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Amperometric Detection of Dopamine in Vivo with an Enzyme Based Carbon Fiber Microbiosensor

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We developed a novel implantable enzyme-based carbon fiber biosensor for in vivo monitoring of dopamine. The biosensor is fabricated using tyrosinase immobilized in a biocompatible matrix consisting of a biopolymer, chitosan and ceria-based metal oxides, deposited onto the surface of a carbon fiber microelectrode with a diameter of ~100 μm. Tyrosinase catalyzes the conversion of dopamine to *o*-dopaquinone, and the reduction of *o*-dopaquinone, which requires a low potential difference, was detected electrochemically. The role of each component in the sensing layer was systematically investigated in relation to the analytical performance of the biosensor. In its optimal configuration, the biosensor demonstrated a detection limit of 1 nM dopamine, a linear range of 5 orders of magnitude between 10 nM and 220 μM, a sensitivity of 14.2 nA·μM^{-1}, and good selectivity against ascorbic acid, uric acid, serotonin, norepinephrine, epinephrine, and 3,4-dihydroxy-L-phenylalanine (L-DOPA). The system provided continuous, real time monitoring of electrically stimulated dopamine release in the brain of an anesthetized rat. Levels of dopamine up to 1.69 μM were measured. This new implantable dopamine biosensor provides an alternative to fast scan cyclic voltammetry for in vivo monitoring of dopamine.

Dopamine is a catecholamine neurotransmitter widely present in the central nervous system, where it modulates several aspects of the brain circuitry. It influences a variety of motivated behaviors, attention span, and neuronal plasticity and plays a critical role in learning and memory.[1,2] Low levels of dopamine have been implicated in pathogenesis of neurological disorders such as Parkinson's disease and schizophrenia.[1,3,4] Given the wide range of physiological and pathophysiological effects of dopamine, precise and accurate measurement of dopamine and its metabolites in biological systems is of great clinical importance. Traditionally, analytical measurement of dopamine has been achieved using in vivo microdialysis to sample dopamine in the extracellular space[4,5] followed by electrochemical,[6] coulometric[7] or fluorescence[8] detection of dopamine in the dialysate. Microdialysis requires a two step process in which the substance of interest is first extracted from the dialysate and purified using capillary electrophoresis[6] and chromatography,[7,9] before the actual measurement. The method is slow and can only measure dopamine fluctuations on the time scale of many minutes to hours. Thus, it is unsuitable for online in situ measurements where dopamine levels are known to fluctuate on the time scale of fractions of seconds.[1] An alternative is to use electrochemical detection methods. Dopamine is electrochemically active and can be measured directly using an electrode held at an appropriate potential. Several electrochemical methods for rapid online in vivo analysis of dopamine with carbon fiber microelectrodes have been described including amperometry,[10] differential pulse voltammetry,[11] and fast scan cyclic voltammetry (FSCV).[3,12]

However, direct electrochemical detection of dopamine has several major limitations including the need for background subtraction and the occurrence of electrode passivation. The oxidation potential for dopamine is large (between 0.5 and 0.7 V) and encompasses the oxidation range of many other substances in the central nervous system. For this reason, it can be difficult to differentiate dopamine from multiple other catecholamine neurotransmitters and from other co-occurring interfering species (e.g., ascorbic acid, nitric oxide, etc.).[13,14] A common strategy to eliminate interferences is to cover the electrode with permselective membranes such as nafion[15,16] or a combination of nafion, metal tetraaminophthalocyanine,[15] and hexacyanoferrates[17] that limit access of the interfering agents to the reactive surface of the

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(1) Robinson, D. L.; Venton, B. J.; Heien, M. L. A. V.; Wightman, R. M. *Clin. Chem.* **2003**, *49*, 1763–1773.
(2) Hefco, V.; Yamada, K.; Hefco, A.; Hritcu, L.; Tiron, A.; Nabeshima, T. *Eur. J. Pharmacol.* **2003**, *475*, 55–60.
(3) Swamy, B. E. K.; Venton, B. J. *Analyst* **2007**, *132*, 876–884.
(4) Wightman, R. M.; May, L. J.; Michael, A. C. *Anal. Chem.* **1988**, *60*, 769A–779A.
(5) Hernández, G.; Shizgal, P. *Behav. Brain Res.* **2009**, *198*, 91–97.
(6) Mecker, L. C.; Martin, R. S. *Anal. Chem.* **2008**, *80*, 9257–9264.
(7) Myers, R. D.; Adell, A.; Lankford, M. F. *Neurosci. Biobehav. Rev.* **1998**, *22*, 371–387.
(8) Nikolelis, D. P.; Drivelos, D. A.; Simantiraki, M. G.; Koinis, S. *Anal. Chem.* **2004**, *76*, 2174–2180.
(9) Sabbioni, C.; Saracino, M. A.; Mandrioli, R.; Pinzauti, S.; Furlanetto, S.; Gerra, G.; Raggi, M. A. *J. Chromatogr., A* **2004**, *1032*, 65–71.
(10) Yavich, L.; Tiihonen, J. *Neurosci. Lett.* **2000**, *293*, 41–44.
(11) Valentini, F.; Palleschi, G.; Lopez Morales, E.; Orlanducci, S.; Tamburri, E.; Terranova, M. L. *Electroanalysis* **2007**, *19*, 859–869.
(12) Cheer, J. F.; Wassum, K. M.; Sombers, L. A.; Heien, M. L. A. V.; Ariansen, J. L.; Aragona, B. J.; Phillips, P. E. M.; Wightman, R. M. *J. Neurosci.* **2007**, *27*, 791–795.
(13) Cao, X.; Luo, L.; Ding, Y.; Zou, X.; Bian, R. *Sens. Actuators, B* **2008**, *129*, 941–946.
(14) Zou, X.; Luo, L.; Ding, Y.; Wu, Q. *Electroanalysis* **2007**, *19*, 1840–1844.
(15) Kang, T.-F.; Shen, G.-L.; Yu, R.-Q. *Anal. Chim. Acta* **1997**, *354*, 343–349.
(16) Jeong, H.; Jeon, S. *Sensors* **2008**, *8*, 6924–6935.
(17) Castro, S. S. L.; Mortimer, R. J.; de Oliveira, M. F.; Stradiotto, N. R. *Sensors* **2008**, *8*, 1950–1959.

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sensor. For example, nafion is anionic, and a nafion electrode coating has been used to exclude negatively charged interfering agents such as ascorbate[16] and nitrite. An alternative strategy to enhance electrode sensitivity and restrict detection of interfering agents is to lower the operating potential difference of the electrode by the use of an enzyme recycling approach. Tyrosinase catalyzes the conversion of dopamine to *o*-dopaquinone, and dopamine can be detected by electrochemical reduction of *o*-dopaquinone at a relatively low holding potential (∼−150 mV), which significantly reduces redox reactions with interfering substances.

Traditionally, tyrosinase catalyzes the conversion of phenol and more complex phenolic compounds to their respective quinone derivatives.[18] Following the same principle, dopamine, which is a catechol-like phenolic compound, can be detected using a tyrosinase-based system.[19−21] The development of several tyrosinase sensors for dopamine has been reported, but they all had a reduced sensitivity.[21−27] As a result, strategies to increase the sensitivity of tyrosinase biosensors are necessary in order to measure dopamine in the concentration range found in vivo. Most tyrosinase-based biosensors for dopamine described to date are characterized by detection limits in the micromolar range and relatively large electrode size. Moreover, their use has not been demonstrated in vivo.[20−22,24,25,27]

Njagi et al.[22] recently described a tyrosinase biosensor for the detection of phenolic compounds, including dopamine, using a ceria−titania biocomposite. This biosensor has a low detection limit, an extended linear range, high sensitivity, and low oxygen dependency. The improved performance of the ceria−titania containing biosensor was attributed to the unique catalytic properties and redox behavior of the ceria-based oxides, their oxygen storage capacity, and their ability to shift between Ce^{3+}/Ce^{4+}oxidation states.[28−30] In the present work, we report the development of an implantable tyrosinase-based biosensor for dopamine and demonstrate the applicability of this sensor to make real-time in vivo measurements of dopamine in the brain of anesthesized rats during high frequency stimulation of the subthalamic nucleus. The biosensor consists of a carbon fiber microelectrode coated with a biocatalytic layer containing tyrosinase embedded in a chitosan biopolymer matrix and a mixture of ceria−titania. A silica sol−gel was also studied as an alternative immobilization matrix. Compared to the FSCV method commonly used for in vivo measurement of dopamine,[1,4,12,31,32] the tyrosinase-based biosensor provided several advantages such as reduced electrode passivation due to the enzymatic/electrochemical cycling of the *o*-dopaquinone at the electrode surface, low detection limits, and low operating potential, which eliminated many common interferences. The biosensor does not contain any other coating membranes, and the materials used are biocompatible, making this sensor particularly attractive for in vivo applications.

EXPERIMENTAL SECTION

Materials. Tyrosinase (EC.1.14.18.1, 3900 U mg^{-1} of solid), dopamine hydrochloride (analytical grade), chitosan (from crab shells; practical grade), cerium(IV) oxide (CeO_2, nanopowder), uric acid, norepinephrine, titanium(IV) oxide (TiO_2, nanopowder, 99.5% rutile), and nomifensine maleate salt were purchased from Sigma (St Louis, MO) and used as received. Carbon wire and Teflon coated silver wire (125 μM OD) were purchased from WPI (World Precision instruments Inc., Sarasota, FL). Ascorbic acid and sodium phosphate (monobasic) were obtained from Fisher Scientific. Sodium phosphate (dibasic, anhydrous) and acetic acid (glacial) were purchased from J. T. Baker (Phillipsburg, NJ). Silver conductive epoxy was obtained from MG Chemicals. Tetramethyl orthosilicate (TMOS) and methyltrimethoxy silane (MTMOS) were obtained from Fluka. We purchased serotonin, 3,4-dihydroxy-L-phenylalanine and dihydroxyphenylacetic acid from Acros Organics and epinephrine from MP Biomedicals. Ketamine/xylazine was obtained through the Dartmouth Medical School animal research facility. All experiments involving tyrosinase were carried out in 0.1 M phosphate buffer solution (PB) at an optimized pH of 6.5. Distilled, deionized water (Millipore, Direct-Q system) with a resistivity of 18.2 MΩ·cm was used for the preparation of the buffer solutions.

Instrumentation. Cyclic voltammetry and amperometric experiments were performed with a CH Instrument Electrochemical Analyzer Potentiostat (CH instruments Inc., Austin, TX). Optimization experiments were carried out using a conventional electrochemical cell with the enzyme modified carbon fiber microelectrode, and all potentials were measured versus a Ag/AgCl reference electrode. Cyclic voltammetric experiments were carried out using a Ag/AgCl wire atop the carbon fiber working electrode as the reference electrode, a platinum wire (BAS MW-1032) as the counter electrode, and a modified carbon fiber electrode (CF) as the working electrode.

Preparation of Carbon Fiber Microelectrodes and Biosensor Fabrication. A carbon wire (∼100 μm) was glued in series to a copper wire using a conductive silver paste and aspirated into a pulled glass capillary tube. Both ends of the capillary tube were sealed using an epoxy resin and cured at a temperature of 120 °C for 5 min. The carbon fiber tip extending from the glass pipet was cut using a scalpel so that it extended up to ∼1.5 mm from the glass seal. About 1 mm of the Teflon coated Ag wire was exposed and chloride-treated to form a Ag/AgCl tip. The Ag/AgCl tip was placed about 2 mm from the carbon fiber tip, and the remainder of the silver reference electrode was glued on to the capillary tube all the way to the electrode terminal. Before modification with the enzymatic layer, microelectrodes were

(18) Andreescu, S.; Sadik, O. A. *Anal. Chem.* **2004**, *76*, 552–560.
(19) Liu, A.; Honma, I.; Zhou, H. *Biosens. Bioelectron.* **2005**, *21*, 809–816.
(20) Tsai, Y.-C.; Chiu, C.-C. *Sens. Actuators, B* **2007**, *125*, 10–16.
(21) Zhou, Y. L.; Tian, R. H.; Zhi, J. F. *Biosens. Bioelectron.* **2007**, *22*, 822–828.
(22) Njagi, J.; Ispas, C.; Andreescu, S. *Anal. Chem.* **2008**, *80*, 7266–7274.
(23) Cosnier, S.; Innocent, C.; Allien, L.; Poitry, S.; Tsacopoulos, M. *Anal. Chem.* **1997**, *69*, 968–971.
(24) Tembe, S.; Kubal, B. S.; Karve, M.; D'Souza, S. F. *Anal. Chim. Acta* **2008**, *612*, 212–217.
(25) Tembe, S.; Karve, M.; Inamdar, S.; Haram, S.; Melo, J.; D'Souza, S. F. *Anal. Biochem.* **2006**, *349*, 72–77.
(26) Tsai, Y. C.; Chiu, C. C. *Sens. Actuators, B: Chemical* **2007**, *125*, 10–16.
(27) Moghaddam, A. B.; Ganjali, M. R.; Niasari, M.; Ahadi, S. *Anal. Lett.* **2008**, *41*, 3161–3176.
(28) Sinha, A. K.; Suzuki, K. *J. Phys. Chem. B* **2005**, *109*, 1708–1714.
(29) Ispas, C.; Njagi, J.; Cates, M.; Andreescu, S. *J. Electrochem. Soc.* **2008**, *155*, F169–F176.
(30) Khan, R.; Dhayal, M. *Electrochem. Commun.* **2008**, *10*, 263–267.
(31) Robinson, D. L.; Hermans, A.; Seipel, A. T.; Wightman, R. M. *Chem. Rev.* **2008**, *108*, 2554–2584.
(32) Robinson, D. L.; Venton, B. J.; Heien, M. L. A. V.; Wightman, R. M. *Clin. Chem.* **2003**, *49*, 1763–1773.

electrochemically treated by repeated over oxidation in 0.1 M PB pH 6.5 in the potential range −0.4 to +1.4 V at 500 V/s until the magnitude of the measured current reached 3 μA.

Preparation of 1.0% chitosan (w/v) and CeO_2/TiO_2 dispersions were as described elsewhere.[22] CeO_2/TiO_2 dispersion (1 μL) was mixed with 1 μL of 200 U/μL tyrosinase (prepared in 0.1 M PB, pH 6.5) and 2 μL of chitosan solution and mixed thoroughly for 5 min. The composite consisting of CeO_2/TiO_2 and chitosan was denoted as CeO_2/TiO_2/Chit. Two aliquots of 2 μL of this aqueous dispersion were sequentially drop coated onto the carbon fiber surface. The first aliquot was allowed to dry before drop coating the second aliquot. After coating, the carbon fiber was allowed to dry for 20 min. To create a control electrode, a similar electrode was fabricated, but the CeO_2/TiO_2 was left out of the tyrosinase/chitosan mixture. A sol−gel solution was prepared by mixing 100 μL of TMOS and 100 μL of MTMOS with 400 μL of deionized water, 440 μL of 1 mM HCl, and 40 μL of PEG 600. This mixture was sonicated for 15 min and stored overnight at 4 °C.[33] The electrodes were thoroughly rinsed with PB before the electrochemical measurements were taken and stored in PB when not in use.

Testing Interferences and Analytical Characterization of the Dopamine Biosensor. The dopamine biosensor was held at −0.15 V, and 200 μM ascorbic acid, 200 μM uric acid, 100 μM serotonin, 50 μM L-DOPA, 5 μM DOPAC, 10 μM norepinephrine, and 10 μM epinephrine were injected in the electrochemical reaction cell consecutively. The response obtained was compared to the response to the addition of 5 μM dopamine. To make sure ascorbic acid was not interfering with the measurements of other catecholamines, selectivity of the biosensor to the interferences was determined individually in the absence of any other interference. The limit of detection was determined according to the $3S_b/m$ criterion,[34] where m is the slope of the linear calibration plot and S_b is the relative standard deviation of the amperometric signal of the blank ($n = 5$).

Animal Studies. The experiments were approved by the Institutional Animal Care and Use Committee of Dartmouth College in accordance with National Institute of Health guidelines for use of animals in research. We studied three Sprague−Dawley rats weighing between 150 and 200 g. The rats were housed in a temperature controlled room (21 °C) under a 12/12 light/dark cycle (light on at 08:00 a.m.). The rats had ad libitum access to food pellets and water prior to surgery.

In Vivo Measurements. Male Sprague−Dawley rats weighing 150−200 g were anesthetized with ketamine/xylazine (100/10 mg/kg) and placed in a stereotaxic apparatus. Supplemental injections of ketamine/xylazine were delivered as needed to maintain an adequate level of anesthesia. A midline scalp incision was made; the skull was exposed, and holes were drilled in the skull. The microbiosensor was inserted unilaterally in the striatum at the following stereotaxic coordinates measured from bregma: +1.2 AP, +2.0 ML, and −4.6 to −6.0 DV. A bipolar stimulating electrode was implanted near the median forebrain bundle at coordinates −4.4 AP, +0.8 ML, and −8.0 to −9.0 DV. The dorsoventral placement of the stimulating electrode was adjusted to maximize dopamine release. Stimulation sequences were generated using a Master-8 programmable stimulator coupled to an Iso-Flex optical isolator (A.M.P.I., Jerusalem, Israel). The stimulator delivered a train of pulses at 150 Hz with a pulse width of 60 μS and 200 μA in magnitude for a total duration of 120 s. Blocking of dopamine reuptake was done by treating the rats with nomifensine maleate (5 mg/kg) 30−40 min prior to in vivo measurements.

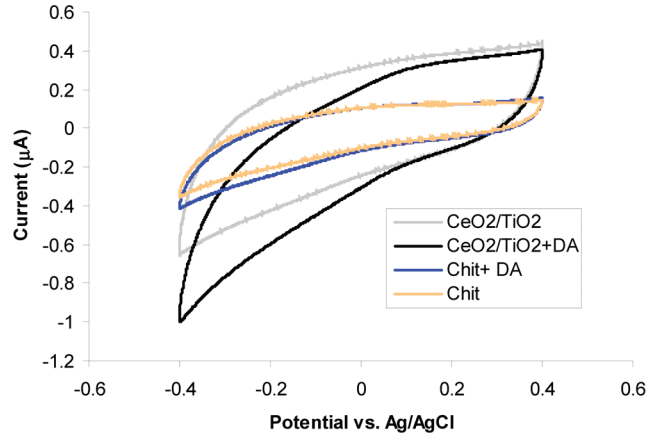

Figure 1. Cyclic voltammograms of tyrosinase modified carbon fiber electrode based on chitosan/CeO_2/TiO_2 (A) and chitosan (B) in the absence and presence of 100 μM dopamine (DA) at a scan rate of 100 mV s^{-1}

At the conclusion of each experiment, the brain was removed and fixed in formaldehyde (4%) in PB for 48 h. Each brain was cryoprotected in 30% sucrose for ~48 h and frozen at −70 °C in Tissue-Tek (Sakura Finetek U.S.A., Inc., Torrance, CA). Subsequently, 50 μm coronal sections were cut through the basal ganglia on a cryostat (Leica CM3050, Leica Microsystems, Inc., Deerfield, IL). The sections were mounted on glass slides and counter stained with cresyl violet. The locations of the stimulating electrode and dopamine biosensor were identified under light microscopy from the insertion track and tissue damage caused by the tips of the stimulating electrode and biosensor and marked on schematic coronal sections of the brain.[35]

RESULTS AND DISCUSSION

Electrochemical Characterization. The tyrosinase-based carbon fiber microelectrodes were first characterized using cyclic voltammetry to test their response to dopamine and identify the role of the metal oxides in the enzyme immobilization matrix. Figure 1 shows the cyclic voltammograms of the enzyme−carbon fiber microelectrodes in the presence and absence of the metal oxide using a chitosan composite mixture as an immobilization matrix. The biosensor responded to dopamine in both the absence and presence of metal oxides. However, in the absence of metal oxides, a slight increase in the "dopamine" reduction current was observed starting from a potential around −100 mV vs Ag/AgCl, which is due to the reduction of *o*-dopaquinone generated in the enzyme catalyzed reaction. The biocompatibility and high porosity of chitosan facilitated loading large amounts of enzyme and also helped retain biocatalytic activity of the enzyme by virtue of

(33) Andreescu, S.; Barthelmebs, L.; Marty, J.-L. *Anal. Chim. Acta* **2002**, *464*, 171–180.

(34) Currell, C. *Analytical Instrumentation Performmance Characteristics and Quality*, 1st ed.; John Wiley and Sons Ltd.: Dartford, U.K., 2000.

(35) Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*, 4th ed.; Academic Press: San Diego, CA, 1998.

Table 1. Summary Table Showing the Analytical Performance Characteristics for the Electrochemical Detection of Dopamine by the Different Carbon Fiber (CF) Microelectrode Configurations[a]

immobilization matrix	biosensor configuration	detection limit (M)	sensitivity (mA M^{-1})	linear range ($M \times 10^{-6}$)	response time (s)
chitosan	tyrosinase/TiO_2/CeO_2/Chit–CF	$1.1(\pm0.1) \times 10^{-9}$	14.2 ± 0.50	0.01–220	<8
	TiO_2/CeO_2/Chit–CF	$1.4(\pm0.2) \times 10^{-7}$	1.7 ± 0.05		~63
	tyrosinase/Chit–CF	$5.3(\pm0.30) \times 10^{-9}$	7.3 ± 0.09	0.05–120	<8
sol–gel	tyrosinase/TiO_2/CeO_2/sol–gel–CF	$4.0(\pm0.2) \times 10^{-8}$	33.4 ± 1.4	0.1–180	~20
	tyrosinase/sol–gel–CF	$8.0(\pm0.3) \times 10^{-8}$	6.4 ± 0.60	0.2–260	~25

[a] Standard deviation from $n = 5$ electrodes prepared in identical conditions.

favorable electrostatic binding to the chitosan amino groups.[36] In the presence of metal oxides, the background capacitive current increases in comparison to the Chit−carbon fiber electrode due to the presence of the metal oxides. When dopamine was added, a significant increase in the reduction current was observed, which was proportional to the amount of dopamine added. The greater response may be attributed to a mediating or catalytic effect of the mixed oxides and may reflect the presence of different oxidation states and reactive species of ceria and their ability to switch between Ce^{3+}/Ce^{4+} under redox conditions.[37] The formation of hydrogen bonds between Ti−O and Ce−O and the amino groups of the chitosan, reported previously for a chitosan/titania horseradish peroxidase electrode,[30] may also contribute to the observed current enhancement.

Biosensor Fabrication and Characterization. To characterize the sensor and select the optimum configuration for in vivo dopamine measurements, four tyrosinase−carbon fiber microelectrodes were fabricated with and without CeO_2/TiO_2 metal oxides (Table 1). The first two were based on chitosan (Chit), which was selected for its biocompatibility and good film-forming properties, strong adhesion, and high mechanical strength.[36] We also investigated a silica sol−gel immobilization matrix instead of chitosan. Sol−gel materials are biocompatible and have good adhesion and favorable electrical properties. In addition, these materials have been used as coatings in implantable devices for drug delivery[38−40] and in neural microelectrodes.[41] All experiments were carried out at an applied potential of −150 mV vs Ag/AgCl, which provides the highest sensitivity for the electrochemical detection of *o*-dopaquinone.[22] Optimization of experimental variables, including pH, enzyme loading, effect of metal oxide, and effect of interferences was performed with the chitosan-based and sol−gel-based carbon fiber microelectrodes.

Analytical Characteristics: Sensitivity, Detection Limit, and Response Time. The analytical performance of the tyrosinase−carbon fiber electrodes with both chitosan and sol−gel in the absence and presence of the metal oxides was evaluated by constant potential amperometry. For both chitosan and sol−gel, inclusion of the metal oxides in the immobilization matrix provided significantly higher sensitivity for dopamine. For example, in the case of chitosan, the senstivity was 14.2 (±0.50) nA/μM in the presence of metal oxides but only 7.3 (±0.09) nA/μM in the absence of metal oxides. The detection limits of these biosensors were 1 and 5 nM, respectively, and the linear range spanned over 4 orders of magnitude from nanomolar to high micromolar dopamine concentrations for both types of biosensors. The detection limit and linear range of the chitosan containing sensors were about 1 order of magnitude better than those reported when an amphiphilic pyrrole monomer was used to entrap tyrosinase on a carbon fiber microelectrode.[23] The response time (t_{90} = time to reach 90% of the maximum response) for all chitosan-based configurations was <8 s. The typical amperometric responses and the calibration curves for the tyrosinase carbon fiber microelectrodes with and without metal oxides are presented in Figure 2. The analytical characteristics of the sensors are summarized in Table 1. When the sol−gel matrix was used, the sensitivity of the biosensors was higher. However, the sol−gel-based bioactive layer was extremely fragile, and the matrix mixture fell off the electrode under low applied mechanical stresses, suggesting that the coating would peel off during insertion into intact animals. Moreover, the response time increased from <8 s to ~20 s due to higher diffusion barriers imposed by this matrix. Since dopamine transients are known to occur in the second to subsecond time scale,[1] this biosensor would be inadequate for monitoring dopamine fluctuations on a biologically meaningful time scale and would be difficult to implant due to the limited mechanical stability. Thus, the chitosan matrix was selected to construct the microbiosensors for monitoring dopamine in vivo.

Ascorbic acid (AA) has been reported to reduce *o*-dopaquinone,[42] the enzyme transduction molecule in this biosensor architecture. To investigate if AA would interfere with dopamine measurements in vivo, the sensitivity of the biosensor in the presence and absence of AA was compared. In the absence of AA, the sensitivity of the biosensor was 14.2 (±0.5) nA/μM while in the presence of AA it was 13.3 (±0.7) nA/μM. The difference in sensitivity lies within the margin of error (~5%), which indicates that the biosensor can perform effectively in the presence of AA.

Enzyme Loading. The enzyme coating deposited onto the tip of a carbon fiber with a diameter of 100 μm creates a very small active surface area for dopamine detection. Therefore, we studied the effect of loading different enzyme concentrations on the

(36) Wang, S. F.; Tan, Y. M.; Zhao, D. M.; Liu, G. D. *Biosens. Bioelectron.* **2008**, *23*, 1781–1787.
(37) Dutta, P.; Pal, S.; Seehra, M. S.; Shi, Y.; Eyring, E. M.; Ernst, R. D. *Chem. Mater.* **2006**, *18*, 5144–5146.
(38) Lopez, T.; Manjarrez, J.; Rembao, D.; Vinogradova, E.; Moreno, A.; Gonzalez, R. D. *Mater. Lett.* **2006**, *60*, 2903–2908.
(39) Gerritsen, M.; Kros, A.; Sprakel, V.; Lutterman, J. A.; Nolte, R. J. M.; Jansen, J. A. *Biomaterials* **2000**, *21*, 71–78.
(40) Salgado, C. L.; Turchetti-Maia, R. M. M.; Pereira, M. M.; Salas, C. E.; Francischi, J. N.; Lopes, M. T. P. *Bioceramics 18, Pts 1 and 2* **2006**, *309–311*, 1035–1038.
(41) Pierce, A. L.; Sommakia, S.; Rickus, J. L.; Otto, K. J. *J. Neurosci. Methods* **2009**, *180*, 106–110.
(42) Ronit, F.; Johann, E.; Ron, G.; Maya, Z.; Itamar, W. *Chem.−Eur. J.* **2007**, *13*, 7288–7293.

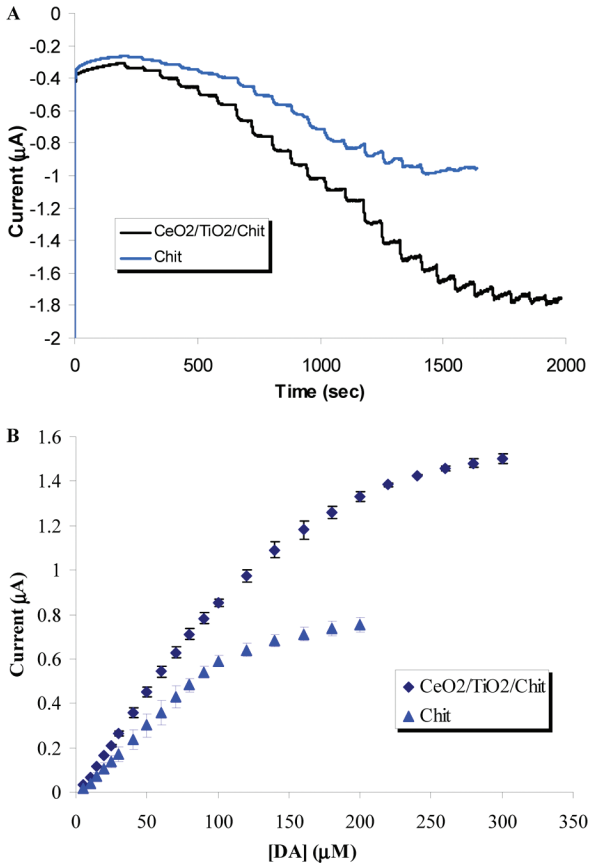

Figure 2. Amperometric responses (A) and the corresponding calibration curves (B) of tyrosinase-based CeO_2/TiO_2/Chit−carbon fiber and Chit−carbon fiber biosensors in aerated buffer solutions (standard deviation from $n = 3$ measurements).

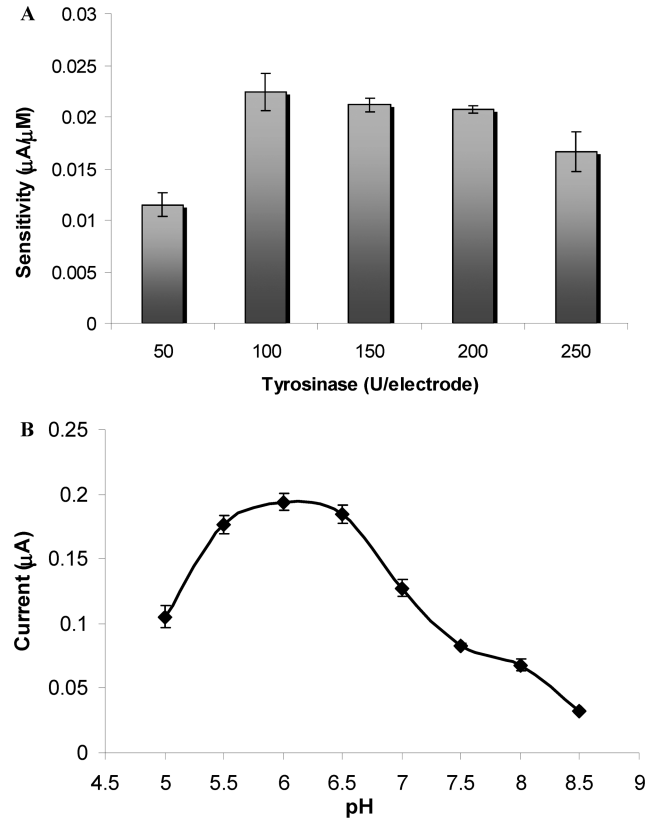

Figure 3. (A) Effect of tyrosinase loading (U/electrode) on the sensitivity of the CeO_2/TiO_2/Chit−carbon fiber microelectrode in oxygenated buffer solutions (standard deviation from $n = 3$ measurements). (B) Effect of pH on the intensity of the current of the CeO_2/TiO_2/Chit−carbon fiber microelectrode to injections of 5 μM dopamine ($n = 3$).

sensitivity of the biosensor. Figure 3A shows the variation of the average sensitivity obtained for biosensors constructed with 50, 100, 150, 200, and 250 U per microelectrode. The optimum sensitivity was achieved with 100 U tyrosinase per electrode. Higher amounts of enzyme did not improve biosensor performance. Higher enzyme concentrations may have saturated the available microelectrode surface and changed the diffusion and permeability characteristics of the layer, making the enzyme less accessible to the substrate or the carbon fiber less accessible to the reaction product. It is worth mentioning that metal oxides facilitate stabilization of the enzyme onto the electrode surface through strong electrostatic interactions.[22,30,43,44] However, when more than 150 U of tyrosinase was loaded onto the electrode, the enzyme leached into the solution, and these sensors were characterized by poor reproducibility of sensitivity.

Effect of pH. The effect of pH on the tyrosinase biosensor was evaluated by measuring the response of the sensor to injection of 10 μM of dopamine in PB at pH values ranging from 5 to 8.5. The optimal biosensor response was found in a pH range from 5.5 to 6.5 (Figure 3B). This optimum pH range is similar to that reported in the literature for other tyrosinase biosensors.[45,46] Since the sensor was being optimized for in vivo measurements, a pH of 7.0 was selected for further analytical characterization.

Control Experiments in the Absence of Enzyme. Due to the known catalytic activity of both ceria and titania with respect to phenolic compounds,[47−49] it was necessary to evaluate the contribution of ceria and titania to the catalytic effect on the sensor response. For this purpose, control experiments were performed with sensors that were fabricated with CeO_2/TiO_2 but no tyrosinase. The response of the sensor to successive additions of dopamine was monitored at an applied potential of −0.15 V vs Ag/AgCl to construct a calibration curve. The sensitivity of these sensors was compared to the sensitivity of sensors fabricated in the same conditions but with tyrosinase present. Interestingly, the carbon fiber electrodes coated with CeO_2/TiO_2/Chit composite responded to injections of micromolar concentrations of dopamine even in the absence of tyrosinase, but the sensitivity of this sensor was low (1.7 ± 0.05 nA/μM) compared to that in the presence of tyrosinase and metal oxides (14.2 ± 0.50 nA/μM). The sensor response to dopamine in the absence of tyrosinase shows that CeO_2/TiO_2 has a catalytic action oxidizing dopamine to *o*-dopaquinone, which was recently described but in an acid environment.[50] To make sure

(43) Li, Y. F.; Liu, Z. M.; Liu, Y. L.; Yang, Y. H.; Shen, G. L.; Yu, R. Q. *Anal. Biochem.* **2006**, *349*, 33–40.
(44) Topoglidis, E.; Cass, A. E. G.; O'Regan, B.; Durrant, J. R. *J. Electroanal. Chem.* **2001**, *517*, 20–27.
(45) Njagi, J.; Andreescu, S. *Biosens. Bioelectron.* **2007**, *23*, 168–175.
(46) Li, Y.-F.; Liu, Z.-M.; Liu, Y.-L.; Yang, Y.-H.; Shen, G.-L.; Yu, R.-Q. *Anal. Biochem.* **2006**, *349*, 33–40.
(47) Lin, S. S.; Chen, C. L.; Chang, D. J.; Chen, C. C. *Water Res.* **2002**, *36*, 3009–3014.
(48) Yang, S. X.; Zhu, W. P.; Wang, J. B.; Chen, Z. X. *J. Hazard. Mater.* **2008**, *153*, 1248–1253.

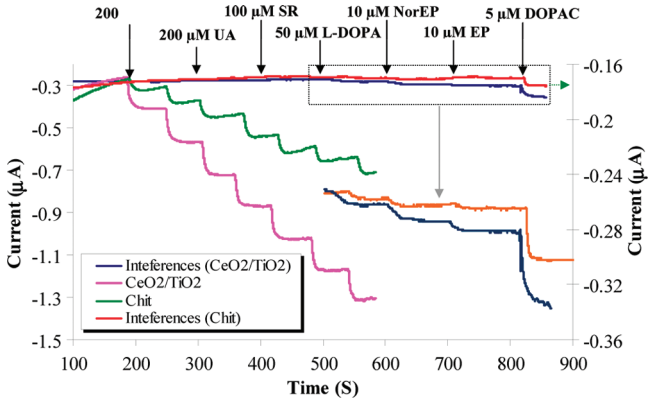

Figure 4. Amperograms showing the selectivity of the dopamine biosensors to AA, UA, SR, L-DOPA, DOPAC, NorEP, and EP. Amperograms show comparison with biosensor responses to successive additions of 5 μM dopamine.

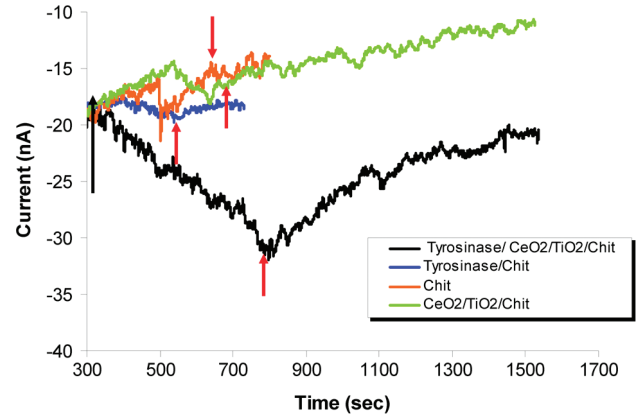

Figure 5. In vivo amperometric responses of the tyrosinase biosensors in the presence and absence of the metal oxides and of the control electrode in the absence of enzyme to dopamine at an applied biosensor potential of −150 mV vs Ag/AgCl during electrical stimulation of the median forebrain bundle. The black arrow shows the onset of the electrical stimulation, and the red arrows indicate when stimulation was stopped.

that the observed response was not from the carbon fiber, another control (without either CeO_2/TiO_2 or tyrosinase) was fabricated, and its response to dopamine was tested. No response to dopamine was observed from this electrode, and therefore, the carbon fibers were not responsible for the response registered with the CeO_2/TiO_2 control. These observations suggest that CeO_2/TiO_2 on the electrode surface contributes to the enhanced response to dopamine of the CeO_2/TiO_2/tyrosinase containing biosensors.[22] This result is important and opens up an interesting area for future research in which these metal oxides can be used as biomimetic materials to fabricate enzymeless sensors to detect phenolic substrates, including dopamine.

Effect of Interferences. Ascorbic acid (AA), uric acid (UA), serotonin (SR), 3,4-dihydroxy-L-phenylalanine (L-DOPA), norepinephrine (NorEP), dihydroxyphenylacetic acid (DOPAC), and epinephrine (EP) are the major interfering species present in biological media with dopamine. The effect of these interfering agents on the biosensors in the absence and presence of CeO_2/TiO_2 was evaluated by holding the electrode at −150 mV vs Ag/AgCl and injecting 200 μM AA, 200 μM UA, 100 μM SR, 50 μM L-DOPA, 10 μM NorEP, and 10 μM EP. No amperometric responses were obtained when AA, UA, and SR were added to the reaction cell. Very low responses of ~1 nA/μM and 0.8 nA/μM were observed for NorEP and EP, respectively. On the other hand, when 5 μM of DA was injected, well-defined and large responses were observed (Figure 4). The selectivity for DA over L-DOPA, NorEP, EP, and DOPAC was calculated as follows: 178.62 ± 10.67, 34.63 ± 3.90, 45.53 ± 3.42, and 5.12 ± 0.60, respectively. To ensure that the low responses of the biosensor to catecholamines (L-DOPA, NorEP, DOPAC, and EP) were not because of the effect of AA, experiments were also performed without AA. No appreciable changes in responses of the catecholamines were observed. The lack of responsiveness of the biosensor to AA and UA can be attributed to the low operating potential of the biosensor, which is below the redox potential of UA and AA.[51] Insensitivity to SR and the low sensitivity to L-DOPA, NorEP, and EP can be attributed to the bulkier structure of these substances, which cannot reach the copper containing active site of tyrosinase. On the other hand, low selectivity of the sensor to DOPAC can be attributed to its similar structure to dopamine. Furthermore, DOPAC is a metabolite of catalyzed monoamine oxidase degradation of dopamine.

Biosensor Stability and Reproducibility. The operational, storage, and continuous stability of the sensor were also tested. The storage stability was evaluated by monitoring the amperometric response to additions of 5 μM dopamine at 3 day intervals over a period of 4 weeks. In the absence of metal oxides, the biosensor stability was 87% after 1 week and then gradually decreased to 55% after 2 weeks. In the presence of metal oxides, the biosensor response was 90% in the first week and then decreased slightly to 75% after 2 weeks. Similar storage stability was reported for a tyrosinase biosensor fabricated by immobilization of ZnO nanoparticles and chitosan.[46] The operational stability was evaluated by measuring the amperometric response to consecutive injections of 5 μM DA injections. In the absence of metal oxides, the residual response was 78% after seven assays and then fell gradually to 40% in the 40th assay. In the presence of metal oxides, the stability was 100% for the first 11 assays after which it decreased to 80% in the 15th assay and 50% in the 40th assay. The observed operational stability is comparable to a tyrosinase biosensor made with layered double hydroxides and chitosan.[52] Continuous stability was investigated by injecting 1 μM DA and monitoring the response current over 2 h. In the presence of metal oxides, the current remained steady for the first 100 min and then gradually decreased to 80% after 2 h. In the absence of metal oxides, the current remained steady for 0.5 h and then decreased to 20% after 50 min (results not shown). These results demonstrate that incorporation of metal oxides enhanced both the operational and storage stability of these biosensors, which can be attributed to the strong adsorption of the enzyme

(49) Liotta, L. F.; Gruttadauria, M.; Di Carlo, G.; Perrini, G.; Librando, V. *J. Hazard. Mater.* **2009**, *162*, 588–606.
(50) Asati, A.; Santra, S.; Kaittanis, C.; Nath, S.; Perez, J. M. *Angew. Chem., Int. Ed.* **2009**, *48*, 2308–2312.
(51) Lu, L.-P.; Wang, S.-Q.; Lin, X.-Q. *Anal. Chim. Acta* **2004**, *519*, 161–166.
(52) Han, E.; Shan, D.; Xue, H.; Cosnier, S. *Biomacromolecules* **2007**, *8*, 971–975.

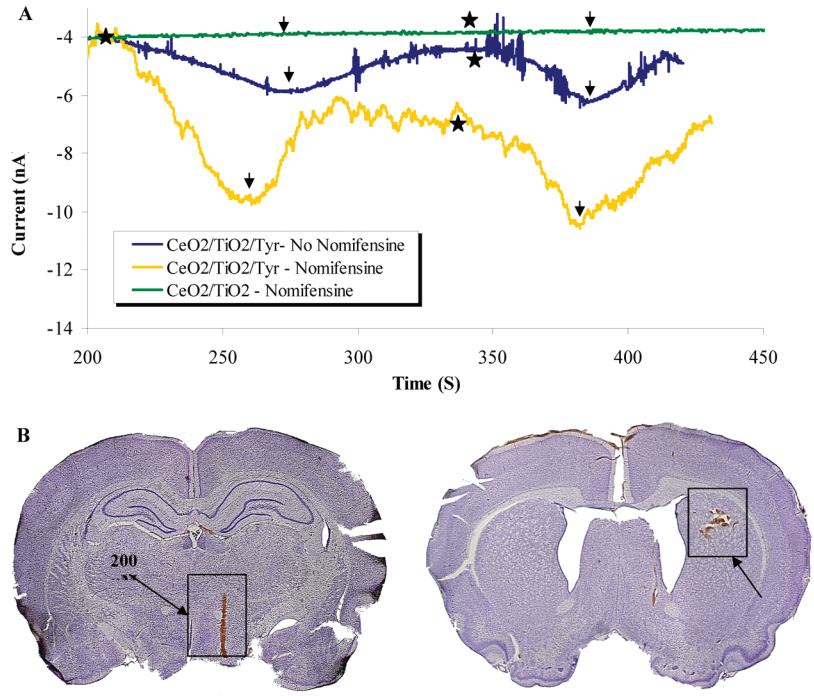

Figure 6. (A) In vivo amperometric responses of the metal oxide tyrosinase biosensors and the control in the presence and absence of nomifensine at an applied potential of −150 mV vs Ag/AgCl during electrical stimulation of the median forebrain bundle. The stars indicate the onset of electrical stimulation while black arrows indicate when stimulation was stopped. (B) Histochemical images of the brain slices for the stimulating and recording sites. The stimulating site was the median forebrain buddle (left side image) while the recording site was the striatum (right side image).

onto the metal oxides, providing a favorable microenvironment to maintain bioactivity. The observed reduction in stability for both types of sensors can also be related to the adsorption of the *o*-dopaquinone onto the biosensor surface, which creates a passivating effect, rather than to enzyme leaching. The reproducibility of the TiO_2/CeO_2/Chit enzyme sensor was evaluated for six identical electrodes prepared independently on different days but following the same electrode construction protocol. The average steady-state current after addition of 5 μM dopamine was 0.095 μA with a relative standard deviation of 2.25%.

In Vivo Dopamine Measurements. The in vivo measurements were carried out using the chitosan-based tyrosinase carbon fiber biosensor over a 2 h period. The results were similar in all three animals tested; for simplicity, only the results from one animal are described. The largest dopamine signal was recorder during evoked dopamine release in the striatum following brief electrical stimulation of the median forebrain bundle (Figure 5). Thus, the dopamine signal was temporally associated with the stimulation sequence. In the absence of CeO_2/TiO_2, very low electrochemical signals were obtained with the tyrosinase biosensor. Electrodes fabricated without tyrosinase were also implanted as a control, and as expected in the absence of CeO_2/TiO_2 and tyrosinase, no amperometric signal was observed during stimulation of the median forebrain bundle. However, in the absence of tyrosinase, the electrode containing only the CeO_2/TiO_2 also demonstrated no signal, which suggests that the presence of enzyme is necessary to obtain quantifiable dopamine signals in vivo; the biosensor is not responding to nondopaminergic interfering agents. Although the in vitro tests of the sensor containing only CeO_2/TiO_2 did respond to dopamine, the sensitivity was low (1.7 ± 0.05 nA/μM) in vitro, and the small dopamine signal generated by such a biosensor could easily be offset by the noise of the sensor in vivo. The biosensors were calibrated in vitro before and after implantation into each rat. The electrodes displayed satisfactory stability after implantation. A slight decrease in sensitivity, from 14.2 (±0.50) nA/μM to 10 nA/μM, was observed in the postmeasurement calibration. Using the premeasurement sensitivity, the amperometric signal observed for the Tyr/CeO_2/TiO_2/Chit−carbon fiber biosensor configuration corresponded to an extracellular value of dopamine of 1.69 (±0.13) μM. The biosensor signals could be generated continuously for over 2 h without any apparent degradation of the signal.

To prove that the signal measured in vivo was from dopamine, a dopamine uptake inhibitor, nomifensine[53] (5 mg/kg), was injected 30−40 min prior to dopamine measurements. The CeO_2/TiO_2 electrodes with and without tyrosinase were tested in the presence and absence of nomifensine. In the presence of tyrosinase, the biosensor displayed a response signal in the presence and absence of nomifensine. However, the signal during high frequency stimulation of the median fore brain bundle was greater after the nomifensine treatment compared to the control stimulation condition (Figure 6A). In the absence of tyrosinase, the metal oxide sensor did not detect any signal in the presence (Figure 6A) or absence (Figure 5) of nomifensine during electrical stimulation. Consequently, incorporation of tyrosinase in the sensor configuration was necessary for the detection of dopamine. Moreover, the temporal specificity of the dopamine response to stimulation and the elevated signal detected

(53) Nicholson, C. *Biophys. J.* **1995**, *68*, 1699–1715.

after nomefinise provide evidence that the sensor was detecting dopamine rather than interfering agents. Histological examination of the brains confirmed that sites of stimulation were closely adjacent to the median forebrain bundle, and the dopamine biosensor was in the striatum in each animal (Figure 6B).

CONCLUSION

We fabricated an implantable enzyme-based biosensor and applied it to measurements of dopamine evoked from the striatum during HFS of the median forebrain bundle in anesthetized rats. The biosensor was engineered using a natural biopolymer chitosan and mixed ceria/titania-based metal oxide nanoparticles, which provided enhanced sensitivity for dopamine. The chitosan ensured a biocompatible environment for the enzyme and an immobilization matrix for the ceria/titania nanoparticles and tyrosinase. A sol−gel composite was also studied as an alternative immobilization matrix, but its performance was less satisfactory since the coating was brittle and broke apart when exposed to only modest physical stress. The chitosan-based biosensor can continuously measure levels of dopamine in the nanomolar range at a low applied potential of −150 mV vs Ag/AgCl, eliminating common interferences without any additional biosensor membrane coating. The sensor exhibited excellent linearity in the concentration range between 0.01 and 220 μM with a R^2 value of 0.9998 and good selectivity for dopamine over serotonin, uric acid, ascorbic acid, L-DOPA, norepinephrine, and epinephrine. The sensitivity of the sensor was 14.2 nA$\cdot\mu$M^{-1}, and the detection limit was 1.0 nM, which was among the lowest values reported for dopamine sensors. The sensor design includes several other unique features, such as reaction product recycling by virtue of an enzymatic/electrochemical recycling process, which minimizes electrode passivation. Moreover, the electrode materials are biocompatible. The response of the biosensor to the induced dopamine production in the rat brain indicates that the sensor operates effectively and has good specificity for dopamine in an in vivo environment. Thus, the materials used in the sensor fabrication and the coupled enzymatic/electrochemical process of dopamine detection make this sensor a good candidate for use as an implantable sensor for real time in vivo monitoring of dopamine.

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