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ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2015

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Platinum-Doped Ceria Based Biosensor for *in Vitro* and *in Vivo* Monitoring of Lactate during Hypoxia

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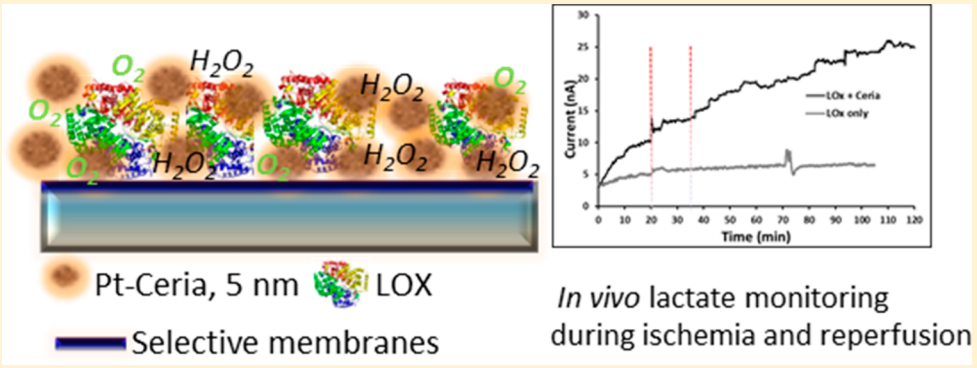
Supporting Information



ABSTRACT: Measurements of lactate concentrations in blood and tissues are an important indication of the adequacy of tissue oxygenation and could be useful for monitoring the state and progress of a variety of diseases. This paper describes the fabrication, analytical characterization, and physiological application of an amperometric microbiosensor based on lactate oxidase and oxygen-rich platinum doped ceria (Pt-ceria) nanoparticles for monitoring lactate levels during hypoxic conditions. The Pt-ceria nanoparticles provided electrocatalytic amplification for the detection of the enzymatically produced hydrogen peroxide and acted as an internal oxygen source for the enzyme, enabling lactate monitoring in an oxygen depleted tissue. *In vitro* evaluation of the biosensor demonstrated high selectivity against physiological levels of ascorbic acid, a storage stability of 3 weeks, a fast response time of 6 s, and good, linear sensitivity over a wide concentration range. *In vivo* experiments performed by placing the biosensor in the hippocampus of anesthetized rats demonstrated the feasibility of continuous lactate monitoring over 2 h ischemia and reperfusion. The results demonstrate that Pt-ceria is a versatile material for use in implantable enzyme bioelectrodes, which may be used to assess the pathophysiology of tissue hypoxia. In addition to measurements in hypoxic conditions, the detection limit of this biosensor was low, 100 pM, and the materials used to fabricate this biosensor can be particularly useful in ultrasensitive devices for monitoring lactate levels in a variety of conditions.

Lactic acid (lactate) is a key metabolite of the anaerobic glycolytic pathway and plays an important role as an energy substrate during neural activation. Blood lactate concentration is a major indicator of ischemia and inadequate oxygenation. Elevated lactate levels are associated with various disorders such as shock, respiratory failure, severe congestive heart failure, and myocardial ischemia.[1] While normal lactate levels in blood range from 0.5 to 2.5 mM, lactate concentrations may exceed 7−8 mM when oxygen delivery is inadequate and insufficient to maintain aerobic metabolism.[2] Currently, lactate levels in blood are measured intermittently from isolated blood samples; a slow process that does not provide timely feedback about the state of patients. Continuous monitoring of lactate, in blood or other tissues, can provide a clinically useful, real-time measurement of the state of ischemia.

Electrochemical microbiosensors can provide real-time measurements of lactate, but sensors that function reliably and maintain accuracy in hypoxic environments are limited. Electrochemical lactate biosensors can be used *ex vivo* with implanted microdialysis or ultrafiltration probes[3] or *in vivo* to avoid the time lag required by microdialysis. *In vivo* biosensors can provide more immediate and direct measurement of lactate concentrations.[4] The reliability of *in vivo* sensors has increased over the last ten years, and their use in monitoring biological processes in real time has grown.[5−8] Several electrochemical lactate biosensors, nearly all based on lactate oxidase (LOX)[6,7] have been described. To be useful during hypoxia, lactate

Received: December 11, 2014
Accepted: January 28, 2015
Published: January 28, 2015

DOI: 10.1021/ac5047455
Anal. Chem. 2015, 87, 2996−3003

biosensors must be functional in conditions of severe oxygen deficiency. Gerhard et al. developed a nafion-based LOX biosensor and showed that the microelectrode response in a normal brain environment (not hypoxic) was ~70−85% of the response in air saturated conditions.[6] Marzouk et al. reported a hypoxia tolerant lactate biosensor[9] that uses an electron mediator, tetrathiafulvalene - tetracianoquinodimethane (TTF-TCNQ) with detection at the redox potential of the mediator, 150 mV. While the use of electrochemical mediators can overcome the problem of oxygen dependency, the risk of leaching of the mediator from the electrode surface to the surrounding tissue has prevented the implementation of this design in clinical applications. Most common configurations still rely on LOX and the detection of the enzymatically generated H_2O_2. However, the sensitivity of these sensors may deteriorate during limited oxygen supply as may occur during ischemia or profound hypoxia since oxygen is a cosubstrate in the enzymatic conversion of lactate to pyruvate.[4,10,11] A biosensor material with the ability to store and release oxygen would improve the function of oxidase-based biosensors in oxygen deficient conditions. Several reports have described using an "oxygen generation system" and an "oxygen chamber" as an integral part of the sensor so that oxygen can be maintained at levels sufficient for an adequate sensor response.[12−14] Electrochemical, LOX containing, needle electrodes fabricated with a polyurethane outer membrane were able to monitor changes in tissue lactate continuously during hemorrhagic shock by delivering oxygenated perfusion buffer over the sensor tip during measurements.[15]

This paper describes a new design for overcoming oxygen limitations of LOX biosensors for the detection of lactate during hypoxia by using Pt-doped ceria nanoparticles to provide oxygen to the enzyme and sustain the oxidative generation of pyruvate and H_2O_2. The catalytic, enzyme mimetic and oxygen storage capacity of pure cerium oxide (ceria) have been demonstrated in multiple biological applications.[16] Ceria can exist in two valence states (Ce^{3+} and Ce^{4+}) depending on the size and redox environment. When the $Ce^{3+/4+}$ ion ratio increases, formation of oxygen vacancies in the nanoceria lattice structure is enhanced. These oxygen vacancies are partly responsible for the powerful catalytic activity of the ceria nanoparticles and may also bind oxygen and create an oxygen reserve within the cerium oxide lattice, which is enhanced by addition of dopants. Our group demonstrated enhanced electrocatalytic activity and the oxygen storage capacity of platinum-doped nanoceria (Pt-ceria).[17] The presence of Pt provides conductivity to the ceria structure, further enhancing the electron transfer process at the surface of nanoparticle. Pt-ceria showed better electrocatalytic activity toward H_2O_2 than pure ceria, which was previously explored as a sensing material for glucose biosensors,[18] and an enhanced oxygen transport and oxygen storage.[17] In this series of studies, we extend our work on nanoceria-based sensing to *in vitro* and *in vivo* measurements of lactate. We engineered a LOX microbiosensor containing Pt-ceria nanoparticles and LOX that is able to measure lactate levels during hypoxia and tested these biosensors in an anesthetized rat model of brain ischemia and reperfusion. We demonstrate that Pt-doped ceria shows promise as a biosensor material for *in vivo* monitoring of oxidase enzyme substrates, particularly in settings where the availability of oxygen may be limiting.

EXPERIMENTAL SECTION

Reagents. Chitosan (practical grade) from shrimp shells, L-lactic acid (99%), lactate oxidase (LOX) L0638-500U, bovine serum albumin (BSA), and potassium phosphate (monobasic) were obtained from Sigma-Aldrich. Sulfuric acid was purchased from Fisher Scientific. L-Ascorbic acid (AA) (99%), o-phenylenediamine (o-PD) (98%), and sodium phosphate dibasic (anhydrous) were purchased from Acros Organics. Ascorbate oxidase (AO) (EC1.10.3.3, 1 KU/vial; from Cucurbita sp.) was obtained from Alfa Aesar. Polyethylene glycol (PEG, Cat No. = 20243-6, MW = 1,500) was purchased from Aldrich. All solutions were prepared with distilled water collected from Millipore Direct-Q with a resistivity of 18.2 Ω cm.

Instrumentation. Amperometry and cyclic voltammetry (CV) experiments were conducted using a CHI 1030A electrochemical analyzer (CH Instruments Inc.). Electrochemical analyses to optimize the biosensor configuration were performed with a conventional cell with enzyme/nanoparticles/chitosan/o-PD/BSA modified platinum wire as the working electrode, an Ag/AgCl as reference electrode, and a platinum wire as a counter electrode. All potentials were referred to the Ag/AgCl reference electrode. The fabrication procedure of the microelectrodes is described in the Supporting Information (SI). The microelectrodes were assembled as shown in Figure S1.

Biosensor Fabrication and Detection. The electrodes were electrocoated in phosphate buffer (PB) pH 7.4 containing 300 mM o-PD by applying a potential of 0.9 V for 30 min. These electrodes were later dipped in a 33.3 mM PEG solution for 2 h followed by drying under N_2 for 4 h. Since o-PD is hydrophobic, the PEG coating was introduced to create a hydrophilic surface on the sensor and to facilitate the adsorption of enzymes. The PEG-coated electrodes were dipped in BSA (5 mg/mL) for 5 min and dried for 45 min in a N_2 environment. Later these electrodes were dipped in the biocatalytic mixture consisting of LOX-CHT-Pt-ceria-AO in a ratio of 3:1:1:0.5 for 5 min followed by drying for 45 min under N_2. This step was repeated 30 times. To ensure the hydration of the polymeric films, electrodes were dipped in PB for an hour prior to testing after which the electrodes were electrochemically cycled from 0 to +1.0 V at a scan rate of 0.1 V/s for 50 cycles. The PB was purged with N_2 for at least 20 min to create an anaerobic environment. Amperometric responses were obtained by applying a constant potential at 0.55 V versus Ag/AgCl.

***In Vivo* Measurements of Brain Tissue Lactate in Rats.** Male Sprague−Dawley rats (Harlan) weighing between 240 and 340 g were used in this study. Experimental protocols 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. The rats were housed in a temperature controlled room (21 °C) under a 12/12 light/dark cycle and given free access to food and water prior to surgery. Each animal was anesthetized with urethane (1200 mg/kg) and chloralose (40 mg/kg). One third of the initial dose was given as a supplement during the study as necessary to maintain anesthesia. Body temperature was controlled by a rectal thermometer and maintained at 37 °C using a heat lamp during the surgery. The surgical procedure was carried out in two steps. In the first step, the right common carotid artery (RCCA) was isolated and exposed. An occluder

DOI: 10.1021/ac5047455
Anal. Chem. 2015, 87, 2996−3003

cuff was placed around the exposed artery and secured in place using suture material passed through the eyelets of the occluder. In the second step, each animal was fixed in a stereotaxic frame (Model 1430, David Kopf Instruments, Tujunga, CA), and a midline incision was made starting caudal to the eyes and rostral to the ears to expose the surface of the skull. A small burr hole was made over the cortex, and the biosensor was implanted unilaterally in the hippocampus at the following stereotaxic coordinates measured from bregma: +3.8 mm AP, +2.0 mm ML, and −1.5 to −2.0 mm DV. The Ag/AgCl reference and platinum auxiliary electrode setup was placed in an anterior cerebral region (ca. A6.0 mm). At the conclusion of each study, animals were euthanized with an excess dose of urethane and chloralose.

In vivo electrochemical measurements were conducted with a DY2116B (Digi-ivy, Inc. Austin, TX) computer controlled potentiostat using amperometry with a constant applied potential at +0.55 V vs Ag/AgCl. Lactate measurements in the hippocampus during ischemia were carried out amperometrically by occluding the RCCA for 15 min followed by reperfusion, which lasted for 60−90 min. For clarity of presentation, data were presented after normalization by adjusting to a common baseline point. Electrochemical *in vivo* data were converted to concentration units of lactate based on the *in vitro* calibrations of the corresponding biosensor, which was performed preceding and following the *in vivo* experiments.

RESULTS AND DISCUSSION

Engineering the Lactate Biosensor: The Role of Pt-Ceria Nanoparticles. First, we used cyclic voltammetry to characterize the oxygen storage and release capabilities of Pt-ceria coimmobilized with LOX. We used doped Pt-ceria nanoparticles with a diameter of ∼4.7 nm prepared by a microemulsion procedure described previously.[19] The particles produced were monodispersed (Figure S2 shows the TEM image of the particles). Figure S3 shows CVs for the bare and Pt/oPD/BSA/CHT-LOX-Pt-ceria-AO (Pt-ceria modified LOX biosensor) in deoxygenated conditions in the presence and absence of 2 mM lactate. In the absence of Pt-ceria, the CV did not show a significant change in the oxidation current upon addition of lactate (as compared to the control) suggesting limited conversion of lactate to H_2O_2 - likely due to the lack of oxygen. In contrast, the Pt-ceria modified LOX biosensor showed a distinct response to lactate under oxygen depleted conditions indicating that the nanoparticles facilitate conversion of lactate to H_2O_2 even in the absence of molecular oxygen in the measurement environment. To evaluate whether the particles alone are responsible for this conversion, a control experiment was performed with a biosensor containing Pt-ceria but without LOX. The CV showed no significant oxidation wave in either oxygenated or deoxygenated PB, indicating that Pt-ceria alone does not react with lactate or take part directly in the oxidation of lactate at the concentration range tested. Therefore, the increase in the oxidation current starting at ∼0.2 V can be attributed to oxidation of enzymatically produced H_2O_2. The observed current can be attributed to the bidirectional redox activity of $Ce^{3+/4+}$ toward H_2O_2 releasing oxygen in the process, and the high catalytic activity of Pt-ceria, which enhances the electrocatalytic oxidation of H_2O_2 at the surface of the electrode (Scheme 1).

These results support the hypothesis, shown in Scheme 1, that H_2O_2 released during oxidation of lactate reacts with cerium oxide to generate ceric oxide and release oxygen in the process. It is this released oxygen that is used by LOX enabling biocatalytic conversion of lactate to generate H_2O_2 at the electrode surface. Moreover, the inherent electrocatalytic activity and conductivity of Pt inserted in the ceria material contributes to the enhanced electrochemical current.

Scheme 1. Mechanism of the LOX Based Detection of Lactate Where the Reaction of Ceria with H_2O_2 Produces Oxygen Replenishing the Oxygen Needed for the Enzymatic Reaction

$$2CeO_2 + H_2O_2 \rightarrow Ce_2O_3 + \mathbf{O_2} + H_2O \qquad 1$$

$$\text{Lactate} + \mathbf{O_2} \xrightarrow{\text{LOX}} \text{Pyruvate} + H_2O_2 \qquad 2$$

Fabrication and Optimization of the Pt-Ceria Based Lactate Biosensor. The Pt-ceria microelectrode was prepared on a 125 μm Pt wire by depositing several polymeric layers to ensure selectivity against interfering compounds and improve adhesion of the enzymatic layer to the microelectrode surface. The first challenge to construct a functional and selective lactate bioelectrode was to load sufficient quantities of LOX onto the 125 μm × 2 mm platinum surface. Electrodeposition of the o-PD layer created a hydrophobic platinum electrode surface. Adsorption of PEG onto the o-PD-platinum electrode provided a comparatively hydrophilic surface to enable adsorption of enzymes. Enzyme immobilization was achieved by sequential dip coating of a mixture of enzyme with Pt-ceria nanoparticles dispersed in chitosan; chitosan was selected as a biocompatible polymeric linker with good adhesion properties.[20] In the absence of PEGylation of the electrode surface, no response to lactate was observed from the Pt-ceria modified LOX microelectrode, suggesting that the enzymatic mixture was not stably attached onto the Pt/oPD surface. In contrast LOX-biosensors modified with PEG showed well-defined current responses to lactate. The PEG layer seemed to be essential to the adsorption of enzyme on the biosensor surface.

Next, the optimum operational conditions were established to improve the stability of LOX immobilization on the electrode surface and the overall sensitivity of the biosensor. Parameters investigated included the following: LOX and Pt-ceria amounts, operating potential and number of dip-coated enzymatic layers. The highest current for the detection of the enzymatically generated H_2O_2 was recorded at 0.55 V, which was chosen as the operational potential for rest of the experiments. The effect of enzyme amount in the enzyme-particle-chitosan mixture was tested using LOX concentrations in the 5 to 100 U/mL. The maximum current response was achieved at the 50 U/mL concentration. Lower amounts generated very low intensity current, while higher loadings did not show a substantially increased response. Figure 1A shows the biosensor response as a function of Pt-ceria concentration (range 1−45 mg/mL) in the enzyme-particle-chitosan mixture after serial additions of 0.1 mM lactate at pH 7.4. The current response increased as the Pt-ceria particle concentration in the mixture increased and reached a maximum for 45 mg/mL. Even though this concentration provided higher responses, these biosensors showed relatively large measurement variability (>13% which can be due to the aggregation of nanoparticles). At concentrations up to 15 mg/mL, the nanoparticles remained well dispersed in the mixture. Therefore, 15 mg/mL was

DOI: 10.1021/ac5047455
Anal. Chem. 2015, 87, 2996−3003

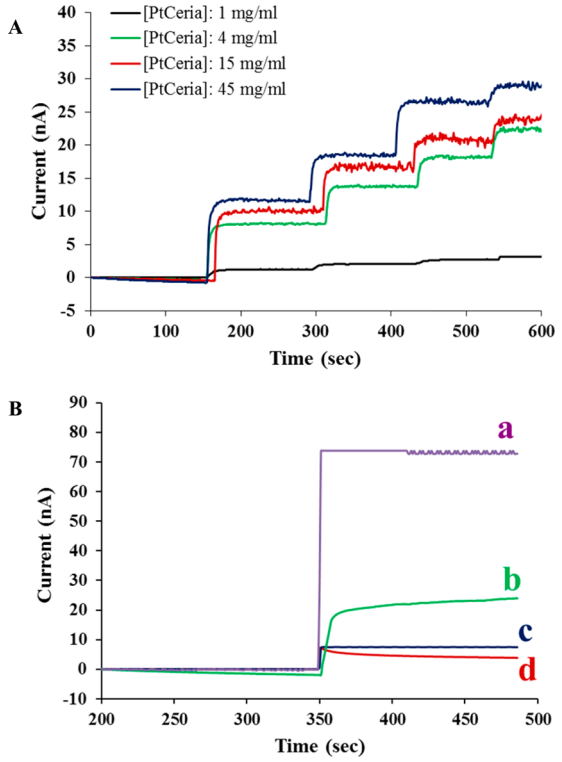


Figure 1. (A) Effect of Pt-ceria loading and on the intensity of the current to additions of 0.1 mM lactate. (B) Current response showing effect of sequential addition of o-PD, BSA, and AO on biosensor surface to reject ascorbic acid interferences. The film assemblies studied are a) Pt/PEG/CHT-LOX-Pt-ceria, b) Pt/oPD/PEG/CHT-LOX-Pt-ceria, c) Pt/oPD/PEG/BSA/CHT-LOX-Pt-ceria, and d) Pt/oPD/PEG/BSA/CHT-LOX-Pt-ceria-AO. The supporting electrolyte solution was 0.1 M PB (pH 7.4).

chosen as the optimum particle concentration. To ensure maximal loading of enzyme and particles on the biosensor surface and achieve higher sensitivity, the number of dippings (5, 10, 30, or 40 times) of the electrode in the enzyme-particle-chitosan mixture was optimized (Figure S4). The current response saturated after 30 dippings, suggesting that the optimal coverage of enzyme-particle-chitosan had formed on the sensing surface.

We further investigated the long-term stability of the optimized biosensors as a function of time when stored in dry-state at +4 °C. Biosensor stability was evaluated amperometrically by measuring the current responses to four consecutive additions of 0.1 mM lactate (Figure S4). The biosensors remained stable for 1 week, after which the response was reduced to 68% after 3 weeks and remained constant thereafter for 5 weeks.

Biosensor Selectivity: Effect of Electrode Coatings in Rejecting Ascorbic Acid Interference. The o-PD, BSA, and AO were used to modify the biosensor surface to prevent current responses originating from AA. Figure 1B shows the effect of o-PD, BSA, and AO in preventing AA interferences. Biosensors without o-PD, BSA, and AO lacked sensitivity against 125 mM AA, which is the physiological level of AA in brain.[21] To impart selectivity the bioelectrode was coated with o-PD to prevent access of AA to the active electrode surface, followed by a layer of PEG to introduce a hydrophilic environment and BSA to stabilize the enzyme and prevent nonspecific interactions. Finally, AO was added to enzymatically remove AA from the active electrode surface. Figure 1B shows the effect of these constituents on the response of the biosensor to 125 mM AA. Since chitosan is negatively charged at physiological pH, it augments rejection of negatively charged AA at the electrode surface.[20,22] Moreover, the number of dippings also affected selectivity of the biosensors. The current response arising from AA decreased 10-fold when the number of dippings was increased from 5 to 30 (Figure S3 in the SI). This decrease in responses to AA may be due to the greater amount of chitosan and AO loaded onto the electrode surface.

Calibration and Analytical Characterization of the Lactate Biosensor. The lactate biosensor was calibrated in both hypoxic and nonhypoxic environments to analyze its performance. For comparison, control experiments were conducted using a similarly prepared biosensor without nanoparticles. Figure 2A shows amperometric responses of

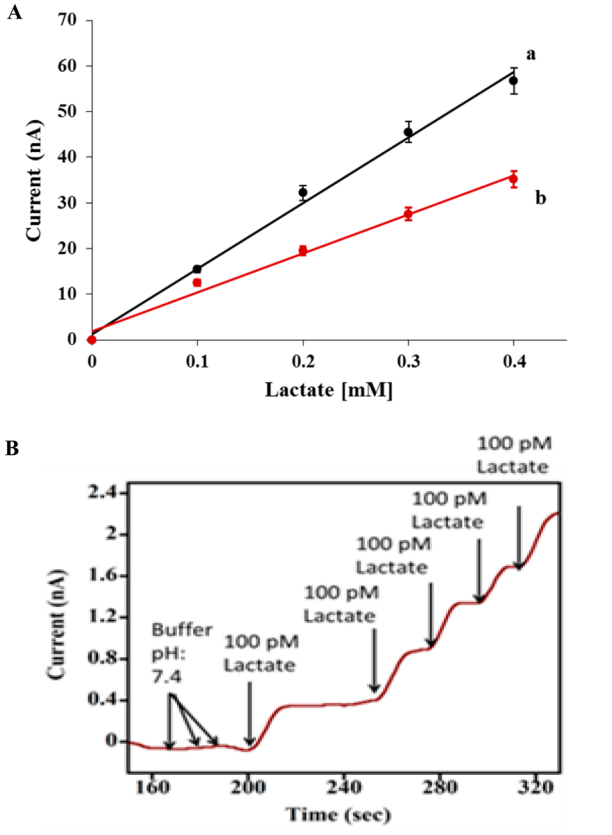


Figure 2. (A) Calibration curves of LOX biosensors with (a) and without (b) Pt-ceria in air saturated PB after 4 additions of 0.1 mM lactate (n = 3 replicates). (B) Typical amperometric responses of the Pt-ceria modified LOX biosensor in the lower calibration curve showing detection limit of the biosensor.

the Pt-ceria based and the control (without Pt-ceria) biosensors in air saturated medium. In the air saturated environment, both the Pt-ceria and the control LOX biosensors responded linearly to the addition of 0.1 mM lactate. The biosensor with Pt-ceria showed greater amperometric responses, due to the electrocatalytic enhancement imparted by the nanoparticles. The response time after addition of lactate was ~6 s. This demonstrates a good diffusion rate of the lactate through the nanocomposite layers. Figure 2B shows an amperometric recording in the lower concentration range to determine the

DOI: 10.1021/ac5047455
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detection limit. The sensor reliably detected lactate at a concentration as low as 100 pM lactate.

In the hypoxic environment, the control biosensors without particles did not show concentration-dependent responses to successive additions of lactate. A negligible current response was observed after the first addition of lactate, and no response was obtained after subsequent additions. The response after the first addition may be due to a small amount of residual surface oxygen in the chitosan matrix. In contrast, the biosensor containing Pt-ceria displayed concentration dependent responses over a wide range from 100 pM to 15.5 mM lactate in similar experimental conditions. The current responses show two distinct linear ranges: one from 100 pM to 0.2 mM in the lower concentration range and a second from 0.5 to 15.5 mM. This may be due to the two-step enzymatic-electrocatalytic process and the reliance of the enzymatic reaction on the ceria mediated release of oxygen (Scheme 1). This process is more prominent at higher lactate concentrations due to a higher oxygen demand. The response time of the sensor in hypoxic conditions was ~9 s. The increase in the response time from 6 to 9 s may be attributed to the time needed for particles to release oxygen (reaction 1 in Scheme 1). The ability of the Pt-ceria based biosensor to function in the absence of oxygen is due to a catalytic recycling process between Pt-ceria and the enzymatically generated H_2O_2 that creates a higher $Ce^{3+/4+}$ ratio and forms oxygen vacancies within the Pt-ceria lattice structure (Scheme 1). Figure 3 shows that the Pt-ceria

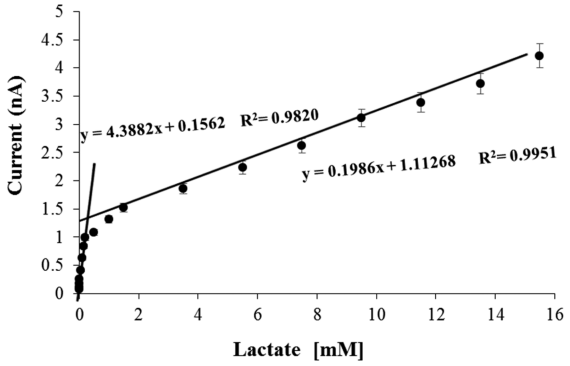

Figure 3. Calibration curves of Pt-ceria biosensors in hypoxic environment. Current responses correspond to 4 additions of 0.1 mM lactate in nitrogen saturated PB (n = 3 replicates).

biosensor is able to sustain the enzymatic reaction and measure lactate up to 15.5 mM in the absence of oxygen, a lactate range that covers both physiological and pathological concentrations of lactate.

***In Vivo* Characterization of the Lactate Biosensor.** Lactate measurements were carried out *in vivo* by placing the biosensor in the hippocampal region of anesthetized rats. Current responses were measured during normoxic conditions, during ischemic conditions-induced by unilateral common carotid artery occlusion and during reperfusion after releasing the arterial occlusion. Three different kinds of biosensor assemblies were tested *in vivo*, sensors modified with (i) Pt/o-PD/PEG/BSA/CHT-LOX-Pt-Ceria-AO (LOX + Pt-ceria), (ii) Pt/o-PD/PEG/BSA/CHT-LOX-AO (LOX alone), and (iii) Pt/o-PD/PEG/BSA/CHT-Pt-ceria-AO (Pt-ceria alone); the other constituents incorporated in the sensor assembly were present in all three biosensor configurations. Biosensors containing Pt-ceria exhibited increased lactate responses during ischemia-reperfusion periods. On the other hand, electrodes fabricated with only LOX were implanted as a control, and as expected in the absence of Pt-ceria, very low electrochemical signals were obtained during the ischemia-reperfusion periods (Figure 4). In the absence of LOX, the electrode containing

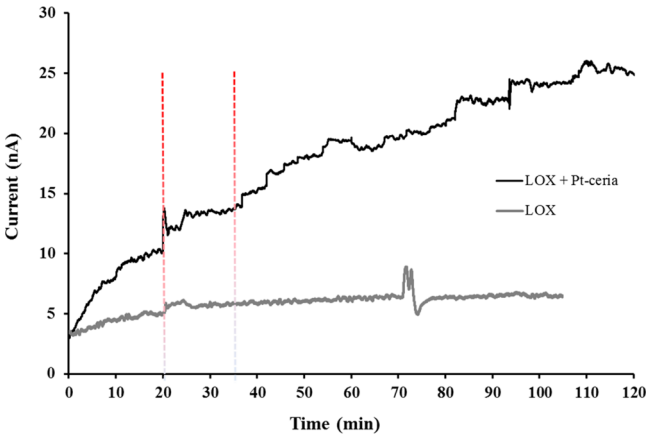

Figure 4. Typical *in vivo* current–time responses of the LOX biosensor with and without Pt-ceria particles showing continuous monitoring of lactate in rat brain hippocampus at an applied biosensor potential of +0.55 V vs Ag/AgCl. The vertical dotted lines indicate the onset of ischemia (20 min) and reperfusion (35 min).

only the Pt-ceria displayed no increased signal during ischemia-reperfusion or nonischemic conditions, which suggests that the presence of LOX enzyme is necessary to obtain quantifiable lactate signals *in vivo*. These results are similar to the *in vitro* tests of the sensors containing Pt-ceria only, which showed no response to lactate.

In vivo amperometric responses of the LOX containing biosensors implanted in the hippocampus modified with or without Pt-ceria are shown in Figure 4. Sensors were allowed to reach a stable baseline after implantation in the hippocampus before inducing ischemia. The current response increased immediately upon inducing ischemia for 15 min and continued to increase throughout the reperfusion period. During some experiments the biosensor response saturated late in the reperfusion period. A rising lactate signal was temporally associated with the ischemia-reperfusion conditions. Similar trends were observed in biosensors modified with and without Pt-ceria, though the overall responses in the LOX biosensors without Pt-ceria were substantially lower. The individual biosensors were calibrated in PB using a five point calibration *in vitro* before and after implantation into each animal. Figure S5 shows corresponding amperograms and calibration curves for the LOX + Pt-ceria biosensors before and after implantation. LOX + Pt-ceria modified biosensors showed 43 (±23) % (n = 3) reduction in sensitivity from 83.7 ± 17.4 nA/mM premeasurement to 56.7 (±9) nA/mM in the post-measurement calibration. Using the premeasurement calibration, the amperometric signal observed for this biosensor configuration *in vivo* corresponded to an extracellular lactate of 128 (±7) μM. The biosensor signals could be generated continuously for over 2 h without any apparent degradation of the signal. Similarly biosensors modified with LOX alone showed a 50 (±10) % (n = 3) reduction in sensitivity in the postmeasurement calibration. Using the premeasurement calibration, the amperometric signal observed for the above

DOI: 10.1021/ac5047455
Anal. Chem. 2015, 87, 2996–3003

biosensor configuration corresponded to an extracellular lactate increase of 17.8 (±8) μM.

Distinct differences in lactate levels were observed with different biosensor designs, and their discrete contributions during ischemia-reperfusion periods were presented in Figure 5.

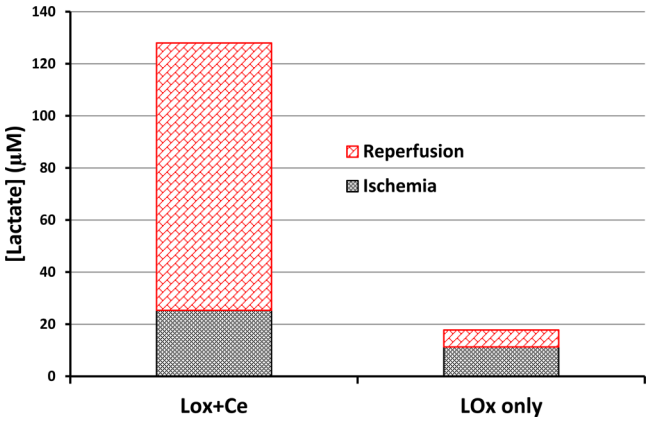

Figure 5. Quantified levels of *in vivo* lactate in rat brain hippocampus of LOX biosensors with and without Pt-ceria during ischemia and reperfusion periods.

There was no detectable increase in lactate levels in the absence of ischemia. This indicates that measured amperometric responses in Figure 4 are due to lactate only. The reduced lactate levels measured during ischemia and reperfusion when Pt-ceria were not present reflect, in our view, a failure of the biosensor to detect changes in lactate concentration secondary to diminished availability of oxygen and substrate limitation in the enzymatic generation of H_2O_2. The contributions of lactate responses calculated with Pt-ceria LOX modified biosensors during ischemia and reperfusion periods were 20% (25 μM) and 80% (103 μM), respectively. Similarly, estimated lactate contributions with LOX only biosensors during the ischemia and reperfusion periods were 63% (11.3 μM) and 37% (6.5 μM), respectively.

DISCUSSION

This study demonstrates the utility of Pt-ceria nanoparticles as an electrocatalytic material for enhancing the sensitivity of implantable bioelectrodes and facilitating operation of oxidase enzymes in hypoxic conditions. The results also demonstrate the potential of this technology for studying quantitatively the release of lactate in brain tissue with high spatial and temporal resolution. Such measurements can facilitate further study and fundamental understanding of the physiology of hypoxic brain injuries and potentially, of hypoxia in other tissues.

Current methods for monitoring the lactate concentration in intact brain include microdialysis[3] in which lactate from blood or tissue diffuses into the dialysate/filtrate and is transported outside of the body where it can be detected, usually by chromatographic methods, flow injection analysis, mass spectrometry, or by an *ex vivo* sensor. Tissue implantation is probably safer than direct implantation of sensors into blood vessels, which carries a risk of thrombosis and embolism. Lactate measurements in tissue can also provide more relevant information on local physiological concentrations, thus allowing detection of local tissue hypoxia as opposed to a body average value obtained by blood analysis. Although the availability of implantable sensors has increased over the last years, sensors that are able to monitor physiologically relevant analytes to assess the status of hypoxia are still limited. Electrochemical biosensors for the detection of lactate have been reported in the literature, but these sensors have seldom been used to measure real-time production of lactate[9,15] in whole animals, and there are few examples of their use in hypoxic tissues.[23] Most lactate biosensors use LOX, which recognizes lactate with high specificity and converts it to pyruvate and hydrogen peroxide (Scheme 1), which is then measured amperometrically. The dependency of this reaction on oxygen has prevented the usage of these biosensors to monitor lactate in hypoxic medium where oxygen availability is rate limiting. In previous work to determine extracellular lactate accumulation in ischemic myocardium, the oxygen dependency of the lactate oxidase reaction was avoided by using an organic charge transfer complex, TTF-TCNQ, to mediate the electron transfer from the enzyme prosthetic group to the electrode surface, thus bypassing the oxygen requirement.[9]

We have proposed a new electrocatalytic mechanism for the detection of lactate that uses redox active nanostructures of 5 nm Pt-doped ceria nanoparticles that, in addition to enhancing conductivity and electrocatalytic activity,[17] also provide a redox cycling process that generates oxygen when in contact with the enzyme. This is the first time this material has been explored or employed for the fabrication of an implantable lactate biosensor. To prepare the electrode, the particles were coimmobilized with LOX in a biocompatible chitosan matrix on the surface of the microelectrode to ensure close contact with the enzyme. We showed that the response of the biosensor increased with the particle and enzyme concentration and with the number of dippings of the electrode into the nanoparticle-enzyme mixture. An optimum configuration was achieved by dip-coating 30 times in a mixture containing 15 mg/mL Pt-ceria and 50 U/mL LOX dispersed in 1% chitosan onto a 125 μm Pt wire coated with selective membranes. A 10-fold increase in selectivity was achieved using a combination of sequentially deposited membranes including o-PD, PEG, BSA, and AA. We showed that a combination of membranes is needed to prevent AA interferences. Incorporation of Pt-ceria in the enzymatic layer significantly increased the electrocatalytic current corresponding to the oxidation of enzymatically generated H_2O_2. The increase in current response can be attributed to the enhanced electrocatalytic oxidation of H_2O_2 by the surface confined particles. We further demonstrate that the Pt-ceria based biosensor functions under oxygen deficient conditions, while sensors without particles under the same conditions showed no quantifiable electrochemical signal. The ability of the biosensor to operate under hypoxic conditions can be attributed to the redox recycling of these particles, which act as an oxygen source and support the enzymatic oxidation of lactate even in the absence of molecular oxygen. Mechanistically, the reaction involves a change in oxidation state from Ce^{4+} to Ce^{3+} as H_2O_2 reacts at the particle surface and releases oxygen, which is taken up by the surface confined enzyme and facilitates continuous biosensor operation in the hypoxic medium. While the intensity of the current is lower than that recorded in air saturated conditions, the sensor is still able to measure lactate with sufficient sensitivity. These results demonstrate the capability of this biosensor to operate in hypoxic conditions and show promise to measure physiological changes of lactate. Table S1 shows a comparison of the performance of the Pt-ceria biosensor versus other LOX based biosensor configurations reported in the literature. The

DOI: 10.1021/ac5047455
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comparative analysis indicates several advantages of the Pt-ceria biosensors including the following: small size, very low detection limit of 100 pM, quick response time, and wide linear range that can be useful for measuring lactate levels in both physiological and pathological conditions. The improved function of the biosensor can be attributed to the environment provided by the catalytic nanoparticles and the biocompatible chitosan. These aspects of the biosensor promote high enzyme loading and stabilized electrostatic binding to the electrode. Moreover, the ceria nanoparticles increase oxygen released in proximity to the enzyme.

To explore the potential of this technology in the biomedical field, we implanted the biosensor in the hippocampus of anesthetized rats and monitored the variation of lactate levels over a period of 2 h during ischemia and reperfusion. It is known that lactate is an indicator of hypoxia in the central nervous system,[24] and devices for real time lactate monitoring can contribute to the assessment of the pathophysiology of tissue hypoxia. The results of the *in vivo* work demonstrate that the Pt-ceria based biosensor is able to provide continuous quantitative monitoring of lactate during brain ischemia. The contrast between the responses measured in the presence and absence of Pt-ceria further supports the importance of the nanoparticles incorporated in the biosensor assembly. Control tests with a Pt-ceria sensor without enzyme (Figure S6) showed that the sensor was not able to measure quantifiable responses *in vivo*. This result indicates that measuring lactate levels during ischemia require both the biocatalytic reaction of LOX and the chemical amplification provided by the Pt-ceria. Although nanoceria particles have intrinsic oxidase activity, the sensitivity of sensors containing particles only is not enough for "enzyme-less" operation *in vivo*. The absence of a response *in vivo* from the sensors without enzyme further validated the selectivity of the measured signals in Figure 4 toward lactate. Moreover, the temporal release of lactate and the elevated current responses detected after inducing ischemia and during reperfusion periods provide evidence that the sensors were detecting lactate and not detecting interfering agents.

Previous studies measuring lactate levels during cerebral ischemia were carried out using flow injection analysis[25] or microdialysis[26] coupled with electrochemical techniques. To our knowledge, no studies have measured lactate levels continuously during ischemia and reperfusion using biosensors implanted in the intact brain of anesthetized rats. Some studies were carried out using a similar approach to measure real-time lactate *in situ*, but these studies were directed toward sleep related issues[27] and memory processing.[28] The continuous, near real-time monitoring of lactate levels during ischemia and reperfusion is highly relevant to clinical problems. Reported levels of basal lactate range between 0.9 and 1.0 mM, and the lactate levels rise almost 2-fold during ischemia and reperfusion.[23,25,29] Our lactate measurements with LOX only sensors during *in vivo* followed similar trends as in earlier studies, with a significant increase in lactate levels during ischemia and a minimal further increase during reperfusion. The present study indicates that sensors modified with Pt-ceria show a greater increase in lactate levels during reperfusion compared to ischemia. Based on our current findings, we suspect that lactate levels rise to values greater than previously appreciated, but with current lactate sensors, these levels cannot be detected due to the inability of the sensors used to operate under hypoxic conditions.

In summary, utilization of this biosensor for continuous monitoring of lactate in hypoxic brain injury demonstrated functionality of the sensor in implantable conditions. However, as with other implanted probes, we also noticed a decrease in the pre- and postmeasurement calibration of ~43%. This is a well-known problem associated with implantation of electrochemical probes[30] and can be due to several factors including biofouling, mechanical damage of the sensing layer during implantation and explantation from the tissue, and to potential degradation of the Pt-ceria surface after sequential redox cycling. Further work will explore the origin of this decrease in sensitivity and the extent of tissue damage at the implantation site.

■ CONCLUSIONS

Enzymatic microbiosensors based on Pt-ceria nanoparticles and LOX were fabricated for measuring lactate levels during hypoxia *in vitro* and *in vivo*. Integration of the oxygen-rich particles in the enzymatic layer allowed operation of the biosensor in hypoxic conditions and provided sufficient sensitivity for continuous lactate monitoring. *In vitro* characterization of the biosensor showed a wide linear range, a very low detection limit, and a quick response time. *In vivo* recording in the hippocampus of anesthetized rats demonstrated the ability of the biosensor to monitor the release of lactate during ischemia and reperfusion. This system can be used in the design of other LOX based lactate biosensors that require increased detection sensitivity and functionality in low oxygen conditions.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and optimization of experimental variables are included as described in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by NIH #R21NS078738-01 and NSF #1200180 grants. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the funding agencies.

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