

Electrochemical Detection for Paper-Based Microfluidics

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We report the first demonstration of electrochemical detection for paper-based microfluidic devices. Photolithography was used to make microfluidic channels on filter paper, and screen-printing technology was used to fabricate electrodes on the paper-based microfluidic devices. Screen-printed electrodes on paper were characterized using cyclic voltammetry to demonstrate the basic electrochemical performance of the system. The utility of our devices was then demonstrated with the determination of glucose, lactate, and uric acid in biological samples using oxidase enzyme (glucose oxidase, lactate oxidase, and uricase, respectively) reactions. Oxidase enzyme reactions produce H₂O₂ while decomposing their respective substrates, and therefore a single electrode type is needed for detection of multiple species. Selectivity of the working electrode for H₂O₂ was improved using Prussian Blue as a redox mediator. The determination of glucose, lactate, and uric acid in control serum samples was performed using chronoamperometry at the optimal detection potential for H₂O₂ (0 V versus the on-chip Ag/AgCl reference electrode). Levels of glucose and lactate in control serum samples measured using the paper devices were 4.9 ± 0.6 and 1.2 ± 0.2 mM (level I control sample), and 16.3 ± 0.7 and 3.2 ± 0.3 mM (level II control sample), respectively, and were within error of the values measured using traditional tests. This study shows the successful integration of paper-based microfluidics and electrochemical detection as an easy-to-use, inexpensive, and portable alternative for point of care monitoring.

The development of microfluidic devices has been spurred at least in part by the desire to produce low-cost point-of-care diagnostics and environmental monitoring devices.^{1,2} For point-

of-care applications, the goal is to provide a total answer where a sample is introduced to the device and data is generated that can be used to make an informed decision. A variety of devices meeting these requirements have been demonstrated. The most common example, by far, has been for DNA analysis where complex functions such as DNA capture, amplification, and separation on a single device have been demonstrated.^{3–7} The Burns group has successfully generated a device with integrated microchannels, heaters, temperature sensors, and fluorescence detectors used to analyze nanoliter-size DNA samples on a single glass and silicon substrate. The device is capable of mixing the solution together, amplifying or digesting the DNA, then separating and detecting those DNA.^{3,7} In another example from the Landers group, a two-stage microfluidic device consisting of C₁₈ reversed-phase monolithic column for DNA extraction efficiency on a single glass slide (3 cm × 2.5 cm) was coupled with a device that was capable of performing polymerase chain reactions (PCR) and DNA separations.⁶ While these examples show the power of microfluidic devices and their potential for solving complex problems, a significant portion of the world's population could never afford the cost of such advanced devices. To this end, there is a growing push to generate microfluidic devices that are extremely low cost (<\$1 U.S.) and require minimal external instrumentation for obtaining quantitative information.

Among the least expensive of the microfluidic devices are the recently introduced paper microfluidic devices. Paper-based microfluidic devices have the potential to be good alternatives for point-of-care testing over traditional glass and polymer based devices because they are easy to use, inexpensive, require small volumes of reagents and sample, provide rapid analysis, are disposable, can be made from renewable substrate materials, and

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are portable.^{8–13} These devices have been used for the simultaneous determination of glucose and protein on a single piece of patterned paper.⁸ The results of the assay were quantified by visually comparing the color intensity of the reaction spots with the developed color intensity. Matching color and color intensity by the naked eye is complicated by many factors including different visual perception of color from one person to another, lighting, and the difference between the colors of a dry printed color on label stock and the colors seen in a reacted (i.e., wetted) paper. In an effort to conduct quantitative analysis for diagnostic tests based on paper microfluidics, several authors have used cameras or scanners to record the color intensity. With the use of this approach, glucose, pH, and protein levels were simultaneously determined by paper-based microfluidic devices.¹³ Camera phones and portable scanners are an attractive format because they can be used by unskilled personnel and provide more accurate results as compared to visual inspection. However, the transmission of data from on-site to experts is still required to analyze the data. Moreover, the intensities of digital images from the camera are affected by lighting conditions and a calibration curve using standards of known color and intensity is necessary for processing data of each imaging device. Finally, camera phones and portable scanners lack the sensitivity and selectivity of traditional analytical instrumentation. Therefore, a detector with high sensitivity and selectivity is still needed for determination of low levels of analytes in biological samples and complex sample matrixes such as blood and plasma.

Electrochemical detection (ECD) is an attractive alternative detection scheme for paper-based microfluidics due to its small size, portability, low cost, high sensitivity, and high selectivity by proper choice of detection potential and/or electrode material.^{14–19} An additional advantage of ECD is simplicity of the instrumentation resulting in low electrical power requirements for in-field use.¹⁹ These advantages have led to the almost universal use of electrochemical detection in hand-held glucometers for monitoring diabetes. Finally, screen-printed carbon electrodes have many attractive advantages for ECD including low cost, disposability, flexibility in design, ease of chemical modification, and ability to produce with minimal outside technology.^{20–22} Here, we present electrochemical based detection for paper-based microfluidic devices for simultaneous determination of glucose, lactate, and

uric acid in biological samples. Electrodes modified with Prussian Blue (PB) for improved selectivity for H₂O₂ detection were characterized to show the viability of the combined approach. Electrodes were subsequently modified with appropriate enzymes for the detection of small molecule markers in human serum samples. Experimentally determined levels measured using the paper microfluidic devices were within the error of the levels measured for the same samples done using traditional clinical diagnostic assays.

EXPERIMENTAL SECTION

Materials and Equipment. D-(+)-glucose, sodium L-lactate, uric acid, glucose oxidase (from *Aspergillus niger*, 215 U/mg) and uricase (from *Bacillus fastidiosus*, 9 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). Lactate oxidase (from *Aerococcus viridians*, 38 U/mg) was obtained from A.G. Scientific, Inc. (San Diego, CA). Potassium phosphate was purchased from Fisher Scientific (Pittsburgh, PA). Acetone was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ). SU-8 3025 negative photoresist was purchased from MicroChem Corp. (Newton, MA). Whatman filter paper 1 was obtained from Cole-Parmer (Vernon Hills, IL). Carbon ink mediated with Prussian blue (C2070424D2) was purchased from Gwent group (Torfaen, United Kingdom). Silver chloride ink (Electrodag 7019) was obtained from Acheson Colloids Company (Port Huron, MI). All chemicals were used as received without further purification. Electrochemical measurements were made using a potentiostat (CHI 1207A, CH Instruments, Austin, TX) at room temperature (22 ± 1 °C).

Preparation of Paper-Based Microfluidic Devices. Photolithography was used to pattern Whatman filter paper 1 according to previously reported methods.^{8,12} Briefly, SU-8 3025 photoresist was poured on the center of the paper and spread over the paper using a spin-coater. The photoresist-covered paper was baked at 95 °C for ~10 min. The paper was then covered with transparency film photomask generated using a standard laser printer and irradiated with a UV lamp. After baking at 95 °C for 1–3 min, unpolymerized photoresist was removed from the paper by submerging the paper in acetone for 1 min and by rinsing the paper with acetone. Next, the paper was dried under ambient conditions for 1 h. Prior to use, paper microfluidic devices were exposed to air plasma (Harrick PDC-32G) for 30 s. Areas covered with photoresist remained hydrophobic while areas without photoresist were hydrophilic.

Design and Preparation of Electrochemical Detector for Paper-Based Microfluidic Devices. The electrodes were screen-printed in house using carbon ink containing PB as the working (WE) and counter electrode (CE) and silver/silver chloride ink as the reference electrode (RE) and conductive pads. The screen-printing emulsion was designed with Corel Draw 9 software and generated by Chaiyaboon Co. (Bangkok, Thailand). The electrode design is shown in Figure 1. The working electrode was designed to be as close as possible to the reference electrode to minimize the effect of uncompensated resistance between the WE and RE (spacing about 0.15 mm). The counter electrode (geometric area about 7.5 mm²) was designed to be larger than the working (geometric area 1.3 mm²) and reference electrodes (geometric area 1.2 mm²) to allow unlimited current transfer in the circuit. All electrodes were designed with the working portion of the electrode on the hydrophilic portion of the paper and the

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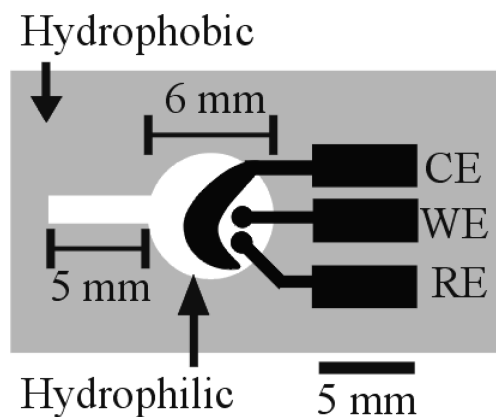


Figure 1. Basic design of the electrochemical detection cell for paper-based microfluidic devices. WE, working electrode; RE, reference electrode; CE, counter electrode.

contact pads on the hydrophilic portion of the paper. The screen printing process consisted of two layers. The first layer was applied with the silver/silver chloride ink and served as the reference electrode and conductive pads. Next, carbon ink containing PB was screened on the paper. Both layers were subsequently cured for 30 min at 65 °C and allowed to cool at room temperature.

Design and Preparation of Paper-Based Microfluidic Devices for Multianalyte Determination. The characterization of electrochemical detection for paper-based microfluidic devices was first studied by dropping 5 μL of 0.1 M potassium phosphate buffer solution (pH 6) on the end of the paper at the detection zone. The solution flowed directly through the electrode zone, and cyclic voltammetry was performed using a CH Instruments 1207A potentiostat. The scan rate dependence was studied at the carbon electrode containing PB. In addition to single analyte devices, multianalyte systems were fabricated using the method described above. A photograph showing the design is shown in Figure 2. Each reaction area was spotted with different enzymes for the specific analytes of interest. The glucose, lactate, and uric acid test zones were prepared by spotting 0.3 μL of glucose oxidase solution (645 U/mL), lactate oxidase (114 U/mL), and uricase solution (27 U/mL), respectively, into the respective test zones. The paper was allowed to dry at room temperature for 10 min. All standard and enzyme solutions were prepared in 0.1 M potassium phosphate buffer (pH 6). For analysis, 5 μL of standard or sample solution was dropped on the center of the paper and subsequently flowed to each test zone. Direct current chronoamperometry was then used for analysis at the screen-printed carbon PB-mediated electrode. The sampling rate for all chronoamperometric analyses was 10 Hz.

Human Serum Sample. Human control serum samples (levels I and II) consisting of lyophilized human serum were obtained from Pointe Scientific (Canton, MI). Levels of analytes such as albumin, cholesterol, creatinine, glucose, lactate, and uric acid were provided by the supplier. Prior to use, we added exactly 5.0 mL of deionized water to the lyophilized human serum. All samples were analyzed using electrochemical detection for paper-based microfluidics without any pretreatment.

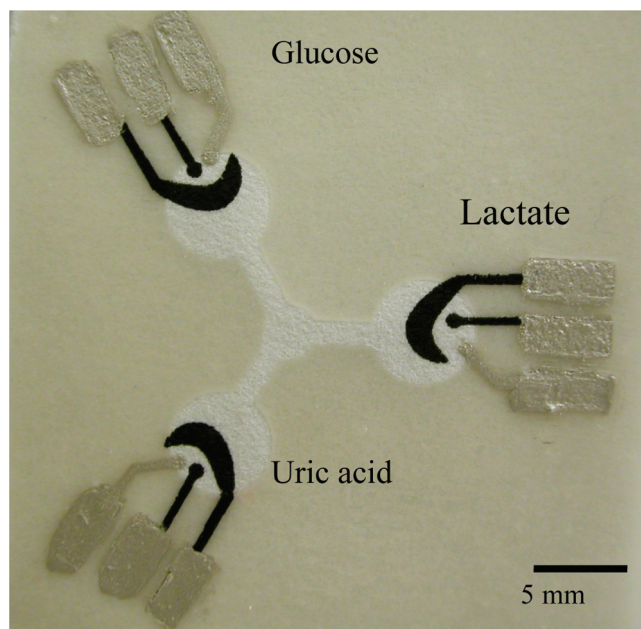


Figure 2. Picture of three electrode paper-based microfluidic devices. The hydrophilic area at the center of the device wicks sample into the three separate test zones where independent enzyme reactions occur. The silver electrodes and contact pads are made from Ag/AgCl paste with the black electrode portions being the PB-modified carbon electrodes. The device size is 4 cm \times 4 cm.

RESULTS AND DISCUSSION

The recent development of paper microfluidics has generated considerable excitement because of their ease of use, very low cost, and portability for point-of-care monitoring. To date, all reported systems have used colorimetric detection. While colorimetric detection is the simplest detection mode, it has several significant problems as discussed previously. Here we report the development of an electrochemical detection scheme for quantitative analysis in paper microfluidics. Electrochemical detection, while more expensive than visual-based colorimetric detection, is still very low cost and requires minimal instrumentation. Here we demonstrate characterization of screen printed electrodes fabricated on paper microfluidic devices followed by their use for the measurement of clinically relevant analytes in a biological matrix using enzyme modified electrodes.

Characterization of Electrochemical Detection for Paper-Based Microfluidic Devices. Before the use of the devices for measuring analytes in biological samples, the functionality of the electrodes was established. The electrodes were screen-printed on the hydrophilic and hydrophobic area of paper with the working area of the electrodes on the hydrophilic portion of the device. To demonstrate proper electrode functionality and isolation

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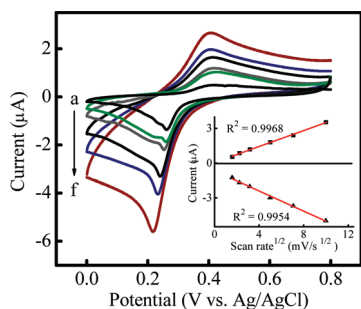
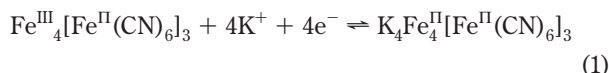


Figure 3. Representative cyclic voltammograms of the PB-modified carbon electrodes at various scan rates ((a) 2.5, (b) 5, (c) 10, (d) 25, (e) 50, and (f) 100 mV/s) in 0.1 M potassium phosphate buffer (pH 6). The relationship between anodic and cathodic currents and the square root of the scan rate is shown in the insert.

of the leads by the hydrophobic photoresist underlayer, characterization of the PB electrodes using cyclic voltammetry of the buffer samples was performed. After dropping solution at the entrance of the microfluidic channel and allowing the test solution to wet the electrode area, cyclic voltammetry was performed. The redox reaction for PB is as follows:



The characteristic voltammograms as a function of scan rate for PB (iron(III) hexacyanoferrate(II))²³ are shown in Figure 3. These results agree well with published results for PB electrodes.²³ Next, the dependence of the PB peak current on the scan rate was studied for the electrochemical system on paper. The insert in Figure 3 shows anodic and cathodic peak currents were directly proportional to the square root of the scan rate between 2.5 and 100 mV/s. It should be noted that the slopes of the forward and reverse sweeps are not identical as would be found at most traditional electrode materials. The differences in slope could be the result of the use of a surface confined redox species (Prussian Blue) coupled with the expected nonideal behavior of these carbon electrodes.²³ The linearity however indicates the mass transfer in this system is a diffusion controlled process similar to the behavior of traditional electrochemical cells.^{23–27} A diffusion controlled process in this system represents the diffusion of potassium ions within the PB lattice in the plating phase of the electrode.^{26,27}

Choice of Detection Potential for Hydrogen Peroxide. The utility of our system was next demonstrated for different oxidase enzymes. Oxidase enzymes catalyze the oxidation of a substrate such as glucose, uric acid, or lactate while reducing oxygen to hydrogen peroxide (H_2O_2). Many electrochemical biosensors using enzymes rely on detection of peroxide for quantification of the analyte. Therefore, it is necessary to have methods for the detection of hydrogen peroxide at a low oxidation potential where few biologically relevant analytes will interfere. The most common electrochemical method is the anodic oxidation of H_2O_2 at a platinum electrode.^{28–30} However, platinum is expen-

sive and requires advanced patterning methods that dramatically increase the cost of fabrication.³⁰ Alternatively, screen-printed carbon electrodes that are significantly cheaper to produce can be used; however, the high overpotential and subsequent interference from matrix species such as ascorbic acid and uric acid represents a significant problem for plain carbon electrodes.³¹ One way to minimize the problem is by using the cathodic reduction of H_2O_2 aided by a catalytic redox mediator (e.g., Prussian Blue (PB),²³ cobalt hexacyanoferrate,³² or horseradish peroxidase).^{20,33–35} For the current study, PB was used as the mediator on the electrode because it has been shown to be a selective catalyst for H_2O_2 reduction.^{36,37} The catalytic reaction occurs in a relatively low potential region (–0.2 to 0.2 V versus Ag/AgCl) where interferences from endogenous compounds such as uric and ascorbic acid are minimal. Moreover, PB is an inexpensive material that is easy to incorporate with the paper-based screen-printed electrode.

The modified electrode was first characterized in the absence of H_2O_2 (Figure 4a, dashed line) and provided the characteristic anodic and cathodic peak of Prussian Blue. Next, the catalytic nature of this electrode to the reduction of H_2O_2 was studied. Figure 4a (solid line) clearly shows a larger cathodic peak in the presence of 1 mM H_2O_2 relative to the background electrolyte. After characterizing the modified electrodes with cyclic voltammetry, a detailed investigation was conducted using chronoamperometry to optimize the detection potential, generate calibration data, and apply the proposed method to real samples. Chronoamperometry was used instead of cyclic voltammetry because it is more sensitive, can achieve lower detection limits, and for long-term applicability, is an easier detection method to implement. Hydrodynamic voltammetry (HDV) was first employed to optimize the detection potential for H_2O_2 in the range of 0.1 to –0.2 V. Analyte solution containing H_2O_2 was deposited in the microfluidic channel and the current measured at a fixed time with different potentials. The cathodic current of the H_2O_2 significantly increased as the detection potentials increased; however, the background current also increased as shown in Figure 4b. Therefore, ratios between H_2O_2 and background current (S/B) were considered. Figure 4c shows the S/B ratio at each potential. The ratio signal had a maximum of 0 V versus the on-chip Ag/AgCl reference electrode so a detection potential of 0 V was selected for further studies. Higher potentials were not investigated because of the concern over interfering reactions with endogenous compounds. Moreover, analytical performance of H_2O_2 using our devices was demonstrated under the optimal detection potential. It obtained that a linear calibration curve between H_2O_2 concentration and the anodic current was between 0 and 0.1 mM (slope = –1.265 $\mu\text{A}/\text{mM}$, intercept = –0.082, $R^2 = 0.9945$). The limit of

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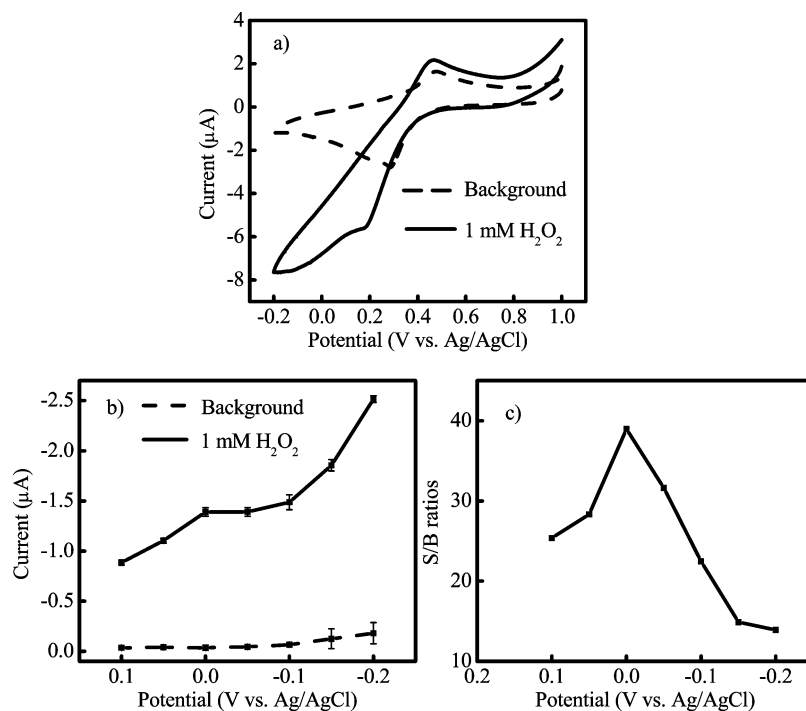


Figure 4. (a) Cyclic voltammograms of the carbon mediator Prussian Blue electrode in the absence (dashed line) and presence of 1 mM H₂O₂ (solid line) at a 100 mV/s scan rate. (b) Hydrodynamic voltammograms of 1 mM H₂O₂ (solid line) and background (dashed line) for 100 s sampling time, from three separate devices. (c) Hydrodynamic voltammogram of signal-to-background ratios extracted from the data shown in part b.

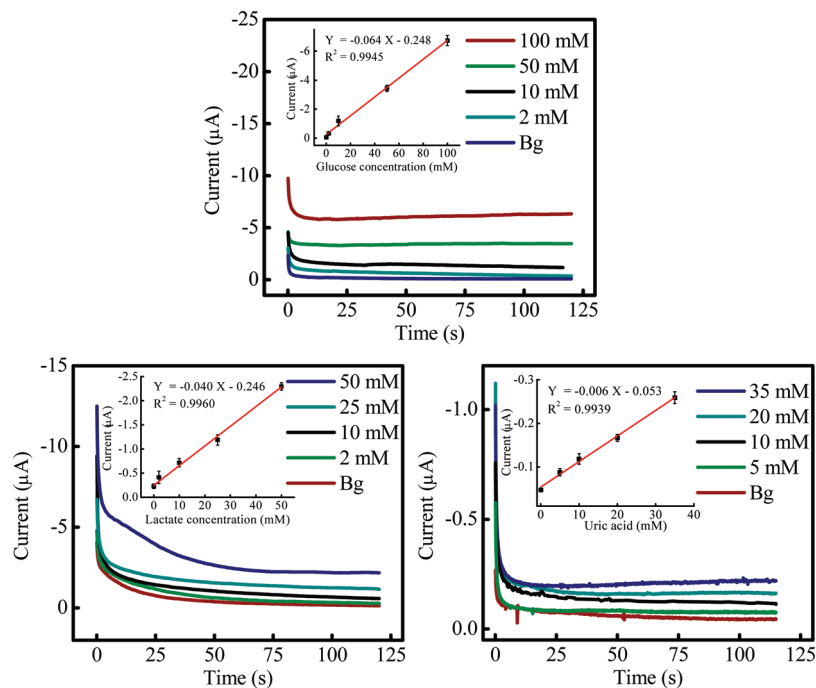


Figure 5. Chronoamperograms of glucose, 0–100 mM; lactate, 0–50 mM; and uric acid, 0–35 mM determination at 0 V versus an on-chip Ag/AgCl. The calibration plots of the anodic currents at 100 s of sampling time for determination of three analytes are shown in the insert, $n = 3$.

detection (LOD) and limit of quantitation (LOQ) were found at 3.6 ± 0.3 and $11.9 \pm 1.1 \mu\text{M}$ (concn \pm SD, $n = 3$), respectively.

Analytical Performance. After determining the optimal detection potential, the anodic current was recorded at 100 s, the apparent steady state current, to generate a linear calibration curve for a three electrode system consisting of glucose, lactate, and uric acid oxidase on each of three electrodes (Figure 5). To

establish a working range for peroxide detection, the relationship of three analytes concentration and the anodic current within a wide concentration range (0–100 mM) was demonstrated. The average and standard deviation in Figure 5 are the mean and relative standard deviation, respectively, from three separate devices. The relative standard deviations of all concentrations of glucose, lactate, and uric acid was less than 14% ($n = 3$),

Table 1. Linear Dynamic Range, Limit of Detection, and Limit of Quantification of the Proposed Method

analyte	linear dynamic range (mM)	concentration (mM \pm SD ^a)	
		LOQ (mM \pm SD ^a)	LOD (mM \pm SD ^a)
glucose	0–100	0.70 \pm 0.06	0.21 \pm 0.02
lactate	0–50	1.19 \pm 0.11	0.36 \pm 0.03
uric acid	0–35	4.60 \pm 0.43	1.38 \pm 0.13

^a SD, standard deviation ($n = 3$).

demonstrating acceptable reproducibility for this type of device. Calibrations of the anodic current against concentrations generated linear functions for all of the analytes within a range between 0 and 100 mM, and the coefficients of determination (R^2) were higher than 0.993. LOD and LOQ were calculated as the concentrations which produced the signal at 3 and 10 times, respectively, the standard deviation of a blank ($n = 10$) and are summarized in Table 1.

The LOD for glucose was found to be substantially lower (0.21 mM) than the camera detection method (0.5 mM).¹³ The normal level of glucose is 3.5–5.3 mM in whole blood, 2.5–5.3 mM in serum, and 0.1–0.8 mM in urine.³⁸ Conventional blood glucometers can detect levels as low as 1.7 mM,³⁹ while more modern glucometers can detect glucose as low as 0.6 mM in urine.³⁰ Our device should therefore be comprehensive for the determination of glucose in all biological samples including serum, blood, and urine. The LOD of lactate was found to be 0.36 mM. Even though our LOD is higher than the LOD of the conventional lactate kit (0.02 mM),⁴⁰ it is sufficient for clinical diagnostics where the normal concentration of lactate is 0.7–1.7 mM in blood, 0.5–1.7 mM in serum, and 5.5–22 mM in urine.³⁸ Uric acid had a measured LOD of 1.38 mM. Commercially available uric acid assay kits can detect as low as 13 μ M.⁴¹ The normal level of uric acid is 0.1–0.4 mM in serum and 1.5–4.4 mM in urine.³⁸ Further improvements in the LOD for uric acid and the other markers can be obtained by improving the enzyme loading on the paper.

Analytical Applications. In order to evaluate the electrochemical paper microfluidic system with real samples, three replicate determinations of glucose, lactate, and uric acid in clinical control samples were carried out using the optimized conditions. The control samples are common systems for determining the accuracy of diagnostic tests in a biologically relevant matrix without worry of blood borne pathogens. The results are shown in Table 2. The paired t -test was used to validate our method versus the control levels for glucose and lactate. No significant difference was found at the 95% confidence level. Thus, the analyzed values of glucose and lactate in human serum can be accepted. We also tried to measure uric acid in the control samples, but the 0.4 mM concentration was below our LOD (1.38

Table 2. Determination of Glucose, Lactate, and Uric Acid in Control Samples

analyte	concentration (mM \pm SD ^a)			
	human serum level I		human serum level II	
	certified value	proposed method	certified value	proposed method
glucose	5.3 \pm 0.3	4.9 \pm 0.6	16.5 \pm 0.7	16.3 \pm 0.7
lactate	1.4 \pm 0.1	1.2 \pm 0.2	3.5 \pm 0.1	3.2 \pm 0.3
uric acid	0.4 \pm 0.1	ND ^b	0.6 \pm 0.1	ND ^b

^a SD, standard deviation ($n = 3$). ^b ND, not detectable.

mM). Therefore, we measured uric acid in spiked samples. The control samples (level I) were spiked with uric acid at 5 and 10 mM and analyzed without any additional treatment. The 5 and 10 mM uric acid spiked samples were measured to contain 5.9 \pm 0.7 and 10.6 \pm 1.3 mM, respectively. After subtracting the level of uric acid in the control samples (level I, 0.4 mM), recoveries of uric acid were obtained in the range of 102–110%. Although our method cannot detect uric acid in the control serum samples, it can be successfully applied to the determination of uric acid in spiked control samples.

CONCLUSIONS

We demonstrate here for the first time the coupling of electrochemical detection and paper microfluidics to provide rapid quantitative measurement of critical health markers in serum. In this work, the biological sample matrix and sample color have negligible effect on the glucose, lactate, and uric acid determination in real sample due to both selectivity of the enzyme reaction and the working electrode material and detection potential (0 V versus Ag/AgCl). Paper-based microfluidic devices have not been previously applied to real biological samples; therefore, we have employed electrochemical detection for paper-based microfluidic devices for the determination of glucose and lactate in real world samples for the very first time, which demonstrates the feasibility of using paper microfluidic devices in medical diagnosis.

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