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Voltammetric Detection of Hydrogen Peroxide at Carbon Fiber Microelectrodes

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Abstract

Hydrogen peroxide is a reactive oxygen species that is implicated in a number of neurological disease states and that serves a critical role in normal cell function. It is commonly exploited as a reporter molecule enabling the electrochemical detection of non-electroactive molecules at electrodes modified with substrate-specific oxidative enzymes. We present the first voltammetric characterization of rapid hydrogen peroxide fluctuations at an uncoated carbon fiber microelectrode, demonstrating unprecedented chemical and spatial resolution. The carbon surface was electrochemically conditioned on the anodic scan and the irreversible oxidation of peroxide was detected on the cathodic scan. The oxidation potential was dependent on scan rate, occurring at +1.2 V vs. Ag/AgCl at a scan rate of 400 $V \cdot sec^{-1}$. The relationship between peak oxidation current and concentration was linear across the physiological range tested, with deviation from linearity above 2 mM and a detection limit of 2 μM. Peroxide was distinguished from multiple interferents, both in vitro and in brain slices. The enzymatic degradation of peroxide was monitored, as was peroxide evolution in response to glucose at a glucose oxidase modified carbon fiber electrode. This novel approach provides the requisite sensitivity, selectivity, spatial and temporal resolution to study dynamic peroxide fluctuations in discrete biological locations.

Keywords

Fast-scan cyclic voltammetry; glucose oxidase; enzyme-modified electrode; biosensor; reactive oxygen species; electrochemical detection

INTRODUCTION

Hydrogen peroxide (H_2O_2) is an important, membrane permeable, reactive oxygen species (ROS) in the brain that is most often regarded as a potential toxin because, under certain conditions, it can form highly reactive hydroxyl radicals that can irreversibly alter DNA, lipid and protein structure[1]. Thus, an imbalance between H_2O_2 generation and metabolism could result in pathological oxidative stress and neuronal degeneration in disease states and aging[2-4]. However, H_2O_2 is also an important player in reductive-oxidative-based signal transduction cascades and thus is essential for normal cell function, especially in the brain[4-6]. Furthermore, H_2O_2 is gaining increasing recognition as a rapid neuromodulatory signaling molecule. Recent studies in brain slices have demonstrated that the classical neurotransmitters, glutamate and γ-aminobutyric acid (GABA), regulate dopamine (DA) release on a subsecond timescale by way of transient H_2O_2 signaling in the dorsal striatum, a key brain region in motor control[7-8].

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Despite a growing interest in H_2O_2, there are few experimental tools available to directly measure dynamic H_2O_2 fluctuations in intact tissue. Molecular imaging with H_2O_2-responsive fluorophores is a powerful, and commonly used, approach for examining the diverse roles that H_2O_2 plays in complex biological environments[9]. However, conventional fluorescent probes lack selectivity for H_2O_2 over other ROS. They are difficult (if not impossible) to use for dynamic *in vivo* measurements, and calibration of the fluorescence intensity of these dyes is not possible, precluding their use in quantitative analyses. In contrast, electrochemical techniques are especially useful for quantifying rapid chemical changes *in vivo*, because small probe sizes and fast sampling rates can be employed[10]. However, the electron transfer kinetics for the irreversible oxidation of H_2O_2 are slow[11-12], thus limiting the development and application of electroanalytical methods for the direct detection of this molecule.

The electrochemical detection of H_2O_2 is of widespread interest to bioanalytical scientists because it is often enzymatically generated at biosensors to serve as an electroactive reporter molecule in the indirect detection of non-electroactive biomolecules such as glucose, glutamate, and acetylcholine[13-19]. Because the electrochemical oxidation of H_2O_2 is electrocatalyzed[12, 20], Pt electrodes are widespread in the construction of these sensors, which normally utilize amperometric detection. However Pt electrodes readily biofoul and, although amperometry provides an accurate measure of the local flux of electroactive species, it lacks selectivity. Thus, when used *in vivo* these devices require several selective exclusion layer coatings in addition to enzymatic coatings. This is a significant drawback because coated electrodes exhibit increased response times due to the time required for analyte to diffuse through the coating, they are difficult to reproducibly fabricate[21]. Furthermore, the stability and selectivity is dependent on coating integrity.

Another electrochemical technique, background-subtracted, fast-scan cyclic voltammetry (FSCV), provides chemical selectivity in addition to temporal resolution and high sensitivity[10]. With this approach, a cyclic voltammogram is generated to serve as a chemical signature for the analyte of interest, allowing discrimination from other electroactive species in the brain22. This technique is commonly used for *in vivo* measurements with carbon fiber microelectrodes, which are advantageous due to their biocompatibility, small size, and ease of fabrication[23]. In this work, we present the first voltammetric recordings of H_2O_2 at single, uncoated carbon-fiber microelectrodes. To overcome the kinetic limitations, the carbon surface is electrochemically conditioned on the anodic scan and H_2O_2 is irreversibly oxidized on the cathodic scan. We verify the identity of our signal by monitoring the selective enzymatic degradation of H_2O_2 in the presence of catalase. Various scan rates are investigated to optimize the detection, and the limits of detection and detectable range of H_2O_2 are established. We demonstrate both *in vitro* and in brain tissue that H_2O_2 can be reliably quantified in the presence of multiple electroactive species that are commonly found in the brain. Finally, we establish that this approach can be used at microelectrodes to detect enzymatically-generated H_2O_2 upon consumption of non-electroactive enzyme substrate.

EXPERIMENTAL SECTION

Chemicals

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI), unless otherwise noted, and used as received. A physiological buffer solution (15 mM Trisma HCl, 3.25 mM KCl, 1.2 mM $CaCl_2$, 1.2 $MgCl_2$, 2.0 mM Na_2SO_4, 1.25 mM NaH_2PO_4, 145 mM NaCl) at pH 7.4 was used in all flow injection analysis experiments. All aqueous solutions were made using doubly distilled deionized water (Barnstead EasyPure II, Dubuque, IA).

Electrode Fabrication

Carbon-fiber microelectrodes were fabricated by aspirating a single 7 μm diameter T-650 carbon fiber (Cytec Industries, West Patterson NJ) into a single borosilicate glass capillary (0.60 mm × 0.40 mm, A-M Systems, Carlsburg, WA). A micropipette puller (Narishige, Tokyo, Japan) was used to taper the glass and form two sealed microelectrodes. The exposed length of carbon fiber was cut to approximately 100 μm. An electrical connection was made by backfilling the capillary with an ionic solution (4 M potassium acetate, 150 mM KCl).

Data Acquisition

All data was collected in an *in vitro*, flow injection system unless otherwise specified. In most experiments, a triangular waveform ranging from -0.4 to +1.4 V with a holding potential of -0.4 V versus Ag/AgCl was applied at a scan rate of 400 $V \cdot sec^{-1}$ and a frequency of 10 Hz using a custom built instrument for potential application to the electrochemical cell and for current transduction (University of North Carolina at Chapel Hill, Department of Chemistry, Electronics Facility). TH-1 software (ESA, Chelmsford, MA) was used for waveform output with a DAC/ADC card (NI 6251 M). A second card (NI 6711) was used for triggering the DACs and ADCs and for synchronization of the electrochemical experiment with flow injection. Signal processing (background subtraction, signal averaging, and digital filtering (4-pole Bessel Filter, 2 KHz) was software-controlled.

Flow Injection

The working electrode was positioned in a custom electrochemical cell using a micromanipulator (World Precision Instruments, Inc., Sarasota, FL). A syringe pump (BS-8000, Braintree Scientific, Braintree, MA) supplied a continuous buffer flow of 3 mL/minute across the working and reference electrodes. Five second bolus injections of analyte to the microelectrode surface were accomplished with a six-port HPLC valve mounted on a two-position air actuator controlled by a digital pneumatic solenoid valve kit (Valco Instruments, Houston TX). The entire apparatus was housed within a custom-built Faraday cage. All experiments were performed at room temperature.

Enzyme Coating of Microelectrodes

0.10 g of bovine serum albumin (BSA) was dissolved in 985 μL ddH_2O by manual agitation at room temperature. 5 μL of glutaraldehyde solution (25%) was added and the mixture was allowed to set for 5 minutes. 1 μL of glucose oxidase (from Aspergillus niger) stock solution (2 U/ μL) was added to 9 μL of the BSA/glutaraldehyde solution in a 500 μL microcentrifuge tube and mixed by pipette agitation giving a final concentration of 0.1% BSA and 0.125% glutaraldehyde. Fabricated microelectrodes were then coated by dipping the microelectrode tip into the enzyme solution three times with 1-minute dry times between dips. Finally, electrode tips were immersed in TRIS buffer (pH 7.4) at room temperature for one hour to allow the enzyme matrix to set.

Brain Slice Experiment

Male Sprague-Dawley rats (n=4, 250-300 g, Charles River Laboratories, Raleigh, NC) were decapitated after being deeply anesthetized with urethane (1.5 g/kg, i.p.). The brain was rapidly removed and kept in cold Tris buffer at pH 7.4 while 400 μm thick coronal slices containing the striatum were prepared with a vibratome (World Precision Instruments, Sarasota, FL). Slices were subsequently placed in a recording chamber and superfused with buffer maintained at 37°C. A bare microelectrode (n=4) was placed at least 100 μm below the surface of the slice, and a glass micropipette pulled from borosilicate glass capillary (1.2 mm × 0.68 mm, A-M Systems, Carlsburg, WA) with an outer tip diameter of 15-20 μm was positioned in the tissue about 1 mm from the recording electrode. Electrode and injector placements were made with

the aid of a microscope (Nikon Instruments Inc., Melville, NY). Buffered solution containing 50 μM H_2O_2 was injected for 1 second at 5 psi with a Picospritzer III (Parker Hannifin Corp., Fairfield, NJ). Animal care was in accordance with institutional guidelines.

Statistics

All values are given as the mean ± standard error of the mean (SEM). One-tailed paired Student's t tests were used to determine statistical differences, designated at $p < 0.05$. Mixtures were resolved with principal component regression using MATLAB (The MathWorks, Natick, MA)[22]. Statistical and graphical analysis was carried out using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

RESULTS AND DISCUSSION

H_2O_2 Cyclic Voltammetry

Cyclic voltammograms were collected in the *in vitro* flow injection system by ramping the potential applied to the carbon fiber electrode from a holding potential of -0.4 V versus Ag/AgCl to 1.4 V and back at 400 $V \cdot sec^{-1}$ every 100 msec, as shown in Figure 1A. The fast scan rate resulted in a non-faradaic background current, shown in Figure 1B (solid line), that was relatively stable over time. The current at the electrode increased slightly upon introduction of a 5 second bolus of 100 μM H_2O_2 to the electrode surface using the flow injection system (dashed line). The non-faradaic background current was subtracted to produce the analyte-specific cyclic voltammograms shown in Figure 1C,D. The oxidation peak for the two-electron, irreversible process was observed at an overpotential on the reverse scan.

Previous voltammetric studies using slow scan rates at carbon, Pt, or Pt + Ir electrodes in physiological buffer solution have established that the electrode must acquire an oxidized surface to accommodate H_2O_2 oxidation[12,20,24]. Electrochemical conditioning of a carbon fiber microelectrode in aqueous solution using fast scan rates and similar potential limits specifically increases the population of carbonyl and hydroxyl functional groups on the carbon surface[25] in an oxidative etching process that constantly renews the electrochemically active surface[26]. Thus, our finding that the H_2O_2 did not oxidize until after the electrode was rapidly scanned to a moderately high anodic potential is not unexpected. To determine the potential limit required to sufficiently oxidize the electrode surface for the oxidation of H_2O_2, the switching potential was varied from 0.8 V to 1.4 V (Figure 2). A significant oxidation peak was observed only at switching potentials greater than 1.2 V. The amplitude of the oxidation current increased as the potential limit was extended, with maximum sensitivity observed using a potential limit of 1.4 V. Thus, this waveform was selected for further characterization.

The scan rate was varied between 100 and 800 $V \cdot sec^{-1}$, typical scan rates for *in vivo* measurements (Figure 3). At the slowest scan rate, the peak oxidation current for 100 μM H_2O_2 occurred at the switching potential, suggesting that oxidation was incomplete prior to switching to the cathodic scan. The cyclic voltammogram is similar to those published previously for the voltammetric detection of H_2O_2 on carbon at slow scan rates[27-28]. When the scan rate was increased the peak oxidation potential, E_p, shifted to the cathodic scan and the peak became well defined. The peak oxidation current was maximized at 400 $V \cdot sec^{-1}$, thus this scan rate was considered optimum.

Signal Validation

In order to pharmacologically verify that the observed current was due to the oxidation of H_2O_2, cyclic voltammograms were collected for 100 μM H_2O_2 before and after the addition of 1 U/mL catalase (from Aspergillus niger), a common enzyme that catalyzes the decomposition of H_2O_2 to water and oxygen. Cyclic voltammograms collected at a

representative electrode are presented in Figure 4. 5 minutes after the addition of catalase, the peak oxidative current was significantly reduced to 6 ± 2 % of the initial current ($p < 0.001$, Student's t-test, n=3). The representative cyclic voltammogram for the catalase-containing H_2O_2 solution (dashed line) shows a reduction of the H_2O_2 signal and no other significant current peaks produced by products of the reaction. 20 minutes after the addition of catalase the current was completely abolished (data not shown). The cyclic voltammogram for a fresh bolus of 100 μM H_2O_2 was identical to that initially obtained, demonstrating that the catalase solution did not simply abolish the current by fouling the electrode.

Characterization of H_2O_2 Electrochemistry

Varying concentrations of H_2O_2 were used to characterize the linear relationship between peak current and solution concentration. Figure 5A shows five representative cyclic voltammograms of physiological concentrations at regular intervals (50 μM to 250 μM). The current versus time traces for these data are presented in Figure 5B. The response time for the detection of 100 μM H_2O_2, defined as the time required to go from 10% to 90% of the peak current, was 0.75 ± 0.05 sec (n=5). The linear plot of peak current versus concentration shown in Figure 5C was compiled from four repetitions of each concentration at six electrodes ($r^2 = 0.95$). Assuming a detection limit of three times the noise, the limit of detection was 1.9 ± 0.1 μM (n=5). Figure 5D shows good linearity up to 2 mM ($r^2 = 0.98$, n=4).

In the brain, other endogenous electroactive neurotransmitters can contribute to the current collected at the carbon fiber surface, thus it is important to be able to distinguish H_2O_2 from potential interferents. The characteristic voltammograms of different neurotransmitters obtained with FSCV are multiple point measures of the species detected, providing qualitative information that enables identification. Dopamine, ascorbate and basic pH shifts are well-characterized at carbon fiber electrodes[22] and these signals are present in discrete brain regions where H_2O_2 might also be detected. To demonstrate that H_2O_2 could be discriminated from these signals, dopamine, ascorbate and H_2O_2 were evaluated at five different concentrations chosen based on estimates of their *in vivo* concentrations[29-30]. Five basic pH shifts were also evaluated, as these signals are often measured in living brain tissue[31-32]. The background-subtracted cyclic voltammograms (Figure 6A-D) were used to construct a calibration set. Mixtures of the four compounds were analyzed using principal component regression (PCR), as described previously[22]. Figure 6E shows the predicted H_2O_2 concentration plotted versus the actual concentration of H_2O_2 in the mixtures at n=6 electrodes. The slope of the regression line was 0.99 ± 0.03 ($r^2 = 0.97$), indicating appropriate identification and quantification of H_2O_2.

Voltammetric Detection of H_2O_2 in a Brain Slice

To demonstrate that the electrode can distinguish H_2O_2 when implanted in tissue, background-subtracted cyclic voltammograms for the oxidation of H_2O_2 were recorded in a brain slice. The microelectrode was inserted at least 100 μm into a section of brain tissue that encompassed the striatum, a region with nerve terminals containing the electroactive transmitter dopamine (a potential interferent). A glass micropipette containing 50 μM H_2O_2 was also positioned in the tissue approximately 1 mm from the carbon fiber electrode. A 1 second pressure ejection was used to introduce the exogenous H_2O_2 to the electrode surface. Example voltammetric recordings are shown in Figure 7. Chemical changes were not recorded until the onset of the pressure ejection (arrow). The individual, background-subtracted voltammograms corresponded to those collected for H_2O_2 *in vitro* as judged by the correlation coefficient ($r > 0.86$).

Enzymatic Production of H_2O_2

To establish whether FSCV could be used to detect enzymatically generated H_2O_2, a carbon fiber microelectrode was modified with glucose oxidase, an oxidoreductase that reduces molecular oxygen to H_2O_2 as a by-product in the highly specific conversion of glucose to gluconolactone. Glucose is a non-electroactive species and thus it cannot be directly detected by FSCV at a bare electrode, as shown in Figure 8 (dashed black line). However, when a glucose sample (125 μM) was introduced to the enzyme-modified microelectrode, a cyclic voltammogram was detected with an oxidation peak occurring at 1.2 V on the cathodic scan (solid black line), consistent with the cyclic voltammogram for H_2O_2 at a bare carbon electrode (solid gray line). This demonstrates that the voltammetric detection of H_2O_2 can be used at enzyme-modified carbon fiber microelectrodes to indirectly monitor nonelectroactive species.

CONCLUSIONS

The voltammetric detection of H_2O_2 at carbon electrodes is challenging due to the slow electron transfer kinetics associated with the irreversible oxidation of peroxide. By using the anodic scan as an electrochemical pretreatment, a rapid, sensitive and selective voltammetric method has been developed for the detection of physiological concentrations of H_2O_2 at uncoated carbon fiber microelectrodes. This strategy will allow for the development of improved biosensors with wide applicability for a variety of biological analytes.

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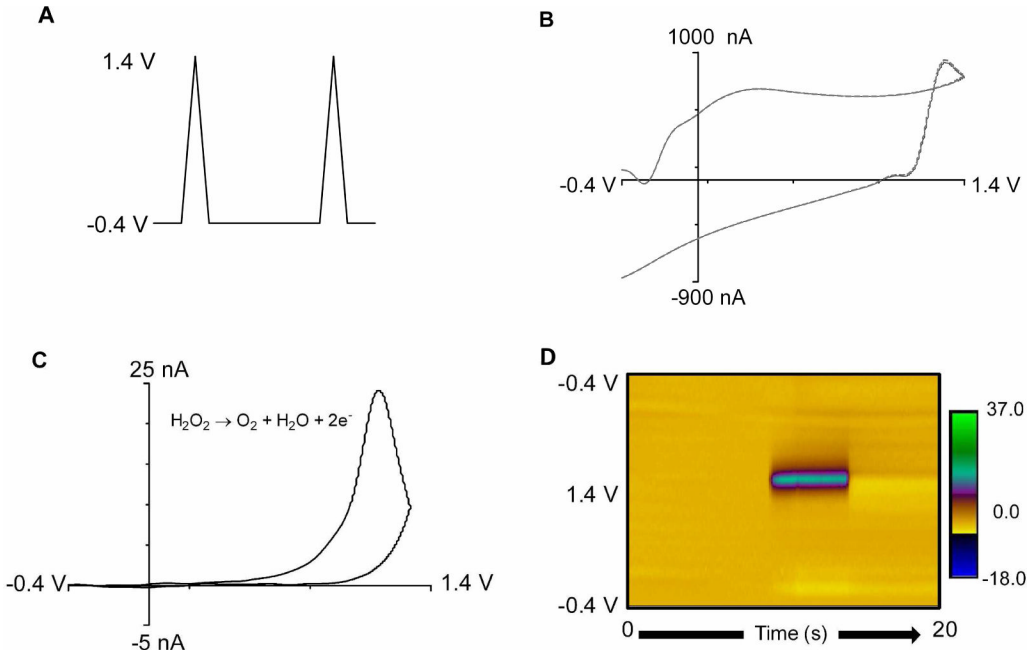

Figure 1.

Fast-scan cyclic voltammetry of H_2O_2. (A) The applied potential was scanned from -0.4 V to 1.4 V and back at 400 V·sec^{-1} every 100 msec. (B) Background current at the carbon surface produced by the rapid scan (solid line). This changed only slightly after 100 μM H_2O_2 was added (dashed line). (C) Background-subtracted cyclic voltammogram of 100 μM H_2O_2 at pH 7.4. At this scan rate the oxidation peak is detected at 1.2 V on the cathodic scan. (D) Color plot containing 200 background-subtracted cyclic voltammograms recorded over 20 sec. The ordinate is the applied potential to the carbon-fiber electrode, the abscissa is time, and the current (nA) is depicted in false color.

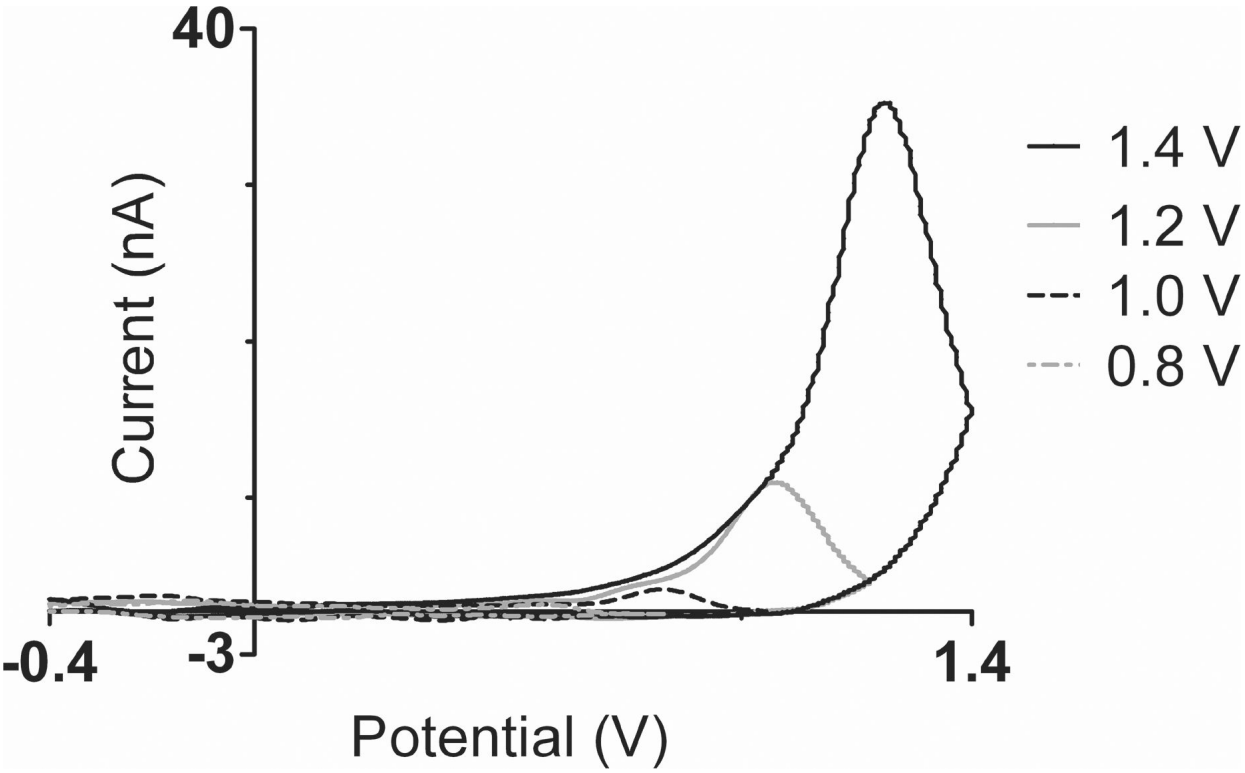

Figure 2.

An oxidized electrode surface is required to accommodate the irreversible oxidation of H_2O_2. A significant H_2O_2 oxidation peak was observed only at switching potentials greater than 1.2 V. The amplitude of the oxidation current increased as the potential limit was extended, with maximum sensitivity observed using a potential limit of 1.4 V.

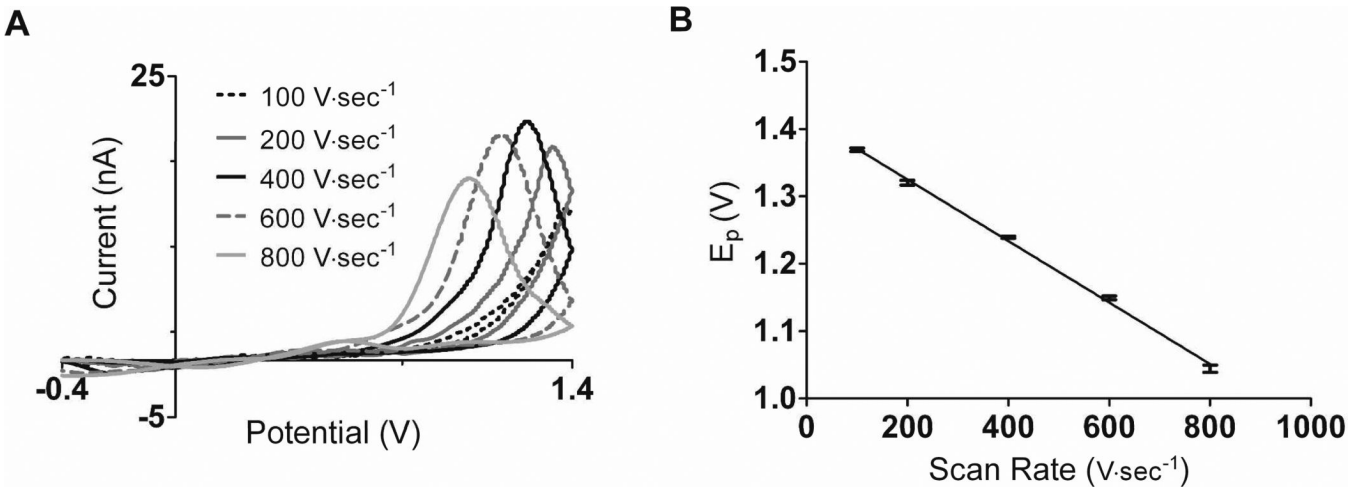

Figure 3.

Effect of various scan rates ($V \cdot sec^{-1}$) on H_2O_2 cyclic voltammetry. (A) Cyclic voltammograms for 100 μM H_2O_2 at typical scan rates for *in vivo* measurements. The oxidation current was maximized at 400 $V \cdot sec^{-1}$. (B) Peak oxidation potential, E_p (V), versus scan rate ($V \cdot sec^{-1}$). $r^2 = 0.99$. Error bars are the SEM (n = 4 electrodes).

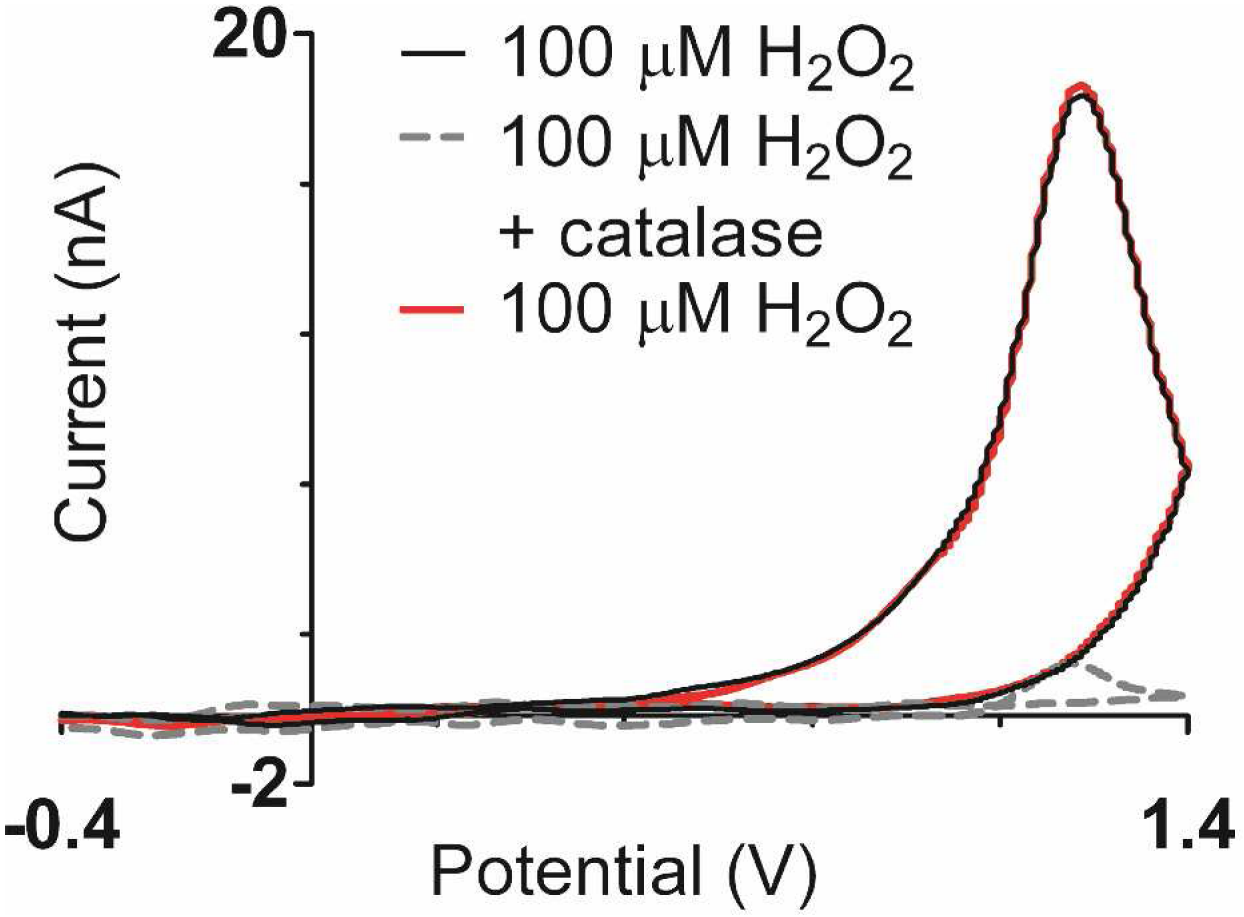

Figure 4.
Enzymatic degradation of H_2O_2. Cyclic voltammograms for H_2O_2 (100μM) before (solid black) and 5 min. after (dashed) the addition of catalase (1U/mL) to the sample. A fresh sample of 100 μM H_2O_2 (red) demonstrates that the integrity of the electrode was not compromised.

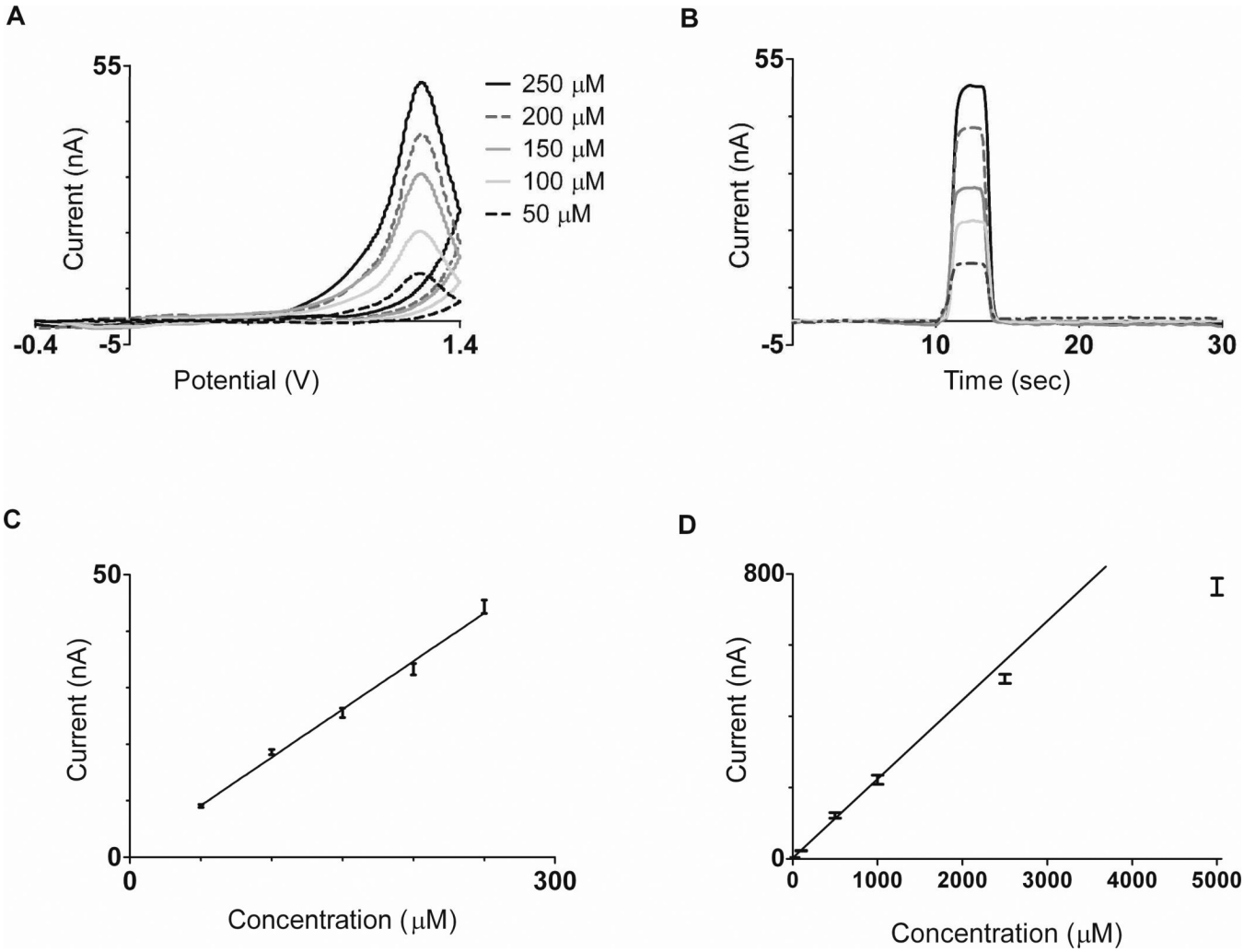

Figure 5.

Characterization of H_2O_2 electrochemistry. (A) Cyclic voltammograms for H_2O_2 solutions ranging from 50 μM to 250 μM. All peak currents occur at 1.2 V. (B) Current (nA) versus time (sec) plot of the same five samples, demonstrating the rapid response time of the electrode. (C) Linear relationship between peak current (nA) versus H_2O_2 concentration (μM). $r^2 = 0.95$, n = 6 electrodes. (D) Current (nA) versus concentration (μM) plot to show linear range. Deviation from linearity occurs at concentrations greater than 2 mM.

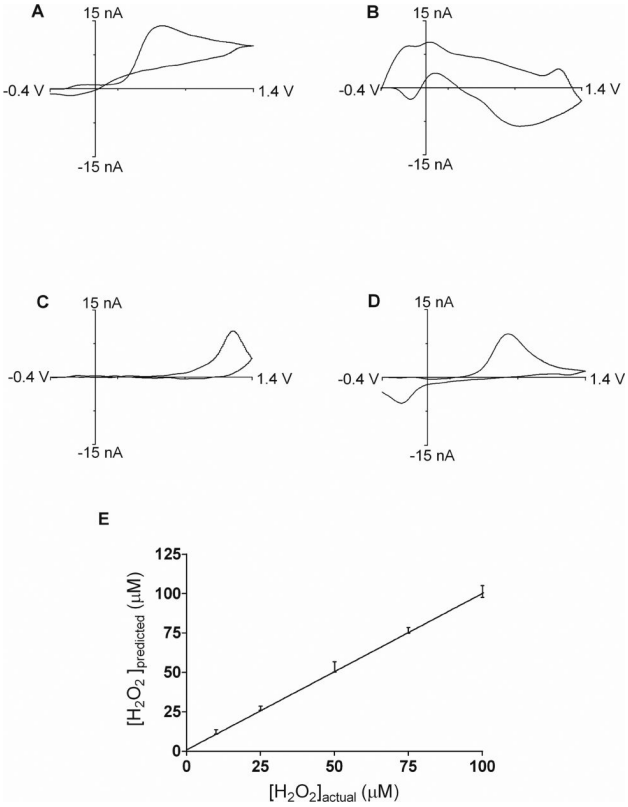

Figure 6.

Selectivity and sensitivity in the presence of multiple interferents. (A-D) Cyclic voltammograms of possible *in vivo* interferents. (A) 50 μM ascorbate (B) 0.2 basic pH shift (7.6) (C) 50 μM H_2O_2 (D) 500 nM dopamine. (E) Predicted concentrations of H_2O_2 as evaluated by PCR compared to known sample concentrations. The diagonal line represents a regression line of 1 to show data variance. The data regression line (not shown) is 0.99 ± 0.03 (r^2= 0.97). Error bars represent the mean ± SEM (n=6 electrodes).

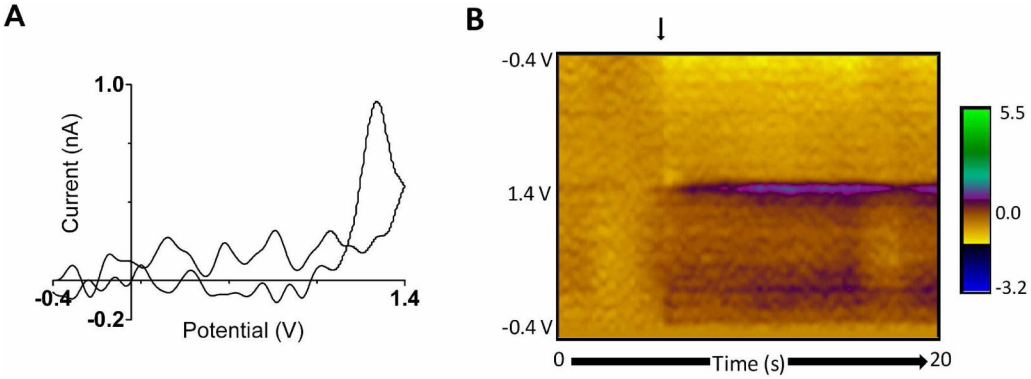

Figure 7.

H_2O_2 detection in a brain slice containing the striatum. A) A background-subtracted cyclic voltammogram collected at the maximum of the current response. The signal corresponds to those collected for H_2O_2 in the *in vitro* flow cell (r>0.86). B) The color plot contains 200 background-subtracted cyclic voltammograms recorded over 20 sec, and allows visualization of interfering species. The ordinate is the applied potential to the carbon-fiber electrode, the abscissa is time, and the current is depicted in false color.

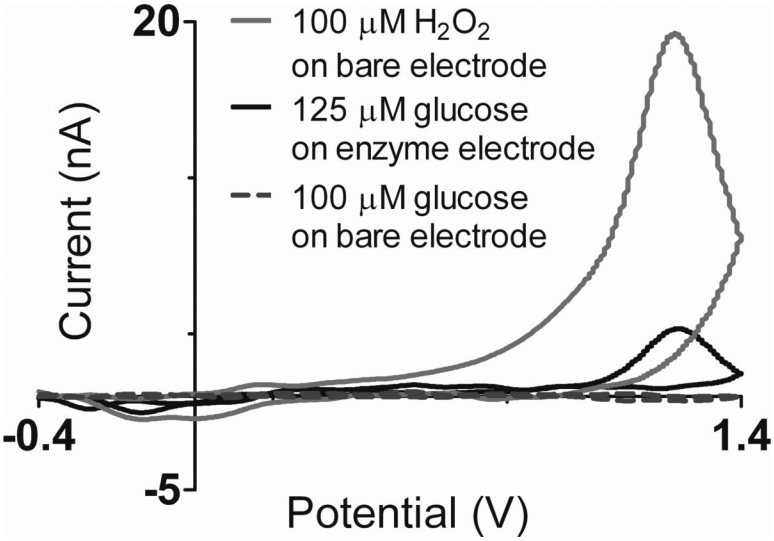

Figure 8.

Enzymatic production of H_2O_2 at a glucose oxidase modified carbon fiber electrode. Cyclic voltammograms of H_2O_2 (grey) and glucose (black, dashed) on a bare carbon-fiber electrode compared to the detection of H_2O_2 produced by enzymatic degradation of glucose (black, solid) at an enzyme-modified electrode.