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Amperometric measurements of ethanol on paper with a glucometer

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ABSTRACT

Recent advances in electrochemical analysis on filter paper exemplify the versatility of this substrate for high performance testing. Its low-cost, light-weight, and environmentally friendly properties make it particularly attractive for applications in addressing health and environmental safety needs in low-resource settings and developing countries. However, the main drawback to sensitive electrochemical testing is the use of a potentiostat, a bench-top instrument that is extremely expensive, thereby negating the some of the benefits of paper-based devices. Hence there is a need to develop paper-devices for use with handheld, portable device readers that can extract quantitative readouts. In this study, we developed a method to use micro-paper electrochemical devices, or μPEDs, with a glucose meter, which are used for personal monitoring of blood glucose levels. Ethanol was chosen as a model target analyte due to its importance in the global issue of road safety. μPEDs were simple in design and could be tested with a potentiostat. We observed that inclusion of the stabilizer trehalose was critical to preparing μPEDs for later analysis. In addition, an NAD^+-dependent enzyme was used to impart selectivity to the biosensor, which also represents a class of enzymes with targets relevant to the health and food industry.

1. Introduction

Screen-printed (SP) biosensors are important tools for quick and sensitive detection of analytes important to health, food, and environmental safety [1–3]. Although conventional substrates for SP biosensors include ceramics and plastics, recent advances in high performance testing on filter paper suggest at its potential as a low-cost alternative for sensitive and quantitative electrochemical analysis [4–8]. Filter paper is particularly attractive in applications in global health [9], as it mitigates several barriers to entry in the developing world being that it is environmentally-friendly and extremely cheap. However, electrochemical testing requires a potentiostat in order to perform the necessary analysis, which can be costly, non-portable, and impractical for point-of-care use. In order for paper-based devices to succeed in the field and low-resource settings, there is an urgent need to develop robust and portable solutions that complement current analytical devices built on paper [10].

Examples of portable device readers for electrochemical analysis are limited, but exist in both the research and commercial spheres. In research settings, the CheapStat is an open-source schematic that provides instructions on building a potentiostat for USD $80 in-house [11]. Although the CheapStat allows for flexible and customizable biosensor design, it would be impractical to mass fabricate in the laboratory. Alternatively, commercial glucometers, used by people with diabetes to monitor blood glucose levels, are portable device readers that perform a single electrochemical test: amperometry. Amperometry is a powerful and simple technique that uses a 3-electrode system. A single potential is applied to an electrolytic solution and, in enzymatic-based biosensors, the resulting net redox reactions generate a Faradaic current that is proportional to the target analyte concentration. The current decays with time according to the Cottrell Equation [12].

$$i(t) = \mathrm{nFAC}(D/(\pi t))^{0.5}$$

Where F is Faraday's constant, n is the number of electrons transferred, A is the surface area of the working electrode, D is the diffusion constant, t is time, and C is concentration.

Glucose meters conduct amperometric sensing when a test strip is inserted and spotted with a blood sample. Although glucometers are not capable of the suite of electrochemical analysis techniques as can the CheapStat or a laboratory potentiostat, they are an excellent example of point-of-care testing. Glucometers are affordable (ranging from USD $30 to $100), robust, low-power, and can be used with little training, for personal care or professional medical monitoring. Of note, iBGStar (Sanofi-Aventis, USA) sells a small glucometer that can be connected to a smart phone for additional healthcare management.

The use of these amperometric device readers with paper electrochemical devices in the literature has been limited. The

Abbreviations: ADH, alcohol dehydrogenase; APDMES, 3-aminopropyldimethylsiloxane; NAD^+, beta-nicotinamide adenine dinucleotide; SP, screen-printed; μPED, micro-paper electrochemical devices

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CheapStat was used to measure glucose, lactose, and uric acid in urine with paper-based electrochemical tests [13]. Nie et al. fabricated paper devices that were compatible with a glucose meter (CVS brand), demonstrating detection of glucose, lactose, cholesterol, ethanol [14]. However, in the latter, the authors were required to replicate the complex electrode design of the commercial test strips with a laser cutter. Furthermore, they did not investigate the long-term use of their paper devices. Given that reagents degrade on filter paper [15,16], reagent stability should be evaluated in order to determine the robustness of the platform.

In order to address these challenges, we developed μPEDs, or micro-paper electrochemical devices, for the detection of ethanol using a commercial glucose meter. The devices were simple in design and easy to fabricate. We used one device design that allowed ethanol concentrations to be measured by both a glucometer and a potentiostat. We demonstrated long-term potential with the stabilizer, trehalose. Ethanol, the consumable form of alcohol, was chosen as the model analyte for this platform for its application in assessing the global issues and challenges associated with road safety in developing nations. Furthermore, we used an NAD^+-dependent enzyme to selectively target ethanol, which represents a large class of enzymes used in the food and dairy industry [17], and is largely underrepresented in the literature for paper-based diagnostics.

2. Methods

2.1. Materials

A commercial glucometer was purchased from a local pharmacy for approximately USD$40 (OneTouch, Lifescan, Inc., USA). Alcohol Dehydrogenase from *Saccharomyces cerevisiae*, beta-nicotinamide dinucleotide (NAD^+), potassium ferricyanide, ethanol, phosphate buffer (PB) was purchased from Sigma. Whatman Grade-1 filter paper was purchased from Fisher Scientific. Trehalose was donated from SriTechnologies (GA, USA). The pH of the phosphate buffer (PB) was adjusted with sodium hydroxide. 3-aminopropyldimethylsiloxane (APDMES) was purchased from Gelest, Inc (PA, USA). Graphite ink was purchased from Ercon, Inc. (MA, USA).

2.2. μPED fabrication

Reagent preparation and fabrication of the μPEDs were based on previously described methods [8,14]. Briefly, circular hydrophobic barriers were patterned onto filter paper (Whatman Grade-1) using a commercial wax printer. The wax-patterned papers were melted on a hot plate for 3 min at 100 °C. Then, graphite was screen-printed using a homemade stencil. The stencil pattern was designed in AutoCad and cut into cellulose acetate film, 0.05 mm thick, with a cutter plotter (Graphtec Craft ROBO Pro, Graphtec America, CA, USA). The patterned sheets were dried on a hot plate for 20 min at 65 °C, then cooled at room temperature for 1 min. An example of resulting μPEDs are shown in Fig. 1D.

2.3. μPED optimization and preparation

In order for ethanol to be successfully detected on μPEDs with a glucose meter, multiple iterations of optimization were performed. μPED designs varied, including by electrode dimensions, reference electrode material, and working electrode surface area. Reagent optimization involved varying the concentration, volume, and ratio (v/v) of the sample solution to the detection reagent solution (which contained ADH, NAD^+, and KCN). The sample volume and time to allow the sample to wet the μPED was also determined to be relevant optimization parameters. Due to the narrow range of currents detectable by the glucose meter, successful optimization of μPEDs was determined if the glucose meter displayed a numerical value within 10 insertions of the same μPED (see Section 2.5).

After optimization, each μPED was spotted twice with 4 μl of 2% wt 3-aminopropyldimethylsiloxane (APDMES), with 15 min to dry between each spotting. To test the response of the tests using a glucose meter, the following stock solution of detection reagent was prepared: 160 Units/ml ADH, 5 mmol L^{-1} NAD^+, 500 mmol L^{-1} KCN (0.1 mol L^{-1} PB, pH 8). Due to the light-sensitive nature of NAD^+ and KCN, these reagents were prepared in the dark. As necessary, trehalose was added to the reagent stock solution to a final concentration of 5% (w/v). Sample solutions of ethanol were prepared in glass vials and diluted in 0.1 mol L^{-1} PB, pH 8.

2.4. Relevant glucometer circuitry

Although the circuitry of a commercial glucometer was proprietary, we gained useful information through visual and electrical analysis using a multimeter. First, the insertion port for the test strip contained five pins (Fig. 1). Pins 1, 2, and 3 connected to the working, counter, and reference electrode of the test strip. Pins 4 and 5 also connected to the test strip and when short-circuited, turned on the glucometer. These latter pins were only necessary for turning on the glucometer and had no electrical effect on the electrodes. When turned on, two pins maintained a constant potential difference of approximately +0.4 V. We screen-printed a strip of carbon paste onto a 0.03 mm acetate film that could short-circuit Pins 4 and 5 (Fig. 1). The short-circuit strip was thin enough to allow insertion of the μPED later.

For each measurement in our study, the glucometer was turned on by short-circuiting Pins 4 and 5. A μPED was then inserted. Upon introduction of a sample, the glucometer immediately began a 5 s countdown. There were four general outputs by the meter: “Lo”, “Hi”, “Er” for error, and a numerical value between 0 and 600 (calibrated to a concentration of glucose).

2.5. Ethanol analysis using a glucometer

After optimization, μPEDs were tested with (1) freshly spotted reagents and (2) dried reagents. For the former, the glucometer was manually turned on by short-circuiting Pins 1 and 2. A μPED was then inserted, followed by spotting a 1:1 mixture of the reagent stock solution and sample (6 μl total). The meter output was recorded. Multiple readings were taken of the same μPED by re-shortcircuiting Pins 4 and 5, then reinserting the μPED. Results were recorded until an “error” message was obtained twice in a row.

For the latter, 5 μl of detection reagent solution was spotted onto each μPED and allowed to dry for at least 1 h at room temperature. To test these μPEDs, the glucometer was first turned on. Sample solution (8 μl) was spotted onto the μPED, left to incubate for 5 s, then inserted into the glucometer, and the results recorded as described.

2.6. Electrochemical testing

The potentiostat (CH Instruments 440) was used to perform amperometry at +0.4 V versus carbon. To mimic the glucometer, amperometry was performed using the potentiostat, but alternating the potential between open circuit potential (0 V vs carbon) and +0.4 V versus carbon every 5 s. The μPEDs and reagents were of the same design and concentrations, respectively.

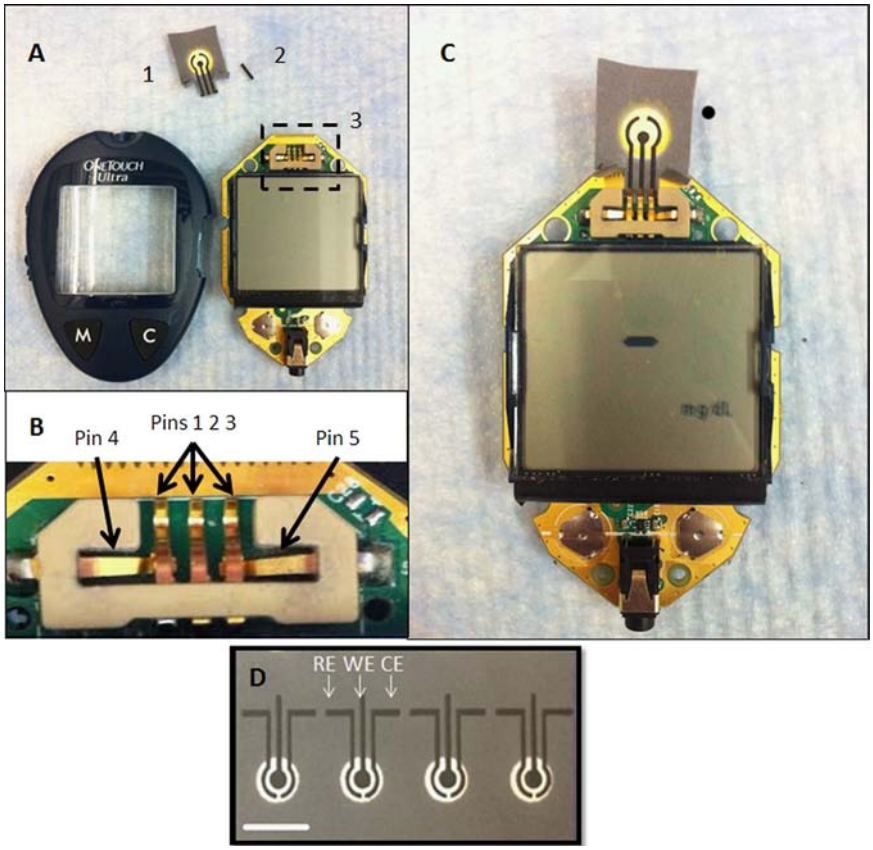

Fig. 1. Schematics of μPED integration with a commercial glucometer. (A) A μPED (1) and a screen-printed carbon "wire" on cellular acetate (2) that when inserted, turn on the glucometer. Housing of glucometer removed to show test strip insertion port (3). (B) Close-up of the test strip insertion port with five gold pins. Pins 1–3 connect to the electrodes of the μPED. Short-circuiting Pins 4 and 5 turn on the glucometer. (C) Demonstration of turning the glucometer on by insertion of the μPED and carbon "wire". (D) Example of screen-printed μPEDs with carbon serving as pseudo-reference electrodes (RE), working electrodes (WE), and counter electrodes (CE). Darkened areas are hydrophobic and do not wick aqueous solutions due to imprinted wax, whereas white areas wick solutions easily. Scale bar = 1 cm.

3. Results

Using a multimeter, we found that the maximum potential applied between two pins of the glucometer was approximately +0.4 V (see Fig. 1). This voltage difference persisted so long as Pins 1 and 2 were connected. Therefore we concluded that the μPEDs were subject to a potential step between open circuit potential (+0 V vs C as measured with the potentiostat) and +0.4 V versus C, when the μPED was disconnected and connected to the glucometer, respectively. With multiple insertions of the same μPED, the potential step was cycled accordingly. More importantly, samples that produced a "HI" output resulted in outputs with a numerical value, decreasing with every insertion. The number of insertions required to go from "HI" to a numerical value was as little as two, to as many as nine. However, this number remained consistent for each batch of prepared μPEDs.

In cases where the first insertion resulted in an output of "HI", the μPED was reinserted for another reading. Fig. 2 shows how the signal for three μPEDs, tested with three different concentrations of ethanol, changed with each insertion. An ethanol response curve was generated by plotting the meter output for μPEDs tested with various concentrations, after a given number of insertions.

This method of using the μPEDs for integration with a glucometer was validated using a potentiostat. Fig. 3 demonstrates the similarity between (1) a μPED subjected to +0.4 V versus carbon continuously and (2) a μPED subjected to a cycle of +0 V versus carbon to +0.4 V versus carbon. In the latter profile, the currents measured at the end of each cycle of the +0.4 V versus carbon phase closely correlated to that of the original profile. Using the Cottrell plots, where the slope correlates to the concentration of the target analyte [12], the

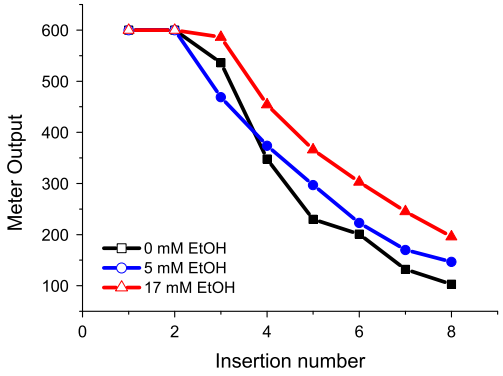

Fig. 2. Sample of data collection with a glucometer and μPED. Meter output recorded after every insertion of a μPED spotted with a sample of ethanol. Time between insertions was approximately 10 s. Unfilled markers indicate meter outputs of "HI.".

mimicked profiles were within 10% of error to that of the original, uncycling profile. Specifically, the slope was $-15.2\ \mu A*s^{1/2} \pm 1.0$ and $-19.8\ \mu A*s^{1/2} \pm 1.0$ for 8.5 mmol $L^{-1}$and 0 mmol L^{-1}ethanol measurements, respectively ($n=3$).

The response to ethanol using μPEDs and a glucometer were satisfactory when reagents were freshly applied (Fig. 4). Also, the linear range of detection became more reliable with more insertions, falling between 2 mmol $L^{-1}$and 8 mmol L^{-1}ethanol. This was because at low insertion numbers, the current generated from higher concentrations of ethanol still had not decayed to within the range of the meter that produced numerical values. We observed

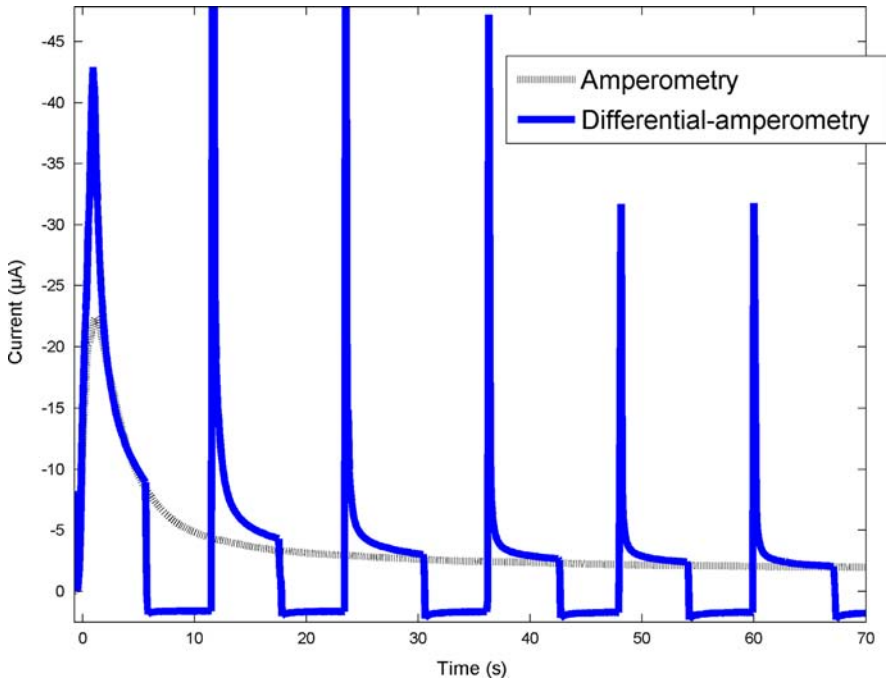

Fig. 3. Representative amperometric response to 8 mmol L^{-1}ethanol using a potentiostat and μPEDs. +0.4 V vs carbon was applied continuously (—) or cycled between +0.4 V and +0 V vs carbon every 5 s.

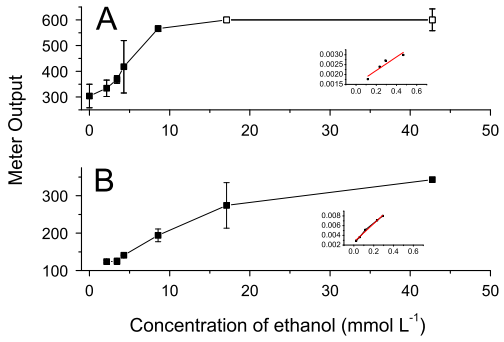

Fig. 4. Glucose meter response to a mixture of ethanol and detection reagent solution (1:1 v/v ratio), applied to μPEDs. Meter response after (A) 3 insertions (~25 s) and (B) 5 insertions. Symbol ▯ denotes outputs that included "HI." Lineweaver-Burke Plots are shown in the corresponding insets, R^2=0.8529 and 0.989 for (A) and (B), respectively. N=3.

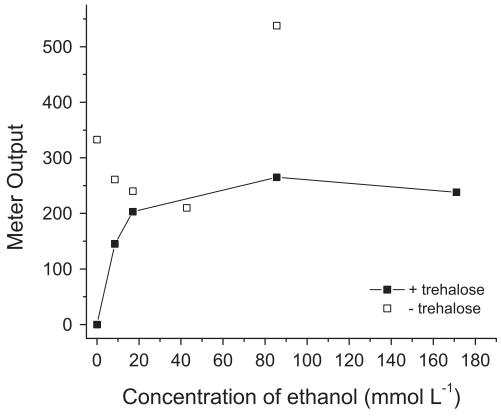

Fig. 5. Representative ethanol response using μPEDs and a glucometer, with and without trehalose after 1 h of storage. Each μPED was incubated with the sample for 5 s prior to insertion into the glucometer. Response was recorded after 6 and 9 insertions without and with trehalose, respectively.

Michaelis-Menten-like behavior, as others have for alcohol SP biosensors [18], and noted that the corresponding Lineweaver-Burke plots after 3 and 5 insertions had R^2=0.8529 and 0.989, respectively. The corresponding limits of detection (3 × 1 STD) were 2.7 mmol $L^{-1}$and 5.2 mmol L^{-1}.The lowest concentration detected without an error message was 2 mmol L^{-1}ethanol. These enzyme kinetics were also observed when the μPEDs were analyzed using a potentiostat (Fig. 6).

In contrast, the response curve using dried detection reagents on μPEDs was largely unsuccessful unless trehalose was added (Fig. 5). We verified that trehalose did not interfere with the signal by measuring the meter response to freshly applied reagents (Fig. 7A). Furthermore, after 48 h of storage at 4 °C with silica beads, the linear range of the biosensor was maintained (Fig. 7B).

4. Discussion

The proprietary circuitry of the commercial glucometer imposed several challenges in developing a paper device that could integrate with the device. The amperometric current from the paper test had to fall within a precise range to which the glucometer was sensitive (i.e. did not produce an error message). Samples that produced a "HI" reading would produce a numerical reading after a certain number of insertions, likely from the decay in current described by the Cottrell Equation. Fortunately, the resulting meter output correlated with the concentration, as measured by a potentiostat (Fig. 3). Given that the performance of the biosensor was influenced by both the presence of a stabilizer and how long the reagents were stored, the method we presented may be a necessary requirement in actual field tests.

The most significant design factors that allowed the μPEDs to fall within the optimal amperometric current range were the addition of APDMES, the width of the electrodes, loading volume, and the mediator concentration. First, as noted by others, APDMES amplified the signal and increased the wicking rate of the sample to the electrodes, resulting in an amperometric response that resembled that of a commercial glucose test strip [14]. APDMES, a silane, is

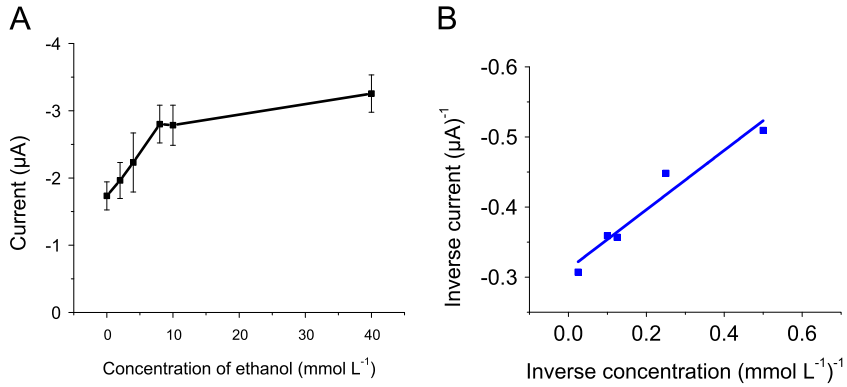

Fig. 6. Ethanol measurements using μPEDs and a potentiostat. (A) Current response and (B) Lineweaver-Burke Plot (R^2=0.9449). Ethanol samples and detection reagents (1 mg/ml ADH, 15 mmol $L^{-1}NAD^+$ in 0.1 M PB) were mixed and applied directly to the μPED with a potential of +0.3 V vs C (t=70 s). Results reported as the mean ± 1 STD, n=4.

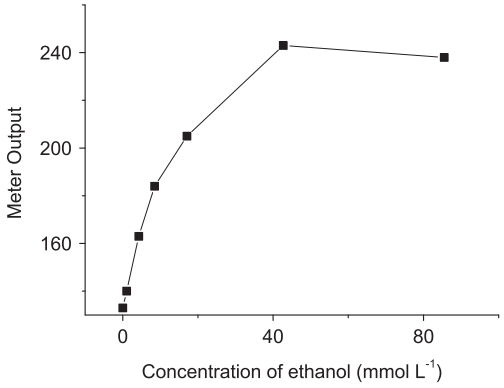

Fig. 7. Representative ethanol response using μPEDs and a glucometer with trehalose included in the detection reagent. Detection reagents were dried for 48 h at 4 °C. Results recorded after 6 insertions. Linear range between 2 mmol L^{-1} and 8 mmol L^{-1}had R^2=0.9482.

believed to activate the cellulose fibers and increase its hydrophillicity, and has been used in similar applications [14]. Second, μPEDs with thinner electrodes produced error messages. Wider electrodes produced "HI" outputs during the initial insertions. Since carbon ink has a non-negligible resistivity, thinner electrodes likely increased the resistance and lowered the current measured by the glucometer, thus producing the error messages. Interestingly, the length of the electrode did not significantly reduce the current. Using carbon inks of lower resistivity or incorporation of high-conducting materials such as graphene [19,20] could also be investigated for signal amplification, but were not done for this study. Additionally, to aid future optimization work, a computational model could be built to predict the current response of μPEDs given input parameters of electrode dimensions, resistivity, and other relevant electrochemical characteristics.

Third, the loading volume and concentration of the mediator influenced whether quantitative outputs could be obtained. We believe that these parameters affected the amperometric current responses through a number of mechanisms: a higher concentration of mediator may impede diffusion, a large loading volume may reduce the resistivity of the electrolyte, and the composition of the buffer may contain agents that influence electrotransfer processes [18]. Interestingly, the composition of the standard glucose solution provided by the manufacturer of the glucometer contains nondescript "viscosity enhancing reagents" (LifeScan Technologies, USA). Amperometry relies heavily on both diffusion and electron transfer kinetics [12], however further investigation is required to confirm these hypotheses.

Despite our promising results, we also had a number of limitations to our method. Although the linear range of ethanol detection remained reproducible, the sensitivity and limits of detection varied between batches of μPEDs and by the insertion number at which the analysis was performed. As such, it would be critical to generate calibration curves to account for batch-to-batch variation as a result of home-made fabrication. We noted that the integrity of the μPEDs can also be affected after multiple insertions. This affected both our sample size and our error between measurements. However, we believe that this did not invalidate our results because we observed similar results with potentiostat analysis of ethanol on μPEDs, where the sample size was larger (Fig. 6). Furthermore these latter results were similar to ethanol measurements on SP biosensors on non-paper substrates [14,18]. Based on work by other authors [14], we believe that further optimization of design parameters can reduce the number of insertions required to elicit a numerical response from the glucose meter. Additionally, challenges with fabrication can be addressed by development and production by industry, as opposed to laboratory grade manufacture of μPEDs that is less robust than the industrial grade.

Nonetheless, after optimization, we successfully developed a sensitive, robust, quick, low-power, low-cost, and quantitative method to measure ethanol using paper devices and a commercial glucometer. The lowest concentration of ethanol detected was 2 mmol L^{-1}, in less than two minutes. The glucometer was inexpensive (USD 40 from a local drugstore) when compared to a benchtop potentiostat (up to thousands of dollars). It was light-weight, easy-to-use, fit in the palm of hand, and low-power. We calculated that the total cost of raw materials for each test was less than USD $0.20, and would likely be less if produced on a smaller scale. With optimization, μPEDs with reagents stored for 48 h at 4 C were successful in detecting ethanol samples.

The glucometer we used allowed us to integrate a μPED simple in design and fabrication, when compared to other glucometers. Nie et al. created paper test strips to integrate with a different commercial glucometer (CVS brand) [14]. They fabricated μPEDs containing four electrodes and two different electrode materials, using a laser cutter to create a screen-printing stencil and mimic the exact dimensions. In contrast, only one ink (and thus one screen-printing step), three electrodes, and a cutter plotter were required in our method. Previous work also did not demonstrate the long-term performance of their method.

The optimized μPED design was compatible with both a glucometer and a potentiostat, and both methods of analysis yielded similar results. Although the design was larger than that of the intended glucose test strips, it was simple to customize and easy to handle by the user. Customization was important as the

performance of the μPED varied by whether the reagents were freshly applied or dried on (Fig. 7). Pre-prepared μPEDs (i.e. with dried reagents) required a 5 s incubation period with the sample prior to inserting into the glucometer, possibly to allow for the reagents to rehydrate and accumulate signal.

Compared to other electrochemical reading devices for μPED integration, a glucometer truly allows for standardized, point-of-care testing. The CheapStat, an open-source and low-cost potentiostat, has been demonstrated to be compatible for analysis of screen-printed biosensors on both plastic and paper [11]. Zhao et al. demonstrated its potential with real urine samples for the detection of uric acid [13]. However, the CheapStat still requires a hardwire connection to a computer for analysis. On the other hand, a glucometer is a stand-alone amperometric device powered by a small battery. They have a user-friendly form factor and are hand-held, inexpensive, and relatively sturdy, making them robust for wide distribution.

We found that the stabilizer trehalose was critical to the potential long-term usability of μPEDs (pre-prepared with dried reagents). μPEDs containing dried reagents could still detect ethanol after storage for more than 48 h at 4 C. The stored biosensors performed with a predictable linear range, so long as trehalose was included during reagent storage (Fig. 7B). Trehalose is a discharride sugar commonly known to stabilize proteins when lyophilized or dried [21,22]. Although we only studied storage at 4 °C, trehalose has been shown to preserve proteins stored under higher temperature and humidity conditions that are often encountered in low-resource settings [16,24]. It is important to note, that even with trehalose, reagent degradation still occurs during the drying process [15]. This was observed in our results by the lower magnitude of the response curve when using fresh and dried reagents. This has also been observed for enzymes and antibodies stored on filter paper [15,16,23]. Future work in demonstrating the robustness of this platform in low-resource settings should look at the overall thermal stability of μPEDs. Finally, this work provides a possible platform to address the 1.2 million people that die from road accidents every year globally, with more than 90% of deaths occurring in the developing world [25]. The legal limit of blood alcohol content in the United States is 0.08% (v/v), which is approximately 17 mmol L^{-1} of ethanol in solution. The lowest concentration of ethanol measured in our study was 2 mmol L^{-1} (0.1 mol L^{-1} PBS), which is approximately 0.01% (v/v). Additional testing is required to evaluate the sensitivity and specificity for detecting ethanol in actual biological solutions. Driving under the influence of alcohol, or drunk-driving, accounts for anywhere from 5% to more than 50% of road-related fatalities, according to a 2009 survey by the WHO [26]. Alcohol can also cause poisoning, which requires immediate treatment but may not be easily detected by symptoms alone. Unfortunately, the number of alcohol-related deaths is likely even higher due to underreporting and a lack of reliable data [26]. Although low-cost and effective alternatives to alcohol testing exist, they are still not cheap enough for low-resource settings. Even breath analysis, which is a well-accepted and low-cost alternative, has associated costs and operator training which restrict their use in many settings [27]. The methods presented in this study could address these shortcomings through a low-cost, rapid, portable method to detect alcohol intoxication.

5. Conclusion

We addressed the need for a portable electrochemical device reader for amperometric measurements on filter paper. Using ethanol as a target analyte with applications in addressing the needs of global road safety, we demonstrated integration of μPEDs with a commercial glucose meter. Key optimization parameters included surface treatments and incubation times; for long-term testing, we found strong evidence that inclusion of the stabilizer trehalose was critical.

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