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Elucidating the Electrochemical Mechanism of N^G-Hydroxy-L-Arginine

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N^G-Hydroxy-L-arginine (NOHA) is a stable intermediate product in the urea cycle that can be used to monitor the consumption of L-arginine by nitrous oxide synthase (NOS) to produce nitric oxide (NO) and L-citrulline. Research has implicated the urea cycle in many diseases and NO has cultivated interest as a potential biomarker for neural health. Electrochemical detection is an established, cost-effective method that can successfully detect low levels of analyte concentrations. As one of the few electrochemically active species in the urea cycle, NOHA shows promise as a biomarker for monitoring disruptions in this biochemical process. In this study, we show that NOHA has an oxidation peak at +355 mV vs Ag/AgCl at a glassy carbon electrode. In addition, cyclic voltammetry studies with structural analogs—alanine and *N*-hydroxyguanidine—allowed us to approximate the oxidation wave at +355 mV vs Ag/AgCl to be a one electron process. Diffusivity of NOHA was found using linear scan voltammetry with a rotating disk electrode and approximated at 5.50×10^{-5} cm^2 s^{-1}. Ample work is still needed to make a robust biosensor, but the results here characterize the electrochemical activity and represent principle steps in making a NOHA biosensor.

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The primary source of nitric oxide (NO) biosynthesis is through the L-arginine metabolite pathway to L-citrulline in the urea cycle.[1–3] In the middle of this pathway, N^G-Hydroxy-L-arginine (NOHA) is a stable intermediate product, which is created in the consumption of L-arginine by nitric oxide synthase (NOS) to produce NO and L-citrulline (Scheme 1).[4,5] Nitric oxide is a neurotransmitter that is difficult to directly monitor because of its short half-life and typically off-gasses.[6,7] Yet, NO is growing more popular as a potential marker for disease monitoring and overall neural health.[8–10]

While various factors influence NO levels outside of biosynthesis production, including diet and exogenous drugs, NOHA is directly correlated to the NO production in pure biosynthesis reactions. The NO and NOHA pathway contains L-arginine, L-citruline, and L-ornithine among other chemicals in the urea cycle. [4,11] Previous work indicated the urea cycle is disrupted in various diseases.[4,5,11–14] As an example, NOHA is a potent arginase inhibitor that has been proven to have antiproliferative and apoptotic actions on the arginase-expressing human breast cancer cells.[4] Another example is NO's effect on tumor growth, where low concentrations of NO produced from endothelial cells caused growth in tumors.[4,5,15,16] It has also been reported that NOHA, in a cultured medium, accumulates up to 20%–30% of the amounts of NO and L-citrulline produced.[5]

Electrochemical detection allows for detection of low levels of analyte concentrations, which is important for distinguishing small changes in biosynthetic processes.[17,18] NOHA has been found to accumulate at around 6 uM in serum samples.[19] In tissue samples, NOHA has been found to accumulate at concentrations of 10–60 nM.[16,20] Detection of small changes in NOHA concentrations could be used as a biomarker for various diseases. In this study are steps towards using NOHA detection at clinically relevant concentrations in the presence of other urea cycle amino acids. To investigate and characterize the electrochemical activity of NOHA, a static cell set up with a glassy carbon working electrode, platinum auxiliary electrode, and Ag/AgCl reference electrode was used.[21] We expand on previous work by providing insight towards the electrochemical mechanism, elucidated through cyclic voltammetry (CV) experiments with structural analogs.[21] Furthermore, diffusivity was also determined through rotating disk electrode CV experiments. To understand the effect of fouling of the electrode in the electrochemical detection of NOHA, a solution of high concentrations of L-arginine, L-ornithine, and L-citrulline was used. Herein, we demonstrate the potential to electrochemically monitor NOHA as a biomarker for urea cycle disruptions.

Experimental

Materials and apparatus.—Phosphate buffer saline solution (PBS) was made in-house and buffered to a pH of 7.3 using chemicals purchased from Fisher Scientific. NOHA was purchased from CALBIOCHEM. L-arginine was procured from Fisher Scientific. L-ornithine and L-citrulline were obtained from Sigma-Aldrich. All experiments, unless indicated, were performed using a Gamry 600 + Potentiostat and a BASi C3 Static Cell Faraday cage. Gamry software was used to record data, and the collected data was graphically displayed using Excel.

Rotating disk electrode (RDE) experiments were performed in a three-electrode assembly within a Pine Research Wavedriver RDE system containing a glassy carbon working electrode, platinum auxiliary electrode, and a Ag/AgCl reference electrode.

For fast scan cyclic voltammetry (FSCV) experiments, carbon fiber microelectrodes (CFE) were prepared by pulling an 11 μm carbon fiber into a glass capillary. The glass capillary was pulled to a fine tip in a pipette puller and sealed using epoxy resin (Epon 828 with a 14% m-phenlylenediamine by weight). The electrode was then polished to a fine tip using a diamond wheel polish at a 45° angle prior to use. More details can be found in published resources.[22,23]

Voltammeric measurements.—Electrodes were purchased from BASi. A platinum auxiliary electrode, a 3 mm diameter glassy carbon electrode (GCE), and a reference electrode of Ag/AgCl were used. Electrodes were submerged in a 20 ml shot glass. In cyclic voltammetry measurements, the potential was swept from −0.2 to +0.6 V vs Ag/AgCl at a scan rate of 50 mV s^{-1}.

Electrode set-up was the same for fast scan cyclic voltammetry, except with the replacement of a CFE working electrode made in-house. Scans were conducted from −1.0 to +1.0 V vs Ag/AgCl at 300 V s^{-1}.[22–25]

Three cyclic voltammograms were performed for each experiment. The second voltammogram was reported for each experiment and was used to analyze the data.

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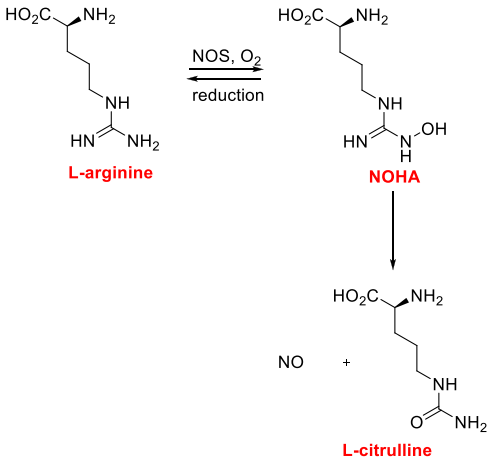


Scheme 1. Oxidation of L-arginine to L-citrulline involving NOHA as a key intermediate.

Serial dilutions.—A serial dilution was performed by starting with a base solution of PBS. NOHA concentration was increased by incrementally adding specific volumes of a concentrated NOHA stock solution. The solution was stirred after the addition of the NOHA for approximately 1 min. Stirring was turned off during the electrochemical measurement. Concentrations of NOHA were measured from 0.5 μM to 148 μM for CV and 50 nM to 148 μM for FSCV. Serial dilutions were done in triplicate with different preparations and electrodes. The displayed graphs are the average of the second scan from each serial dilution, and the standard deviations are the averages of these triplicate measurements.

Results and Discussion

Electrochemical detection of NOHA.—Cyclic voltammetry experiments with a glassy carbon electrode were performed for a serial dilution of NOHA from 0.5 to 148 μM. NOHA in PBS had an oxidation peak at +355 mV vs Ag/AgCl. A reduction peak was observed at +188 mV vs Ag/AgCl (Fig. 1a). NOHA undergoes a quasi-reversible reaction indicated by the ratio between the oxidation and reduction peak currents not equaling one. Having a linear relationship between concentration and peak current is important for creating a future NOHA sensor. Therefore, calibration curves were made for current vs concentration of NOHA at both the oxidation (Fig. 1b) and reduction peak (Fig. 1c). The oxidation peak ($R^2 = 0.99$) of NOHA had a linear relationship between the peak current and the concentration, while the reduction peak ($R^2 = 0.92$) had a logarithmic relationship. The sensitivity of the NOHA's oxidation peak was 5.39 nA/uM, while the reduction peak was 1.98 nA/uM.

Investigating structural analogs to NOHA.—In order to determine the electron transfer mechanism of NOHA oxidation, structural analogs, specifically, alanine (Fig. 3) and *N*-hydroxyguanidine (Fig. 4), were tested. We hypothesized that oxidation would occur at either the amino acid wherein the amine functional group is oxidized to the imine or at the hydroxyguanidine terminus is oxidized to a carbamide (Scheme 2).

The identified structural analogs, alanine and *N*-hydroxyguanidine, were tested with CV under the same conditions as NOHA. No electrochemical activity was observed in the voltammogram of alanine (Fig. 2). *N*-hydroxyguanidine is a known electrochemically active species,[26,27] and a clear oxidation and reduction peak was observed from the *N*-hydroxyguanidine at +355 mV and +250 mV vs Ag/AgCl respectively (Fig. 3a). The calibration curve of the oxidation peak can be linearly fitted with similar sensitivity compared to NOHA (Fig. 3b). The sensitivity of *N*-hydroxyguanidine was 4.09 nA/uM and NOHA 5.39 nA/uM. Additionally, the peak current has a linear relationship with respect to the concentration of *N*-hydroxyguanidine as was observed with NOHA.

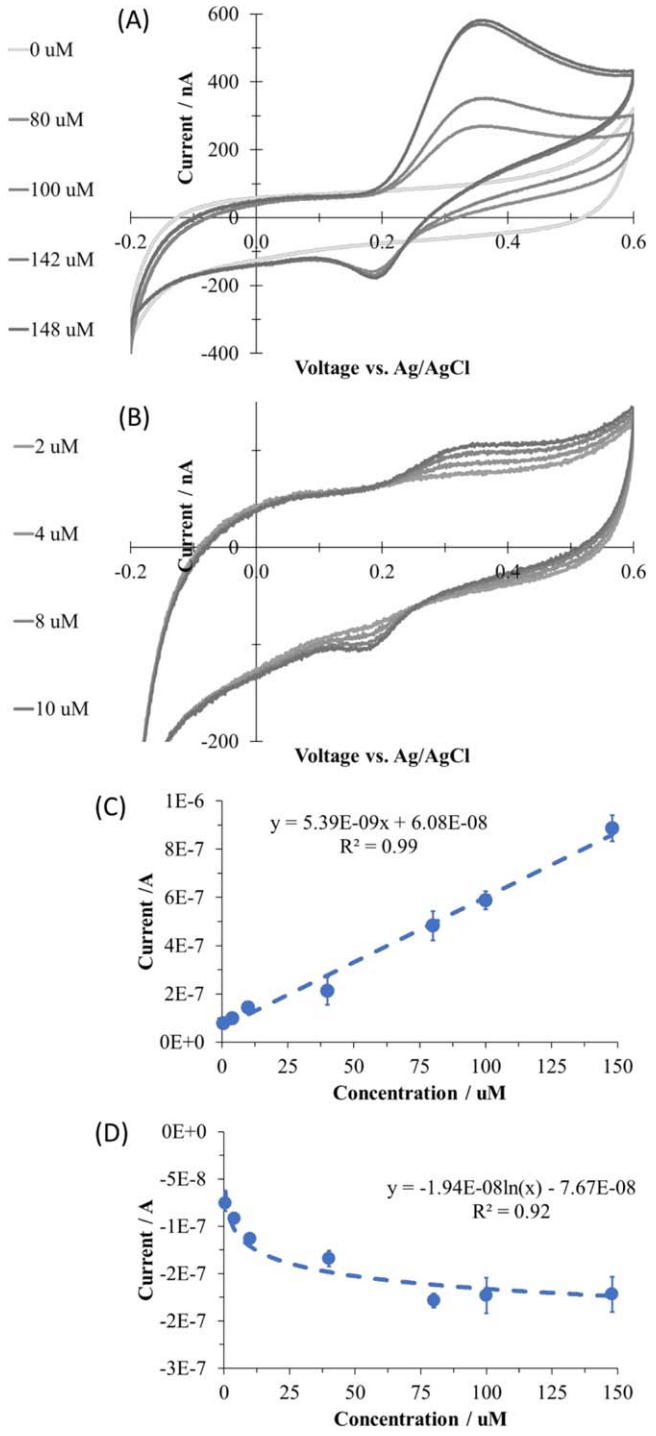


Figure 1. A NOHA serial dilution from 0.5 to 148 μM in PBS. Oxidation peak of NOHA observed at +355 mV vs Ag/AgCl and a reduction peak at +188 mV vs Ag/AgCl at (a) high concentrations and (b) low concentrations. NOHA calibration curve for serial dilution from 0.5 to 148 μM in PBS. Both the (c) oxidation peak current at +355 mV vs Ag/AgCl and (d) reduction peak current at +188 mV vs Ag/AgCl were calibrated in a linear and logarithmic fit respectively. Data was collected in triplicate and error bars represent standard deviation. Reproduced, in part, with permission from.[19] Copyright 2018, The Electrochemical Society.

Based on these findings (Figs. 2 and 3), we propose a mechanism for the electron transfer of NOHA (Scheme 3). Cyclic voltammetry experiments of alanine in PBS with a bare GCE revealed that the

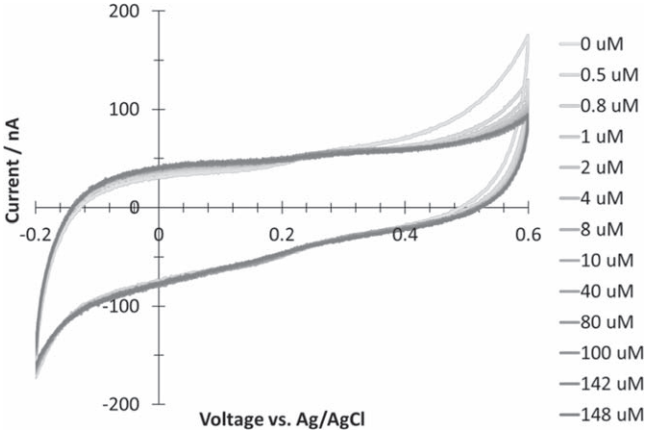
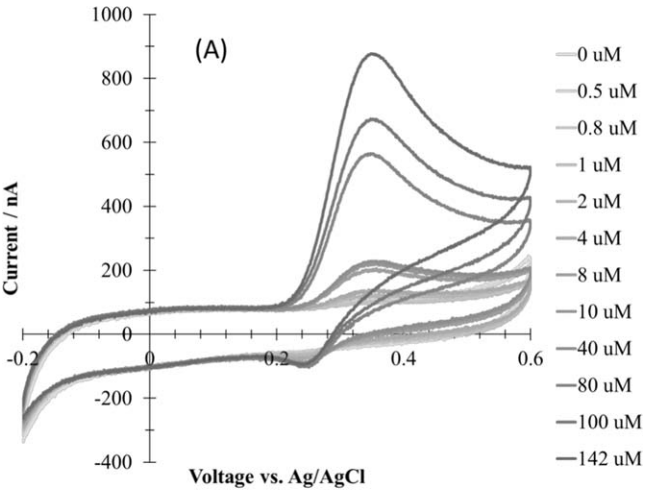


Figure 2. Cyclic voltammatograms of alanine from 0.5 to 142 μM revealed no faradaic electrochemical activity supporting that alanine is not electrochemically active.

compound is electrochemically inactive (Fig. 2). Typically, the GCE needs to be modified with a redox active metal like Ni(II) or Cu(II) to activate electrochemically inactive amino acids like alanine.[28–32] Experiments with *N*-hydroxyguanidine showed a quasi-reversible oxidation wave around +355 mV (Fig. 3), consistent with the CV of



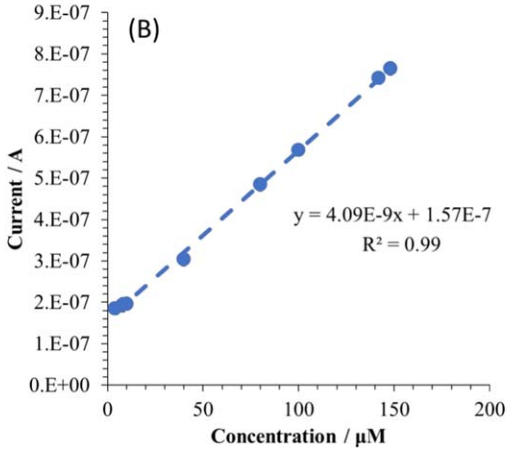
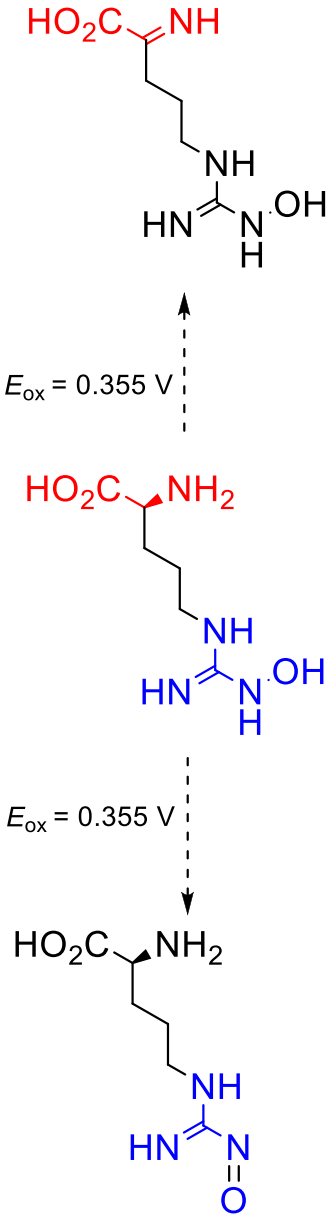


Figure 3. (a) Cyclic voltammatograms of *N*-hydroxyguanidine from 0.5 to 142 μM revealed an oxidation wave at +355 mV vs Ag/AgCl and (b) a calibration curve of the oxidation current vs concentration of *N*-hydroxyguanidine.



Scheme 2. Two possible electron oxidation products of NOHA.

NOHA. This indicates that only the hydroxyguanidine unit of NOHA is undergoing a quasi-reversible oxidation.

Previous work proposed a mechanism for the oxidation of hydroxyguanidine derivatives,[27] they observed two separate and irreversible oxidation events in organic solvent and at higher potential. We observed a one-step, one electron oxidation event in a buffered aqueous medium. The proposed one-step, one electron transfer is due to the quasi-reversible nature of hydroxyguanidine at physiological pH. We propose the oxidized species is susceptible to hydrolysis, which leads to the quasi-reversible reduction event (Scheme 3).

Rotating disk electrode experiments and diffusivity calculations.—For this work to be translatable in a clinical setting, detection of NOHA in biological fluids will be needed. Biological fluids are often a mixture of proteins, cells, and small molecules.[33] Filtration of the biological fluids might be needed to obtain a clear detectible signal of NOHA on a bare glassy carbon electrode surface. Membrane filtration is one technique that could be used to separate small molecules from larger proteins and cells.[33] Design of an

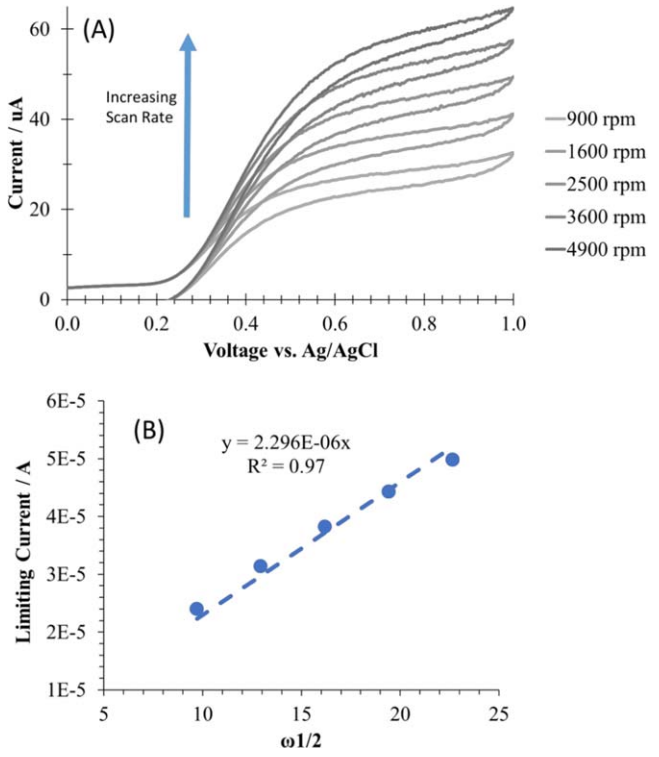
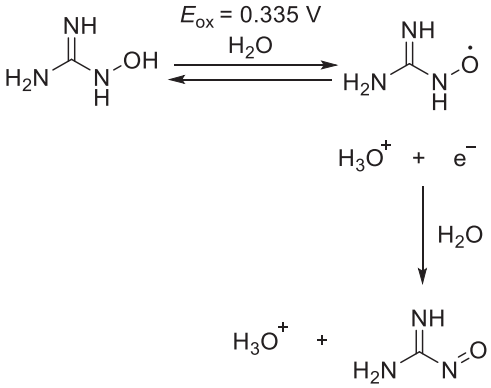


Figure 4. (a) Rotating disk electrode experiments were done from 900–4900 rpm at a constant concentration of 160 μM of NOHA in 7.3 pH PBS. (b) Calibration curve for RDE experiments from 900–4900 rpm comparing the limiting current with the square root of the angular velocity shows a linear relationship.

appropriate membrane or filtration system would require knowing the diffusion of NOHA. To the best of our knowledge, the diffusivity of NOHA has not been calculated in a PBS solution or other aqueous medium. We used a rotating disc electrode with the Levich equation (Eq. 1) to determine the diffusivity of NOHA.

Rotating disk electrode (RDE) experiments were done from 900–4900 rpm at a constant concentration of 160 μM in order to calculate the diffusivity of NOHA in PBS (Fig. 4a). Rotating disk electrode experiments were used because forced convection allows for the assumption of steady state mass transport to the surface of the electrode. As expected, an increase in the limiting current was observed with increasing spin speed. The RDE experiments done show a linear relationship between the limiting current and the square root of the angular velocity with a slope of 2.296×10^{-6} $As^{1/2}$ ($R^2 = 0.97$) (Fig. 4b).



Scheme 3. Proposed electrochemical mechanism. Oxidation of alanine did not occur but oxidation of N-hydroxyguanidine exhibited an oxidation event at 355 mV, similar to NOHA.

Using the Levich equation (Eq. 1) the current, i, is related to the number of electrons transferred, n, Faraday's constant, F, the area of the surface of the electrode, A, the diffusivity, D, angular velocity, ω, and the kinematic viscosity, ν.

$$i = 0.62nFAC^*D^{2/3}\omega^{1/2}\nu^{-1/6} \quad [1]$$

The validity of the Levich equation indicates the hydrodynamic boundary layer cannot be large and the angular velocity cannot result in turbulent flow. Within our experimental settings of 900–4900 rpm, both the low and high angular velocity conditions are within the acceptable range. Therefore, Levich equation can be rearranged to calculate the diffusivity, if the slope of the limiting current vs the square root of the angular velocity is known and linear (Eq. 2).

$$D = \left(\frac{Slope}{0.62nFAC^*\nu^{-1/6}}\right)^{3/2} \quad [2]$$

From Fig. 4b, the slope of the limiting current and the square root of the angular velocity was found to be 2.296×10^{-6} $As^{1/2}$. Therefore, resulting diffusivity for a single electron transfer is 5.50×10^{-5} $cm^2\ s^{-1}$ (Eq. 2). The calculated diffusivity for NOHA is similar to the diffusivity of urea in water.[34]

Detection of NOHA in the presence of urea cycle amino acids.—Detection of NOHA in the presence of other substances is necessary for producing a robust biosensor in the future. Using a glassy carbon electrode, cyclic voltammetry experiments were performed for a serial dilution of NOHA from 0.5 to 148 μM in a solution of 100 μM L-arginine, 10 μM L-citrulline, and 100 μM L-ornithine to mimic the primary amino acids in the urea cycle (Fig. 5a). High concentrations of L-arginine, L-citrulline, and L-ornithine were selected to confirm that NOHA was still detectible

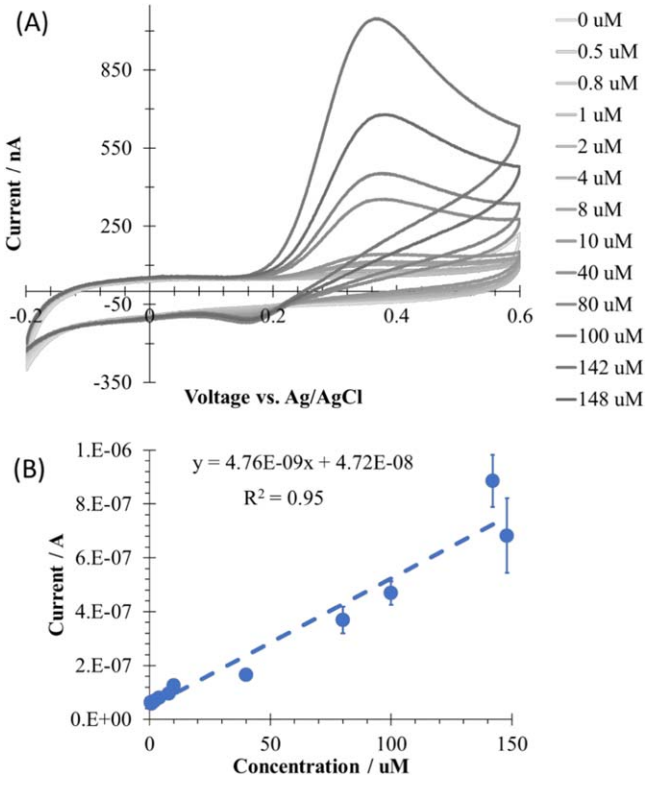


Figure 5. (a) Cyclic voltammatograms of NOHA in an amino acid solution from 0 to 148 μM revealed an oxidation peak at +360 mV vs Ag/AgCl. (b) A calibration curve of the oxidation current versus concentration of NOHA shows a linear relationship. The specificity of the oxidation of NOHA is 4.76 nA/uM in the presence of an amino acid solution.

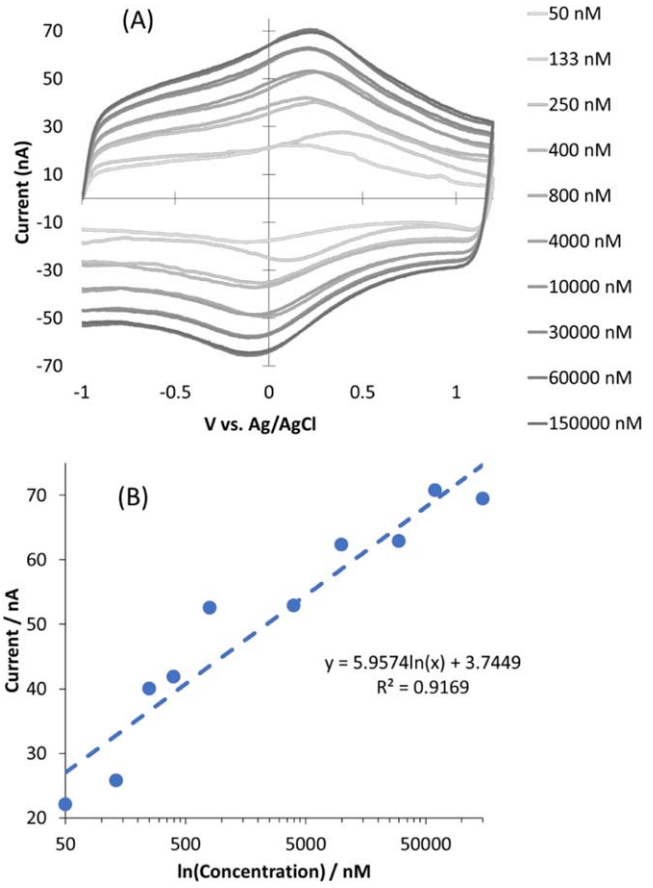


Figure 6. (a) Fast scan cyclic voltammatograms of NOHA in an amino acid solution from 50 nM to 150 μM revealed an oxidation peak at +250 mV vs Ag/AgCl. (b) A calibration curve of the oxidation current versus concentration of NOHA shows a logarithmic relationship.

even in a mixture with minimal loss of sensitivity. To confirm L-arginine, L-citrulline, and L-ornithine do not contribute to the signal observed with NOHA, we did initial scans with amino acids and no NOHA added. We did not observe any oxidation or reduction peaks (Fig. 5a). These findings are corroborated by others for L-arginine, L-citrulline, and L-ornithine as electrode modifications are often needed to get a signal.[32,35]

The oxidation peak of NOHA was found to have shifted by +5 mV to +360 mV vs Ag/AgCl compared to when NOHA was in a PBS solution. Data suggests that NOHA has a linear relationship between concentration and peak current even in the presence of other substances that could foul the electrode (Fig. 5b). The sensitivity of the oxidation peak of NOHA in the presence of L-arginine, L-citrulline, and L-ornithine is 4.76 nA μM^{-1}. This is promising for future work in detecting NOHA to monitor the consumption of L-arginine in the urea cycle.

Electrochemical detection of NOHA using fast scan cyclic voltammetry.—Since many of the chemicals in the urea cycle are not electrochemically active, we investigated fast scan cyclic voltammetry (FSCV) at a carbon fiber microelectrode as a way of increasing the limit of detection. The oxidation peak of NOHA with FSCV was observed at +250 mV vs Ag/Ag/Cl (Fig. 6a). Compared to CV experiments (Fig. 1), the shift of the oxidation peak was in a negative potential direction, counterintuitive to standard practice. Future work will be dedicated to understanding the directional shift in the oxidation peak. We observed a decrease in the limit of detection to 50 nM (Fig. 6a), indicating that NOHA can be detected at physiologically relevant concentrations. Fast scan cyclic voltammetry is typically used to measure swift changes in concentration. Swift changes of NOHA are not expected, and therefore, other methods like chronoamperometry or differential pulse amperometry could also be investigated to help lower the limit of detection of NOHA. In addition, a logarithmic calibration curve for NOHA can be fit over the physiologically relevant range (50 nM to 150 μM) (Fig. 6b).

Conclusions

We have demonstrated that NOHA is electrochemically detectable on a glassy carbon electrode under buffered aqueous conditions. NOHA's oxidation peak was found to be at 355 mV with a sensitivity of 5.39 nA/uM. Additionally, CV experiments with structural analogs revealed that oxidation is only occurring at the hydroxyguanidine unit of NOHA. Preliminary results show that oxidation peak of NOHA shifts by 5 mV to 360 mV when in an amino acid solution that could foul the signal. Further preliminary results indicate that NOHA can be calibrated over the physiological relevant concentration using FSCV. NOHA has been found in both biofluids and tissue samples. In this work we demonstrate detection capabilities for both of these sample types. This is promising for future work in detecting NOHA in physiological samples to monitor the urea cycle.

Acknowledgments

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