Published in final edited form as:

Analyst. 2016 March 07; 141(5): 1796–1806. doi:10.1039/c5an02587g.

Electrochemical Detection of Peroxynitrite using Hemin-PEDOT Functionalized Boron-Doped Diamond Microelectrode

Serban F. Peteu^{a,b}, Brandon Whitman^a, James J. Galligan^c, and Greg M. Swain^a

^aDepartment of Chemical Engineering and Materials Science, 428 S. Shaw Lane, Michigan State University, East Lansing, Michigan 48824-1226, United States

^bDepartment of Chemistry and the Neuroscience Program, 578 S. Shaw Lane, Michigan State University, East Lansing, Michigan 48824-1322, United States

^cDepartment of Pharmacology and Toxicology, and the Neuroscience Program, B440 Life Sciences Building, Michigan State University, East Lansing, MI 48824-1317, United States

Abstract

Peroxynitrite is a potent nitroxidation agent and highly reactive metabolite, clinically correlated with a rich pathophysiology. Its sensitive and selective detection is challenging due to its high reactivity and short sub-second lifetime. Boron-doped diamond (BDD) microelectrodes have attracted interest because of their outstanding electroanalytical properties that include a wide working potential window and enhanced signal-to-noise ratio. Herein, we report on the modification of a BDD microelectrode with an electro-polymerized film of hemin and polyethylenedioxythiophene (PEDOT) for the purpose of selectively quantifying peroxynitrite. The nanostructured modified polymer layer was characterized by Raman spectroscopy and scanning electron microscopy (SEM). The electrochemical response to peroxynitrite was studied by voltammetry and time-based amperometry. The measured detection limit was 10 ± 0.5 nM ($S/N=3$), the sensitivity was 4.5 ± 0.5 nA/nM and the response time was 3.5 ± 1 s. The hemin-PEDOT BDD sensors exhibited a response variability of 5% or less (RSD). The stability of the sensors after a 20-day storage in 0.1 M PB (pH 7.4) at 4 °C was excellent as at least 93% of the initial response to 50 nM PON was maintained. The presence of PEDOT was correlated with a sensitivity increase.

1. Introduction

Peroxynitrite (PON) is a reactive nitrogen species from a family of compounds that also includes nitric oxide (NO) and the nitrogen dioxide radical (NO₂). PON is an anion with the chemical formula ONOO⁻ and is generated in the reaction between nitric oxide and superoxide. It is a strong oxidant that can damage subcellular organelles, membranes and enzymes through its actions on proteins (nitration of tyrosine residues of proteins), lipids, and DNA. It is a highly reactive metabolite, a potent oxidative and nitrosative agent *in vivo* and it is known clinically to exert a variety of deleterious and cytotoxic effects in cells and tissues [1–3]. PON detection methods have been recently reviewed [5–8]. Quantification of the analyte is complicated by a variety of intrinsic obstacles, including a short sub-second lifetime, inherent difficulties to reproduce its true *in vivo* kinetics in model experiments [4,5,8] and the complexities of a real environment. PON typically reacts with more than one

target per unit time due to its high reactivity [8-11]. Indeed, at physiological pH, PON undergoes two main degradation routes: protonation into its conjugated acid ONOOH ($pK_a = 6.8$) followed by the formation of the very reactive radicals, $NO_2^\bullet$ and $OH^\bullet$ or follow-up reactions with CO_2 , thiols, metals, etc. [10].

The most widely used methods for PON detection are fluorescence-based techniques [12–16]. Studies of the biochemical roles of the PON precursors, nitric oxide and superoxide anion, have been performed using electrochemical detection methods for real-time, label-free and direct measurement of these reactive species [17–19]. There are some reports of the direct detection of PON by electroanalytical techniques reported in the literature [14–28]. Amatore and co-workers studied the electrochemical oxidation of PON by steady-state and transient voltammetry with platinized carbon microelectrodes [15–18]. Xue et al. used manganese phthalocyanine-modified ultramicroelectrodes for the sensitive and selective detection of peroxynitrite anion, released from cultured neonatal myocardial cells induced by ischemia-reperfusion [20]. Chemically-modified platinum microelectrodes coated with manganese tetraaminophthalocyanine films were utilized for PON detection in alkaline solution (pH 10.2) where it is more stable [21]. More recently, it was shown that improved catalytic PON activity could be achieved by depositing nanostructured metalloporphyrin or metallophthalocyanine films onto glassy carbon or carbon fiber electrodes [25–28].

Boron-doped diamond (BDD) electrodes generally show substantial improvements in linear dynamic range, detection limit, response stability and reproducibility as compared to the conventional sp^2 carbon electrodes, like glassy carbon [29–33]. BDD also exhibits a lower background current and a wider potential window in aqueous solutions than sp^2 carbon electrodes, which allows for the detection of electroactive species with less interference from water decomposition [30–33]. Organic substances that have been determined so far with diamond electrodes include adenosine [33,35], catecholamines [36], glucose [37], nucleic acids [38] and the list is constantly growing. Inorganics detected with BDD include nitrate [39], nitrite [36], as well as metal ions such as Cd^{+2} and Pb^{+2} [40], Ag^+ [41] and Mn^{2+} [42]. The modification of diamond electrodes for more sensitive detection of analytes was performed, for example, by the electrodeposition of polymer composite films for monitoring dopamine [43] and glucose [44].

Motivated by properties of BDD and by the catalytic properties of hemin-modified interfaces, we prepared hemin-PEDOT-BDD microelectrodes and used them to detect PON. We report on the electrochemical detection figures of merit for PON generated from the donor, 3-morpholino-sydnnonimine (SIN-1) [45,46]. The objectives of this work were to investigate the electrocatalytic performance of a thin film of hemin-PEDOT electropolymerized on a BDD microelectrode for the sensitive and selective quantification of PON. We sought to determine the performance of these microsensors in terms of the response time, detection limit, sensitivity, reproducibility and stability, and how these detection figures of merit correlate with the ratio of PEDOT to hemin used in the sensor. Our long-term goal is to use this amperometric sensor *in vitro* to study inhibitory neuromuscular transmission in the gastrointestinal tract in a diet-induced obesity animal model where inflammation is present.

2. Experimental

2.1. Materials

The iron protoporphyrin IX (hemin), ethylenedioxythiophene, tetrabutylammonium tetrafluoroborate and dichloromethane were purchased from Sigma Aldrich (St Louis, MO). The peroxyxynitrite donor 3-morpholinodnnonimine (SIN-1), was obtained from Cayman Chemical (Ann Arbor, MI). The ultrapure water used for the solution preparations was from a Barnstead ultrapure water system Model D3750 with a resistivity of 18 M Ω -cm. All other chemicals were reagent grade quality and used as received.

2.2. Microelectrode preparation

The preparation of diamond microelectrodes has been reported in detail elsewhere [29-32]. Briefly, a boron-doped diamond thin film was deposited on a sharpened 76 μ m diameter Pt wire (both ends) using microwave-assisted chemical vapor deposition (1.5 kW, 2.54 GHz, ASTeX, Woburn, MA). The wire was prepared for growth by (i) ultrasonic cleaning in acetone for 20 min and (ii) ultrasonic seeding from a mixture of detonation diamond (3-6 nm with 30 nm aggregates as per the supplier) and DMSO (0.5 w/v%, International Research Center, Raleigh, NC) for 30 min. The wires were then rinsed 3x with ultrapure water, air dried and placed in the deposition reactor for pump down to a base pressure of 15 mtorr. Three wires were coated during a deposition run with each wire producing two microelectrodes. The wires were mounted horizontally on a quartz plate with the ends extending past the quartz. This placement allowed for uniform temperature across the wire and access of reactive gas phase species to all regions of the exposed wire. The deposition was performed using a 1% CH₄/H₂ source gas mixture containing 10 ppm diborane (B₂H₆) diluted in H₂ for doping. The growth pressure was 35 torr and the microwave power was 650 W. The deposition time was 6-9 h producing a final film thickness in the 3-5 μ m range [29-32]. After growth, the substrates were cooled in the presence of atomic hydrogen to an estimated temperature of < 400 °C. This was done by stopping the CH₄ and B₂H₆ gas flows with the plasma (H₂) still ignited. The power and pressure were slowly reduced over a 30 min period to 150 W and 10 torr to cool the specimens. Post-growth cool-down in atomic hydrogen is essential for removing any adventitious sp² carbon impurity, eliminating any surface reconstruction and for maintaining a stable H surface termination.

The design of the insulated microelectrode is schematically shown in Figure 1. After growth, the diamond-coated Pt wire was cut in half forming two microelectrodes. The cut end of the wire was then affixed to a Cu wire current collector using Ag epoxy for conductivity and super glue for mechanical strength. The diamond microelectrode was then insulated by carefully melting the end of a polypropylene pipette tip in a micropipette puller. This causes the polymer to flow over the surface of the diamond-coated Pt wire forming a tight seal. The process produces a conically-shaped microelectrode. The exposed length of the diamond-coated wire is not easily controlled by this process but is generally in the 500-800 μ m range.

2.3. Hemin-PEDOT film formation

Two types of hemin-PEDOT films were prepared. The film labeled A was electropolymerized from a monomer solution of 1.5 mM hemin and 4.5 mM

ethylenedioxythiophene (EDOT) in 0.1 M tetrabutyl-ammonium tetrafluoroborate with dichloromethane as the solvent (so-called type A). The film labeled B was prepared with 1.5 mM hemin and 13.5 mM EDOT (3× higher concentration) in 0.1 M tetrabutyl-ammonium tetrafluoroborate. Dichloromethane was again the solvent. Forty potential sweeps from −1.5 to +1.5 V were applied in deoxygenated solution to deposit the polymer. Deoxygenation was accomplished with a 20-min N₂ purge. The solution was blanketed with the gas during the polymer film formation. There was a progressive increase in the redox currents during each voltammetric cycle, indicative of the growth of a hemin-PEDOT film on the immersed microelectrode surface. After each modification step, the microelectrodes were thoroughly rinsed with ultrapure water and then dried under a stream of N₂. To enhance selectivity, the hemin-PEDOT-modified microelectrode was covered with a polyethyleneimine (PEI) membrane by dip coating three times in a 1.5% PEI aqueous solution (Sigma-Aldrich). PEI is a polymeric amine with high charge density that screens against cation permeation and also prevents fouling [24].

2.4. Generation of peroxynitrite anion

Synthetic PON stock solutions need a higher pH (such as pH 9.5) where the analyte is more stable and can be detected. By contrast at physiological pH, PON is prone to fast decomposition. This is why the 3-morpholino-sydnimine (SIN-1, stored at −20 °C) was used for its generation at pH 7.4. A stock solution was formed by mixing SIN-1 with 0.1 M phosphate buffered saline solution (PBS) of pH 7.4 at room temperature. Then, as the solution equilibrates with the air, SIN-1 liberates superoxide anion and nitric oxide spontaneously in solution. PON is continuously generated in this manner for 25-30 minutes at room temperature, until its concentration reaches a maximum, as evidenced by UV-Vis absorbance [8,24,25]. This steady-state PON concentration lasts for about 30 minutes. It was during this period that all the measurements reported herein were made. Figure 2 shows that in an aerobic aqueous solution, SIN-1 decomposes readily to SIN-1A, which in the presence of an oxidant, like oxygen, forms the unstable SIN-1A radical cation. The latter liberates NO and eventually forms the stable end-product, 3-morpholino-iminoacetonitrile (SIN-1C), as shown in the figure. The literature indicates a ratio of 1/100 between the concentration of PON generated and the concentration of SIN-1 in solution in the 30-min steady state region [45,46] as measured with electron (paramagnetic) spin resonance spectroscopy [47, 48] and UV-Vis spectrophotometry [8,24,25]. The lifetime of SIN-1 freshly made solution is typically about 120 minutes [24,27]. For the continuous amperometric measurements, a stock solution of 250 mM SIN-1 in PBS, pH 7.4, was prepared and stored in a leak-tight sealed vial. For the cyclic voltammetric measurements, a stock solution of 1500 mM SIN-1 in 0.1 M phosphate buffer (PB), pH 7.4, was prepared and stored. The PON concentration in solution, generated from SIN-1, has been measured using a UV/Vis spectrophotometric assay at $\lambda = 302$ nm ($\epsilon = 1705 \text{ mol}^{-1} \text{ cm}^{-1}$) [5,8, 24, 25]. The PON solutions used herein were prepared by adding a known aliquot of the SIN-1 stock solution to an oxygenated (or air-equilibrated) PB solution and assuming a 1/100 ratio of PON to SIN-1. SIN-1 solutions were typically kept on ice to minimize any spontaneous degradation.

2.5. Instrumentation

Raman—The Raman spectra were collected using inVia Raman Microprobe (Renishaw) equipped with a diode-pumped solid-state laser DPSS (300 mW max. output power, 532 nm line). Spectra were acquired using a 1- μ m spot size with an integration time of 10 s. The spectrometer was calibrated (wavelength position) with internal silicon standard.

Scanning Electron Microscopy—Images of the film morphology were obtained using a JSM-6610LV (field emission) scanning electron microscope (JEOL Ltd., Tokyo, Japan) housed at MSU's Center for Advanced Microscopy. The images were constructed from both secondary and back-scattered electrons using an accelerating voltage of 12 kV. The microscope was equipped with energy dispersive x-ray microanalysis (EDS).

Electrochemistry—Cyclic voltammetry and continuous amperometry were performed in a 10 mL single-compartment glass cell. The three-electrode system consisted of the Hemin-PEDOT-BDD working electrode, an Ag/AgCl (3 M KCl) reference electrode, and a platinum wire counter electrode. The electrodes were connected to CH Instruments 832A (Austin, TX) electrochemical workstation. The cell was housed in an electrically grounded Faraday cage in order to reduce the electrical noise. All measurements were performed at room temperature 23 ± 1 °C unless otherwise specified.

3. Results and Discussion

3.1 Hemin-PEDOT film formation and characterization

The BDD microelectrode was immersed in the hemin-EDOT monomer solution in the presence of organic solvent with supporting electrolyte. It was then cycled 20 times between -1.5 and $+1.5$ V at 50 mV s^{-1} . vs. Ag/AgCl under a N_2 gas blanket. The hemin-PEDOT film is formed by electropolymerization with the hemin molecules incorporated into the PEDOT network as shown in Figure 3A [25,26,49]. The oxidized conductive polymer turns back into a neutral (undoped) semiconductive form upon reduction. A continuous increase in the anodic and cathodic peak currents is seen with cycle number as shown in Figure 3B. The progressive increase in current is reflective of growth of an electroactive PEDOT layer [25,50,51]. In other words, the polymer layer increases in thickness with cycle number. The electropolymerization basically involves an electrogenerated cation radical on the anodic sweep as the reactive species. Polymer formation then proceeds through a series of radical coupling reactions and electrochemical reoxidations [50]. Radical formation and chain growth cause the anodic charge at potentials positive of 0.1 V, and the corresponding cathodic charge to increase with cycle number.

3.3. SEM Images

SEM was used to study the conducting polymer film morphology. Characteristic images are shown in Figure 4. The lower magnification image (left) shows that the surface of the hemin-PEDOT polymerized layer is fractal and rough with peaks and valleys at the microscale. The higher magnification image (right) reveals the typical nanostructured 'cauliflower' morphology that is characteristic of a 3D branched-multiglobular film. Individual nodules are present with a diameter of 100-300 nm [25, 52].

3.4 Raman Spectra

Raman spectroscopy was carried out to probe the microstructure of a hemin-PEDOT layer (A) and a hemin-only layer (B), both formed on a BDD microelectrode. Spectrum A in Figure 5 exhibits characteristic bands of PEDOT. The spectrum of the polymer is dominated by an intense band at 1431 cm^{-1} characteristic of the $C_{\alpha} = C_{\beta}$ band of the thiophene ring [53]. The spectrum also consists of peaks at 1268 , 1367 , 1513 and 1557 cm^{-1} , which are assigned to the stretching mode of the ethylenedioxy group, the C-C stretching mode in the thiophene ring, the $C_{\alpha} = C_{\beta}$ stretching and the quinoid structure, respectively [53,54]. For hemin, spectrum B contains an intense peak at 1625 cm^{-1} reflective of the porphyrin core vibrations and C=C stretching. The 753 cm^{-1} peak is typical of hemin, as is the C-C asymmetric stretch visible at 1554 cm^{-1} [55]. Spectrum C for BDD is reflective of a heavily boron-doped film (Mott transition to metallic behavior) with a threshold carrier concentration of at least $3 \times 10^{20}\text{ cm}^{-3}$ [56,57]. When the boron concentration induces metallic conductivity within the boron dopant band, an increasing deformation appears in the spectrum for the zone-center optical phonon around 1332 cm^{-1} , and simultaneously a continuum with two new bands emerges around 1220 and 500 cm^{-1} [56-59]. The onset of metallic conductivity in BDD can be followed by the change of the zone-center optical phonon (ZCP) (i.e., the diamond Raman line) [55,59]. This normally occurs at 1332 cm^{-1} but is red-shifted to $1300\text{-}1320\text{ cm}^{-1}$ and acquires an asymmetric Fano lineshape due to the presence of free charge carriers [60-62]. The asymmetry is caused by a quantum mechanical interference between the ZCP and the continuum of electronic states induced by the presence of the dopant [60-62]. The shift to lower wavenumber of the ZCP is due in part to a greater fraction of C-B bonds due to the heavy doping rather than C-C bonds of diamond. This lowers the effective reduced mass, hence the lower wavenumber band. The shift of the ZCP is accompanied by broad lower wavenumber scattering with maxima at 1220 and 500 cm^{-1} [56-59]. There is still some uncertainty as to the assignment of these two peaks. The peak at 500 cm^{-1} has been assigned to boron dimers and to clustered boron atoms due to the heavy doping level [63-64]. The origin of the peak at 1220 cm^{-1} is still the subject of research. It has been assigned to the presence of defects arising from the heaving boron doping. The 1536 cm^{-1} peak is consistent with the presence of some more amorphous sp^2 -bonded carbon resulting from the high boron doping level [56,59,65,66].

3.5 Electrochemical performance

Voltammetry—Cyclic voltammetry was used to assess the redox behaviour of the hemin-PEDOT-BDD microelectrode. Figure 6 shows cyclic voltammograms recorded with different concentrations of PON from 50 to 400 nM. An oxidation peak at $+1.35\text{ V vs. Ag/AgCl}$ is seen that increases proportionally with the concentration. PON oxidation occurs concomitantly with the oxidation of Fe^{+3} to Fe^{+4} in the hemin. The peak on the reverse sweep at $+1.15\text{ V}$ is due to the reduction of Fe^{+4} back to Fe^{+3} . Previous studies [25] examined the ratio I/I_0 ; that is the peak current of the voltammetric wave of the modified electrode in the presence of PON (I) relative to the current measured in absence of the analyte at same potential (I_0). The I/I_0 depends on the scan rate and gradually decreases as the scan rate increased for all concentrations studied [25,26]. This behavior is typical of an electrocatalytic process [67] where the oxidation of PON is mediated by the hemin

polymeric film. The catalytic process as described above was not observed with films of protoporphyrin-only (lacking the iron) suggesting the critical role played by the bound iron atom in the hemin-based films. The oxidation and reduction peaks for the hemin-only electrode are shown in Figure 7A. The overall catalytic reaction mechanism for peroxynitrite oxidation is shown in Figure 7B. The observed peaks are assigned to the redox couples $\text{Fe}^{+3}/\text{Fe}^{+2}$ (~ 0.1 V) and $\text{Fe}^{+4}/\text{Fe}^{+3}$ (~ 1.25 V). The iron center is necessary for the oxidative catalytic turnover of PON [25,26,67-69]. Radi et al. [70] and Groves et al. [71,72] reported fast reactions between manganese-based porphyrins with PON, and implicitly invoked a mechanism driven by direct interaction of PON with the metallic center. Therefore, the presence of the iron metal center is critical for mediating the inner electron transfer from the PON substrate to the oxidizing porphyrin ring, which acts as an “antenna” for oxidizing equivalents from the electrode [70,71].

Amperometry—The detection of PON was also accomplished using continuous amperometry with the hemin-PEDOT BDD microelectrode poised at +1.35 V vs. Ag/AgCl. The current was recorded in response to varying aliquots (5, 10, 50, 100 μL) of the SIN-1 stock solution added to the electrochemical cell containing a magnetically-stirred PB solution at pH 7.4. As seen in Figure 8A, the limiting current scales proportionally with an increase in the PON concentration. Recall the PON concentration was estimated from the SIN-1 solution concentration and the 1/100 ratio of PON to SIN-1 generated under steady-state conditions [5,8,24,25,45,46]. The arrows indicate the point where the aliquot was added. The lowest detectable concentration in Figure 8A is 10 ± 0.5 nM ($S/N=3$) with a 3.5 ± 1 s response time, which is defined as the time to reach 90% of the maximum current. The response variability was 5% RSD based on at least three measurements at each concentration. The concentration limit of detection (LOD) was calculated from the minimum detectable signal (y_{LOD}), mean (y_{blank}) and standard deviation (s_{blank}) of replicate blank readings and the sensitivity (m) according to the following two equations:

$$y_{\text{LOD}} = y_{\text{blank}} + 3s_{\text{blank}} \quad [1]$$

$$C_{\text{LOD}} = \frac{y_{\text{LOD}} - y_{\text{blank}}}{m} = \frac{3s_{\text{blank}}}{m} \quad [2]$$

The slope of the regression line (sensitivity) from Figure 8C is 2.4 nA/nM. It should be noted that this is on the low end of the range of sensitivities found for different sensors (4.5 ± 0.5 nA/nM).

Using equations 1 and 2, a theoretical LOD (C_{LOD} , $S/N=3$) of 3 nM was calculated. The actual LOD determined experimentally was 10 ± 0.5 nM ($S/N=3$). This value compares favorably with other PON-sensitive electrochemical sensors reported on in the literature. Summary of the reported detection limits is presented in Table 1.

3.6 Sensitivity and the effect of PEDOT

The sensitivity of differently modified BDD microelectrodes to PON was compared. Figure 9 shows response curves plotted for the unmodified, hemin-only, PEDOT-only, hemin-PEDOT type A and hemin-PEDOT type B BDD microelectrodes. Recall that the type B electrode consisted of a PEDOT layer formed from 3× higher concentration of the EDOT monomer in solution as compared to type A electrode. The sensitivities were as follows: 0.05-0.06 nA/nM for the unmodified, 0.8-0.9 nA/nM for hemin-only; 0.7-0.8 nA/nM PEDOT type A-only; 1.9-2.1 nA/nM for hemin-PEDOT type A; and 5.0-5.5 nA/nM for hemin-PEDOT type B BDD microelectrodes. Clearly, the increased loading of PEDOT leads to increased sensor sensitivity. This is likely because of a greater number of hemin molecules in the thicker PEDOT layer available for coordination with PON. Polythiophene is attractive for modified electrodes because of the rich functionalization afforded by its monomer ring. This polymer also offers good electrical conductivity and high stability [72-75]. The 3,4-ethylenedioxythiophene or EDOT (Fig. 2) has been especially preferred for several reasons. The two oxygen atoms coupled to the thiophene rings permits this monomer to be oxidized at lower potentials. PEDOT offers high electrical conductivity and a narrow bandgap; being easily oxidized over a wide anodic potential window [52, 72-75]. PEDOT is a highly conductive polymer that mediates a faster electron transfer within the catalytic film. Together, the hemin and PEDOT provide a catalytic and electrically conducting layer for PON oxidation [25,26].

If this is the case and PEDOT is a determining factor in the enhanced signal, then an increase in the PEDOT loading in the film should result in a higher sensitivity. Indeed, a 3-fold increase in the EDOT monomer content used for the film formation (i.e., greater PEDOT loading) produced a 2.8 fold increase in the sensitivity to PON as indicated in Figure 9 (hemin-PEDOT film B compared with hemin-PEDOT film A).

3.7 Sensor selectivity, reproducibility and stability

To improve the response selectivity, the hemin-PEDOT film was covered with a polyethyleneimine (PEI) layer. PEI is a polymeric amine with high charge density that screens against cations [24]. The selectivity of the hemin-PEDOT-PEI BDD microelectrode for PON was evaluated in the presence of several potential interfering electroactive species, namely norepinephrine, serotonin and uric acid. All of three compounds would undergo diffusion limited oxidation at BDD in PBS solution at the PON detection potential of 1.35 V vs. Ag/AgCl. These tests were performed using a 140-fold higher concentration of the interfering analyte as compared to PON. The results are shown in Figure 10. These data indicate the response of each interferent constitutes only 6-7% when compared with the PON response, which is considered to be 100%. As expected, the PEI layer aids in the rejection of the cationic norepinephrine and serotonin (pH 7.4). These are interferents that would be encountered in in vitro studies in the gut wall – our long-term goal for this sensor. Surprisingly, there is also good rejection of the urate ion. Perhaps the anion accessibility to the underlying diamond surface, where it would be expected to undergo oxidation at the PON detection potential, is blocked by the hemin and PEDOT. Future work will involve more detailed studies of sensor selectivity to different potential anionic interferents. The reproducibility of the hemin-PEDOT-PEI BDD microelectrode was assessed using 50 nM

PON. Five microelectrode sensors were prepared and the continuous amperometric response to PON was measured. A relative standard deviation (RSD) of 5.8% was determined indicating good sensor reproducibility. To assess the longer-term response stability, five microelectrode sensors were stored in 0.1 M PB (pH 7.4) refrigerated at 4 °C. The sensors were stored in glass vials with the tops wrapped using parafilm. After 20 days, the microelectrode sensors were removed and used in continuous amperometry to measure 50 nM PON mixed with 0.1 M PB (pH 7.4). All five sensors retained greater than 93% of their initial responses to 50 nM PON.

4. Conclusions

We report for the first time on the functionalizing a boron-doped diamond (BDD) microelectrode with a hemin-PEDOT electropolymerized film to quantify peroxynitrite. The morphology of the hemin-PEDOT film was characterized with SEM and Raman spectroscopy. The nominal response time for a 10 nM injection of PON was 3.5 ± 1 s ($n = 3$). By comparison, the response time for a carbon fiber microelectrode modified with the same hemin-PEDOT layer was 8 ± 1 s ($n = 3$). The noise for the BDD microelectrode sensor was generally around 5 nA as compared to 50 nA for the hemin-PEDOT carbon fiber microelectrodes of similar geometric area. The detection limit for the hemin-PEDOT BDD microelectrode was 10 ± 0.5 nM ($n = 3$) and the sensitivity was 4.5 ± 0.5 nA/nM ($n = 3$). The hemin-PEDOT BDD sensors exhibited a response variability of 5% or less (RSD). The stability of the sensors after a 20-day storage in 0.1 M PB (pH 7.4) at 4 °C was excellent as at least 93% of the initial response to 50 nM PON was maintained. In conclusion, BDD microelectrodes function as a suitable platform for the hemin-PEDOT layer. BDD microelectrodes exhibited a faster response time and were 10× less noisy than similarly modified carbon fiber microelectrodes. The hemin-PEDOT polymer layer functions to catalyze the oxidation of peroxynitrite exhibiting low detection limits, good response sensitivity and excellent response reproducibility and stability. Increasing the loading of PEDOT increases the response sensitivity of the sensor. Work is now underway to use the hemin-PEDOT-PEI BDD sensors in *in vitro* tissue preparations (ileum and colon) in order to better understand the link between inflammation and obesity on gut function.

Acknowledgements

The authors gratefully acknowledge financial support from the National Institutes of Health through grant R01 DK094932.

References

1. Beckman JS, Beckman TW, Chen J, Marshall PA, Freedman BA. Proc. Nat. Acad. Sci. 1990; 4(87): 1620. [PubMed: 2154753]
2. Radi R, Beckman JS, Bush KM, Freeman BA. J. Biol. Chem. 1991; 7:4244. [PubMed: 1847917]
3. Radi R, Beckman JS, Bush KM, Freeman BA. Arch. Biochem. Biophys. 1991; 2(288):481. [PubMed: 1654835]
4. Beckman JS, Koppenol WH. Am. J. Physiol. 1996; 5(271):C1424. [PubMed: 8944624]
5. Pacher P, Beckman JS, Liaudet L. Physiol. Rev. 2007; 1(87):315. [PubMed: 17237348]
6. Ferrer-Sueta G, Radi R. ACS Chem. Biol. 2009; 4:161. [PubMed: 19267456]
7. Liu PT, Hock CE, Nagele R, Wong PYK. Am. J. Physiol: Heart Circ. Physiol. 1997; 272:H2327.

8. Peteu SF, Boukherroub R, Szunerits S. Biosensors Bioelectronics. 2014; 59:359. [PubMed: 24681525]
9. Szabo C, Ischiropoulos H, Radi R. Nat. Rev. Drug Discovery. 2007; 6:662. [PubMed: 17667957]
10. Keller A, Mohamed A, Droese S, Brandt U, Fleming I, Brandesca RP. Free Radic. Biol. Med. 2004; 38:1257.
11. Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM. Free Radic. Biol. Med. 1999; 27:146. [PubMed: 10443931]
12. Lin KK, Wu SC, Hsu KM, Hung CH, Liaw WF, Wang YM. Org. Lett. 2013; 15:4242. [PubMed: 23924291]
13. Tian J, Chen H, Zhuo L, Xie Y, Li N, Tang B. Chem. – Eur. J. 2011; 17:6626. [PubMed: 21590826]
14. Zhang Q, Zhu Z, Zheng Y, Cheng J, Zhang N, Long YT, Zhend J, Qian X, Yang Y. J. Am. Chem. Soc. 2012; 134:18479. [PubMed: 22978649]
15. Amatore C, Arbault S, Guille M, Lemaitre F. Chem. Rev. 2008; 108:2585. [PubMed: 18620370]
16. Bedioui F, Quninton D, Griveau S, Nyokong T. Phys. Chem. Chem. Phys. 2010; 12:9976. [PubMed: 20680199]
17. Amatore C, Arbault S, Bruce D, De Oliveira P, Erard M, Vuillaume M. Chem.–Eur. J. 2001; 7:4171. [PubMed: 11686596]
18. Amatore C, Arbault S, Bouton C, Coffi K, Drapier JC, Ghandour H, Tong Y. ChemBio Chem. 2006; 7:653.
19. Amatore C, Arbault S, Bruce D, De Oliveira P, Erard M, Vuillaume M. Faraday Discuss. 2000; 116:319. [PubMed: 11197488]
20. Xue J, Ying X, Chen J, Xian Y, Jin L, Jin J. Anal. Chem. 2000; 72:5313. [PubMed: 11080882]
21. Kubant R, Malinski C, Burewicz A, Malinski T. Electroanal. 2006; 18:410.
22. Quinton D, Girard A, Kim LTT, Raimbault V, Griscom L, Razan F, Griveau S, Bedioui F. Lab Chip. 2011; 11:1342. [PubMed: 21321748]
23. Cortes, J. Sandoval, Grabados, S. Gutierrez, Alatorre, A., Jimenez, J. A. Lopez, Griveau, S., Bedioui, F. Electroanal. 2007; 19:61.
24. Koh WCA, Son JI, Choe ES, Shim Y-B. Anal. Chem. 2010; 82:10075. [PubMed: 21090633]
25. Peteu SF, Peiris P, Gebremichael E, Bayachou M. Biosens. Bioelectron. 2010; 25:1914. [PubMed: 20129772]
26. Peteu SF, Bose T, Bayachou MB. Anal. Chim. Acta. 2013; 780:81. [PubMed: 23680554]
27. Oprea R, Peteu SF, Subramanian P, Qi W, Pichonat E, Happy H, Bayachou M, Boukherroub R, Szunerits S. Analyst. 2013; 138:4345. [PubMed: 23730686]
28. Hosu IS, Vasilescu A, Peteu SF, Raditoiu V, Railian S, Zaitsev V, Turcheniuk K, Boukherroub R, Szunerits S. RSC Advances. 2015; 5:1474.
29. Cvacka J, Quaiserova V, Park JW, Show Y, Muck A Jr. Swain GM. Anal. Chem. 2003; 75:2678. [PubMed: 12948136]
30. Park J, Show Y, Quaiserova V, Galligan JJ, Fink GD, Swain GM. J. Electroanal. Chem. 2005; 1(583):56.
31. Park J, Quaiserova-Mocko V, Patel BA, Novotny M, Liu AH, Bian XC, Galligan JJ, Swain GM. Analyst. 2008; 133:117.
32. Zhao H, Bian XC, Galligan JJ, Swain GM. Diam. Rel. Mater. 2010; 19:182.
33. Duran B, Brocenski RF, France M, Galligan JJ, Swain GM. Analyst. 2014; 139:3160. [PubMed: 24802953]
34. Chailapakul O, Amatatongchai M, Wilairat P, Grudpan K, Nacapricha D. Talanta. 2004; 64:1253. [PubMed: 18969738]
35. Xie S, Shafer G, Wilson CG, Martin HB. Diam. Rel. Mater. 2006; 15:225.
36. Granger MC, Xu J, Strojek JW, Swain GM. Anal. Chim. Acta. 1999; 397:145.
37. Ivandini TA, Sato R, Makide Y, Fujishima A, Einaga Y. Diam. Rel. Mater. 2004; 13:2003.
38. Prado C, Flechsig G-U, Gründler P, Foord JS, Marken F, Compton RG. Analyst. 2002; 127:3.
39. Welch CM, Hyde ME, Banks CE, Compton RG. Anal. Sci. 2005; 21:1421. [PubMed: 16379380]

40. Sonthalia P, McGaw E, Swain GM. *Anal. Chim. Acta.* 2004; 522:35.
41. Chatterjee A, Wiltshire R, Holt KB, Compton RG, Foord JS, Marken F. *Diam. Rel. Mater.* 2002; 11:646.
42. Saterlay AJ, Foord JS, Compton RG. *Analyst.* 1999; 124:1791. [PubMed: 10746310]
43. Martin-Romero FJ, Gutierrez-Martin Y, Henao F, Gutierrez-Merino CJ. *J. Fluorescence.* 2004; 14:17.
44. Meij JT, Haselton CL, Hillman KL, Muralikrishnan D, Ebadi M, Yu L. *Mol. Pharmacol.* 2004; 66:1043. [PubMed: 15258257]
45. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. *Biochem. Pharmacol.* 2005; 70:343. [PubMed: 15963955]
46. Ashki N, Hayes KC, Bao F. *Neuroscience.* 2008; 156:107. [PubMed: 18662749]
47. Lee MCI, Shoji H, Komatsu T, Yoshino F, Ohmori Y, Zweier JL. *Redox Report.* 2002; 7:271. [PubMed: 12688508]
48. Imaram W, Gersch C, Kim KM, Johnson RJ, Henderson GN, Angerdorsfer A. *Free Rad. Biol. Med.* 2010; 49:275. [PubMed: 20406679]
49. Guo Z, Liu H, Jiang C, Zhu Y, Wan M, Dai L, Diang L. *Small.* 2014; 10:2087. [PubMed: 24585690]
50. Roncali J. *J. Mater.Chem.* 1999; 9:1875.
51. Arias-Pardilla J, Giménez-Gómez PA, de la Peña A, Segura JL, Otero TF. *J. Mater. Chem.* 2012; 22:4944.
52. Zanardi G, Terzi F, Seeber R. *Anal. Bioanal. Chem.* 2013; 405:509. [PubMed: 22941065]
53. Lefrant S, Baltog I, Lamy de la Chapelle M, Baibarac M, Louarn G, Journet C, Bernier P. *Synth. Metals.* 1999; 101:184.
54. Selvaganesh SV, Mathiyarasu J, Phani KLN, Yegnaraman Y. *Nanoscale Res. Lett.* 2007; 2:546.
55. Liu D, Huang GM, Yu YQ, He Y, Zhang HL, Cui H. *Chem. Commun.* 2013; 84:9794.
56. Szirmai P, Pichler T, Williams OA, Mandal S, Bäuerle C, Simon F. *Phys. Status Solidi B.* 2012; 249:2656.
57. Gonon P, Gheeraert E, Deneuville A, Fontaine F, Abello L, Lucazeau G. *J. Appl. Phys.* 1995; 78:7059.
58. May PW, Ludlow WJ, Hannaway M, Heard PJ, Smith JA, Rosser KN. *Diam. Rel. Mater.* 2008; 17:105.
59. Hikavy A, Clauws P, Maes J, Moshchalkov VV, Butler JE, Feygelson T, Williams OA, Daenen M, Haenen K. *Phys. Status Solidi B.* 2006; 203:3021.
60. Pruvost F, Deneuville A. *Diam. Rel. Mater.* 2001; 10:531.
61. Gheeraert E, Gonon P, Deneuville A, Abello L, Lucazeau G. *Diam. Rel. Mater.* 1993; 2:742.
62. Fano U. *Phys. Rev.* 1961; 124:1866.
63. Bernard M, Baron C, Deneuville A. *Diam. Rel. Mater.* 2004; 13:896.
64. Bourgeois E, Bustarret E, Achatz P, Omnès F, Blase X. *Phys. Rev. B.* 2006; 74:094509.
65. Phaner M, Hong Z, Bian X, Galligan JJ, Swain GM. *Diam. Rel. Mater.* 2011; 20:75.
66. Brocenschi RF, Rocha-Filho RC, Li L, Swain GM. *J. Electroanal. Chem.* 2014; 712:207.
67. Jensen MP, Riley DP. *Inorg. Chem.* 2002; 41:4788. [PubMed: 12206706]
68. Liu D, Bao F, Prough DS, DeWitt DS. *J. Neurotrauma.* 2005; 22:1123. [PubMed: 16238488]
69. Ferrer-Sueta G, Batinic-Haberle IS, Spasojevic I, Fridovich I, Radi R. *Chem. Res. Toxicol.* 1999; 12:442. [PubMed: 10328755]
70. Lee J, Hunt JA, Groves JT. *J. Am. Chem. Soc.* 1998; 120:6053.
71. Lee J, Hunt JA, Groves JT. *J. Am. Chem. Soc.* 1998; 120:7493.
72. Ferrer-Sueta G, Vitturi D, Batinic-Haberle I, Fridovich I, Goldstein S, Czapski G, Radi R. *J. Biol. Chem.* 2003; 278:27432. [PubMed: 12700236]
73. Ahonen HJ, Lukkari J, Kankare J. *Macromolecules.* 2000; 33:6787.
74. Lapkowski M, Pron A. *Synth. Met.* 2000; 110:79.
75. Groenendaal L, Zotti G, Aubert PH, Waybright MS, Reynolds JR. *Adv. Mater.* 2003; 15:855.

Figure 1. Schematic of the BDD microelectrode architecture after insulation with a polypropylene micropipette tip and the electrochemical formation of the hemin-PEDOT layer on the exposed microelectrode.

Figure 2.
The oxygen-dependent mechanism of peroxynitrite generation from 3-morpholino-sydnimine (SIN-1). Reproduced from reference [27] with permission of The Royal Society of Chemistry.

Figure 3.

The hemin-PEDOT film (A) is formed by the electropolymerization of EDOT with hemin. (B) Cyclic voltammograms recorded during the formation of the hemin-PEDOT conducting polymer film. The electropolymerization was performed over 20 cycles at 50 mV s^{-1} between -1.5 and $+1.5 \text{ V vs. Ag/AgCl}$ in a hemin-EDOT monomer solution. The measurements were made with the solution under a N_2 blanket. The curves show increasing anodic and cathodic currents with increasing cycle number indicative of polymer growth.

Figure 4. (Left) SEM image of the hemin-PEDOT film on a BDD microelectrode at low magnification. The image reveals a nodular morphology of the conducting polymer that covers the entire microelectrode surface. (Right) The higher magnification SEM image shows the typical nanostructured ‘cauliflower’ morphology characteristic of a 3D branched-multiglobular polymer film with features within the 100–300 nm range.

Figure 5. Raman spectra of (A) a hemin-PEDOT BDD microelectrode, (B) a hemin-only BDD microelectrode, and (C) an unmodified BDD microelectrode. $\lambda_{\text{ex}} = 532$ nm. Integration time = 10 s. The hemin-only microelectrode was performed by the same potential cycling as the hemin-PEDOT microelectrode without any EDOT monomer.

Figure 6.

Cyclic voltammetric i - E curves recorded for different concentrations of PON in 0.1 M PB (pH 7.4) from 50 to 400 nM at a hemin-PEDOT BDD microelectrode. Scan rate = 100 mV/s. The PON concentration was estimated from the known concentration of SIN-1 and assuming a 1/100 ratio of PON to SIN-1 under steady-state conditions [8,24,25,46,47].

Figure 7. (left) Cyclic voltammogram of 1.5 mM hemin in dichloromethane with 0.1 M tetrabutylammonium tetrafluoroborate at an unmodified BDD microelectrode (blue curve). The initial scan is shown. The background voltammogram in the absence of any added hemin is also shown (black curve). Scan rate = 100 mVs⁻¹. (right) Schematic showing the catalytic redox reaction mechanism for the hemin-mediated oxidation of PON.

Figure 8.

(A) Continuous amperometric *i-t* curves recorded at a hemin-PEDOT BDD microelectrode for varying concentrations of PON generated by adding aliquots of a SIN-1 solution of known concentration to a mechanically-stirred 0.1 M PB (pH 7.4) solution. (B) Response curve shown for PON concentrations from 0-800 nM generated from the steady-state oxidation current at each concentration. (C) Response curve shown for PON over a more narrow concentration range with a linear regression fit. All currents were measured at +1.35 V vs. Ag/AgCl.

Figure 9.

A comparison of the sensitivity of differently modified BDD microelectrodes for PON recorded in 0.1 M PB (pH 7.4). Response curves are shown for unmodified, hemin-only, PEDOT-only, hemin-PEDOT type A and hemin-PEDOT type BDD microelectrodes. The hemin-PEDOT type B film was formed using a 3× greater EDOT concentration in solution as compared to hemin-PEDOT type A. The PON concentration was estimated from the known concentration of SIN-1 in solution and assuming a 1/100 ratio of PON to SIN-1 under steady-state conditions [8,24,25,46,47]. All currents were measured at +1.35 V vs. Ag/AgCl.

Figure 10. Selectivity of the hemin-PEDOT-PEI BDD microelectrode during a continuous amperometric measurement of PON (50 and 500 nM) in 0.1 M PB (pH 7.4) in the absence and presence of three potential interfering species: norepinephrine, serotonin and uric acid. The concentration of each was 70 μM . The detection potential was +1.35 V vs. Ag/AgCl. Arrows indicate the time at which a solution of the interferent was added to the PON solution.

Table 1

Detection limit for the electrochemical detection of peroxynitrite as reported in the literature.

Electrode Design	Detection Limit	Reference
The response for platinized carbon (C) fiber microelectrodes (μ Es) deconvoluted into the signals for reactive oxygen and nitrogen species.	9 fM at cell level	[17-19]
Film of manganese tetraaminophthalocyanine polymerized onto Pt μ Es	5 μ M	[23]
Film of manganese tetraaminophthalocyanine polymerized onto C fiber μ Es	18 nM	[20]
Conducting polymer manganese poly-dithienylpyrrole-benzoic acid on Pt μ Es	1 nM	[24]
Hemin adsorbed on reduced graphene oxide (rGO) formed on glassy carbon electrode (GCEs)	5 nM	[27]
rGO cobalt phthalocyanine film on GCEs	1.7 nM	[28]
Hemin-PEDOT modified BDD μ Es	3-10 nM	this work