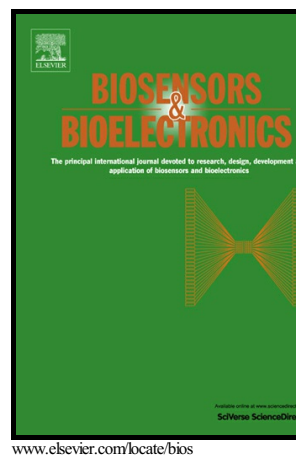


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David P. Hickey, Russell C. Reid, Ross D. Milton, Shelley D. Minter



PII: S0956-5663(15)30409-7
DOI: <http://dx.doi.org/10.1016/j.bios.2015.09.013>
Reference: BIOS7980

To appear in: *Biosensors and Bioelectronics*

Received date: 1 July 2015
Revised date: 4 September 2015
Accepted date: 6 September 2015

Cite this article as: David P. Hickey, Russell C. Reid, Ross D. Milton and Shelley D. Minter, A self-powered amperometric lactate biosensor based on lactate oxidase immobilized in dimethylferrocene-modified LPEI, *Biosensor and Bioelectronic*, <http://dx.doi.org/10.1016/j.bios.2015.09.013>

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A Self-Powered Amperometric Lactate Biosensor Based on Lactate Oxidase Immobilized in Dimethylferrocene-Modified LPEI

David P. Hickey^[a], Russell C. Reid^[b], Ross D. Milton^[a], Shelley D. Minteer^{[a]*}

^[a]Department of Chemistry, University of Utah

^[b]Department of Mechanical Engineering, University of Utah

315 South 1400 East, Salt Lake City, Utah 84112, United States

E-mail: minteer@chem.utah.edu

Abstract

Lactate is an important biomarker due to its excessive production by the body during anaerobic metabolism. Existing methods for electrochemical lactate detection require the use of an external power source to supply a positive potential to the working electrode of a given device. Herein we describe a self-powered amperometric lactate biosensor that utilizes a dimethylferrocene-modified linear poly(ethylenimine) (FcMe₂-LPEI) hydrogel to simultaneously immobilize and mediate electron transfer from lactate oxidase (LOx) at the anode and a previously described enzymatic cathode. Operating as a half-cell, the FcMe₂-LPEI electrode material generates a j_{max} of $1.51 \pm 0.13 \text{ mA cm}^{-2}$ with a K_M of 1.6 ± 0.1 and a sensitivity of $400 \pm 20 \text{ } \mu\text{A cm}^{-2} \text{ mM}^{-1}$ while operating with an applied potential of 0.3 V vs. SCE. When coupled with an enzymatic biocathode, the self-powered biosensor has a detection range between 0 mM and 5 mM lactate with a sensitivity of $45 \pm 6 \text{ } \mu\text{A cm}^{-2} \text{ mM}^{-1}$. Additionally, the FcMe₂-LPEI/LOx-based self-powered sensor is capable of generating a power density of $122 \pm 5 \text{ } \mu\text{W}$

cm^{-2} with a current density of $657 \pm 17 \mu\text{A cm}^{-2}$ and an open circuit potential of $0.57 \pm 0.01 \text{ V}$, which is sufficient to act as a supplemental power source for additional small electronic devices.

1. Introduction

Advancements in the expanding field of modern sports medicine have led to a growing interest in the development of methods to detect lactate in a continuous and convenient manner.(Nikolaus and Strehlitz 2008) Additionally, studies in the wider medical community have linked lactate concentration to various biological processes and medical conditions including tumor cell metastases and head trauma.(Cureton et al. 2010; Hirschhaeuser et al. 2011; Rassaei et al. 2014) Lactate is produced when endogenous glycogen in the muscle tissue is broken down through the glycolytic pathway to pyruvate which is subsequently reduced by lactate dehydrogenase.(Robergs et al. 2004) Throughout this process, the rate of lactate production far exceeds the rate of consumption and thus there is a buildup of lactate concentration that is proportional to the extent of physical exertion.(Falk et al. 1991; Green et al. 2004) Lactate is subsequently circulated throughout the body via the monocarboxylate transporter and can be detected in sweat, tears, urine, saliva, and serum (at typical concentrations of 20 mM, 3 mM, 0.1 mM, 0.5 mM, and 1 mM, respectively).(Goodwin et al. 2007; Johnson and Edwards 1937; Meyer et al. 2007; Robergs et al. 2004; van Haeringen and Glasius 1977) The relative abundance of lactate in a broad range of accessible biological solutions and its physiological significance as a biomarker for both physical exercise and stress make lactate an important target in the field of biosensors.

Many of the existing methods for the electrochemical detection of lactate utilize a first generation biosensor approach in which an enzyme, such as lactate oxidase (LOx), catalyzes the oxidation of lactate to produce H_2O_2 as a byproduct that is then detected amperometrically at a platinum electrode.(Faridnia et al. 1993; Goran et al. 2011; Lamas-Ardisana et al. 2014; Lei et al. 2012; Malon et al. 2014; Mascini et al. 1987) A similar approach uses the enzyme lactate dehydrogenase (LDH) and detects nicotinamide adenine dinucleotide hydrate (NADH), but this approach requires the addition of the oxidized form of NADH, called NAD^+ , because it does not occur in sufficiently high concentrations in biological fluids.(Azzouzi et al. 2015; Nesakumar et al. 2014) Both of these methods require the use of a standard three electrode cell with a reference, counter, and working electrode, for which a relatively large potential must be applied to facilitate the detection of peroxide or NADH. The large applied potential also results in artificially inflated current caused by the unwanted oxidation of biological contaminants. A possible solution to this problem is to utilize high current density redox mediators to facilitate electron transfer from the flavin adenine dinucleotide (FAD) active site of LOx to the electrode surface at a lower potential than that required for H_2O_2 oxidation.(Casero et al. 2014; Gamero et al. 2012; Qin et al. 2012) Additionally, such materials can be coupled with a biocathode to provide the electrochemical driving force required for lactate detection; such a device can then function as a self-powered lactate sensor to eliminate the need for an external power source.

Self-powered biosensors allow for the use of a simplified two electrode cell and do not require an externally applied potential to operate. Additionally, such devices are powered by biological fluids and are therefore ideal as implantable sensors. The concept for a self-powered biosensor was first described by Katz et al. as a potentiometric device for the detection of both glucose and lactate.(Katz et al. 2001) Subsequent research efforts on self-powered sensors have

primarily focused on the amperometric detection of glucose, however very little research has been performed on self-powered lactate biosensors due to the low current densities generated by peroxide oxidation.(Meredith and Minter 2011; Wang 2012; Zhou and Dong 2011; Zhou and Wang 2012) Some recent work has utilized tetrathiafulvalene (TTF) and Prussian Blue as redox mediators to minimize the applied potential necessary for adequate detection.(Jia et al. 2013; Pribil et al. 2014) However, the currents generated by such methods are still not enough to power their own operation, and thus further research into high-current density lactate sensors is needed.

Ferrocene-modified polymers such as linear poly(ethylenimine) (LPEI) have been previously used to simultaneously immobilize and mediate electron transfer of FAD-dependent oxidoreductase enzymes.(Heller 1990; Meredith et al. 2011a) Rapid rates of self-exchange for ferrocene compounds allow for efficient electron shuttling between an enzymatic active site and the electrode surface. Additionally, the use of polymethylated ferrocene moieties has been shown to allow for a lower oxidative overpotential relative to unmethylated ferrocene which minimizes inaccuracies caused by interferents such as ascorbic acid.(Meredith et al. 2013) In the work presented here, we report the use of a dimethylferrocene-modified LPEI (FcMe₂-LPEI) to immobilize LOx onto a carbon electrode as a lactate sensor. The biosensor was characterized by constant potential amperometry to determine its sensitivity and limit of detection as well as for the determination of optimal pH and temperature. Additionally, the current density generated was substantial enough to couple with a previously reported bilirubin oxidase (BOD) based biocathode to make a biofuel cell capable of providing the electrochemical driving force required for lactate detection without an external power source.(Meredith et al. 2011b) A scheme of this self-powered biosensor is shown in **Figure 1**.

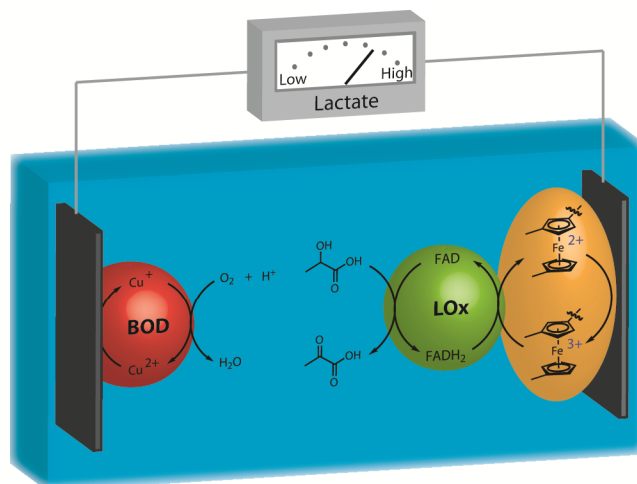


Figure 1 – Schematic of a self-powered lactate sensor consisting of a bilirubin oxidase-based cathode and a ferrocene-mediated lactate oxidase-based anode.

2. Materials and Methods

2.1. Chemicals

All chemicals were of reagent grade and were, unless otherwise specified, used without further modification. Sodium lactate, lactic acid, phosphoric acid, boric acid, acetic acid, sodium hydroxide, and lactate oxidase (LOx) from *Pediococcus sp.* were obtained from Sigma Aldrich. The LOx (Sigma L0638, activity 200 U/ml) was dissolved in a phosphate buffer at pH 6.5, divided into aliquots, and kept at -20 °C until immediately before being used. Ethylene glycol diglycidyl ether (EGDGE) was obtained from Polysciences, Inc. Billirubin oxidase (BOx) was a gift from Amano Enzyme, Inc. Tetrabutylammonium bromide (TBAB)-modified Nafion was prepared as described previously.(Treu and Minteer 2008)

2.2. Electrode preparation

Test samples were prepared using 3 mm diameter (0.0707 cm^2) glassy carbon electrodes from CH Instruments, Inc. and 0.25 cm^2 buckypaper electrodes from National Composites Center (C-grade MWNT, 27 gsm). Glassy carbon electrodes were used for basic lactate sensor characterization and buckypaper was used to demonstrate sensor performance on a material that could be integrated into a wearable device such as a lactate patch sensor or a contact lens biofuel cell.(Jia et al. 2013; Reid et al. 2015) Glassy carbon electrodes were thoroughly polished before use and buckypaper electrodes were cleaned by oxygen plasma for 5 minutes using a PDC-32G plasma cleaner from Harrick. A previously developed redox polymer was used to mediate electron transfer between LOx and the electrodes. The redox polymer used here was a dimethylferrocene-modified linear polyethyleneimine (FcMe₂-LPEI) and was prepared as previously reported.(Meredith et al. 2011a) Solutions of 10 mg mL^{-1} FcMe₂-LPEI in deionized (DI) water, 200 U mL^{-1} LOx in phosphate buffer, and 2-6% v / v EGDGE in DI water were prepared immediately prior to use. These three solutions were then combined in a volumetric ratio of 56 /24/3 of FcMe₂-LPEI/LOx/EGDGE and thoroughly mixed. Then, $3 \text{ }\mu\text{L}$ and $25 \text{ }\mu\text{L}$ was pipetted onto each glassy carbon and bucky-paper electrode, respectively, and allowed to cure overnight.

Biocathodes for the self-powered lactate sensor consisted of 3.18 mm-thick carbon felt from Alfa Aesar coated with a solution of anthracene-modified multi-walled carbon nanotubes (An-CNTs), BOD, 50 mM phosphate buffer at pH 6.5, and TBAB-Nafion. The An-CNTs were produced as previously reported.(Giroud and Minteer 2013; Meredith et al. 2011b) To prepare 3 cathodes with a geometric area of 1 cm^2 each, 7.5 mg of BOD was dissolved in $750 \text{ }\mu\text{L}$ of phosphate buffer. To that solution, 37.5 mg of An-CNTs was added and the mixture was vortexed for 1 minute, sonicated for 30 seconds and vortexed and sonicated two more times for

the same time durations.(Milton et al. 2015) 250 μ l of TBAB-Nafion was added and the solution was vortexed and sonicated one more time. Finally, 300 μ l of solution was coated onto each cathode and allowed to dry for 3 hours.

2.3. *Electrochemical methods*

All electrochemical experiments were performed in triplicate with triplicate prepared electrodes. All uncertainties correspond to the standard deviation of those triplicate measurements. All electrochemical experiments were performed with moderate stirring and using either a CHI600 series potentiostat from CH Instruments or a DY2300 potentiostat from Digi-Ivy. A 50 mM phosphate buffer adjusted to pH 6.5 with 4 M NaOH was used for all experiments except for pH dependence where a Britton-Robinson buffer (40 mM borate, 40 mM phosphate, 40 mM acetate) was used for its buffering range from pH 2-12. A platinum mesh counter electrode and a saturated calomel electrode (SCE) reference were used for all experiments except the self-powered sensor tests. All voltammetric experiments were performed with a scan rate of 2 mV s⁻¹ and amperometric experiments were all accomplished using an applied voltage of approximately 50 mV above E_{ipa} (0.3 V vs. SCE). The temperature dependence data was collected while using a Cole Parmer Polystat recirculator to control the solution temperature. All current densities were calculated using the geometric surface area of the electrode face (i.e. 0.707 cm² for glassy carbon electrodes, and 0.25 cm² for all Bucky paper electrodes).

Power curves for the self-powered lactate sensors were generated voltammetrically by performing linear sweep voltammetry on the cell while using the FcMe2-LPEI/LOx anode as the reference and counter electrode, and the An-CNT/Laccase cathode as the working electrode. In order to mimic a constant-resistance device, the voltammetrically-determined power curves were

used to calculate the effective resistance at each corresponding current density. The lactate calibration curve from the self-powered sensor was then generated by taking the voltammetrically-derived current response at the same calculated resistance (2.5 k Ω) using different lactate concentrations.

3. Results and Discussion

3.1. Electrochemical Characterization of Lactate Sensor

Lactate biosensors were prepared by cross-linking FcMe₂-LPEI onto a carbon electrode with ethylene glycol diglycidyl ether (EGDGE) in the presence of LOx. The FcMe₂-LPEI redox polymer used here was previously shown to effectively mediate electron transfer between the FAD active site of an enzyme, glucose oxidase (GOx), and the surface of an electrode in the context of a glucose biosensor/biofuel cell.(Meredith et al. 2013) The cross-linked FcMe₂-LPEI film forms a hydrogel that swells to several times its original volume which facilitates sufficient diffusion of substrate through the polymer matrix. Additionally, the swelling capability of the polymer film allows for high segmental mobility of the redox-active side chains which in turn facilitates a high rate of electron transfer via electron self-exchange between ferrocene moieties.(Hickey et al. 2014)

A comparative cyclic voltammogram is shown in **Figure 2A** of the FcMe₂-LPEI/LOx film on a glassy carbon electrode in the absence and presence of lactate. A characteristic reversible redox couple for dimethylferrocene is present in the cyclic voltammetry with an oxidation potential of 0.240 V vs. SCE and results in a mediated catalytic current density (j_{max}) of 386 $\mu\text{A cm}^{-2}$. The low oxidation potential of the ferrocene moiety relative to that of peroxide allows for a significantly lower applied potential for operation of the biosensor. A calibration

curve for lactate detection by FcMe₂-LPEI films, shown in **Figure 2B**, indicates a linear response range from 0 mM lactate up to 5 mM lactate with a reproducible detection limit of 3 μ M lactate. Additionally, the calibration curve was fitted to traditional Michaelis-Menten kinetic parameters which resulted in a calculated j_{max} of $460 \pm 14 \mu\text{A cm}^{-2}$ and a Michaelis-Menten constant (K_M) of $2.3 \pm 0.3 \text{ mM}$. While this sensor would not be suitable under the current conditions for the detection of lactate in undiluted physiological sweat samples where lactate concentrations can be higher than 20 mM, the combination of a large value of j_{max} and a small K_M allows for a high sensitivity within physiological concentrations of lactate found in tears, urine, and serum under typical conditions. Under intense physical exertion, however, lactate concentration in these fluids can approach 10 mM, which is still within the detection range for this sensor but at a lower sensitivity.

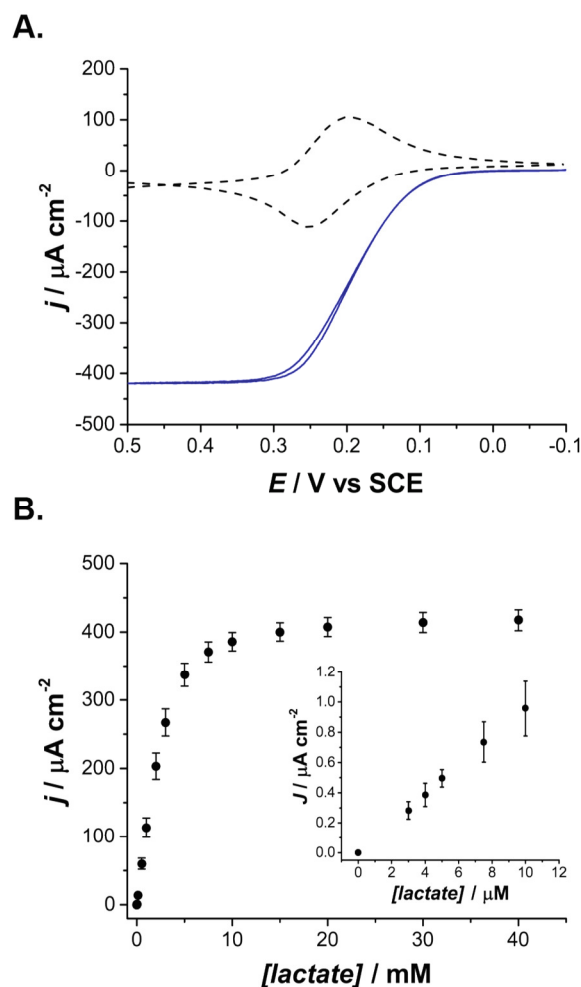


Figure 2. (A) Catalytic cyclic voltammogram of FcMe₂-LPEI/LOx film on a glassy carbon electrode in the absence (dashed) and presence (solid) of 40 mM lactate; performed at 2 mV sec⁻¹. (B) Calibration curve of FcMe₂-LPEI films with the lower concentration range inset (where error bars represent one standard deviation from the mean, n=3). Experiments were performed using a 3 mm glassy carbon electrode and 50 mM phosphate buffer at pH 6.5 and 25 °C.

With a working sensor in hand, we sought to determine the effect of both temperature and pH on the activity of the FcMe₂-LPEI/LOx films to ensure reasonable activity under the desired operating conditions. Plots of j_{max} as a function of temperature and pH for FcMe₂-LPEI/LOx films are shown in **Figure 3**. These pH and temperature profiles indicate that FcMe₂-LPEI/LOx films reach maximum catalytic activity at pH 9 and between 37 and 40 °C. These results are consistent with the reported literature on the activity of free LOx in solution, which indicates that

the FcMe₂-LPEI polymer used to immobilize the enzyme does not significantly interfere with its characteristic activity. (Lowinsohn and Bertotti 2008) Additionally, it should be noted that under physiological conditions (pH 7.4 and 37 °C), the electrode film is within 85% of its maximum activity with respect to both temperature and pH. The ability to maintain activity under these conditions is crucial when considering the possibility of using FcMe₂-LPEI/LOx as an implantable sensor.

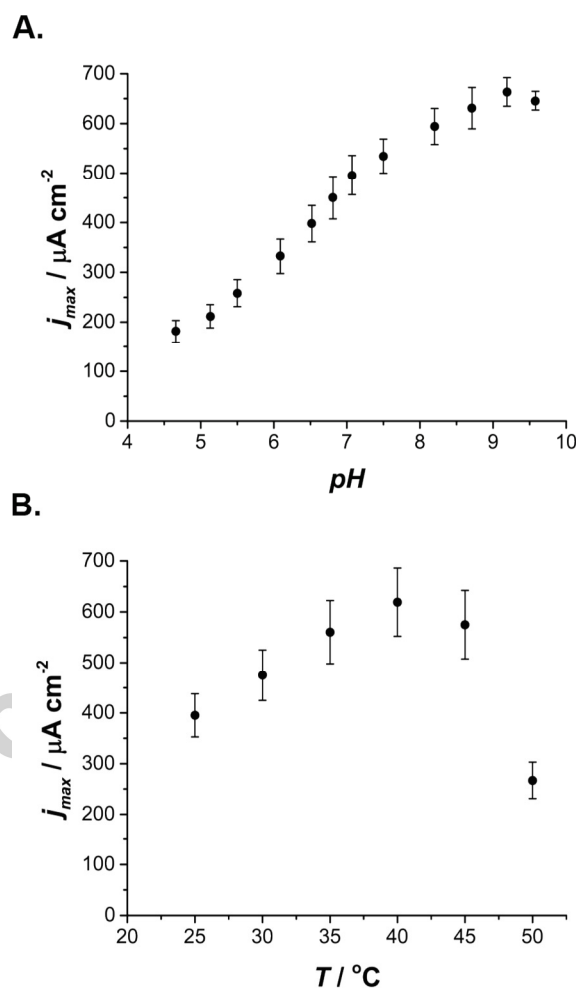


Figure 3. Temperature (A) and pH (B) profiles of FcMe₂-LPEI/LOx films on 3 mm glassy carbon electrodes. Experiments were performed using either 50 mM phosphate buffer at pH 6.5 (A) or 40 mM Robinson buffer with variable pH (B), and 40 mM lactate at 25 °C. Error bars represent one standard deviation from the mean, n=3.

3.2. Scaled Sensor on Bucky Paper

Fundamental characterization of the lactate sensor was performed on a glassy carbon electrode to minimize the possibility of anomalous effects seen with some high surface electrode materials. However, once the sensor was sufficiently characterized, we turned to the use of a high surface area electrode in order to translate the FcMe₂-LPEI/LOx film into a practical material for lactate detection. Buckypaper is a carbon paper comprised of multi-walled carbon nanotubes (MWCNT) pressed together to form a sheet that is bendable and yet maintains the excellent porosity, surface area, and resistivity of other carbon paper electrodes. In addition, forming MWCNTs into a sheet does not remove their ability to be chemically functionalized to detect a wide range of analytes. This makes buckypaper an attractive electrode material for implantable and wearable sensors requiring flexibility for adhering to non-planar surfaces or for repeated bending while in use.

Amperometric response profiles to lactate were generated for FcMe₂-LPEI/LOx films on buckypaper to serve as a comparison of the kinetic parameters between the two types of electrode materials. The resulting calibration curve, shown in **Figure 4**, was fitted as above to give a j_{max} of $1650 \pm 190 \mu\text{A cm}^{-2}$ and a K_M of $1.6 \pm 0.1 \text{ mM}$ with a detection limit of $1 \mu\text{M}$ lactate. The values of these kinetic parameters indicate that the use of a high-surface area buckypaper electrode has the effect of increasing the maximum current and sensitivity without significantly affecting the apparent binding constant of the enzyme. The ability to generate such high current densities is important as it allows for the high sensitivity needed to differentiate various lactate concentrations in a practical self-powered biosensor. Other considerations for practical biosensor development are the selectivity and shelf-life of the device.

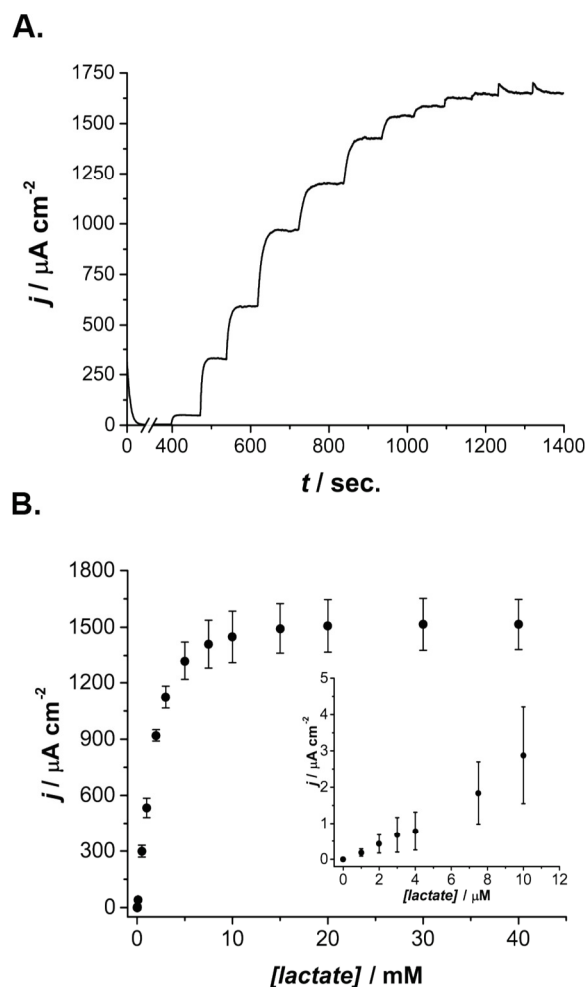


Figure 4. Amperometric response (A) and the resulting calibration curve (B) of FcMe₂-LPEI/LOx films on buckypaper electrodes to various concentrations of lactate. Experiments were performed using 50 mM phosphate buffer at pH 6.5 and 25 °C. Error bars represent one standard deviation from the mean, n=3.

Constant potential amperometry was used to determine the response of FcMe₂-LPEI/LOx films to a commonly tested biological interferent, ascorbate.(Nikolaus and Strehlitz 2008) The resulting calibration curve for ascorbate is shown in **Figure 5A**. An amperometric response ranging from $124 \pm 6 \mu\text{A cm}^{-2}$ to $185 \pm 14 \mu\text{A cm}^{-2}$ under physiological concentrations of ascorbate. The absolute magnitude of the sensor's response to ascorbate is less than 50% of the corresponding response to a change of 1 mM lactate. In addition, the range for amperometric

responses of FcMe₂-LPEI/LOx films to ascorbate does not change significantly with respect to the lactate sensitivity of the sensor.

The stability of FcMe₂-LPEI/LOx films on buckypaper was determined by preparing films and storing them at 4 °C for various lengths of time before testing their amperometric response to lactate. The storage stability of FcMe₂-LPEI/LOx films is shown in **Figure 5B**, and indicates that such films on buckypaper do not significantly lose any activity even after storage for up to 21 days. It should be noted that films may be stable for significantly longer than this, but further long-term storage stability tests are needed.

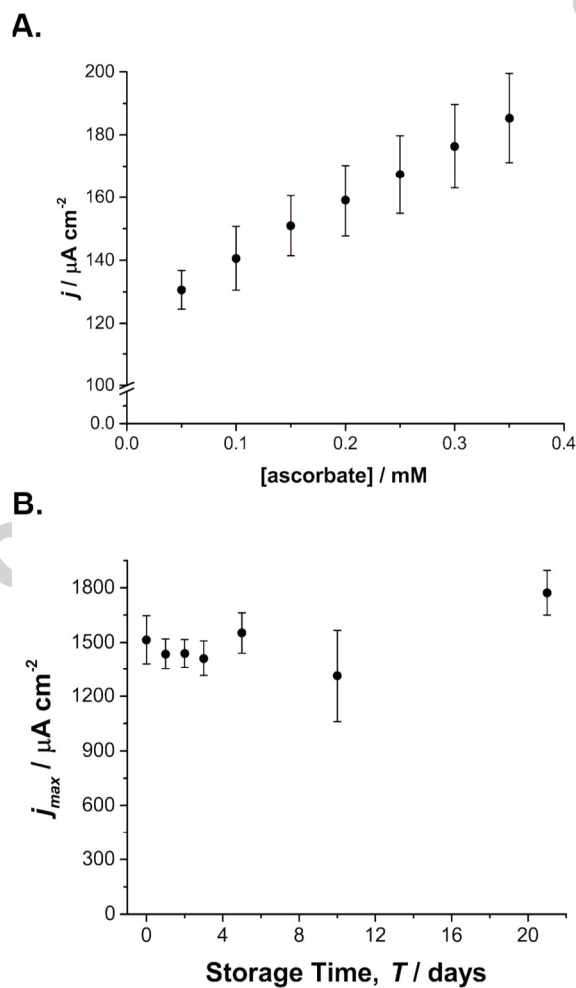


Figure 5. Amperometric response of FcMe₂-LPEI/LOx films on buckypaper to ascorbate (A) and storage stability of FcMe₂-LPEI/LOx films on buckypaper electrodes (B). For storage stability, films were stored at 4 °C and activity to both ascorbate and lactate was determined using 50 mM phosphate buffer at pH 6.5 and 25 °C. Error bars represent one standard deviation from the mean, n=3.

3.3. Self-Powered Lactate Sensor

The improved sensitivity of the FcMe₂-LPEI/LOx films on buckypaper coupled with the high current densities that were produced were sufficient to utilize the newly developed lactate sensor in a self-powered configuration. A self-powered lactate sensor was constructed by coupling the FcMe₂-LPEI/LOx film with a previously described enzymatic biocathode that uses anthracene-modified carbon nanotubes (An-CNTs) as a means of immobilizing the enzyme, bilirubin oxidase (BOD), for the reduction of molecular oxygen. In this configuration, the An-CNT/BOD cathode spontaneously reduces O₂ to water under ambient aqueous conditions which in turn generates the positive potential required for the lactate-sensing anode. (Meredith et al. 2011b) The current is measured when the electrodes are connected over a fixed resistance to determine the concentration of lactate.

Power curves of the FcMe₂-LPEI/LOx|An-CNT/BOD self-powered sensor at various concentrations of lactate are shown in **Figure 6** along with the corresponding calibration curve. A considerable transport limitation area can be seen in the low resistance region of the power curves at every lactate concentration studied which indicates that there is slow diffusion of product away from the electrode at either the anode or the cathode. However, a linear response of current density to lactate concentration is observed in a concentration range that is consistent with the anodic half-cell. The open circuit potential for all non-zero concentrations of lactate was in the range from 0.567 V to 0.580 V (with an average of 0.57 ± 0.01 V) while the current density of the self-powered sensor reached as high as $650 \mu\text{A cm}^{-2}$ in the presence of 5 mM

lactate. While the linear range of the sensor limits the analytical solutions that can be tested, the high current and power density generated under physiological concentrations of lactate could allow for the use of this material either as an implantable or wearable biofuel cell. It should also be noted that the limiting component to this device is certainly the cathode material. The relatively low current density generated at the cathode provides a limit to the sensitivity that can be achieved, and thus any improvements on this device should be aimed at the development of more effective cathode materials.

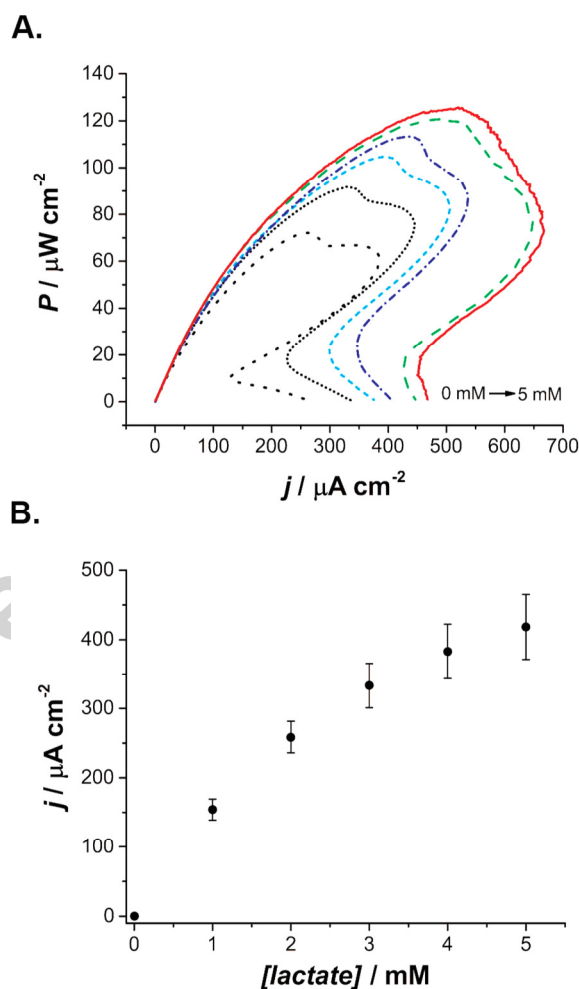


Figure 6. Power curves (A) and corresponding calibration curve (B) for FcMe₂-LPEI/LOx/An-CNT/BOD self-powered lactate sensors. Experiments were performed using 50 mM phosphate buffer at pH 6.5 and 25 °C. Error bars represent one standard deviation from the mean, $n=3$.

4. Conclusions

Self-powered biosensors allow for the electrochemical detection of a biological analyte without the need of an external power source to supply a potential to the working electrode. We have presented a ferrocene-mediated lactate sensor capable of generating sufficient current density to operate as the anode of a self-powered lactate sensor. The amperometric sensor was prepared by immobilizing LOx onto an electrode surface with a cross-linked film of FcMe₂-LPEI. The FcMe₂-LPEI/LOx biosensor material exhibits maximum catalytic activity under near physiological pH and temperature and can be stored for up to 21 days without significant loss of activity. We coupled this material with an enzymatic biocathode to construct a self-powered lactate biosensor with a linear amperometric response range between 0 and 5 mM lactate and an open circuit potential of 0.57 ± 0.01 V. Additionally, the self-powered sensor was capable of generating a maximum power density of $122 \pm 5 \mu\text{W cm}^{-2}$ and a maximum current density of $657 \pm 17 \mu\text{A cm}^{-2}$. A possible approach moving forward is to couple this self-powered sensor to a triboelectric or piezoelectric generator as a supplemental power supply to enhance the operational stability of an applied device. (Hansen et al. 2010; Ramadoss et al. 2015; Yang et al. 2013) Future studies must still be performed to determine the potential toxicity effects of long-term use of such devices, and ongoing research is aimed at utilizing these high-current density materials to engineer practical self-powered biosensors and biofuel cells into both wearable and implantable devices.

Acknowledgments

The work presented here was funded by the National Science Foundation (Grant #1158943).

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Highlights

- A self-powered amperometric lactate sensor was constructed using a ferrocene-modified linear poly(ethylenimine)/lactate oxidase hydrogel anode and a previously reported enzymatic biocathode
- Maximum current and sensitivity for the amperometric lactate sensor were $1.51 \pm 0.13 \text{ mA cm}^{-2}$ and $400 \pm 20 \text{ } \mu\text{A cm}^{-2} \text{ mM}^{-1}$
- The self-powered lactate sensor generated $122 \text{ } \mu\text{W cm}^{-2}$ of power with a sensitivity of $45 \pm 6 \text{ } \mu\text{A cm}^{-2} \text{ mM}^{-1}$
- The lactate sensor was shown to maintain activity even after 21 days of storage at $4 \text{ }^{\circ}\text{C}$