: 368, 321, 3676, 46,300 Hz)

preferable for a handheld device. These impedance techniques are sensitive enough to detect analyte concentrations in the low pg/mL range and can to quantify whole cells, proteins, neurotransmitters, DNA, or aptamers [14, 15].

2 Materials

2.1 Equipment

The equipment required to carry out these experiments include a 660C CH Instruments Electrochemical Analyzer (CHI, Austin, TX, USA), an analytical scale such as the Ohaus EX224 analytical

balance, and a VWR Digital Vortex Mixer (VWR, Randor, PA, USA), referred to as vortexer. The CH Instruments accessories used include the 2 mm gold disk working electrode (CHI 101), a silver–silver chloride wire (CHI 112) which was removed from its glass casing and used as the reference electrode, and a platinum wire (CHI 115) used as the counter electrode.

2.2 Electrochemical Reagents

All chemical reagents are purchased from Sigma-Aldrich, St. Louis, MO, USA unless otherwise stated. The following are the general and immobilization solutions created to run the electrochemical experiments described in Subheading 3.

1. 10 mM phosphate buffer saline (PBS, EMD Chemicals, Billerica, MA, USA) is made by dissolving one tablet in 1 L of deionized (DI) water.
2. 100 mM Potassium hexacyanoferrate (III) salt is dissolved in 10 mM Phosphate Buffer Saline to create the redox probe solution. 10 mL of 100 mM redox probe is more than sufficient for these studies (*see* **Note 1**). For the rabbit blood protocol, higher concentrations of redox probe are required, 5 mL of 1000 and 200 mM ferricyanide solutions were also prepared.
3. The self-assembling monolayer solution, 1 mM 16-mercaptohexadecanoic acid (16-MHDA), is made by dissolving the white powder in ethanol. Store this solution in a dark drawer until needed.
4. Based on the total volume needed (depending on the number of GDEs to be immobilized), determine the volume needed of *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) to make the solution 10 mM (each GDE requires 100 μL of this solution). Use a 10 μL Gastight 1801 Syringe from Hamilton Company (Reno, NV, USA) to draw up the correct amount of EDC from the septum-covered EDC bottle. Next, weigh out the appropriate mass of the white powder *N*-hydroxysulfosuccinimide sodium salt, henceforth called NHS (Toronto Research Chemicals, Toronto, Ontario, Canada) to make an 80 mM solution and add to the EDC solution. Lastly, add the required volume of PBS and vortex. The purpose of this solutions is to prime the end of the MHDA coming off the GDE for enzyme attachment. The solution is stored in the 4 °C refrigerator until needed.
5. The enzyme sensitive to norepinephrine, phenylethanolamine *N*-methyl transferase (PNMT), was purchased from Calzyme (Tulelake, CA, USA). 1 mg of this lyophilized powder was suspended in 1 mL of 10 mM PBS (this is enough to immobilize 10 GDEs). The solution is stored in the 4 °C refrigerator until needed. If desired, a NanoDrop instrument can be used to verify the concentration of the enzyme (or any protein).

6. The blocking solution is 99% ethanolamine diluted to 1% ethanolamine in DI. Store this solution in a dark drawer until needed.
7. The solutions tested contained two parts DL-norepinephrine hydrochloride to one part PNMT cofactor, S-(5′-adenosyl)-L-methionine chloride dihydrochloride (SAM) in PBS. The whole blood used in these experiments is obtained from White New Zealand rabbits, stabilized with K2-EDTA from Bioreclamation LLC (New York, USA). The process for making these solutions is described in the next section. Store these solutions in the 4 °C refrigerator until needed.

3 Methods

3.1 NE and Whole Blood Solution Preparation

The following steps are required to create the purified (no blood) NE + SAM solutions via serial dilution (*see* **Note 2**). The desired concentrations for the EIS and Z–t NE + SAM gradients: 0, 1, 5, 10, 50, 100, 500, 1000, 5000, 10,000 pg/mL of NE, where 0 pg/mL is the blank (D0 = PBS only) and 10,000 pg/mL is the highest concentration (D9). However, since each measured/tested solution is 100 μL in total volume: 50 μL is a mixture of NE + SAM dissolved in PBS, while the other 50 μL is 100 mM redox probe. So, in our calculations, we must take into account that whatever concentration of NE is present in the gradient we create, it is effectively cut in half when the redox probe is added and the electrochemical measurement is taken. Therefore, the NE + SAM solutions which are actually made are 0, 2, 10, 20, 100, 200, 1000, 2000, 10,000, 20,000 pg/mL to account for the fact that each solution will be diluted by half in the final tested solution.

1. Create the stock solution: 0.5 mg of NE plus 0.25 mg of SAM (half of the NE mass) in 1 mL of PBS. Vortex at a speed of 1500.
2. Determine the volume required for each solution in the gradient (1 NE + SAM gradient = 1 GDE). If 50 μL of each NE + SAM solution in PBS is required to create one test solution, the total volume required depends on how many replicates will be measured. For example, if we want to have three replicates (requires 3 GDEs) of each solution, we need at least 150 μL of each NE + SAM gradient solution. However, in a serial dilution, part of one solution is used and diluted with more PBS to make the next smallest dilution. Therefore we must make more than 150 μL of each NE + SAM solution. These calculations take some trial and error, but in general it takes at least double the amount of what is needed to run the electrochemical tests to make the next gradient. In this case, every dilution in the NE + SAM gradient is 350 μL: 150 μL is

used to run three electrochemical tests, and 200 μL may be used to make the next dilution. Now, we can calculate what mass of NE and SAM will be required and what volume of additional PBS will be needed to create 350 μL of each gradient solution.

(a) First, we start with the stock solution (0.5 mg/mL = 500,000,000 pg/mL) to create 350 μL of D9 = 20,000 pg/mL. Using the following equation, we can determine what volume of the stock is required to make the D9 solution:

$$C_1 V_1 = C_2 V_2,$$

where C_1 is the concentration of the stock solution, C_2 is the concentration of D9, V_1 is the volume required from the stock to create D9, and V_2 is the amount of D9 we need to make.

(b) Since we want to know V_1, this equation can be rearranged to

$$V_1 = \frac{C_2 V_2}{C_1}.$$

(c) In this example, we plug in 500,000,000 pg/mL for C_1, 20,000 pg/mL for C_2 (the units for the concentrations must match), 350 μL for V_2 to obtain the volume of the stock needed to create D9:

$$V_1 = \frac{(20000\ \text{pg/mL}) * (350\ \mu\text{L})}{500,000,000\ \text{pg/mL}} = 0.014\ \mu\text{L}.$$

(d) Based on this calculation, 0.014 μL of the stock solution is added to 349.986 μL of PBS (the desired 350 − 0.014 μL = 349.986 μL) creates 350 μL of D9. However, if you do not have a pipette which can reliably measure out 0.014 μL, simply increase the 350 μL volume until you get a V_1 which can be measured out.

(e) The next calculation to make D8 from D9 will be the same, but instead C_1 = 20,000 pg/mL, C_2 = 10,000 pg/mL, V_2 is still 350 μL, and as long as the calculated V_1 is below the 200 μL we have allotted to make the next dilution, these calculations can be repeated for the remainder of the solutions. If V_1 ever exceeds 200 μL, increase V_2 to adjust, but be sure that increasing V_2 leaves enough of the stock solution to run the three electrochemical tests.

For the blood solution measurements (*see* **Note 3**), a different NE + SAM gradient must be made. Four sets of blood solutions were created: 5, 10, 25, and 90% whole blood solutions.

1. The 5, 10 and 25% whole blood solutions were structured the same way, still 100 μL total volume: 25 μL of 200 mM ferricyanide, 25 μL of 4× NE + SAM in PBS solution, 50 μL of whole blood in PBS. In these cases, instead of multiplying the desired concentration of NE by 2, as in the purified case previously described, the NE + SAM gradient is instead: 0, 4, 20, 40, 200, 400, 2000, 4000, 20,000, 40,000 pg/mL. Also, 50 μL of the whole blood solution is only 50% of the total volume, if the desired effective whole blood concentration is 5%, then 50 μL of a 10% blood solution must be added to the NE + SAM and redox probe solutions. If we wanted to make 2 mL of a 10% blood solution (which would end up as the 5% blood solution) we would mix 200 μL of whole blood with 1.8 mL of PBS. The calculations to create the NE + SAM gradient, as described in the purified case, still apply here. In this n = 1 case, we needed only 25 μL of each dilution. However, because this gradient can be used for 5, 10, and 25% $n = 1$ measurements, 75 μL of each dilution is required, thus $V_2 = 400$ μL (75 μL for the measurements, and 325 μL to make the next solution).
2. The 90% whole blood solutions were structured differently, though still 100 μL total volume: 5 μL of 1000 mM ferricyanide, 5 μL of 20× NE + SAM in PBS, and 90 μL of 100% whole blood. In this case, instead of multiplying the desired concentration of NE by 2, as in the purified case previously described, the NE + SAM gradient is instead: 0, 20, 100, 200, 1000, 2000, 10,000, 20,000, 100,000, 200,000 pg/mL. In this $n = 1$ case, we needed only 5 μL of each dilution, thus $V_2 = 50$ μL (5 μL for the measurements, and 45 μL to make the next solution).

3.2 Electrochemical Cell Setup

A three electrode electrochemical cell is used to make impedance measurements in both the Z–t and EIS cases. The three electrode materials include: one 2 mm gold disk working electrode (GDE), one Ag/AgCl wire ($d = 0.5$ mm) reference electrode, and one platinum wire ($d = 0.5$ mm) counter electrode (all purchased form CH Instruments, Austin, TX, USA). *See* **Note 4**.

1. As shown in Fig. 2, a makeshift well is created by cutting off the tapered end of a 1 mL pipette tip, and the end which interfaces with the pipette slides over the GDE to make a water tight seal.
2. The reference and counter electrode wires are bent into the well and Scotch taped to the outside of the pipette tip to prevent movement. The wires should be ~2 mm from the top of the GDE surface.

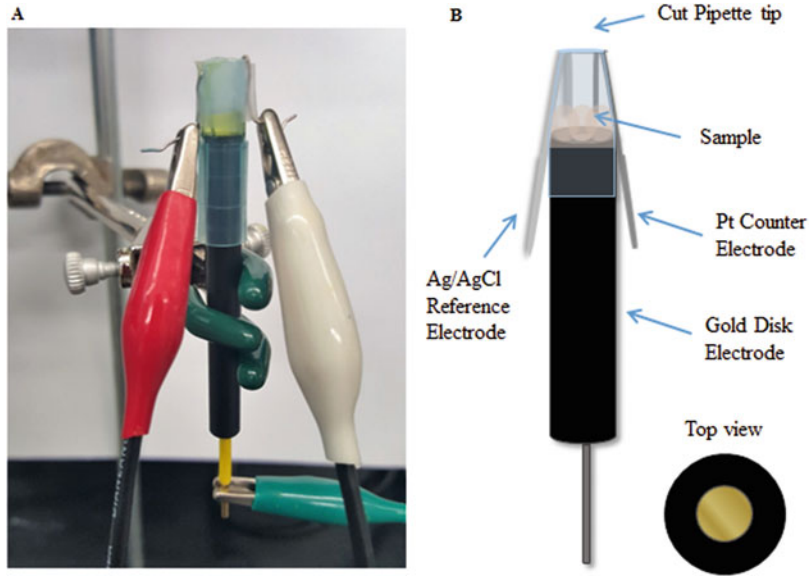

Fig. 2 (**a**) Picture of the actual electrochemical three-electrode system setup on a ring stand. The *yellow solution* is the redox probe + target + cofactor solution. The *red clip* is connected to the Pt counter wire electrode, the *white clip* is connected the reference Ag/AgCl electrode and the *green clip* is connected to the brass connection to the working *gold disk* electrode. (**b**) is a schematic of the electrochemical cell setup including all three electrodes and makeshift well (with a sample inside)

3. The alligator clips from the electrochemical analyzer are connected to the wire electrodes directly and the brass connection for the GDE. In the CHI system, green connects to the working electrode (GDE), red connects to the counter electrode (Pt wire), and white connects to the reference electrode (Ag/AgCl wire), the black clip if available is ground.
4. The 100 μL sample or test solution can be pipetted into the makeshift well and should make contact with all three electrodes, and is ready for measurement.

3.3 Sensor Preparation/ Immobilization [14, 15]

This immobilization process is used for all the electrochemical techniques used in this work: purified and whole blood EIS, and Z–t.

1. The first step of the immobilization process is to ensure the GDE surfaces are clean. The GDEs may be brand new and the sulfur from its protective cap must be removed, or the remnants of a previous functionalization must be removed. This is accomplished using 0.05 μm alumina oxide wetted with DI water on a fine polishing pad (kit purchased from CHI). The GDE surface is rubbed on the polishing surface in a figure eight motion applying only a little more pressure than you might use to write. The GDEs are well rinsed and dried with a Kimwipe tissue, then

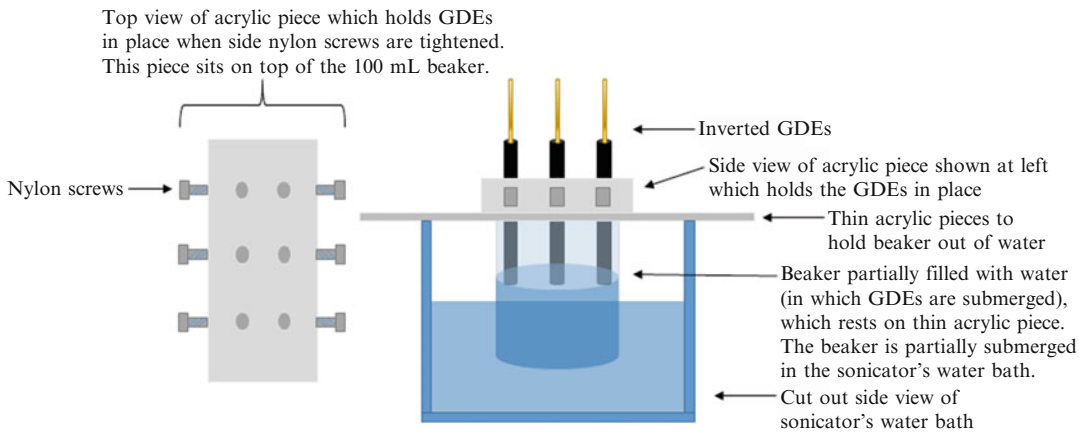

Fig. 3 A schematic of the sonicator apparatus to clean the GDEs. The *left side* is a *top view* of the GDE holder with screws on the side. The *right image* shows how the beaker and acrylic pieces sit on the sonicator's bath, and the beaker's water level nearly matches the water level in the sonicating bath

placed in a beaker with fresh DI. It is important that the GDEs remain in clean DI as gold will become dirty sitting in air.

2. Once all the GDEs you plan to use are clean, they must be sonicated for 20 min in DI. As shown in Fig. 3, we have a homemade acrylic apparatus with holes for the GDEs to be placed through. In the side of this apparatus, the holes have been threaded so nylon screws can be used to tighten the GDEs into a secure position. The bottom of this acrylic piece has been cut so it can securely sit on top of a 100 mL beaker. This beaker is filled with DI, and the GDEs are inverted into the DI. This acrylic/beaker apparatus is placed on another acrylic piece which has been cut out to hold the beaker in the sonicator bath. Sonication for 20 min is used to remove any remaining contaminants and alumina oxide. Once finished sonicating, the GDE can be stored in DI in full 1 mL pipette tips which have been parafilmed at the tapered end.
3. Before the immobilization occurs, the GDEs are checked that they are actually clean. This is accomplished by running a cyclic voltammogram and EIS on each GDE in the echem cell setup described in the previous section. 100 μL of 100 mM ferricyanide is pipetted into the makeshift well and a CV is run using the following parameters:
    - Initial E: −1 V; High E: 1 V; Low E: −1 V.
    - Initial Scan Polarity: Negative.
    - Six segments.
    - Scan rate: 0.01 V/s.
    - Sensitivity: 1E-03 A/V.

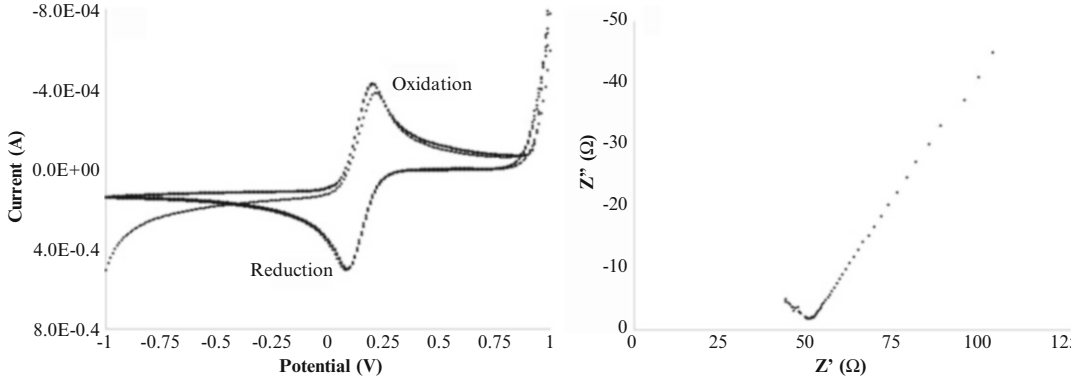

Fig. 4 (**a**) This CV of 100 mM ferricyanide is used to calculate the formal potential of the cleaned GDE. The graph shown in (**b**) is an example of a clean and unmodified GDE after running EIS on 100 mM ferricyanide using the formal potential calculated using the CV information

Once the CV is complete, the formal potential must be calculated: the voltages at which the oxidation and reduction max current occurs are averaged. As shown in Fig. 4a, the oxidation peak occurs at 0.2 V, and the reduction peak occurs at 0.08 V. Therefore, the formal potential is calculated as follows:

$$\mathrm{FP} = \frac{(\text{Oxidation Potential} + \text{Reduction Potential})}{2}$$

$$= \frac{(0.20\,\mathrm{V} + 0.08\,\mathrm{V})}{2} = 0.14\,\mathrm{V}.$$

Next, the GDE is rinsed with DI and tapped on the edge of the waste container to remove excess water. The GDE is put back into the echem cell setup, a new 100 μL of 100 mM ferricyanide is pipetted into the makeshift well and EIS is run using the following parameters:

- Initial E: 0.14 V (formal potential of GDE).
- High frequency: 100,000 Hz; Low frequency: 1 Hz.
- Amplitude: 0.005 V.

Figure 4b shows the Nyquist results for a clean GDE. The general Warburg shape of the Nyquist seen in Fig. 4 indicates a diffusion dominated system and the real impedance on the *x*-axis is around or less than 100 Ω means that the GDE is sufficiently clean. The GDEs should again be stored in DI in a parafilmed pipette tip when not in use. If the real impedance is higher, and/or the Nyquist plot looks like a Randles (electron transfer dominated system) instead of a Warburg (Randles is shown in Fig. 5), re-polish and sonicate the GDE and make sure the wire electrodes are clean.

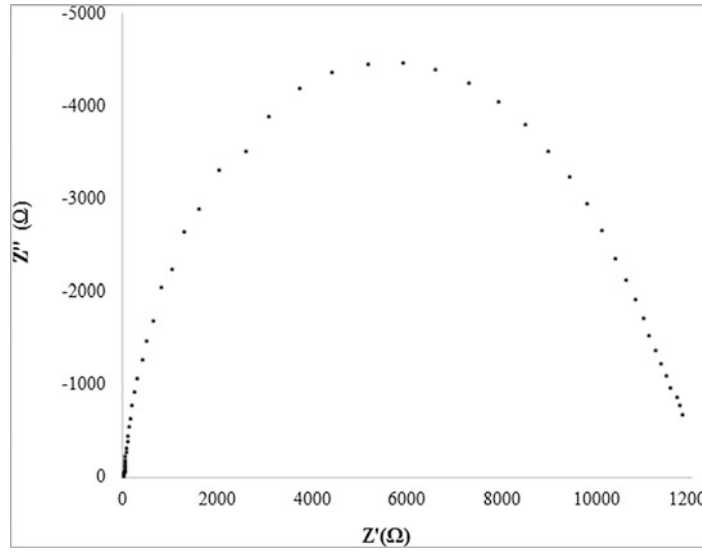

Fig. 5 This post-MHDA EIS Nyquist plot shows how the impedance significantly increases and changes shape as a result of the GDE surface being coated with the self-assembling MHDA layer

4. Now that the GDEs have been confirmed to be clean, the immobilization process can commence. The alkane-thiol self-assembling monolayer solution is 1 mM 16-MHDA in ethanol; 100 μL of this solution is required for each GDE. 1 mL pipette tips are cut to create incubation wells (one pipette tip for each GDE). Once the incubation wells are placed onto each GDE, 100 μL of the 1 mM 16-MHDA solution is pipetted into the wells, the tops of the wells are covered with Parafilm, and the GDEs are allowed to incubate for 1 h at room temperature in a drawer (the 16-MHDA solution is light sensitive).
5. While the 16-MHDA is incubating, cut more 1 mL pipette tips for the next four incubation periods. Once the 16-MHDA incubation is complete, remove and discard the incubation well and carefully rinse the GDE with DI (*see* **Note 5**). Then place a new cut pipette tip over each GDE. Do not add any liquid, not even DI. Parafilm the top of the empty pipette tip and store the GDEs in a drawer overnight to allow the self-assembly process to occur.
6. The following day, EIS (using the same formal potentials used in **step 3**) is run on each GDE with a test solution of 100 μL 100 mM ferricyanide. This time, the Nyquist curve should have a Randles shape, as shown in Fig. 5, with significantly higher impedances on both axes. Generally, the real impedance should be higher than 10^{-3} Ω. Recover the GDEs with the empty dry parafilmed pipette tips while the next solution is prepared.

7. Pipette the EDC/NHS solution using a new pipette tip incubation well, parafilm the top and allow to incubate at room temperature for 1 h. If the solution is not going to be used right away, store it at 4 °C until needed (use the same day). While waiting for the EDC/NHS solution to incubate, it is recommended that the remaining solutions be prepared (enzyme and ethanolamine solutions).
8. At the end of the EDC/NHS incubation time, rinse the GDEs with DI and place a new cut pipette tip on the GDE. Pipette 100 μL of the PNMT enzyme solution onto each GDE, parafilm the top and allow to incubate at room temperature for 1 h. If there is any remaining solution, it can be frozen at −20 °C until the next immobilization. Avoid repeated freeze–thaw cycles. While the enzyme is incubating, it is recommended that the NE + SAM concentration gradients be made, as described in Subheading 3.2.
9. After the PNMT incubation, discard the incubated enzyme solution, rinse the GDEs with PBS, place a new cut pipette tip on the GDEs and pipette 100 μL of the 1% ethanolamine solution onto each GDE. Parafilm the top and allow to incubate at room temperature for 30 min. The purpose of this solution is to block unbound MHDA and unbound GDE surface so the target molecule will not nonspecifically bind; only target that binds to the enzyme will generate a signal.
10. Once the ethanolamine solution has finished incubating, perform the final rinse with PBS, and (using whole 1 mL pipette tips) store the GDEs in PBS at 4 °C until needed. Allow the GDEs to remain at 4 °C for at least 15 min before beginning the first concentration gradient. Once functionalized, it is recommended that each GDE be used to run one gradient within 24 h, but this depends on the stability of the protein used to functionalize the GDE surface.

3.4 EIS Measurements

The purified NE + SAM gradient EIS measurements will be very similar to the EIS measurements described in **step 3** of Subheading 3.2 (*see* **Note 6**). The parameters and set up are the same.

1. The first solution to be run is a blank (50 μL of D0 and 50 μL of 100 mL redox probe).
2. Once a measurement is completed, the GDE is carefully rinsed with PBS.
3. The previous solution is discarded before the next solution is placed in the same makeshift well for the next reading (D1, etc.) until the full gradient is completed on the same GDE (lowest concentration first, highest concentration last).

Table 1
This table shows the raw EIS.txt data for one concentration on one GDE pasted into Excel

		1.00	pg/mL	
Sept. 30, 2011 17:18:08				
A.C. impedance				
File: h:_labelle\data\pnmt&sam\pnmt&sam&ne\eis\immob#3\[ne]gradient\gde2_red\gde2_red_d1_2.txt				
Data source: experiment				
Instrument model: CHI660C				
Header:				
Note:				
Init E (V) = 0.24				
High frequency (Hz) = 1e + 5				
Freq/Hz	Z′/ohm	Z″/ohm	Z/ohm	Phase/deg
96680.00	78.03	−24.58	81.81	−17.50
81050.00	77.83	−25.68	81.96	−18.30
66410.00	79.96	−29.41	85.20	−20.20
54690.00	81.08	−32.65	87.40	−21.90
45900.00	82.64	−37.02	90.55	−24.10
…	…	…	…	…

This table extends to all frequencies measured (lowest value is 1.184 Hz)

The blood EIS measurements were conducted in the same way as the purified EIS measurements with the exception that the electrochemical cell setup was also rinsed with PBS between each measurement to rinse out residual blood (*see* **Note 3**).

3.5 EIS Data Analysis

1. Once all the GDE gradients have been run, it may be necessary to convert the raw data files (in the CHI system these are .bin files) to .txt files using the CHI software (*see* **Note 7**). The raw .txt data is copied and pasted into an Excel workbook. The raw data should have five columns: Frequency (Hz), Z′ (Ω), Z″ (Ω), Z (Ω), and Phase (deg). *See* Table 1.

2. The complex Z (Ω) column for each NE + SAM concentration is extracted from the raw data (ten columns total, one for each D0–D9). *See* Table 2.

3. Two columns are calculated at each frequency for all concentrations: natural logarithmic slope (complex impedance is left alone and the natural log of NE concentration is taken) and R^2 of the natural log fit. The slope of the ln[NE + SAM] versus complex impedance provides the general responsivity of the sensor while the R^2 provides insight as to the reproducibility of the sensor. *See* Table 2.

Table 2
This table includes all *Z* (complex impedance) values for all concentrations on one GDE pasted into another area of the same Excel for easy viewing

Electrode 2													
												Ln	
	0.001	1.00	5.00	10.00	50.00	100.00	500.00	1000.00	5000.00	10000.00	pg/mL		
Freq(Hz)	D0, Z/ohm	D1, Z/ohm	D2, Z/ohm	D3, Z/ohm	D4, Z/ohm	D5, Z/ohm	D6, Z/ohm	D7, Z/ohm	D8, Z/ohm	D9, Z/ohm		SLOPE	R-sq
96680.0	78.23	81.81	81.26	76.58	90.05	83.10	81.67	84.45	82.55	79.12		0.227	0.0842
81050.0	78.37	81.96	81.46	76.94	89.95	83.31	81.96	84.61	82.67	79.37		0.230	0.0910
66410.0	81.56	85.20	84.71	80.10	93.43	86.68	85.27	87.99	86.08	82.72		0.244	0.0978
54690.0	83.76	87.40	87.01	82.54	95.69	88.95	87.69	90.34	88.41	85.12		0.256	0.1088
45900.0	86.96	90.55	90.15	85.73	98.93	92.25	91.03	93.62	91.79	88.34		0.266	0.1158
...	...	...	...	...	...	...	...	...	...	...	...	...	...

The top of these columns indicate the concentration name and value. This table also shows the calculated columns for natural logarithmic slope and R^2 calculations for all concentrations at each frequency. The Excel SLOPE function is used where the y argument is an array of all the complex impedances at each frequency, while the x argument is the natural log of the array of concentrations listed at the top of each column of complex impedances. The Excel RSQ function is used to generate the R^2 calculation for each frequency, where the y and x arguments are the same arrays used in the slope calculation

4. An optimum frequency (which is ideally the frequency where the highest slope and R^2 occur) is determined from replicated GDEs. Firstly, in our experience, the highest slope and R^2 rarely coincide at the same frequency. Thus the optimal frequency is a tradeoff between high response (slope) and reproducibility (R^2), *see* Table 3. But it is more important to have a predictable system than a higher signal, thus, the optimal frequency is selected based more on the reproducibility rather than response. A word of caution, a 0.99 R^2 on a line with a slope of 0.1 Ω/ln(pg/mL) means you have a wonderful fit to a very flat line (no signal), which is not helpful. Additionally, each GDE will have a slightly different optimal frequency for various reasons including the fact that each GDE is a little different which will be evident from the difference in the blanks from GDE to GDE. Typically, a small range of frequencies (~10 frequencies), where hopefully the optimal frequencies from all the GDEs fall into, are focused on. As a note, the ten frequencies completely depends on the protein and target being measured, thus using a different protein to detect the same target may yield a different optimal frequency.
5. Also for each of the ten frequencies, a calibration curve is made: a plot of the average complex impedance versus ln[NE + SAM], and a logarithmic fit is applied to the data. The calibration curves from all ten frequencies are compared and the final optimal frequency decision is made (again, usually a trade-off of slope in favor of R^2). In the case of PNMT detection of NE + SAM, on a basis of an $N = 12$ calibration curve analysis (Fig. 6), 371.1 Hz was selected to be the optimal frequency [15].

   The equation for the calibration curve is also important because it could be potentially used to program a handheld device. Currently, the self-monitoring blood glucose meters (which use the amperometric-it technique) measure current for literally a couple of seconds, plug that in as the *y*-variable and solve for *x* (concentration of glucose), which is then displayed on the screen. This calibration curve could work in a similar fashion to convert the measured impedance at a particular frequency to concentration of norepinephrine.
6. Additionally, once a calibration curve is selected, the lower limit of detection can be calculated using the following equation [16, 17]:

$$\mathrm{LLD} = 3.3 * \frac{\text{STDEV of blanks}}{\text{slope of calibration curve}} = 3.3 * \frac{594.444\,\Omega}{20.899\,\frac{\Omega}{\mathrm{pg/mL}}}$$

$$= 94.18\,\mathrm{pg/mL},$$

   where LLD is the lower limit of detection (the smallest reliable measurement practically possible), STEDV is the standard deviation of the blank concentration from several GDEs.

Table 3
This table shows the section of 1 GDE where the highest ln R^2 frequently occurs (as seen in other GDEs)

Electrode 2													
												Ln	
	0.001	1.00	5.00	10.00	50.00	100.00	500.00	1000.00	5000.00	10000.00	pg/mL		
Freq (Hz)	D0, Z/ohm	D1, Z/ohm	D2, Z/ohm	D3, Z/ohm	D4, Z/ohm	D5, Z/ohm	D6, Z/ohm	D7, Z/ohm	D8, Z/ohm	D9, Z/ohm		SLOPE	R-sq
...	...	...	...	...	...	...	...	...	...	...		...	...
2148.00	585.90	597.60	605.80	609.70	621.60	621.80	630.30	628.70	628.30	625.10		2.995	0.8907
1758.00	705.20	718.40	728.80	735.20	747.10	747.20	757.10	755.20	754.90	752.40		3.533	0.8987
1465.00	834.90	854.00	862.60	870.80	885.10	886.40	897.30	894.00	894.80	891.50		4.186	0.9076
1172.00	1023.00	1045.00	1057.00	1066.00	1082.00	1085.00	1098.00	1096.00	1099.00	1092.00		5.1949	0.9173
966.80	1222.00	1245.00	1260.00	1268.00	1291.00	1291.00	1309.00	1305.00	1304.00	1298.00		5.8125	0.8889
810.50	1427.00	1452.00	1473.00	1479.00	1508.00	1510.00	1532.00	1529.00	1527.00	1519.00		7.1434	0.88856
664.10	1748.00	1779.00	1802.00	1811.00	1842.00	1843.00	1867.00	1863.00	1863.00	1854.00		8.0881	0.8980
546.90	2067.00	2102.00	2134.00	2139.00	2181.00	2183.00	2218.00	2213.00	2212.00	2198.00		10.2621	0.8858
459.00	2429.00	2468.00	2508.00	2511.00	2561.00	2564.00	2608.00	2602.00	2600.00	2583.00		12.1617	0.8806
371.10	3022.00	3074.00	3118.00	3128.00	3180.00	3182.00	3229.00	3220.00	3221.00	3207.00		14.0331	0.9035
312.50	3451.00	3502.00	3568.00	3561.00	3641.00	3645.00	3715.00	3710.00	3709.00	3675.00		18.2048	0.8676
253.90	4171.00	4231.00	4316.00	4303.00	4404.00	4410.00	4502.00	4493.00	4493.00	4450.00		22.7940	0.8624
...	...	...	...	...	...	...	...	...	...	...	...	...	...

The cells highlighted in red indicate the highest R^2 value for this particular GDE in this experiment, this is the optimal frequency for this GDE. The ~10 frequencies highlighted in yellow are the frequencies to be further analyzed; the next analysis compares the same frequency range of multiple GDEs across multiple experiments/days. For these ten frequencies, additional analysis is conducted. For each of the ten frequencies, the average complex impedance for all GDEs at that frequency is calculated along with standard deviations and RSD, as shown in Table 4

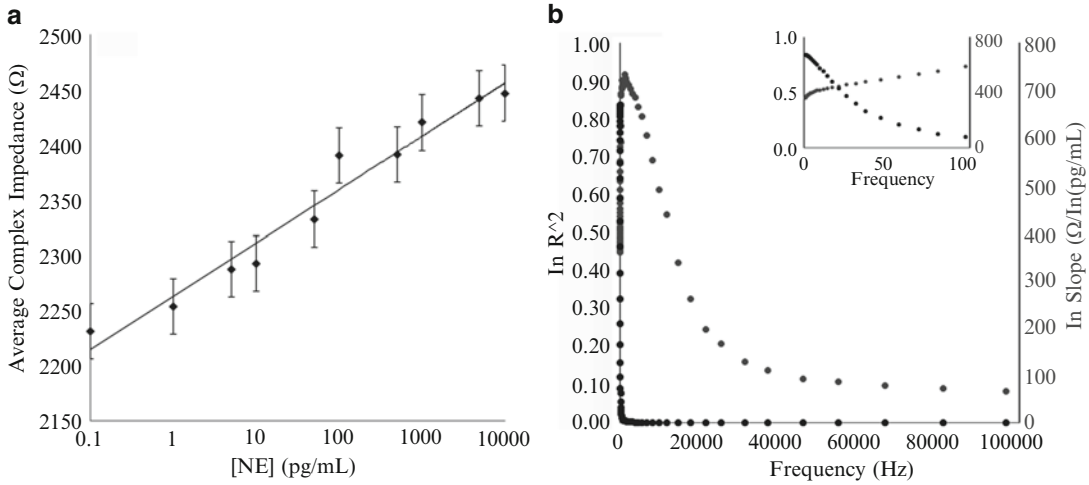

Fig. 6 (**a**) An $N = 12$ calibration curve of purified NE detected using PNMT-immobilized GDEs. The natural logarithmic fit for this curve is Complex Impedance = 20.899*ln([NE]) + 2263.4, with an R^2 of 0.9626. In (**b**), an $N = 1$ comparison of ln R^2 and slope are plotted with respect to frequency. From the inset zoomed in graph, it is easy to tell that the peak R^2 frequency does not coincide with the peak slope. Thus to pick an "optimal frequency" when peak ln R^2 and slope are not possible, a trade-off is required

Analysis for the whole blood data is conducted in a similar manner. However, the nature of the analysis is more to confirm that the optimal frequency is still valid, and to determine in which blood solutions reliable signal can still be obtained. One way to compare the different solutions of whole blood is to plot R^2 at the optimal frequency versus % whole blood versus slope at the optimal frequency (second *y*-axis), as shown in Fig. 7 [15]. Another way is to plot R^2 and slope versus frequency to see if a better frequency exists, as shown in Fig. 6. The same LLD calculation could be applied for each blood solution, but without replication, a standard deviation cannot be calculated for the blank solutions.

3.6 Z–t Measurements [15]

The goal of the first set of Z–t measurements is to determine the minimum amount of assay time required to obtain a reliable impedance measurement. This technique would ideally take less than 10 s and could be used in a handheld device to rapidly quantify norepinephrine concentration.

1. The first set of Z–t measurements are made the same way the EIS measurements are made, except that the following parameters are used:
   - Initial E: 0.14 V (formal potential of GDE).
   - Amplitude: 0.005 V.
   - Frequency: 368 Hz (closest option to the optimal frequency determined in EIS data analysis).
   - Run time: 90 s.

Table 4
This table shows how 12 GDEs are compared at one frequency within the frequency range of interest shown in Table 3

Exprmnt #	GDE #	[pg/mL] Freq/Hz	0.1 Z/ ohm	1 Z/ohm	5 Z/ohm	10 Z/ ohm	50 Z/ ohm	100 Z/ohm	500 Z/ohm	1000 Z/ ohm	5000 Z/ ohm	10000 Z/ ohm	Ln SLOPE	Ln *R*-sq
1	#1	371.1	1195	1175	1213	1260	1248	1290	1309	1340	1391	1365	42.985	0.9069
1	#3	371.1	2490	2513	2573	2553	2678	2697	2803	2815	2833	2790	78.555	0.9106
2	#1	371.1	3034	3052	3088	3136	3152	3183	3211	3248	3252	3253	49.932	0.9621
3	#1	371.1	2749	2794	2805	2838	2880	2880	2896	2917	2893	2895	25.019	0.8718
3	#2	371.1	3022	3074	3118	3128	3180	3182	3229	3220	3221	3207	32.312	0.9035
3	#3	371.1	2235	2264	2295	2326	2327	2343	2371	2381	2381	2376	23.842	0.9170
6	#4	371.1	2095	2124	2133	2111	2167	2192	2204	2212	2217	2222	28.285	0.9078
7	#1	371.1	2175	2288	2400	2382	2389	2373	2369	2434	2422	2433	40.646	0.6884
7	#2	371.1	1125	1158	1261	1087	1066	1564	1265	1465	1671	1802	127.587	0.6228
7	#4	371.1	1746	1720	1814	1820	1842	1829	1823	1816	1821	1828	17.231	0.4957
8	#1	371.1	2522	2443	2340	2444	2585	2641	2680	2653	2730	2684	62.659	0.6196
8	#4	371.1	2389	2442	2409	2432	2481	2513	2535	2542	2477	2508	26.383	0.6512
		AVG	2231.42	2253.92	2287.42	2293.08	2332.92	2390.58	2391.25	2420.25	2442.42	2446.92	46.29	0.79
		STD	622.97	633.01	615.44	646.81	670.56	596.76	653.71	624.21	591.06	574.56	31.18	0.16
		RSD	27.92%	28.08%	26.91%	28.21%	28.74%	24.96%	27.34%	25.79%	24.20%	23.48%	67.35%	20.26%

There were originally more GDEs in this table, but for various legitimate reasons, those GDEs were removed. When compared in this fashion it is easier to see when one or more GDEs are causing the average, standard deviation and relative standard deviation to become skewed, especially when looking at several frequencies simultaneously. The AVERAGE Excel function was used to average the complex impedance data in each column. The STDEV function was used to calculate the standard deviation of each column. The relative standard deviation was calculated by dividing the STD by the AVG and expressed as a percent. Both STD and RSD can be used to determine how similar the complex impedance from the selected GDEs. Generally, a smaller STD or RSD means there is little difference between the numbers selected, which is desirable, and is reflected in the R^2 value

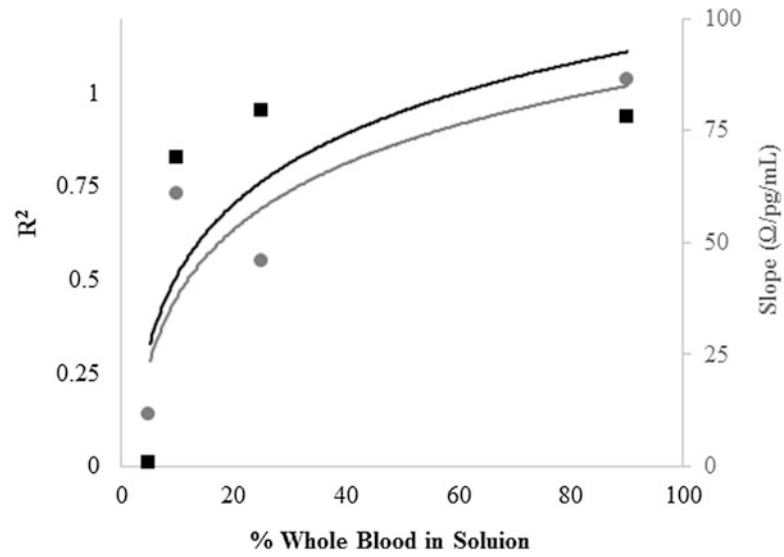

Fig. 7 The R^2 (*squares*) and slope (*circles*) of the natural logarithmic fits (for impedance versus [NE]) at 371.1 Hz for each whole blood solution (5, 10, 25, 90%) is shown. Adapted from [15]

2. The tests are repeated at a "dead" frequency, a very high frequency like 46,300 Hz, choose a frequency well above 10,000 Hz since our experience shows biological signals occur below 5000 Hz. Try to avoid using harmonics of your frequencies of interest, just in case.

The purpose of the second set of Z–t experiments is to compare the two frequencies nearest the optimal frequency found in the EIS data analysis (the hardware in the Electrochemical analyzer prevented the use of the exact 371.1 Hz frequency, the optimal frequency used to create the calibration curve in Fig. 6).

1. The second set of Z–t experiments were run with the same parameters as the first Z–t experiment, but the run time was 500 s to accommodate the measurement of a solution with multiple injections of 500 pg/mL of NE + SAM. The multiple injections were used in order to determine if this technique could be used for multiple measurements, whereas with EIS, once a sample is measured, the electrodes are rinsed and a new sample is applied to be measured. Additionally, this data can be used to determine the kinetics involved in the enzyme–cofactor–substrate system. The Z–t measurements were started with 50 μL of 100 mM ferricyanide and the 500 pg/mL NE + SAM solution was injected at 30, 40, 85, 145, and 325 s time points after initialization of the Z–t assay.
2. The second Z–t experiment was also replicated using PBS to instead of the 500 pg/mL NE + SAM solution to investigate the injection artifact, as depicted in Fig. 8.

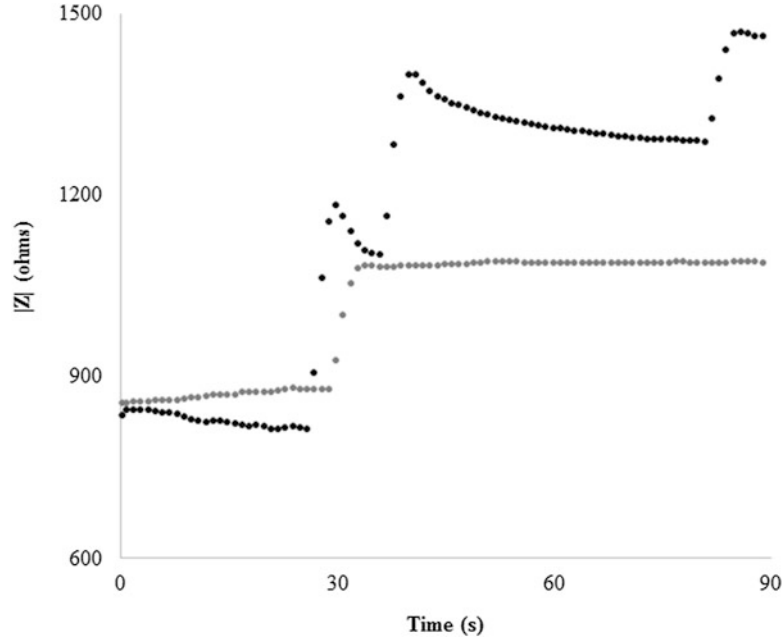

Fig. 8 The *black markers* depict the electrochemical behavior of the enzyme–-cofactor–substrate system when NE + SAM in PBS is injected at the times when a bell-like curve is seen (~30, 45, and 90 s). The time it takes the system to again stabilize after each injection can be analyzed to determine enzyme kinetics, but this also shows that the sensor is still capable of detecting analyte after a couple of uses. The *gray markers* indicate one injection of PBS on a different GDE at about the same time the first NE + SAM injection takes place. These data help to remove the injection artifact as well as the influence of dilution and the electrochemical behavior of the PBS solvent

3.7 Z–t Data Analysis

For the first Z–t discrete experiments where the solutions were run the same way the EIS solutions were run, the goal of data analysis is to determine the minimum time required to run the assay reliably (at the optimal frequency determined in the EIS data analysis).

1. To find this minimum time, again, the complex impedance (Ω) is plotted against the concentration of NE (pg/mL) at various times which are selected based on the same natural logarithmic slope and R^2 calculations performed in the EIS data analysis. As with selecting an optimal frequency in the EIS analysis, selecting an optimal time for Z–t data is often a trade-off between slope and R^2.
2. In this data set, the calibration curves for 3, 24 and 89 s were compared because they offered the highest slope and R^2 for the natural logarithmic fit (Fig. 9) [15]. In this case, no trade-off was necessary as the quickest time offered the best slope and R^2 fit. Therefore, Z–t can be run for 3 s to reliably quantify NE concentration, which rivals the self-monitoring blood glucose meter assay time.

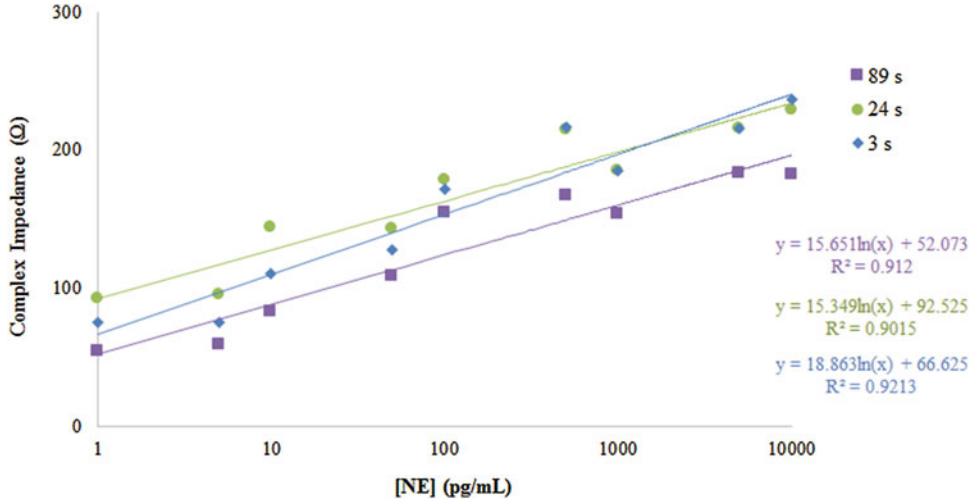

Fig. 9 These Z–t results at 368 Hz compare the impedance readings at different times during the measurement: 89 s (*purple squares*), 24 s (*green circles*), and 3 s (*blue diamonds*). Adapted from [15]

Since the Z–t hardware cannot exactly match the optimal frequency, the two closest frequencies were analyzed (368 and 321 Hz) via periodic injection and continuous measurement in the second set of Z–t experiments.

1. The bar graph in Fig. 10 shows the adjusted peak height (NE + SAM signal minus the PBS signal) [15]. From the data collected, the adjusted peak heights of each injection were compared and it was found that 368 Hz showed the most promise for a predictable and reliable frequency to be used in a final handheld device to detect norepinephrine.

4 Notes

1. The Redox probe solutions remain stable up to 1 month at room temperature in an amber bottle to prevent photodegradation. The 1000 mM ferricyanide solution will need additional mixing on the vortexer at the highest speed the mixer can reach to completely dissolve. Make sure all chemicals have been completely dissolved and the solutions are well mixed using the vortexer before use.
2. It is highly recommended that all solutions are used the day they are made, especially as the NE + SAM solutions become discolored and unusable if left in a 4 °C refrigerator overnight.
3. When vortexing whole blood solutions, use a low speed (~1500 RPM), and wear a respirator as an added precaution. It is also recommended that low-binding pipette tips are used

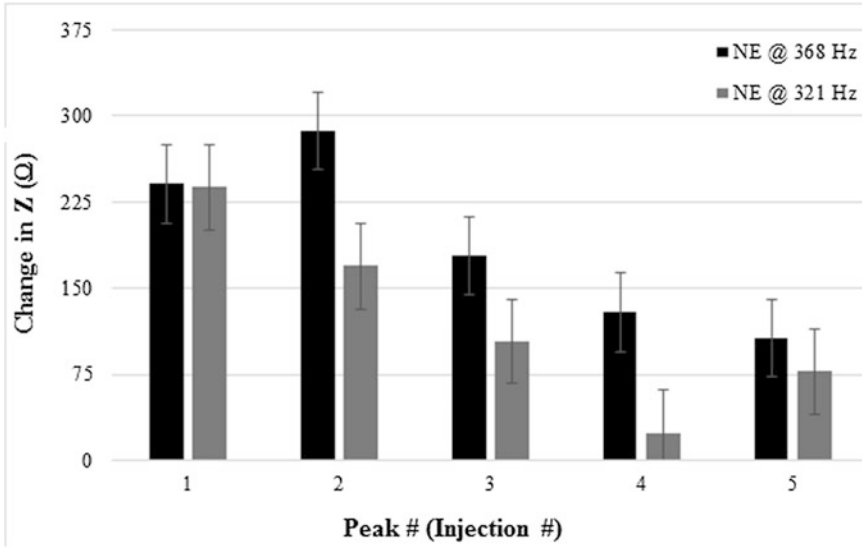

Fig. 10 This bar chart depicts the average peak height ($N = 2$) at each injection point without the injection artifact (subtracted the PBS injection data) at the two frequencies closest to the optimal frequency determined in the EIS data analysis. Adapted from [15]

in the whole blood solution handling, as blood will stick in regular pipette tips. It is strongly recommended that biosafety and bloodbourne pathogens training is obtained before performing these experiments.

4. The Pt and Ag/AgCl wires come with brass connectors at the ends. It is recommended that these brass connectors be cut off with wire cutters so the alligator clips can be directly attached to the wires. Corrosion resulting from the storage solutions preferentially occurs at the brass interface and will eventually interfere with impedance readings. For best results, the GDE should be polished as described in Subheading 3.2. The Pt and Ag/AgCl wires should be cleaned with an abrasive pad after each experiment and stored in DI water, and 3 M KCl plus 10 mM $AgNO_3$ in DI solutions, respectively. A well-cared for Ag/AgCl wire is white when ready for use and becomes a dull gray after extensive use.
5. Do not rinse the gold directly, that will strip off the MHDA or anything else that has bound to the GDE surface. Instead, hold the GDE upside down so the gold faces the inside of a waste container, then slowly rotate the GDE as you spray DI near the middle of the black part of the GDE,allowing water to gently run down and off the GDE.
6. When running electrochemical measurements, beware of solution temperature changes and try to minimize variation.

For example, the gradients once made should be stored in the 4 °C refrigerator, and these solutions should be kept cold while running the experiments. Also, as the GDEs are removed from the refrigerator before performing experiments, make sure they have time to reach room temperature before first use.

7. When performing data analysis and pasting .txt data into Excel, make sure that the data is put into separate columns. This may take some effort using the text to columns function in Excel using the delimited option and selecting the separated by commas option. Also, one cannot take the natural log of the 0 pg/mL NE + SAM concentration, so there are two options: remove the blank from analysis, or use a small number such as 10^{-5} pg/mL as the blank concentration.

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