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Real-Time Monitoring of Cellular Bioenergetics with a Multi-Analyte Screen-Printed Electrode

Jennifer R. McKenzie^{†,‡}, Andrew C. Cognata[†], Anna N. Davis[†], John P. Wikswø^{‡,§}, and David E. Cliffler^{†,‡,*}

[†]Department of Chemistry, Vanderbilt University, Nashville, TN 37235, United States

[‡]Vanderbilt Institute for Integrative Biosystems Research and Education, Vanderbilt University, Nashville, TN 37235, United States

[§]Departments of Physics and Astronomy, Biomedical Engineering, and Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37235, United States

Abstract

Real-time monitoring of changes to cellular bioenergetics can provide new insights into mechanisms of action for disease and toxicity. This work describes the development of a multi-analyte screen-printed electrode for the detection of analytes central to cellular bioenergetics: glucose, lactate, oxygen, and pH. Platinum screen-printed electrodes were designed in-house and printed by Pine Research Instrumentation. Electrochemical plating techniques were used to form quasi-reference and pH electrodes. A Dimatix materials inkjet printer was used to deposit enzyme and polymer films to form sensors for glucose, lactate, and oxygen. These sensors were evaluated in bulk solution and microfluidic environments, and found to behave reproducibly and possess a lifetime of up to six weeks. Linear ranges and limits of detection for enzyme-based sensors were found to have an inverse relationship with enzyme loading, and iridium oxide pH sensors were found to have super-Nernstian responses. Preliminary measurements where the sensor was enclosed within a microfluidic channel with RAW 264.7 macrophages were performed to demonstrate the sensors' capabilities for performing real-time microphysiometry measurements.

Real-time monitoring of cellular metabolism can enhance the knowledge gained during traditional toxicology studies by detecting metabolic changes as they occur.¹ Advances in instrumentation allow metabolic measurements to be performed in real-time and with high throughput using electrochemical methods.^{2–5}

*Corresponding Author: d.cliffler@vanderbilt.edu. Tel: +1-615-343-3937. Fax: +1-615-343-1234.

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Supporting Information: The supplementary section provides additional descriptions and figures to support statements made in the main document and further background and detail to interested readers. It includes photographs of the microfluidic channels and enzyme modifications, cyclic voltammograms of the unmodified, commercially printed sensor in ferricyanide, representative plots of data from which reported values listed in the paper were derived, and data from sensor storage studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Electrochemistry offers many advantages over other analytical methods employed in the study of biological systems. Rapid and continuous measurements permit observation of real-time changes in diverse biological systems, from single cells⁶ to patients in a clinical setting.⁷ Additionally, as demonstrated by Wang et al., electrochemical sensors can enable label-free, real-time intracellular and extracellular measurements without perturbing the system under investigation.⁶ This capability is important for the incorporation of metabolic dynamics into systems biology models,⁸ for closed-loop control of cellular systems,⁹ and for monitoring the health and drug response of organs-on-chips.^{10,11}

Temporal resolution can be improved by co-locating the sensors with the cells to prevent mixing due to diffusion. Placing both sensors and cells within a microfluidic environment decreases the distance from cells to sensors¹² and increases sensitivity by lowering sample volumes, minimizing the dilution of cellular metabolic products such as lactate into a large extracellular volume, and maximizes the concentration changes with cellular consumption and production, e.g., glucose, oxygen, lactate and acid.^{13,14} Electrochemical measurements are often coupled with microdialysis and electrophoretic separations to enable near-real-time detection of metabolites.^{5,7} Mecker et al. combined microdialysis with electrochemical detection of dopamine for near-real-time detection from cultured neurons.⁵ The addition of a sensor for catechol further improved these measurements by allowing simultaneous detection of multiple analytes, which can provide a more detailed investigation of the biological system under study.¹⁵

The combination of multi-analyte detection at or near cells under study yields more knowledge than can be gained from studying only one analyte. The Amatore group regularly performs simultaneous detection of reactive oxygen and nitrogen species released from cells, which makes it possible to differentiate between oxidative burst mechanisms controlling release of those products.^{4,6} In another example, Feuerstein et al. measured glucose and lactate amperometrically from a microdialysis probe and combined these measurements with subdural electrocorticographic recordings to measure how cellular energetics were related to acute brain injury.⁷

Other methods combine amperometric and potentiometric sensors to measure changes to cellular bioenergetics in real-time.^{14,16} The multi-analyte microphysiometer (MAMP) employs amperometric glucose, lactate, and oxygen sensors and a pH-sensitive light-addressable potentiometric sensor (LAPS) to measure real-time changes caused by the metabolism of cells immobilized in a microfluidic chamber.¹⁴ The unique combination of these analytes allows for the monitoring of both aerobic and anaerobic respiration. Glucose is consumed in both forms of respiration and converted to pyruvate. In aerobic respiration, pyruvate and consumed oxygen enter the TCA cycle, with final products of ATP and dissolved CO₂, the latter of which is released into the extracellular space and acidifies the media. In anaerobic respiration, pyruvate is converted to lactic acid via lactate dehydrogenase, releasing lactate and protons.

The MAMP was created by modifying a Cytosensor Microphysiometer,¹⁷ developed by Molecular Devices and capable of detecting shifts in pH in 2D cultures, with additional sensors for the detection of glucose, lactate, and oxygen.¹⁴ When combined with a

multichamber multipotentiostat to allow simultaneous multi-analyte measurement in up to eight microfluidic chambers,³ the MAMP can simultaneously monitor several different aspects of cellular respiration in real-time.

This 4-analyte system has been used in numerous studies, including the discrimination of toxins based on their metabolic effects,¹⁸ exploring the metabolic pathways affected by cholera toxin exposure,¹⁹ the effects of nutrient deprivation on neuronal bioenergetics,²⁰ and immune cell activation.²¹

A new MAMP platform would allow for a wider range of studies and enable simplified, high-throughput experiments. Planar screen-printed electrodes (SPEs) have found utility in environmental and food analysis methods due to their simple fabrication, versatility, high reproducibility, and low cost.²² In addition, SPEs are planar, simplifying the design of a microfluidic chamber.

Towards the goal of creating a next-generation MAMP, a SPE with five modifiable Pt electrodes was designed. Each electrode was modified, creating a 4-analyte sensor for glucose, lactate, oxygen, and pH against a Ag/AgCl quasi-reference, allowing for simultaneous detection within one microfluidic channel. A materials inkjet printer was employed to deposit enzyme and polymer films in a reproducible manner. The sensors were evaluated in bulk and microfluidic volumes in order to determine the sensitivity and lifetimes of the resulting films. Finally, this sensor was enclosed in a microfluidic flow chamber with macrophages cultured on a membrane and perfused with culture media to demonstrate its feasibility as a microphysiometry platform.

Methods

Reagents and Apparatus

The SPE was designed in-house and printed by Pine Research Instrumentation. Silver nitrate, sulfuric acid, ammonium, potassium chloride, potassium ferricyanide, 50 mM phosphate buffer (pH 7), sodium L-lactate, and potassium carbonate anhydrous were obtained from Fisher Scientific (Waltham, MA). 30% hydrogen peroxide, oxalic acid dihydrate, bovine serum albumin (BSA), and 25% glutaraldehyde were obtained from Sigma-Aldrich (St. Louis, MO). Iridium tetrachloride, iron trichloride, Nafion®, and Pt wire for fabricating counter electrodes were obtained from Alfa Aesar (Ward Hill, MA). A Veeco Dektak 150 profilometer with a 12.5 μm diamond-tipped stylus was employed to trace surface topography of the SPE. Concentrated hydrochloric acid and sodium hydroxide were obtained from EMD Millipore (Billerica, MA). Stabilized glucose oxidase (GOx) with activity of 153 U/mg and stabilized lactate oxidase (LOx) with an activity of 11.3 U/mg were obtained from Applied Enzyme Technologies (Pontypool, UK). Aqueous reference electrodes and a 660A Electrochemical Analyzer for sensor modification were from CH Instruments (Austin, TX). A previously described multichamber multipotentiostat was used to perform simultaneous amperometric and potentiometric measurements.³ A FujiFilm Dimatix DMP-2800 Series materials printer and all related consumables were obtained from Fujifilm Dimatix, Inc. (Santa Clara, CA).

RAW 264.7 gamma NO⁻ macrophages (CRL-2278) were obtained from the American Type Culture Collection (Manassas, VA). 20% Glucose, RPMI-1640 and gentamicin-sulfate were obtained from Life Technologies (Carlsbad, CA). Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). Dubecco's Phosphate Buffered Saline (DPBS) was obtained from Corning Cellgro (Manassas, VA). Custom RPMI (1 mM phosphate, no glucose or sodium bicarbonate) was obtained from the Vanderbilt Molecular Biology Core.

Description and Testing of the Unmodified Sensor

The SPE consists of four layers: a white ceramic substrate, a printed Pt layer, and two identical printed clear ceramic layers. The composition of the metal ink is proprietary to Pine Research Instrumentation; however it differs from other common SPE methods, in that no binders are required to adhere the metal film to the ceramic. There are three Pt disk electrodes with areas of 1.8 mm² intended for glucose, lactate, and pH detection, one band electrode with a total area of 0.08 mm² intended for oxygen detection, and a band electrode with an area of 19 mm² modified to act as a Ag/AgCl quasi-reference in a 2-electrode system. The areas of the three disk electrodes are defined by the insulating ceramic layer. Due to the small area of the oxygen electrode and the 100 μm resolution of the ceramic printing process, the oxygen electrode area was not defined by the insulating layers. The electrodes are labeled **1-5** in Figure 1c.

Stylus profilometry was used to determine the thickness of each layer, as well as the contours of the sensor, in order to design an effective microfluidic chamber. A 12.5 μm diamond-tipped stylus applying 6.5 mg of force was used to trace the surface of the SPE, including over the electrical contacts, the insulating ceramic layer, and the electrode surfaces. An example of a typical scan path and resulting profilometry scan are shown in Figure 1 a and b. Figure 1c details the six scan locations and resulting thickness, as well as illustrates the contours of the sensor.

Cyclic voltammetric measurements were carried out using a CHI 660A Electrochemical Analyzer to determine the reproducibility of the custom sensors. The four working electrodes were scanned in 5 mM ferricyanide with 100 mM KCl at a scan rate of 0.1 V/s. The largest electrode on the SPE was used as a counter electrode, and measurements were against an external Ag/AgCl (3 M KCl) reference electrode.

Electrochemical Sensor Modifications

Two electrochemical modifications were performed to modify the SPEs for four-analyte measurements. These modifications utilized an external aqueous Ag/AgCl (3 M KCl) reference and Pt mesh counter. All electrochemical modifications were performed prior to application of polymer and enzyme films to prevent bath solutions from damaging the sensors. Prior to any modifications, each electrode was electrochemically cleaned by cycling in 0.5 M sulfuric acid.

Preparation of Ag/AgCl quasi-reference electrodes on electrode **1** were performed by modifying a method previously developed for evaporated Pt electrodes.²³ A solution

containing 0.3 M silver nitrate in 1 M ammonia was dispensed into a pipet basin using a 0.2 μm syringe filter. Both preconditioning and plating steps occurred during stirring at 300 rpm. The 19 mm² Pt electrode was first preconditioned by holding the potential at +0.95 V vs. Ag/AgCl (3 M KCl) for 30 s, and then plated at a constant current of 6.5 mA/cm² for 450 s. The resulting Ag films were then treated by submersion and agitation in 50 mM FeCl₃ for 60 s to form a AgCl layer. After oxidation, the sensors were stored in phosphate buffer (PB) with 120 mM KCl at 4 °C until the next modification.

Solution preparation of pH-sensing iridium oxide (IrOx) films was adapted from previously published methods.²⁴ Briefly, 37.5 mg IrCl₄ was weighed in an amber bottle. 25 mL of DI water was added and the solution stirred. After 15 min, 250 μL of cold 30% H₂O₂ was added and the solution stirred for 10 min prior to adding 125 mg oxalic acid dihydrate. After 10 min, potassium carbonate anhydrous was used to adjust the pH of the solution from ~1.5 to 10.5. The resulting iridium oxalate solution was stored in the dark at room temperature for 5 days to develop, and then stored at 4 °C.

Preparation of pH-sensitive IrOx films on **5** was performed on one 1.8 mm² Pt disk electrode on each sensor. The solution was dispensed into a pipet basin using a 0.2 μm syringe filter. Both preconditioning and plating steps occurred during stirring at 300 rpm. The electrode was preconditioned by holding the potential at -0.6 V vs. Ag/AgCl (3 M KCl) for 60 s, followed by a constant potential of +0.6 V vs. Ag/AgCl (3 M KCl) for 7 min. After plating, sensors were returned to PB with 120 mM KCl at 4 °C until the next modification.

Application of Enzyme Films

Enzyme and polymer films were used to modify the remaining Pt electrodes for detection of glucose, lactate, and oxygen. In previous MAMP studies, enzyme and polymer films were prepared by mixing the enzyme solutions and glutaraldehyde cross-linker together immediately prior to hand-casting films on the electrode surface.² This method can result in inconsistent enzyme coatings with less reproducibility. The planar nature of the SPEs permitted the use of a materials inkjet printer to deposit enzyme solutions in a controlled manner, allowing for greater reproducibility and mass production.

Preparing enzyme solutions for use in a materials inkjet printer required changes from the previously published protocols.²⁰ To counteract bubble formation by BSA, the BSA-PB solution was lowered to a concentration of 31 mg/mL BSA. The BSA-PB solution was then used to dissolve either stabilized GOx or LOx. Enzyme solutions were gently mixed to limit bubble formation. A 0.25% glutaraldehyde solution was prepared by diluting 25% glutaraldehyde in 50 mM PB, pH 7. A dilute Nafion[®] solution was prepared with equal volumes of 5% w/w formulation of perfluorosulfonic acid-PTFE co-polymer (Nafion[®]) and 200 proof ethanol. The solutions were then dispensed into Dimatix printer cartridges via a 0.2 μm syringe filter and blunt stainless steel needle.

GOx and LOx films were printed in a 4 mm² square over the 1.8 mm² disk electrodes **2** and **4**, respectively. A drop voltage of 23-25 mV, a drop spacing of 35 μm and application of 2 layers of enzyme solution, followed by 1 layer of cross-linker were used. Two layers of the dilute Nafion[®] solution were printed in a 0.5 mm $\times$ 1 mm rectangle over the 0.08 mm² Pt

electrode **3** to form the oxygen sensing electrode. In some studies, layers of Nafion[®] were also applied over the GOx films to form a diffusion barrier. This modification strategy contrasts with a recently published study where cross-linker was added to the enzyme ink prior to printing, and the diffusion layer was applied via spin-coating.²⁵

After printing, sensors were stored dry at 4 °C for three days, and once wetted, stored in PB, pH 7 with 120 mM KCl. Once loaded, printer cartridges were stored at 4 °C. Prior to next use, the print head of each cartridge was removed and sonicated for 60 s in DI water to clear the nozzles.

Bulk Calibration of Sensor Arrays

After modification, sensor arrays were tested in 1 mM phosphate buffer with 120 mM KCl. A previously described multichamber multipotentiostat was used to simultaneously measure glucose, lactate, oxygen, and pH versus the Ag/AgCl quasi-reference at each sensor. The potentiostat features six individual channels which each measure all four analytes, allowing for simultaneous detection of up to 24 sensors in one experiment, with sampling performed once a second.³ Glucose and lactate were measured amperometrically by biasing the potential at +0.6 V vs. Ag/AgCl quasi-reference to oxidize the hydrogen peroxide produced by their respective enzyme films. Oxygen was measured amperometrically by biasing the potential at -0.45 V vs. Ag/AgCl quasi-reference to reduce dissolved oxygen. An open-circuit potential (OCP) measurement vs. Ag/AgCl quasi-reference was used to measure the change in potential at the IrOx pH-sensing electrode. An Orion pH meter and probe were used to monitor the change in pH of the solution to compare to OCP at the IrOx electrode. Glucose and lactate were added to the stirred solution using a 20% glucose stock and 500 mM sodium-L-lactate stock solution. pH was modulated with additions of dilute HCl. Oxygen was measured, but not calibrated, to avoid altering GOx and LOx activity by modulating oxygen tension throughout the experiment.

Bulk calibrations for glucose, lactate, and pH were performed in two manners: simultaneous mode, where each analyte was added concurrently at each 5min step, and sequential mode, where each analyte was added in 5min intervals until the desired concentration was reached before beginning additions for the next analyte. In simultaneous mode, glucose, lactate, and acid were added for each step in 20 s intervals. In sequential mode, lactate was added first, followed by glucose, and acid.

Microfluidic Calibration of Sensor Arrays

Originally, a PDMS channel was fabricated to create a 23 μ L channel and was employed in a previous study where macrophages were cultured on the roof of the channel and sealed to the sensor to allow for real-time detection of superoxide production.²⁶ While these devices allowed for real-time detection of metabolites, they were difficult to seal without crushing the cells or blocking the electrodes with PDMS or bubbles, limiting reproducibility. For this work, two custom acrylic housings were designed to house either a single SPE or a six-sensor array of SPEs. Further description and photographs of the devices can be found in the Supplemental Material. Figure 2 details how fluid moves over the electrodes and provides a cross section view of the housings with and without cells. Calibrations in the microfluidic

channels were performed using the same measurement parameters as the bulk measurements. Harvard Apparatus PicoPumps and a VICI five-position valve were used to deliver calibration solutions to a sensor sealed in the acrylic housing. Due to limited availability of valves, only one channel was calibrated at a time.

Microphysiometry Performed with Microfluidic Channel

Microphysiometry experiments aim to enclose adherent cells within a microfluidic chamber with sensors to achieve real-time bioenergetic responses of cells to stimuli. A previous study was conducted with the SPE cultured cells on the roof of a disposable PDMS housing to measure real-time release of superoxide from stressed immune cells,²⁶ but a new approach was required to enclose cells in close proximity to sensors in the acrylic microfluidic housing. Macrophages were plated on cell-culture treated, 3 μ m pore polycarbonate (PC) membranes at a density of 2×10^5 cells/mL and cultured for 2 days. The membrane was situated directly on top of a SPE. To prevent direct flow from the inlet tube of the housing from disrupting adhered cells, an unmodified membrane was added between the cell membrane and lid of the housing. The top of the six-sensor housing was then sealed against the membranes, immobilizing cells and the SPE within a 23 μ L chamber. Once assembled, the bioreactor was perfused with a low-buffered (1 mM phosphate, bicarbonate-free) version of RPMI-1640 with 5 mM glucose at 15 μ L/min. A stop-flow method was employed with a repeating pattern of 200 s at 15 μ L/min, followed by 40 s at 0 μ L/min. Cessation of flow allows for detectable levels of glucose and oxygen consumption and lactate and acid production. After basal metabolism was measured, cells were perfused with 10 μ g/mL alamethicin to initiate cellular death through formation of pores in the cell membrane. After cell death, normal media is returned to obtain the electrochemical signals with contributions from cellular metabolism.

Results and Discussion

Characterization of Unmodified Sensor

The contours of the unmodified sensor were measured using stylus profilometry. As shown in Figure 1a and b, by scanning the tip over the surface, it was observed that the areas with ceramic were relatively smooth compared to the exposed electrode areas. The sensor is printed with a Pt nanoparticle ink, resulting in the rough surface of the electrode areas. A raised pattern was also observed when scanning over the areas where Pt was present under the ceramic layer. Repeated scans over the entire sensor surface were performed to characterize this pattern and are shown in Figure 1c.

The Pt contacts at the top of the SPE were found to have a curved surface, with the edge of the contact having a height of $8.5 \pm 0.6 \mu\text{m}$ and the center a height of $5.3 \pm 1.3 \mu\text{m}$. This suggests that the surface tension of the printed Pt nanoparticles suspended in liquid forms a meniscus, and this shape is maintained after baking. The metal films are thinner than those typically achieved through thick-film screen-printed processes. Unlike carbon SPEs, which create a carbon ink by mixing carbon with a polymer binder,²⁷ the proprietary process performed by Pine Research Instrumentation creates Pt films on ceramic using minimal amounts of binder present in the ink. The RMS roughness of the underlying ceramic

substrate and the Pt layers were 732 ± 70 nm (N=2) and 673 ± 73 nm (N=3), respectively, and the smoother ceramic insulating layer was 359 ± 70 nm (N=3).

The printed meniscus has a multiplicative effect on the ceramic layers printed on top. This can be seen in the scan of the chip over the Pt wires between the contacts and exposed electrodes, where the contours of the printed Pt are still observed, and are in fact increased to 9.5 ± 1.2 μ m. These ridges suggest that a successful microfluidic channel must seal well against these contours to prevent fluid leakage.

The printed ceramic layer was used to define the electrode areas of the three 1.8 mm² diameter disk electrodes. Under these conditions the liquid ceramic beaded up at the edges of the electrode prior to baking. This effect, in conjunction with the thickness of the ceramic layer, formed a 35 μ m well with the electrode at the bottom, which were taken into account when designing a microfluidic chamber.

Electrochemical testing of the quality of the printed electrodes was performed by cyclic voltammetry in K₃Fe(CN)₆. As shown in Supplemental Figure 2, the three Pt disk electrodes and single small Pt bar electrode have similar current densities. The average cathodic peak potential, E_{pc} , was 0.182 ± 0.001 V, and the average anodic peak potential, E_{pa} , was 0.253 ± 0.003 V, exhibiting good reversibility ($E_p = 70.5$ mV), N = 4.

Sensor Modification

The SPE was modified for detection of glucose, lactate, oxygen, and pH against a Ag/AgCl quasi-reference. Supplemental Figure 3 shows 50 $\times$ images of the sensor films. The printed enzyme films are not visible over the rough Pt surface, but the edges of the square printed films are visible in Supp. Figure 3d-f. Initially, silver quasi-references were fabricated using a Cy-less Silver-plating solution, but these films proved unstable when used in the 4-analyte detection scheme. Silver from silver nitrate was deposited using a constant current method and the resulting films were evaluated by assessing film thickness and stability. Stylus profilometry was used to scan across three locations of both unmodified and silver-plated 19 mm² electrodes. The average height of the unmodified electrode was subtracted from each modified sensor, with the average thickness calculated to be 5.1 ± 0.6 μ m (N = 5), comparable to thickness obtained from the original plating method.²³ The calculated RMS roughness was 2.0 ± 0.3 μ m, significantly greater than the underlying Pt layer. When tested in PBS, fabricated Ag/AgCl quasi-references were found to have an OCP of 76 ± 19 mV vs. Ag/AgCl (3 M KCl). The films were tested after one month of use in various applications performed in cellular media, and the OCP was 72 ± 14 mV vs. Ag/AgCl (3 M KCl). The drift over a period of twelve hours when the films were new was -40 nV/s and -100 nV/s after a month of use, or a total change of 1.7 mV and 4.3 mV over the measurement, which was comparable to drifts observed using a similar method on evaporated Pt films.²³

After formation of the quasi-reference, electrode **5** was cycled in sulfuric acid and then plated with an IrOx film. Variations were observed in film color, ranging from dark blue films to films with a gradient of color from blue to colorless across the disk electrode. This effect was also observed when the method was repeated on commercially available smooth Pt disk electrodes.

After both electrodeposition methods were completed, the remaining three electrodes were cycled in acid to remove adsorbed species, rinsed with DI water, and left to dry in air. GOx, LOx, and Nafion[®] films were then deposited using a Dimatix materials inkjet printer. Initially, after drying the sensors were stored in PBS at 4 °C. In these studies, the linear range of the glucose sensor was only 5 mM. This range was found to increase to at least 28 mM glucose by keeping the sensors dry at 4 °C for several days after printing. One sensor array was stored dry, and individual sensors were tested on day 5, 13, and 26 (Supplemental Figure 4). Loss of sensitivity attributed to enzyme degradation began to occur after seven days of cold, dry storage. While the glucose sensors were still operational after three weeks' dry storage, the lactate sensor exhibited total loss of sensitivity after two weeks. While loss of sensitivity during dry storage is not uncommon,²⁸ there are many other examples of enzyme-based sensors with longer lifetimes, which in some cases have been shown to be stable for 18 months when stored desiccated at 4 °C.²⁷ Sensor stability over time is dependent on the method used to immobilize the enzyme, and is most successful when the tertiary structure of the protein can be stabilized to prevent denaturation.²⁹ Four common methods for achieving this aim are enzyme entrapment in a polymer, cross-linking, covalent attachment to the electrode, or microencapsulation within an electrode.^{29–31} In the case of the glucose sensor with an 18 month lifetime, GOx was added to the carbon ink used to prepare the thick-film SPEs, which enabled extended sensor lifetimes. The sensors discussed in this study were stabilized by cross-linking with glutaraldehyde, which provides enough stability for lifetimes longer than a month when stored in buffer, but not enough to allow long-term, dry storage. It is unclear if the materials printer could be used to prepare sensors using alternate stabilization methods, especially those requiring electrochemical procedures.

Following this study, all sensors were stored dry for three days after printing, followed by continued cold storage in PBS for up to six weeks. The increase in linear range observed in the glucose sensor is attributed to the robust activity of the GOx enzyme, which requires the co-consumption of oxygen. The dry storage of the enzyme electrodes likely causes some of the GOx to denature, lowering the effective enzyme loading of the film and decreasing the oxygen requirements, which then enables a larger linear range. LOx has a much lower activity, and is known to be more sensitive to unfavorable storage or handling.³²

Several variations were attempted in the development of printed sensors, including changes in enzyme loading and additional coatings. GOx was printed at concentrations of 2.5 mg/mL and 0.25 mg/mL, where the 0.25 mg/mL sensors were tested with and without additional layers of Nafion[®]. LOx was printed at four different concentrations: 2 mg/mL, 10 mg/mL, 20 mg/mL, and 25 mg/mL. The resulting linear ranges and limit of detection (LOD) are listed in Table 1. Figures of merit for GOx and LOx sensors used in a recent work with the original MAMP²⁰ are listed for comparison. The hand-cast LOx films featured a LOD comparable to LODs found for the 20 mg/mL LOx SPE sensors.

Enzyme loading was found to be critical for modulation of LOD and linear range for both GOx and LOx sensors. For example, the linear range of the GOx sensors more than doubled when the enzyme loading was reduced by a factor of ten but the calculated LOD also increased. This knowledge allows tailoring of enzyme films to match critical metrics in a given study. For example, in cases where the user only needs to measure over small range,

such as 0 to 5 mM glucose, but wants to observe smaller changes, it may be advantageous to use higher enzyme loading. This contrasts with the hand-case GOx sensor where the enzyme loading was sufficiently high to limit the linear range of the sensor, but did not exhibit the decrease in LOD observed with higher enzyme loadings in the SPE printed films.

While lowering the amount of entrapped enzyme was successful in increasing detection ranges for both types of sensors, in the case of the lactate sensor with 2 mg/mL LOx, subsequent attempts to use the sensor yielded little to no sensitivity, suggesting that the stability of the entrapped LOx was poor, shortening the sensor lifetime. An enzyme loading of 10 mg/mL LOx yielded a linear range between 1 and 2 mM lactate, and yielded sensor lifetimes of at least six weeks.

Some variations were noted between the sensors that were compiled over several printing sessions. In the case of the 0.25 mg/mL GOx sensors with Nafion[®], the linear range varied from 28 mM glucose to as high as 75 mM glucose. Similarly, the linear range of the 10mg/mL LOx sensors varied from 1 to 2 mM lactate. The lowest linear range obtained for these sensor sets is listed in Table 1.

The oxygen sensor was modified with two printed layers of 2.5% Nafion[®]. The average signal obtained from this sensor during bulk measurements was 275 ± 130 nA. For further comparison, oxygen measurements at the original MAMP sensor head have typically been on the order of 20 – 90 nA.^{3,19} As the SPE oxygen electrodes were designed to have the same total area as those in the MAMP, it is likely the increased surface area from the rough surface as well as the larger microfluidic volumes of the SPE contributes to larger currents.

Some sensor arrays were calibrated in simultaneous mode and sequential mode to determine the selectivity of each sensor. As shown in Supplementary Figure 5, both the LOx and GOx sensors were selective to their respective substrates. Additionally, resulting linear ranges and LODs for the glucose and lactate sensors were not found to be significantly different between the two calibration modes.

The IrOx sensors, however, did respond to additions of glucose and lactate, where additions of either lead to an increase in OCP. This effect increased the calculated sensitivity found when all four electrodes were calibrated in simultaneous mode, where the pH sensors yielded a sensitivity of -106 ± 15 mV/dec ($N = 12$) in simultaneous mode and -73 ± 7 mV/dec ($N = 12$) in sequential mode. The value yielded in sequential mode is consistent with other hydrated IrOx films,³³ including other films prepared on Pt substrates.³⁴ Due to the low levels of glucose and lactate observed in microphysiometry, these effects are not significant for this study, but will be discussed further in a future work.

The addition of the IrOx sensors enables a departure from the Cytosensor-based MAMP. As previously discussed,³ the combination of the amperometric measurements with the silicon-based LAPS for sensing pH in the Cytosensor MAMP resulted in increased noise in the pH signal, with standard errors near 25%. Moving to a pH sensor that can be measured with the same electronics vastly reduces the noise in the signal. When an IrOx and LAPS sensor were tested in the original MAMP, sensitivities were comparable at -39 mV/dec and -32 mV/dec ($N = 1$) respectively. While the phenomenon has not been investigated fully, it seems likely

that the IrOx sensor on the SPE will also yield sub-Nerstian sensitivities when calibrated in a cellular environment.

Microfluidic Calibration of the Multi-Analyte Sensor

Originally, microfluidic measurements were performed in the six-sensor array; however, inefficiencies in flow splitting, a lack of microfluidic valves to enable switching between calibrants, and leaking from the Diba fittings rendered microfluidic simultaneous calibrations at all six channels difficult. To address this, a single-channel microfluidic device was devised.

A six-sensor array was modified with each of the previously described sensor modifications, with a 2.5 mg/mL GOx film and a 20 mg/mL LOx film. The array was calibrated in a bulk solution of PBS in ten steps in simultaneous mode with additions of glucose, lactate, and acid.

Each sensor was then detached from the array to allow for calibration in the microfluidic chamber. A Harvard PicoPlus pump was used to pump calibrants at 100 $\mu\text{L}/\text{min}$, with a VICI valve to enable fluid switching. This calibration was repeated for each of the six sensors. The resulting calibration curves from bulk and microfluidic measurements were compared. A representative comparison is shown in Figure 3. Across all six sensors, the glucose calibrations performed in bulk yielded lower current magnitudes. At 16 mM glucose, the highest concentration, signal at the electrode during bulk calibrations was on average $44 \pm 5\%$ lower than the current observed during the microfluidic measurement. This trend was not observed in the lactate sensor, where the average difference between the bulk and microfluidic signals was $6 \pm 3\%$. Oxygen signals were also 50% higher on average in the microfluidic channels, as well as less noisy. The average deviation in baseline oxygen signal in the microfluidic channel was 9nA, versus an average deviation of 17nA in the bulk solution. Differences in baseline noise are attributed to the noise from magnetic stirring compared to the noise from a syringe pump. The combination of lower oxygen levels in the bulk and decreased response of the glucose sensor may be tied to the high enzyme loading of GOx in the tested sensor.

The average OCP measured with the IrOx sensor at pH 7.01 using bulk and microfluidic methods was $0.16 \pm 0.01\text{mV}$ and $0.19 \pm 0.01\text{mV}$ respectively, an average difference of $18 \pm 5\%$. This shift in OCP between bulk and microfluidic measurements is likely due to how those environments affect the relationship between the quasi-reference and the electrodes. The average sensitivity for bulk and microfluidic measurements in this test was $-118 \pm 3\text{mV}/\text{dec}$ and $-122 \pm 18\text{mV}/\text{dec}$.

Microphysiometry Measurements Performed with Microfluidic Devices

Adherent cell cultures were immobilized within the microfluidic sensing chambers to enable real-time measurements of glucose, lactate, oxygen, and acidification. The cells were perfused with 5 mM glucose RPMI in a repeating pattern of 200 s at 15 $\mu\text{L}/\text{min}$ and 40 s at 0 $\mu\text{L}/\text{min}$. Figure 4 displays a 20min portion of these measurements before and after treatment with alamethicin to initiate cell death. Glucose (4a) and oxygen (4c) consumption is observed as decreases in current magnitude. Lactate (4b) production is observed as an

increase in current magnitude. Acid production (4d) is observed as a positive increase in OCP. When compared to the peaks observed for each sensor after cessation of cellular activity (dashed lines), it is clear that cellular bioenergetics may be monitored with the 4-analyte SPE.

Conclusions

A screen-printed sensor was designed and modified for simultaneous detection of glucose, lactate, oxygen, and pH. Electrochemical methods were employed to form Ag/AgCl and IrOx films on Pt electrodes to create quasi-references and pH-sensitive films. A materials inkjet printer was successfully used to deposit enzyme and polymer solutions to create reproducible glucose, lactate, and oxygen sensors. The enzyme loadings for both GOx- and LOx-based sensors proved critical to limits of detection and linear ranges of the sensors, with higher linear ranges and LODs found with lower enzyme loadings. Lower LODs could be achieved by increasing the enzyme loading at the cost of linear ranges due to enzyme kinetics.

Preliminary microphysiometry measurements were performed by enclosing the developed sensor in an acrylic microfluidic chamber with macrophages cultured on a polycarbonate membrane. A repeating pattern of flow/stopped flow was employed to measure the consumption of oxygen and glucose and the release of acid and lactate, collectively the cellular bioenergetics of the extracellular environment. Once the cellular activity was eliminated by killing the cells, each of the peaks was significantly reduced, allowing the signals due to cellular activity to be separated from signals resulting from the flow pattern. Future work will focus on improved cellular immobilization strategies and integrating low-cost programmable pumps and valves to enable automated pumping and sensor calibration for up to six replicate sensors simultaneously.

An advantage of the screen-printed sensor is the versatility the user has to alter and improve sensors for the required tasks. All of the electrodes feature a Pt electrode that can be modified with enzyme, polymer, ionophore, and metal films in a variety of configurations, and the sensor designs employed here could benefit from further improvements. Due to SPE footprint and surface contours, the smallest microfluidic housing possible for the sensor is 23 μL . The spacing and size of the working electrodes of the SPE were based on a combination of the original MAMP sensor head and single-analyte SPEs produced by Pine Research Instrumentation. The original MAMP had a 3 μL chamber, thus the current version, with a 23 μL chamber, dilutes the cellular response and decreases the temporal resolution. Future sensors will feature smaller disk electrodes on the order of 500 μm , or switch to bar electrodes. In addition to the large footprint of the current sensor, the insulating ceramic layer and ceramic-defined disk electrodes could be modified to limit thickness and contours, enabling smaller channel heights and lower chamber volumes. The inclusion of an additional Pt electrode to allow for separation of the reference and counter electrodes is another possibility for improvement. It may also be feasible to replace the electroplated Ag reference electrode with a Ag film formed with the materials inkjet printer, as recently demonstrated.³⁵ One downside of the ceramic SPE is the limited ability to perform microscopy simultaneously with electrochemical measurements, which is one traditional

method of assessing cellular viability, however the numerous studies performed with microphysiometry demonstrate that measuring bioenergetics is sufficient for assessing viability.

The sensor modifications developed here are highly adaptable, and could easily be employed in a new electrode designs, including sensors prepared on optically transparent substrates, allowing for multiplexing with optical methods. Additionally, novel and improved sensors currently in development can replace the sensors described here, for example, osmium-based enzyme sensors that are longer lasting and have lower biasing potentials, which make them resistant to interfering compounds. Another possibility is the use of ionophore-based pH sensors. Both of these options are currently in development for use with the SPE and may be beneficial to future microphysiometry studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Contours of the SPE based on profilometry. (A) Profilometry scan of a portion of the multi-analyte SPE. . Profilometry tip was scanned from left to right across the surface of the SPE as indicated by the red line. (B) The resulting scan plotted in μm distance vs. μm height. (C) Resulting diagram of SPE contours compiled from profilometry data, as well as individual electrode designations and sensor width.

Figure 2. Schematic of the working area of the microfluidic flow chambers including a) the area within the o-ring and direction of flow, as well as the cross-section of the chamber with (b) and without a polycarbonate membrane with adhered cells (c).

Figure 3.

Representative calibration curves for a) glucose b) lactate and c) pH from one sensor of a six-sensor array tested under bulk (◆) and microfluidic (◇) conditions. d) Measured oxygen in bulk and in microfluidic are indicated with labels.

Figure 4.

Real-time detection of (a) glucose (b) lactate (c) oxygen and (d) pH at sensors and macrophages sealed together in a microfluidic flow chamber. Signals obtained from the cells before and after cell death are labeled above. Each cycle is 240s: 200 s at 15 $\mu\text{L}/\text{min}$, 40 s 0 $\mu\text{L}/\text{min}$, and repeats over the course of three hours.

Table 1
Summary of sensor enzyme loadings with resulting linear ranges and LOD. Comparative values from a previously work with the original MAMP are included for comparison

Enzyme ID	[Enzyme], mg/mL	Diffusion Barrier	Linear Range, mM	LOD, μ M	N =	Printing Sessions	Source
GOx	0.25	None	0-40 mM	270 ± 70	3	1	This work
GOx	0.25	2.5% Nafion	0-28 mM	340 ± 140	19	4	This work
GOx	2.5	None	0-13 mM	33 ± 1	12	2	This work
GOx [*]	20	Hand Cast Nafion	0-5 mM	580 ± 230	6	N/A	Ref 21
LOx	2	None	0-1.5 mM	14 ± 3	3	1	This work
LOx	10	None	0-1.5 mM	14 ± 4	16	3	This work
LOx	20	None	0-0.75 mM	8 ± 4	6	1	This work
LOx	25	None	0-0.75 mM	5 ± 3	6	1	This work
LOx	20	None	0-0.2 mM ^{**}	14 ± 12	6	N/A	Ref 21

^{*} This sensor used GOx from Sigma-Aldrich.

^{**} LOx sensor only calibrated over this range.