Microsensors for the Electrocatalytic Quantification of Peroxynitrite

Serban F. Peteu and Mekki Bayachou*

Department of Chemistry, Cleveland State University, 2121 Euclid Avenue, Cleveland Ohio 44115.
*Corresponding author, email: m.bayachou”at”csuohio.edu

Abstract

Peroxynitrite [1] ($ONOO^-$) constitutes a major cytotoxic agent, implicated in an increasing number of patho-physiological conditions [2-7], which stimulates a tremendous interest for its real time *in situ* monitoring. The current methods including fluorescent spectroscopy, immuno-histochemistry, or probe nitration, are inherently complex and difficult to apply *in situ*. We have investigated simple metallo-porphyrins electropolymerized into thin films on carbon (C) macro- and micro-electrodes as a catalytic platform for peroxynitrite detection technique [8, 9]. The carbon electrodes were modified and characterized by surface analysis techniques, including field emission scanning electron microscopy with elemental analysis. The response of the modified electrodes in the presence the peroxynitrite was investigated by cyclic voltammetry, and showed typical electrocatalytic oxidation of this analyte. The modified electrodes were then used in amperometric determination of peroxynitrite in standing solutions, as well as in a flow-cell setting. Preliminary results show a detection limit in the high nanomolar range. More work is underway for improving the sensitivity, selectivity of these metalloporphyrin-modified microelectrodes towards peroxynitrite detection. The performance of these sensors is being assessed towards quantifying the potential uncoupling of electron transfer and nitric oxide (NO) biosynthesis by the Nitric Oxide Synthase (NOS) enzymes on NOS-modified electrodes, which yields to peroxynitrite as a side product. This real-time quantification of peroxynitrite would bring a better understanding of the NOS catalytic reaction.

Keywords: electrocatalysis, peroxynitrite, hemin (iron protoporphyrin), C-microelectrode, flow injection, NOS.

1. Introduction

Many publications investigated the independent functions of superoxide [10] and nitric oxide [11]. However, their recombination product, peroxynitrite seems to be lately under heavy scrutiny [2-6]. More specifically, the peroxynitrite anion ($ONOO^-$) is deemed to be a major cytotoxic agent, being formed throughout the human body by the fast reaction of $NO^\bullet$ with $O_2^{\bullet-}$ [9] and displaying a wide range of biochemical reactivity: (a) nitrating proteins (tyrosine residues), carbohydrates and nucleic acids, (b) oxidizing lipids, thiol groups, Fe/S and Zn/S centers, oxyhemoglobin to methemoglobin, and (c) freely crossing the cytoplasmic membrane of red blood cells when protonated [12, 13]. As a result, ONO_2^- has been linked to several pathological conditions such as acute ischemia-reperfusion injury, arthritis, carcinogenesis, Alzeimer’s, Parkinson’s, and AIDS. Recently, several groups investigating the water soluble manganese and iron porphyrins, have found these to catalytically decompose peroxynitrite [14-18]. Interestingly, elsewhere metallo-porphyrins have been electropolymerized as adherent films on carbon electrodes [19, 20]. Also, the hemin-modified carbon electrodes have been employed for the catalytic reduction of dioxygen [21], the reduction of organohalides [22], the electrochemical study and quantification of tryptophan and its derivatives [23], or as a sensor for superoxide [24]. The purpose of this work is investigating for the first time the electrocatalytic performance of hemin electropolymerized thin films on carbon macro- and micro-electrodes for the oxidation of peroxynitrite. Additionally, new hemin-modified poly(3,4-ethylenedioxi-thiophene)-functionalized microelectrodes were assessed for the first time as possible peroxynitrite-sensitive sensors. These sensors are to be used to assess PON on NOS-modified electrodes, i.e. in the study of the NOS biocatalysis in confined environments.

$$NO^\bullet + O_2^{\bullet-} \rightarrow ONO_2^-$$

2. Experimental Details

2.1. Peroxynitrite Synthesis, Chemicals. Several synthetic methods have been reported for the preparation of peroxynitrite [25-29]. Herein, the method employed was the two-phase displacement reaction by the hydroperoxide anion, in the aqueous phase, on the isoamyl nitrite, the organic phase [30]. The peroxynitrite, with a final concentration of 750 mM, was packaged in 5 mL aliquots at –20°C. The iron protoporphyrin IX (hemin) and protoporphyrin were purchased from Sigma-Aldrich, St Louis Mo. The deionized water was obtained from a Barnstead ultrapure water system, with the resistivity of at least 18.2 MΩ cm. All other chemicals were reagent grade and were used as received.

2.2. Electrodes, Apparatus and Methods. Films of electropolymerized porphyrins were deposited on carbon macro- and micro-electrodes [19, 20, 29]. The cyclic voltammetry experiments and the time-based amperometric dose-response tests were performed in a three-electrode configuration cell on a CHI Instruments Model 440 electrochemical station. The working electrode being polarized at 750 mV unless otherwise noted, and all graphed data points represent an average of at least three different experiments, unless mentioned otherwise. A flow-cell was fabricated in house.

3. Results and Discussion

3.1 Response of the hemin-modified carbon macro-electrode to peroxynitrite For the hemin glassy carbon (GC) macro-electrode, the oxidation current increased with increasing peroxynitrite concentration, as evidenced in the **Figure 1a**. The Lineweaver-Burk double reciprocal analysis of the data yielding a Michaelis-Menten constant of 0.70 mM is illustrated in Figure 1b. The catalytic current increases as a function of substrate's concentration, with the onset of saturation kinetics observed above 0.25 mM. By comparison, the catalytic decomposition of peroxynitrite by micromolar amounts of water-soluble Fe(III) porphyrin complexes was recently reported [16], with a Michaelis-Menten kinetics maintained for a ONO_2^- concentration range of (40-610) μM at 5 μM catalyst concentration, and a K_m in the range of 0.3—0.62 mM. The response of the hemin-GC in the presence of peroxynitrite was investigated as a function the of the scan rate, from 0.01 V/s to 3 V/s for three different concentrations of peroxynitrite: 0.55 mM, 0.90 mM, and 1.45 mM. Consistent with an electro-catalytic process initiated within the cyclic voltammetry time scale the ratio I/I_0 decreases as the scan rate increases [29, 31]. Also, the catalytic efficiency increased significantly with the pH, as previously shown [29, 31].

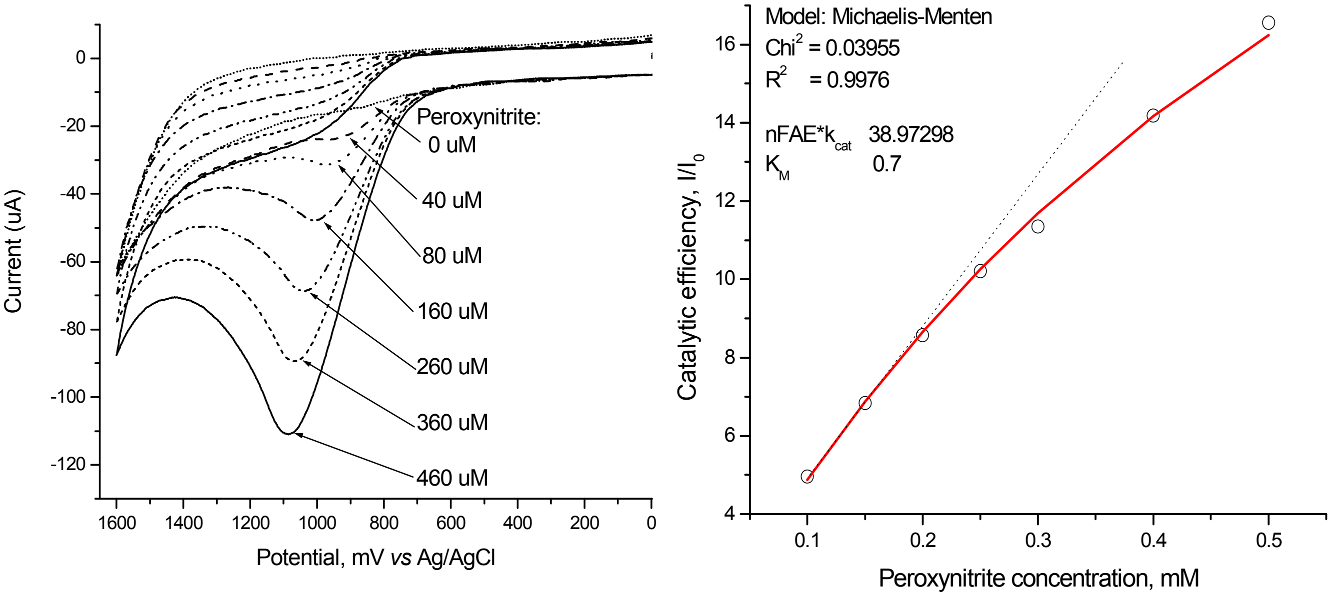


Figure 1a, b. Voltammograms of Hemin/GC in pH 10.5 CAPS buffer, at 150 mV/s scan rate, for different peroxynitrite concentrations from 0.04 to 0.46 mM (a, left). The Lineweaver-Burk double reciprocal analysis of the data yielding a Michaelis-Menten constant of 0.70 mM (b, right).

To further evaluate the electrochemical characteristics of the hemin-modified glassy carbon as potential peroxynitrite-sensitive electrodes, several time-based amperometry tests were performed. The current was measured in response to varying amounts of peroxynitrite standard solution, successively added to the magnetically stirred, deaerated 10.5 pH buffer solution. A hemin-modified carbon macro-electrode had a sensitivity of 0.35 nA/μM, which is 5 fold higher than bare [29, 31].

3.2. Miniaturization. Several glass-encased carbon fiber microelectrodes were prepared, both bare C-fiber and modified with a hemin film, and tested with peroxynitrite by amperometry in a home-made flowcell, as shown in the **Figure 2a-e**. After these tests, the same bare and hemin-modified fibers were imaged with the FESEM. In this amperometric test, the sensitivity for the hemin-modified fiber was 2 fold higher than that of a bare, and work is now ongoing to optimize its response to peroxynitrite. One of the benefits of using a flow-cell is the convective transport of analyte, which implies a reduction in the noise and faster response times. From the charge/peak area *vs.* ONO$_2^-$ concentration, it follows that the sensitivity of the hemin modified microelectrode is 9.4 nC/µM, which is 4 fold bigger than the bare microelectrode.

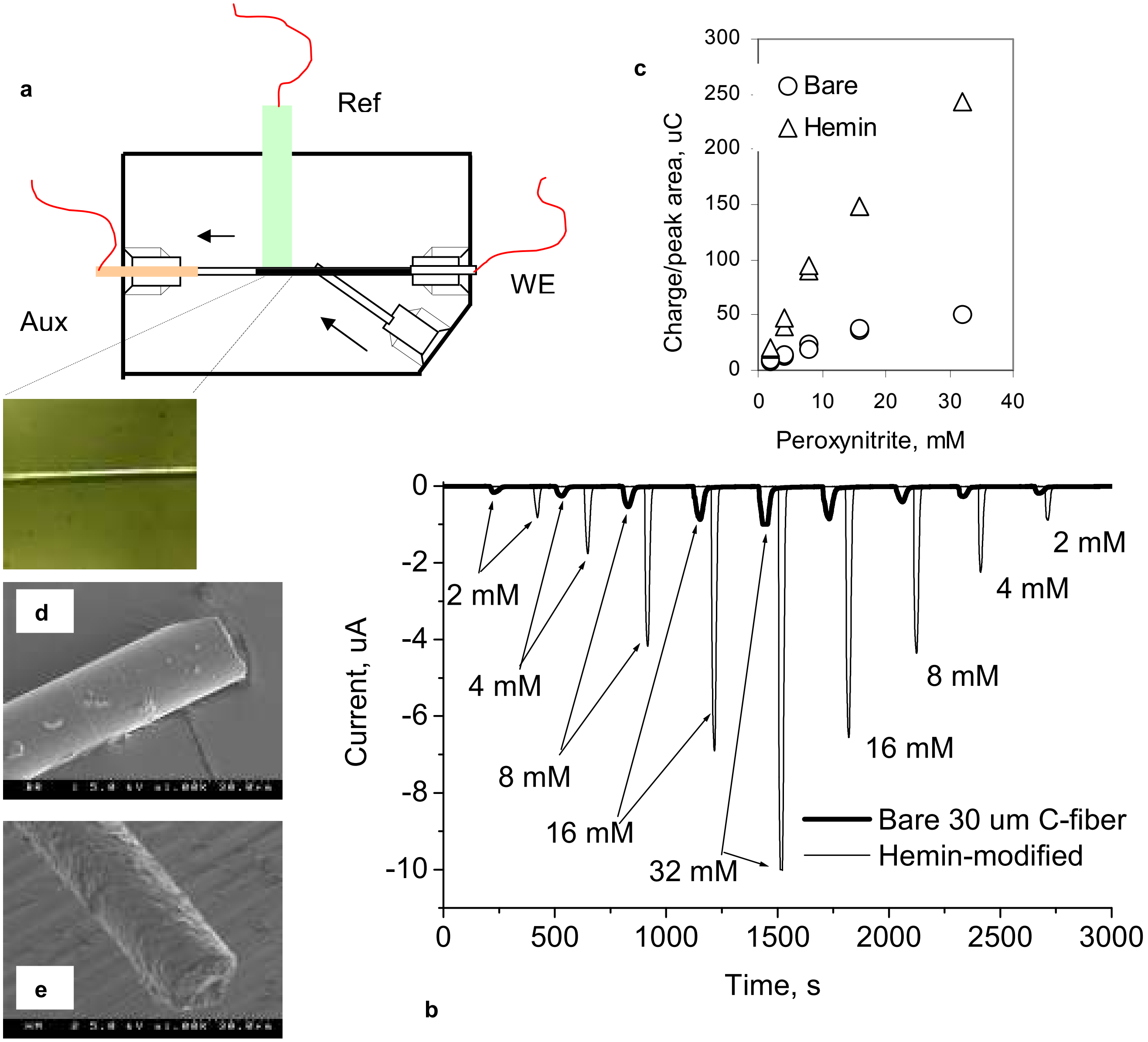
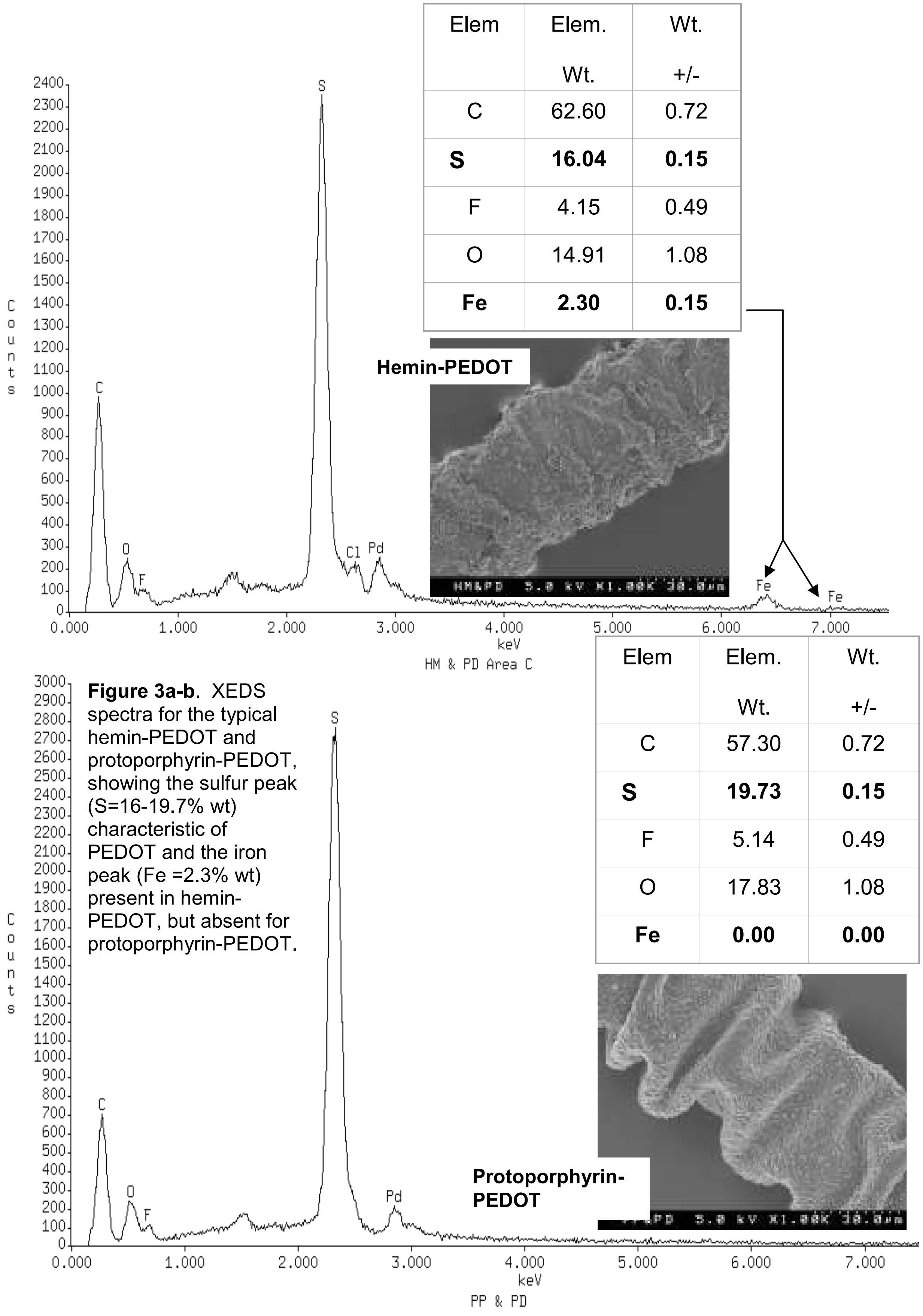


Figure 2 a-e. Flow-cell injection analysis with carbon fiber microelectrodes: (a) flow cell geometry, (b) the peroxynitrite peaks, and (c) the calibration curves for the charge/ peak area vs. ONO$_2^-$ concentration, recorded for a hemin-modified microelectrode (e) compared with the bare (d).

In order to further increase the sensitivity of the hemin-modified carbon fiber microelectrodes, we set out to investigate the inherently conductive (3,4-ethylenedioxy-thiophene) (EDOT) functionalized with hemin, by electrodeposition on a 30 µm dia. CFE. There are previous reports of electroactive polymers (such as polypyrrole, polyaniline or polythiophene) in (bio)analytical electrochemical sensors. These ‘synthetic metals’ exhibit high conductivity, are mediating rapid electron transfer, can be synthesized under mild conditions and deposited onto



Elem	Elem. Wt.	Wt. +/-
C	62.60	0.72
S	**16.04**	**0.15**
F	4.15	0.49
O	14.91	1.08
Fe	**2.30**	**0.15**

Elem	Elem. Wt.	Wt. +/-
C	57.30	0.72
S	**19.73**	**0.15**
F	5.14	0.49
O	17.83	1.08
Fe	**0.00**	**0.00**

Figure 3a-b. XEDS spectra for the typical hemin-PEDOT and protoporphyrin-PEDOT, showing the sulfur peak (S=16-19.7% wt) characteristic of PEDOT and the iron peak (Fe =2.3% wt) present in hemin-PEDOT, but absent for protoporphyrin-PEDOT.

Moreover, the X-ray energy dispersive spectroscopy (XEDS) was used as a sensitive analytic tool to study and ascertain the surface composition of the catalyst layers. The X-rays penetrate a substantial distance into the sample and excite the electrons (photoelectrons). A small fraction of these electrons from the top 5 nm film make it outside and are detected, in terms of their kinetic energy. The XEDS spectra for the hemin-PEDOT and protoporphyrin-PEDOT are both showing the sulfur peak (S=16-19.7% wt) characteristic of PEDOT, as seen in **Figure 3.** The iron peak is present in hemin-PEDOT (Fe =2.3% wt), but absent for protoporphyrin-PEDOT (Fe=0.0% wt).

To further evaluate the electrochemical characteristics of the hemin-modified glassy carbon as potential peroxynitrite-sensitive electrodes, several time-based amperometry tests were performed. The current was

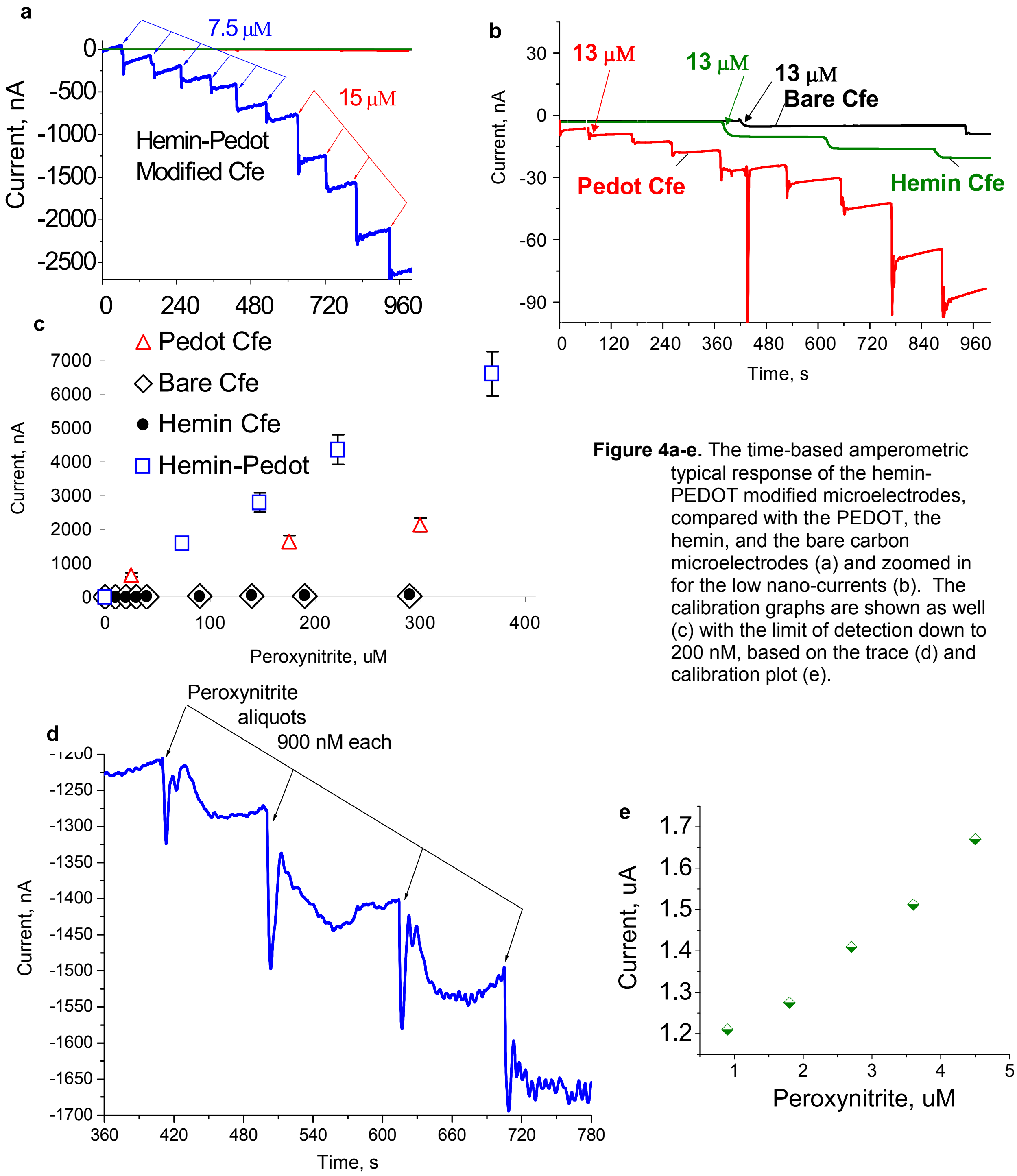


Figure 4a-e. The time-based amperometric typical response of the hemin-PEDOT modified microelectrodes, compared with the PEDOT, the hemin, and the bare carbon microelectrodes (a) and zoomed in for the low nano-currents (b). The calibration graphs are shown as well (c) with the limit of detection down to 200 nM, based on the trace (d) and calibration plot (e).

measured in response to varying amounts of peroxynitrite standard solution, successively added to the magnetically stirred, deaerated 10.5 pH buffer solution. A typical dose-response current trace is shown in the **Figure 4**. Each arrow indicates when a ONO_2^- aliquot was added to this solution. The calibration curves from these amperometric tests are illustrated in Figure 4a with a detail for the 0-40 μM analyte concentration range(b) and an amperometric test with diluted peroxynitrite (d, e) for the limit of detection. Based on this test with diluted analyte, the limit of detection based for a 3/1 response-to-noise ratio was determined as low as 200 nM peroxynitrite. The typical 90% response times for the modified carbon fiber electrodes were between 5 s and 30 s, depending on the fiber length, and the thickness of the catalyst film, which in turn depends on the duration of the electrodeposition.

4. Conclusion

Herein, a hemin electropolymerized film was found to act as an electrocatalyst mediating the oxidation of peroxynitrite, for the first time to the best of our knowledge. This electro-catalytic oxidation and the preliminary dose-response time-based amperometry show for the first time that the hemin-modified electrodes could be used as potential sensors for the sensitive peroxynitrite detection. In conclusion, optimized hemin-PEDOT modified C-fiber microelectrodes were utilized for the first time to detect ONO_2^-, with a response time as low as 5 s and a limit of detection down to 200 nM as evidenced by the time-based dose-response amperometry. These hemin-PEDOT modified C-fiber microelectrodes showed a sensitivity of 13 nA/μM (or 13 pA/nM), over 50 times higher than the hemin-modified CFE and 130 times higher than the bare C-fibers.

Some selectivity tests were performed, and the preliminary results showed that peroxynitrite was detected in the presence of nitric oxide, without significant interference from it. More work is underway using other electro-calalysts, to improve the detection limit and the selectivity. These sensors are to be used to assess peroxynitrite on NOS-modified electrodes, i.e. in the study of the NOS biocatalysis in confined environments, and work is underway to identify the diffusion and reaction pathway of this electrocatalysis.

5. Acknowledgements

The authors would like to thank Mr. John Sears for help with the FESEM at the Case Western University and to Mr. Ermias Gebremichael for fabricating and testing some of the modified electrodes, while in training in our laboratory. This work is supported by grants from the US Department of Energy, Basic Energy Sciences, Catalysis Science Grant DE-FG02-03ER15462, and by startup funds from Cleveland State University.

CITED REFERENCES

1. The IUPAC recommended name for peroxynitrite anion is oxoperoxynitrate (-1) and hydrogen oxoperoxonitrate for peroxynitrous acid (Nomenclature of Inorganic Chemistry. Recommendations, p.1, Blackwell, Oxford, London). The term 'peroxynitrite' is employed to indicate the total concentration of $ONOO^-$ + HOONO. The individual concentrations $ONOO^-$ and HOONO are governed by the pKa of 6.8 for the peroxynitruous acid (Prior, Squadrito, Am. J. Physiol., Lung. Cell. Mol. Physiol. 12, 268, 699-722).
2. Beckman, J.S., *Oxidative damage and tyrosine nitration from peroxynitrite.* Chem. Res. Toxicol., 1996. **9**: p. 836-844.
3. Douki, T.C., Ames, B. N., *An adduct between peroxynitrite and 2'-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2'-deoxyguanosine.* Chem. Res. Toxicol., 1996. **9**: p. 3-7.
4. Daiber, A.M., M.; Ullrich V.;, *New aspects in the reaction mechanism of phenol with peroxynitrite: the role of phenoxy radicals.* Nitric oxide: biology and chemistry, 1998. **2**: p. 259-269.
5. Sodum, R.S.F., E. S., *Analysis of peroxynitrite reactions with guanine, xanthine, and adenine nucleosides by high-pressure liquid chromatography with electrochemical detection: C8-nitration and -oxidation.* Chem. Res. Toxicol., 2001. **14**: p. 438-450.
6. Editorial, *Editorial. Forum: reactive species of peroxynitrite.* Chem. Res. Toxicol., 1998. **11**: p. 1.
7. Trujillo, M.N., M.; Alvarez, M.N.; Peluffo, G.; Radi, R.;, *Peroxynitrite biochemistry: formation, reactions and detection.* Analusis, 2000. **28**: p. 518-527.
8. Xue, J.Y., X.; Chen, J.; Xian, Y.; Jin, L.;, *Amperometric ultramicrosensors for peroxynitrite detection and its application toward single myocardial cells.* Analyt Chem, 2000. **72**: p. 5313-5321.

9. Amatore, C.A.; Bruce, D.; de Oliveira, P.; Erard, M; Vuillaume, M., *Characterization of the electrochemical oxidation of peroxynitrite: relevance to oxidative stress bursts measured at the single cell level.* Chem. Eur. J., 2001. **7**(19): p. 4171-4179.
10. Akaike, T.N., Y.; Ijiri, S.; Setoguchi K.; Suga, M.; Zheng, Y. M.; Dietzhold, B.; Maeda, H.;, *Pathogenesis of influenza virus-induced pneumonia: involvement of both oxide and oxygen radicals.* Proc. Natl. Acad. Sci. USA, 1996. **93**: p. 2448-2453.
11. Lancaster, J.R., *Simulation of the diffusion and reaction of endogenously produced nitric oxide.* Proc. Natl. Acad. Sci. USA, 1994. **91**: p. 8137-8141.
12. Ischiropoulos, H.B., J.;Crow, J.; Royall, J.; Kooy, N.;, Methods Enzymol., 1995. **7**: p. 367-373.
13. Radi, R., *Peroxynitrite reactions and diffusion in biology.* Chem. Res. Toxicol., 1998. **11**: p. 720-721.
14. Groves, J.T.M., S. S.;, *Peroxynitrite-induced DNA strand scission mediated by a manganese porphyrin.* J Am Chem Soc, 1995. **117**: p. 9578-9579.
15. Ferrer-Sueta, G.B.-H., I. S.; Spasojevic, I.; Fridovich, I.; Radi, R., *Catalytic scavenging of peroxynitrite by isomeric Mn(III) N-Methylpyridylporphyrins in the presence of reductants.* Chem. Res. Toxicol., 1999. **12**: p. 442-449.
16. Jensen, M.P., Riley, D. P.;, *Peroxynitrite decomposition activity by iron porphyrin complexes.* Inorg. Chem., 2002. **41**: p. 4788-4797.
17. Lee, J.H., J. A.; Groves, J. T.;, *Mechanisms of iron porphyrin reactions with peroxynitrite.* J. Am. Chem. Soc., 1998: p. 7493-7501.
18. Crow, J.P., *Manganese and iron porphyrins catalyze peroxynitrite decomposition and simultaneously increase nitration and oxidant yield: implications for their use as peroxynitrite scavengers in vivo.* Arch. Biochem. Biophys., 1999. **371**(1): p. 41-52.
19. Macor, K.A.S., T. G.;, *Prophyrin electrode films prepared by electrooxidation of metalloprotoporphyrins.* J. Am. Chem. Soc., 1983. **105**: p. 5601-5607.
20. Younathan, J.N.W., K. S.; Meyer, T. J.;, *Electrocatalytic reduction of nitrite and nitrosyl by iron(III) protoporphyrin IX dimethyl ester immobilized in an electropolymerized film.* Inorg. Chem., 1992. **31**: p. 3280-3285.
21. Zheng, N.Z., Y.; Osborne, P.G.; Li, Y.; Chang, W.; Wang, Z., *Electrocatalytic reduction of dioxygen on hemin based carbon paste electrode.* J. Appl. Electrochem., 2002. **32**(129): p. 129-133.
22. Nakashima, N.T., T.; Owaki, H.; Murakami, H.; Sagara, T., *Catalytic reduction of organohalides at hemin and hemin-imidazole polymer films on pyrolytic graphite electrodes.* Coll. Surf. A: Physicochem. Eng. Aspects, 2000. **169**: p. 163-170.
23. Nan, C.G.F., Z. Z.; Li, W. X.; Ping, D. J.; Qin, C. H.;, *Electrochemical behavior of tryptophan and its derivatives at a glassy carbon electrode modified with hemin.* Analyt. Chim. Acta., 2002. **452**: p. 245-254.
24. Chen, J., Wollenberger, U., Lisdat, F., Ge, B., Scheller, F. W., *Superoxide sensor based on hemin modified electrode.* Sens & Act B Chem, 2000: p. 115-120.
25. Prior, W.A.C., X.J.; Jin, X.; Koppenol, W.H.; Ngu-Schwemlein, M.; Squadrito, G. L.; Uppu, P. L.; Uppu, R.M., Free Rad. Biol. Med., 1995. **18**(1): p. 75-83.
26. Hughes, M.N., Nicklin, H.G., *Autooxidation of hydroxylamine in alkaline solutions.* J. Chem. Soc. A: Inorg. Phys. Theor., 1971: p. 164-168.
27. Reed, J.W.H., H.H.; Jolly, W.L., *Chemical synthesis with a quenched flow reactor. Hydroxytrihydroborate and peroxynitrite.* J. Am. Chem. Soc., 1974. **96**: p. 1248-1249.
28. Plumb, R.C., Edwards, J.O., *Color centers in UV-irradiated nitrates.* J. Phys. Chem., 1992. **96**(8): p. 3245-3247.
29. Peteu, S.F., Bayachou, M., *Hemin as an electrocatalyst for peroxynitrite's oxidation.* Submitted, 2005.
30. Uppu, R.M., Prior, W. A., *Synthesis of peroxynitrite in a two-phase system using isoamyl nitrite and hydrogen peroxide.* Analytical Biochem., 1996. **236**: p. 242-249.
31. Bayachou, M., Peteu, S.F., *Nano-structured electroactive polymer with metallo-porphyrins as electrocatalysts for peroxynitrite's oxidation.* Invention Disclosure to Cleveland State University, 2005.