Electrochromic Sensor for Multiplex Detection of Metabolites Enabled by Closed Bipolar Electrode Coupling

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ABSTRACT: Biosensors based on converting electrochemical signals into optical readouts are attractive candidates as low-cost, high-throughput sensor platforms. Here, we described a closed bipolar electrode (CBE)-based two-cell electrochromic device for sensing multiple metabolites, using the simultaneous detection of lactate, glucose, and uric acid as a model system. In the two-cell configuration, an analytical cell contains a redox mediator combined with a specific oxidase, e.g., lactate oxidase, glucose oxidase, or uricase, to form an electrochemical mediator–electrocatalyst pair that supports redox cycling. A closed bipolar electrode couples the electron transfer event in the analytical cell to an electrochromic reaction in a separate reporter cell, such that the magnitude of the color change is related to the concentration of metabolites in the analytical cell. To demonstrate multiplex operation, the CBE-based electrochromic detector is modified by integrating three sets of detection chemistries into a single device, in which simultaneous determination of glucose, lactate, and uric acid is demonstrated. Device sensitivity can be tuned by using reporter cells with different volumes. Furthermore, the analytical cell of this device can be fabricated as a disposable, paper-based carbon electrode without any pretreatment, demonstrating the potential to screen phenotypes that require multiple biomarkers in a point-of-care format.

KEYWORDS: bipolar electrode, biosensors, colorimetric detection, multiplex sensing, paper analytical device

The rapid development of functional biomaterials and advanced fabrication technologies has enabled researchers to design biosensors with integrated biomolecular components, yielding improved sensitivities and fast response times. For example, electrochemical biosensors typically consist of integrated devices which combine a biorecognition element with electrochemical transduction to provide analytical information about biomolecules. The biomolecular recognition event should be simple and fast and display high sensitivity, but most importantly it should be selective. A number of different transducers have been developed, differing in the type of signal generated, including those based on potentiometry, voltammetry/amperometry, such as CV (cyclic voltammetry), differential pulse voltammetry (DPV), anodic stripping voltammetry (ASV), and impedimetric detection. Impedance-based measurements are an especially popular choice to determine the interaction between bioanalyzer and the working electrode. However, few electrochemical biosensors have successfully made the transition from benchtop instruments to portable point-of-care devices. The challenge lies not only in building a sensitive and specific detector, but also in integrating different parts together in a miniature device, with all parts working together to produce a signal free from interferences. Furthermore, multiplex sensing is desirable, especially in biomedical applications, since accurate diagnosis of many disease conditions requires a combination of biomarkers.

In traditional electrochemical biosensors, a (bio)chemically modified electrode recognizes and reacts with biomolecules, producing an electrochemical signal which is captured and read out. In order to detect multiple analytes simultaneously, the detector needs to acquire and analyze multiple chemically distinct signals in parallel. Thus, an important goal is to design an electrochemical biosensor with high-throughput capacity to detect multiple biomarkers in a single measurement. To accomplish this, we employ an architecture in which separate analytical working electrodes are associated with a distinct biomolecular recognition motif, so that single analyte detection is performed separately in each cell. A second, and critically important, part of the strategy relies on transforming the electrochemical detection event to an optical signal, such as plasmonic, fluorescence, or a colorimetric change. Colorimetric detection is particularly interesting, because it represents a direct approach to developing an analytical...
which can be read out by a number of different detectors, e.g., CCD camera, smartphone, or even by direct visual inspection. This approach is attractive, because the electrochemical and optical signals are spatially separated, and the chemical systems constituting the analytical and reporter cells can be changed to achieve optimum performance. The question remains how to couple the analytical (electrochemical) and reporter (optical) cells. Bipolar electrodes (BPEs) are a special type of electrode which can couple electrochemical detection to optical sensing in different cells. In principle, when a potential gradient is provided between two driving electrodes, and an electrically floating metallic element is placed in the electrified fluid, redox reactions are coupled at opposite poles of the BPE, even without external connections. Crooks and co-workers, for example, have developed a variety of BPE-based devices for sensing and screening applications. In addition to these conventional open BPEs, closed bipolar electrode (CBE) systems place the anode and cathode in different solutions, such that they are chemically and fluidically isolated. Two distinct redox half reactions in different solution environments are coupled at the cathodic and anodic poles of the CBE by electron transport through the BPE. Therefore, CBE systems enable two redox systems to be independent from each other, thereby minimizing cross-talk or interference between the two systems. Demonstrating these principles, Zhang and co-workers demonstrated fluorescence-enabled electrochemical microscopy based on CBE structures, and Xu and co-workers coupled ECL detection with CBEs to detect biomarkers of protein and DNA. In our laboratory, CBEs including interdigitated electrode arrays and microchannels have been coupled with fluorescence for chemical sensing. We also developed and characterized a single channel CBE-based electrochromic detector, which showed good performance in both aqueous and nonaqueous environments. In this latter design, the analytical reactions were isolated in an analytical cell and analyzed by monitoring the redox-induced color change in a BPE-coupled reporter cell via intercell electron transfer through the CBE.

Here, we exploit the CBE-enabled electrochromic detection principle to achieve a sensor exhibiting multiplex detection for biomedically relevant metabolites (glucose, lactate, and uric acid). Electrons generated in the analytical cell are transferred through the CBE to a reporter cell, where they mediate reduction of methyl viologen (MV$^{2+}$), causing a colorless-to-purple color change. The color change can be recorded by smartphone, followed by RGB analysis. Multiplex detection of multiple analytes simultaneously is also accomplished within a single device. In one implementation of the multiple cell concept, a single analyte is determined at different concentra-

![Figure 1. (a) Schematic illustration of the CBE-enabled electrochromic sensor architecture. (b) Reaction scheme for lactate detection in the analytical cell.](acsensors.7b00292)
trations by comparing the color change of the analyte with standards, making the device useful as a threshold detector. Furthermore, because a substrate-specific enzyme is used in the detection, good target molecule selectivity can be achieved during the detection. Chronoamperometric current profiles confirm that only the specific enzyme substrate contributes to the observed color change. In addition, the sensitivity of the device can be adjusted by using reporter cells with different volumes to detect analytes in different concentration ranges. Finally, good performance is obtained when the device is coupled with paper-based carbon electrodes, supporting its applicability to point-of-care applications.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** Methyl viologen, glucose, glucose oxidase, lactate, lactate oxidase, uric acid, and uricase were obtained and used as received from Sigma-Aldrich. Potassium chloride and potassium ferricyanide were obtained from Fisher Scientific. All chemicals were analytical grade and used as received without further purification. All solutions were prepared using 18.2 MΩ cm deionized (DI) water by Milli-Q Gradient System. Tape (Scotch), paper-based carbon electrodes (CH Instruments, Inc.) and indium tin oxide (ITO) coated glass slides with different conductivities (Delta Technologies and Sigma-Aldrich) were used for device fabrication.

**Device Assembly.** For single analyte detection, the structure of the electrochromic sensor device was similar to the geometry reported elsewhere. Circular analytical cells (⌀10 mm) and reporter cells (⌀1 mm) were fabricated by placing tape (50 μm thick) around the periphery to act as a spacer between the analytical cell working electrode (WE), or counter/quasi reference electrode (CE/QRE) and the ITO CBE, respectively (Figure 1a). The observation window in the reporter cell was opened in the tape spacer and filled with indicator solution, e.g., 10 mM methyl viologen in 0.1 M KCl; and the analytical cell was opened and filled with analyte solution, i.e., metabolites of varying concentrations in aqueous solution mixing with 0.5 mM Fe(CN)₆³⁻/⁴⁻ (nonredox mediator) and the specific oxidase enzyme in 0.1 M phosphate buffered saline solution. The concentration for glucose oxidase, lactate oxidase, and urate oxidase were 4, 6, and 10 U/mL, respectively. Analytical and reporter cells were connected by a CBE, which was composed of the top ITO electrodes in both analytical and reporter cells connected to each other by a Cu wire. For multiple analyte detection, the device consisted of three sets of independent electrochromic sensors to monitor the detection of three different analytes simultaneously. In this design, the WE, and CE/QRE were shared by three sets of sensors, while each sensor had its own ITO BPE bridged between the WE, and CE/QRE for electron transfer without interference. Different target analytes were placed in different analytical cells, while the same indicator solution was used to fill all reporter cells. In some experiments, the ITO WE, was replaced by a paper-based carbon WE, to perform multiple analyte detection, while the remaining parts of the device were unchanged. To effect multiplex sensing, three-channel detection with three metabolites was performed within a single device, and the color change in each reporter cell was captured independently.

**Electrochemical and Colorimetric Measurements.** Electrochemical measurements were performed on a commercial potentiostat (CHI 842C, CH Instruments). In a conventional three-electrode cell, the half wave potentials for Fe(CN)₆³⁻/⁴⁻ and MV²⁺/⁺ are 360 mV and −645 mV vs Ag/AgCl, respectively. The interfacial potential at each pole of the CBE, ca. 1.0 V for a WE, potential of 2.0 V, provided sufficient overpotential to observe the coupled redox reactions. Potentials ranging from 2.0 to 3.0 V were applied on WE, to drive the redox reaction in the analytical cell and to trigger the color change of electrochromic indicator in the reporter cell through a CBE. For smartphone-based detection, images were acquired with an iPhone 6 (Apple) equipped with a macro lens (Olloclip) at 21× magnification at a height of 16 mm above the observation window of the reporter cells. The built-in camera was operated in manual mode with the following parameters: ISO sensitivity, 100; shutter speed, 1/20 s; and auto white balance. Images were captured in RAW-format and then were analyzed by ImageJ software to obtain the red-green-blue (RGB) color information. The green channel intensity was used for further analysis, because it exhibited the highest linear dynamic range and the largest concentration sensitivity.

**RESULTS AND DISCUSSION**

**Single Analyte Sensing.** In our previous work, model redox-active analytes, ferricyanide and ferrocene, were used to characterize the CBE-based electrochromic sensor device. In order to extend the device for use as a biosensing platform, essential molecular indicators of metabolism, such as lactate, glucose, and uric acid, were measured using a similar approach. Unlike ferricyanide and ferrocene which undergo reversible redox reactions themselves, these biomolecules are not redox active. Therefore, electrochemical mediators were employed to couple enzymatic recognition reactions to the electrode (Figure 1b). Here, ferricyanide was combined with a specific oxidase, e.g., lactate oxidase, glucose oxidase, or uricase, to form biochemically specific mediator—electrocatalyst pairs.

In order to detect lactate, the sample was pretreated by mixing lactate with lactate oxidase and ferricyanide solution, and then detection was performed after 20 min incubation. Detection was accomplished by the reaction of lactate with lactate oxidase (oxidized form) to produce pyruvate, converting the lactate oxidase back to its reduced form. Reduced lactate oxidase then reacts with ferricyanide to regenerate oxidized lactate oxidase and ferrocyanide. In order to complete the redox cycle, a sufficiently oxidizing potential is applied to WE, to convert ferrocyanide back to ferricyanide. Owing to the regeneration of lactate oxidase (oxidized form) and ferricyanide, reaction occurs as long as lactate is present in the mixture. Redox chemistry in the analytical cell is then coupled through the CBE to the reduction of methyl viologen in the reporter cell, with the resulting MV²⁺ → MV⁺ reaction producing a color change in reporter cell. Although the scheme outlined here was demonstrated with lactate, clearly, the strategy is general for nonredox active analytes, provided they can be coupled to a redox active species with the aid of enzymes and electrochemical mediators.

Figure 2a shows images of color changes in the reporter cell when lactate at different concentrations was detected with WE, = +3.0 V, for which the potentials at the analytical cell—CBE interface and the CBE-reporter cell interface are both ca. 1.5 V. A control experiment was performed with the sample containing no lactate (0 mM), in which no color change was detected in the reporter cell, as shown in Figure 2a. Increasing lactate concentrations caused a purple color in the reporter cell corresponding to oxidation of lactate in the analytical cell. Because the control experiment showed no color change in the reporter cell, electrochemical interference in the sample was negligible. The difference between nonzero lactate concentrations and the control experiment was also reflected in the measured chronoamperometric currents. Since enzymatic reaction occurs only in the presence of lactate, higher currents were achieved for lactate detection, compared with the background current obtained in the without lactate. The induced electron transfer through the CBE triggered the reduction of colorless MV²⁺ to purple MV⁺ (with perhaps also some contribution from the yellowish MV), which resulted in the color change in the reporter cell. Lactate samples with different concentrations in the range of 0 to 10 mM were
detected at the same potential, and produced color changes for which the green channel RGB value, $\Delta I_G$ is plotted in Figure 2b. As expected, the color change increases with increasing lactate concentration, with the response approaching saturation above 1 mM. As shown in the inset of Figure 2b, a linear response was observed in the concentration range 0 to 1 mM with slope $= 7.74 \times 10^4$ M$^{-1}$ and limit of detection, LOD $\sim 180 \mu$M.

The successful detection of lactate in the CBE-enabled electrochromic device suggests that the device can be used to detect other metabolites. Thus, glucose (Figure S1, Supporting Information) and uric acid (Figure S2) were tested using the same strategy with the same device. Obvious color changes were observed when different concentrations of glucose and uric acid were placed in the analytical cell, and in both cases, a linear response was observed up to 1 mM. Furthermore, LODs of 0.18 and 0.11 mM were obtained for glucose and uric acid, respectively, all of which are sufficient for routine clinical monitoring.

**Multiple Analyte Detection.** In many cases, clinic diagnoses can be improved by relying not on detection of a single biomarker, but by measuring multiple biomarkers. Thus, it is desirable to integrate multiple analyte determinations into a single electrochemical biosensing platform. As shown in Figure 3a, the CBE-enabled electrochromic device was modified for multiple simultaneous determinations using the design principles developed for single analyte detection. In this design, the device has three individually addressable channels, one for each of three analytes. Each channel possesses an independent BPE, while the WE and CE/RE are shared among the three channels. Each analyte reacts in its own specially populated analytical cell at a common applied potential on the WE. Redox reactions in the analytical cells are coupled through the independent CBEs, and induce reactions in the corresponding reporter cell (CE/RE).

The integrated electrochromic device was next used to simultaneously detect 2.5 mM glucose, 2.5 mM lactate, and 2.5 mM uric acid as a model target analyte mixture, comparing results against a control experiment in which only ferricyanide and enzyme were added. In the control experiment, no color change was observed below $E_{appl} = +3.2$ V at WE, while in the presence of target analytes, color changes were observed at or above $E_{appl} = +2.4$ V. Figure 3b shows the color change for detection of three metabolites and the relevant controls at $E_{appl} = +3.0$ V. An obvious purple color can be seen in the presence of analytes, confirming that multiple analytes can be determined simultaneously and independently by color change in a single multiplex device.

Clearly, the concentration response of the three analytes indicates that they respond linearly over different concentration ranges. Thus, to extend the results to provide semiquantitative information, samples with glucose of 0 mM (control), 2.5 mM, and 25 mM were placed in the three analytical cells. Potentials ranging from +2.0 V to +3.4 V were applied to support redox cycling and the color change in reporter cells, as shown in Figure 4a.b. The control experiment, blue squares (Figure 4b), did not show an obvious color change in the range $E_{appl} = +2.0$ V to +3.0 V, indicating negligible electrochemical reaction from interference over this range. However, obvious change of intensity difference was observed in the experiment at $E_{appl} > +3.4$ V was applied, illustrating the limit of the applied potential window. In contrast, the channel containing 2.5 mM glucose, red circles (Figure 4b), exhibited a monotonically increasing color change with applied potential across the entire potential range. A similar trend was observed for 25 mM glucose, green diamonds (Figure 4b), although the color change was larger than for 2.5 mM glucose at all applied potentials in the range. Interestingly, 25 mM glucose exhibited a substantial $\Delta I_G$ even at the lowest applied potential, +2.0 V, suggesting that measurements at higher concentration ranges could be extended to smaller values of $E_{appl}$. Furthermore, comparison to Figure 2 illustrates that larger potentials tend to produce saturation at lower analyte concentration and vice versa. Therefore, analytes with different concentrations can be distinguished visually from the color change in the reporter cells. These measurements suggest a further extension of the applications of this device in which multiple determinations could be made in a sample of unknown analyte concentration.
assessing whether the concentration is within normal range by comparing the color change of the unknown with color changes obtained from upper limit and lower limit standards preloaded into the device.

**Selectivity.** Selectivity in these measurements is conferred by the specific enzymes used, making it possible to detect a target analyte in the presence of extraneous interferences. Since the enzyme preferentially reacts with its target substrate, a selective response, e.g., the measurement of lactate in the presence of glucose, can be obtained through either the amplified chronoamperometric current in the analytical cell or the resultant color change in the reporter cell. Three samples were prepared and detected within the CBE-enabled electrochromic detector, including ferricyanide, lactate oxidase, and glucose (Sample I); ferricyanide, lactate oxidase, and lactate (Sample II); and ferricyanide, lactate oxidase, glucose, and lactate (Sample III). These three samples were tested individually, and the potential required to observe detectable color change in each sample was recorded separately. A detectable color change was not observed (negative control) in sample I until $E_{\text{appl}} = +2.2$ V. However, color changes in samples II and III were observed at $E_{\text{appl}} = +1.8$ V. We attribute the difference in threshold driving potential for color change to the presence of lactate, the canonical substrate for lactate oxidase. The chronoamperometric current plots associated with the detection of three samples at $+1.8$ V is shown in Figure 5. Current produced by sample I is lower than the currents achieved in samples II and III and is indistinguishable from the background due to the absence of lactate and its subsequent enzymatic reaction. Lactate was detected in samples II and III, because the reaction with lactate oxidase results in an amplified current which subsequently produces a color change in the

**Sensitivity and Reporter Cell Geometry.** The above results indicate that color development can saturate, effectively defining the upper concentration range of the device. We hypothesized that color change might vary as a function of reporter cell volume. Since the number of $\text{MV}^{2+} \rightarrow \text{MV}^+$ indicator reactions scales with the concentration of indicator compound. An experiment with different reporter cell volumes was performed by varying the diameter of the detection region. In these experiments, 2.5 mM lactate was detected at different driving potentials in reporter cells of different volumes, as shown in Figure 6. While the color change in the reporter cell becomes darker at higher potentials for all three reporter cell volumes, the smallest cell exhibits the largest color change and the largest cell exhibits the least at any given potential. On the other hand, small reporter cells saturate at lower potentials than larger reporter cells. These experiments demonstrate that the detector sensitivity can be controlled by adjusting the volume of the reporter cell within the constraints imposed by tests with different detection ranges.

**Paper Analytical Cells.** Paper-based devices benefit from being portable, disposable, and inexpensive, dictating their use in POC devices where possible. In light of this, the ITO WE$_a$ was replaced with a paper-based carbon WE$_a$ (Figure 7a), all other components remaining the same. A mixture of 2.5 mM glucose, 2.5 mM lactate, and 2.5 mM uric acid was used as model analyte, while only ferricyanide and the relevant enzyme were added in the control experiment. For the control experiment, no color change in the reporter cell was observed until $E_{\text{appl}} > +2.0$ V; however, detectable color change in the reporter cell was observed starting from $E_{\text{appl}} = +1.6$ V. Figure

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**Figure 4.** (a) Color change in the reporter cell for 0 mM, 2.5 mM, and 25 mM glucose detection at different applied potentials. (b) Change in the green channel signal intensity relative to the green value of white background, i.e., 255 ($\Delta I_c$) from RGB analysis of images in panel (a): 0 mM (control, blue squares), 2.5 mM (red circles), and 25 mM (green diamonds).

**Figure 5.** Chronoamperometric currents obtained in the analytical cells from ferricyanide, lactate oxidase, and glucose (Sample I, black); ferricyanide, lactate oxidase, and lactate (Sample II, red); and ferricyanide, lactate oxidase, glucose, and lactate (Sample III, blue). (Inset) Color changes observed in the reporter cells for Samples I, II, and III detection.
7b presents the observed reporter cell color changes for both analyte detection and control experiments at $E_{appl} = +2.0 \, \text{V}$. In all three cases, an obvious color change was observed in the reporter cell compared to the control, in a manner qualitatively similar to the results observed with the ITO WE. These results confirm that detection can be performed with a paper-based carbon WE, without significant loss of performance, pointing the way to the development of CBE-enabled electrochromic sensors with disposable paper cells for POC applications.

### CONCLUSIONS

In conclusion, split-cell CBE-based electrochromic multiplex sensors have been developed for metabolite detection and tested by application to lactate, glucose, and uric acid mixtures. In this design, the mediator and specific enzyme make it possible to detect nonredox active molecules by enzymatically converting target analytes to redox-active products in an analytical cell, which can then produce electrochemical signals. Because the electrochemical reaction in the analytical cell is CBE-coupled to the electrochromic reduction of MV$^{2+}$ in the reporter cell, the presence of target molecule in the analytical cell can also be monitored by observing the color change in the reporter cell. Analyte detection based on colorimetry in the reporter cell is confirmed by analytical cell chronoamperometric currents and is quantitative with analyte concentration over specific ranges, typically <1 mM. The CBE electrochromic sensor is capable of multiplex operation by integrating three sets of detection chemistries into a single device, rendering it capable of detecting multiple analytes with different concentrations simultaneously. Analytes with unknown concentrations can be determined semiquantitatively by bracketing them in a range of color change produced by standards of known concentration. The CBE device is also selective to target analyte due to the activity of the specific enzymes in each channel. Chronoamperometric currents and colorimetric signals agree, in that detection requires the canonical substrate for the enzyme to produce signal for both. Sensitivity can be adjusted over limited ranges by varying reporter cell volume. Finally, devices with paper-based WE were used for detection of multiple analytes simultaneously, providing an inexpensive method for further device simplification. Altogether, this CBE based electrochromic detector constitutes a low-cost and simple electrochemical biosensing platform for simultaneous multiple-analyte detection. It is sufficiently versatile that it could be extended to a variety of applications by changing the detection chemistry, the only requirement being a recognition reaction that produces an electroactive product that can be coupled to the reporter cell through the CBE.

### ASSOCIATED CONTENT

#### Supporting Information
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Additional figures, including the color change and RGB analysis of glucose and uric acid at different concentrations (PDF)

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**Notes**
The authors declare no competing financial interest.

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