Supplementary Information for:

Integration of Paper-based Microfluidic Devices with Commercial Electrochemical Readers

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EXPERIMENTAL SECTION

Chemical Reagents

Graphite ink (E3456) and silver ink (E1660) were purchased from Ercon Inc (Wareham, MA) and Conductive Compound (Hudson, NH), respectively. D-glucose, L-lactate, cholesterol, ethanol (≥99.5%), Triton X-100, β-Nicotinamide adenine dinucleotide hydrate, glucose oxidase (136,300 units/mg, *Aspergillus niger*), lactate oxidase (>20 units/mg, *Pediococcus species*), alcohol dehydrogenase (≥300 units/mg, *Saccharomyces cerevisiae*), cholesterol oxidase (≥50 units/mg, *Brevibacterium* sp.), and potassium ferricyanide were purchased from Sigma–Aldrich, and used as received. Organic silane 3-aminopropyldimethyl-ethoxysilane used for surface modification was purchased from Gelest. Inc. (http://www.gelest.com/), and used as received. Single-donor human plasma was purchased from Innovative Research, Inc. (http://www.innov-research.com/innov2010/), and used as received.

Glucometers

True Track™ blood glucometers were purchased from CVS/Pharmacy, and calibrated following the instruction manual before use. According to the manufacturer, the test measures glucose concentrations over the range 20 to 600 mg/dL (~ 1.1-33.3 mmol/L).[1]

Fabrication of EμPADs

Microfluidic Channels. Paper-based microfluidic channels were fabricated by patterning paper (Whatman 1 Chr) using wax printing.[2] We printed a piece of paper using a solid-wax printer (Xerox phaser 8560), and baked it at 150 ^{o}C for 2 min in an

oven. The printed wax melted and diffused into paper to form the hybrophobic barriers that defined the paper channels. We produced about 150 – 200 EμPADs on one US letter-sized page of chromatography paper. The printing step takes approximately 1-2 seconds per page.

Circuits and Electrodes. We fabricated electrodes by manually screen-printing graphite ink (or silver ink, for wires) on patterned paper.[3] The circuits on paper were designed to fit into the port of the glucometer by mimicking the commercial configuration. We generated a stencil by cutting the designed pattern into vinyl stencil film (Grafix®Frisket film) using a laser-cutter (VersaLASER VLS3.50, Universal Laser Systems Inc.). We placed the stencil on top of patterned paper, and filled the openings of the stencil with silver ink to produce conducting wires. We removed the stencil, and baked the paper device on a hotplate at 100 ^{o}C for 30 minutes. After the drying of silver ink, we screen-printed another layer of graphite electrodes from graphite ink following the same procedures. The graphite electrodes had a small portion of the electrodes overlapping with conducting wires.

Geometry of EμPADs. A typical EμPAD we used had dimensions of about 7 mm in width, and about 100 μm in height (determined by the thickness of the paper), and varying length for different measurements, Figure SI1. The width of silver wires was approximately 1.0 mm. A typical working and counter electrode had a width of 0.4 mm, and a typical reference electrode had a width of 1.5 mm; they all had a thickness of about 100 μm. The detailed dimensions of EμPADs used for different measurements are summarized in Table SI1.

Figure SI1. Schematic diagram shows the dimensions of an EμPAD. Table SI1 gives values for dimensions A to G. WE: working electrode; CE: counter electrode; and RE: reference electrode.

Figure SI1.

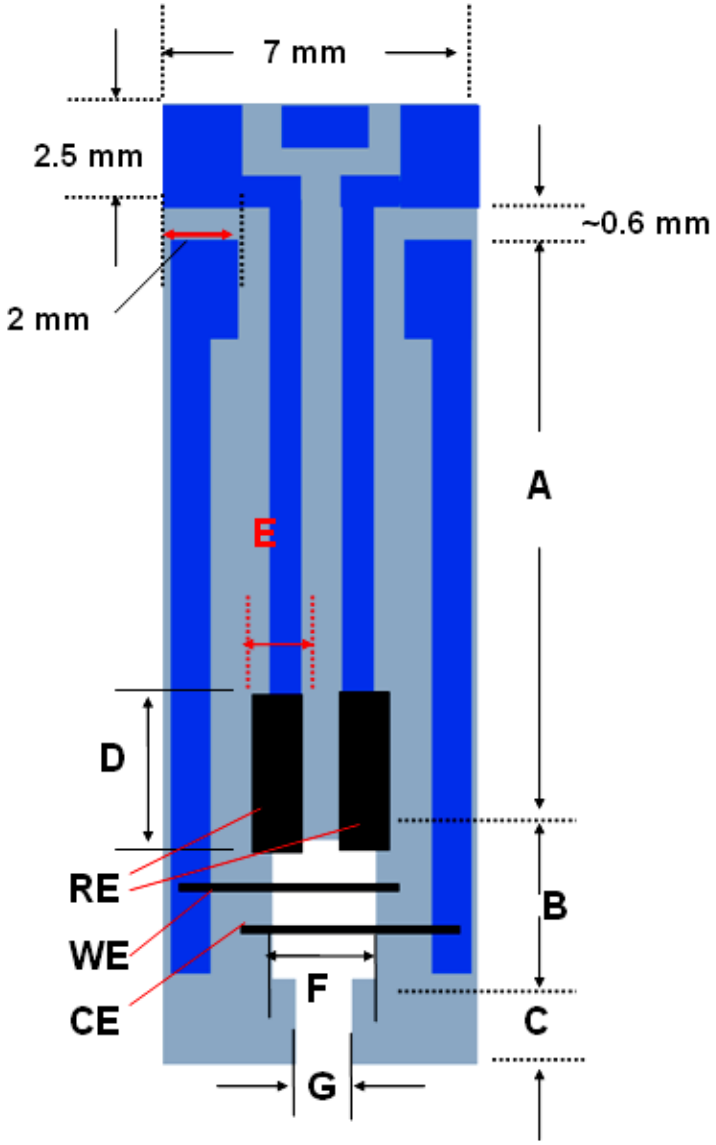


Table SI1. Dimensions of EμPADs Used for the Electrochemical Analysis.

Analyte	A (mm)	B (mm)	C (mm)	D (mm)	E (mm)	F (mm)	G (mm)
Glucose	14.0	4.0	0	4.0	1.5	1.2	—
Ethanol	15.0	4.5	2.0	4.0	1.5	3.0	1.0
Cholesterol	15.0	4.5	1.0	4.0	1.5	3.0	1.0
Lactate	12.0	4.0	0	2.5	1.0	2.5	—

Surface Treatment of Devices. We treated the paper channel and electrodes with an aqueous solution of 2 wt% 3-aminopropyldimethyl-ethoxysilane (APDES) before loading enzyme (or other reagents) in an EμPAD. We typically spotted 2 μL of an aqueous solution of 2 wt% APDES in the EμPAD. The solution was distributed to the paper channel by capillary wicking. After the solution dried at room temperature for about 10 minutes, we spotted another 2 μL of an aqueous solution of 2 wt% APDES in the EμPAD. The device was ready for use after the solution dried at room temperature for about 10 minutes. For the measurement of analytes in blood, we treated the EμPAD with 2 wt% APDES three times following the same procedures described above.

Measurement of Analytes in EμPADs Using Glucometers

All assays were performed in EμPADs using a glucometer as an electrochemical reader. For the use of EμPAD stored with chemical reagents, we inserted an EμPAD into the port of the glucometer, and dipped the exposed end of the EμPAD in a solution of analytes. The fluids wet the paper channel by wicking, and dissolved chemical reagents stored in the paper channel. After the meter beeped, we immediately took the end of the EμPAD out of the solution. After countdown of 10 seconds, the glucometer displayed the result of measurement on its LCD screen. When an EμPAD that did not have stored chemical reagents was used, we mixed all chemical reagents in a 0.6-mL centrifuge tube (the mixing can be also conducted on any clean substrate, such as a plastic thin film, or the surface of a table), and allowed the chemicals to proceed to completion for 60 s in the dark. We then applied the solution

to the EμPAD by dipping the exposed end of the EμPAD in a drop of this reacted solution. A typical volume of fluids required to completely wet the paper channel was 1~2 μL, depending on the surface area of the paper channel. This volume determines the amounts of samples needed for the assays; and it can be reduced to about 0.5-1.0 μL by shortening the length of the paper channel. All measurements were conducted under ambient conditions.

Assays

Assays of D-glucose. Chemical reagents required for the analysis of glucose were stored in a dry form on EμPADs treated with a 2 wt% APDES aqueous solution. We spotted 1 μL of a solution of 250-mM potassium ferricyanide in water on the paper channel. The solution was allowed to dry for 20 minutes at room temperature in the dark (ferricyanide in solution is sensitive to light). We then spotted 1 μL of a PBS buffer solution (pH 7.0) containing 500 units/mL of glucose oxidase on the paper microchannel. The device was ready for use after the solution dried for 20 minutes at room temperature in the dark. We did preliminary tests of the storage stability of the system. When the devices were stored at ~ 4 ^{o}C for about three weeks in the dark, the devices performed well. We did, however, observed that the readout of the measurements decreased with time when the devices were stored at room temperature. We believe that the addition of reagents (e.g., trehalose) that stabilize the activity of proteins can certainly improve the storage stability of the system. Stock solutions of D-glucose with a concentration of 10,000, and 40,000 mg/dL were prepared in a PBS buffer (pH 7.0), and allowed to mutarotate overnight before use. Solutions of glucose in human plasma with concentrations ranging from 0 to 1000 mg/dL (approximately 0

to 55.6 mM) were prepared by diluting the stock solutions with human plasma. Before use, the human plasma was confirmed to have no detectable glucose in it using commercial glucose test strips. Each measurement was performed by dipping the inlet of an EμPAD into a small drop of a solution of glucose in human plasma. For the analysis of blood samples with unknown concentration of glucose, we obtained human blood from the same healthy volunteer (male, ~30 years of age) about two hours after his breakfast. Briefly, we placed the end of a steel lancet against the tip of finger, and lanced the fingertip. We allowed a small drop of blood to form before applying the blood to EμPADs (or commercial test strips).

Assays of L-lactate. For the measurement of L-lactate, we mixed a solution of L-lactate in human plasma with a solution containing lactate oxidase and ferricyanide in a 0.6-mL centrifuge tube (the mixing can be also conducted on any clean substrate, such as a plastic thin film, or the surface of a table). A separate step was necessary, since the time interval required to complete this enzymatic reaction was greater than the 10-second waiting-time set in glucometers as default. Briefly, we prepared a solution containing 200 units/mL of lactate oxidase in a PBS buffer (pH 7.0), and a solution containing 800-mM potassium ferricyanide in water. We pipetted 25 μL of each of these two solutions, and mixed them thoroughly in a 0.6-mL centrifuge tube. A 20 μL of the resulting solution was then mixed with 20 μL of L-lactate solution in a same centrifuge tube. The reaction was allowed to proceed toward completion for 60 s at room temperature in the dark, before being applied to the EμPADs. We prepared L-lactate solutions with desired concentrations by diluting a 100-mM lactate standard (Biovision, Lactate Assay kit) with water. These L-lactate aqueous solutions were

then used to prepare solutions of L-lactate in human plasma with concentration ranging from 1.1 mM to 11 mM by mixing 2 μL of these solutions with18 μL of human plasma. The human plasma purchased from Innovative Research, Inc. (http://www.innov-research.com/innov2010/) itself contains 1.1 mM L-lactate before the addition of any L-lactate.

Assays of Cholesterol. For the measurement of cholesterol, we mixed a solution of cholesterol in artificial human plasma with an aqueous solution containing cholesterol oxidase and ferricyanide in a 0.6-mL centrifuge tube. Artificial human plasma was used for these specific assays of cholesterol due to the presence of large amount of cholesterol in human plasma (or whole blood) purchased from Innovative Research, Inc. (http://www.innov-research.com/innov2010/). The artificial human plasma was prepared according to the recipe reported previously.[4] The artificial human plasma contained 8.036 g/L sodium chloride, 0.352 g/L sodium bicarbonate, 0.225 g/L potassium chloride, 0.238 g/L disodium phosphate ($Na_2HPO_4 \cdot 3H_2O$), 0.311 g/L magnesium chloride ($MgCl_2 \cdot 6H_2O$), 0.293 g/L calcium chloride, and 0.072 g/L sodium sulfate all mixed in Millipore-purified water. All inorganic reagents were purchased from Sigma–Aldrich.

We prepared a solution containing 100 units/mL of cholesterol oxidase in a PBS buffer (pH 7.0), and a solution contaning 500-mM potassium ferricyanide in water. We took 10 μL of each of these two solutions, and mixed them thoroughly in a 0.6-mL centrifuge tube. A 20 μL of the resulting solution was then mixed with 20 μL of cholesterol solutions with concentrations ranging from 0 to 400 mg/dL. The reaction was allowed to proceed for 60 s in the dark before being applied to the

EμPADs. We prepared a stock solution of cholesterol (400 mg/dL) by dissolving 40 mg of cholesterol in 1 mL of Triton X-100 and 9 mL of plasma solution as reported previously[5]. Cholesterol solutions containing the desired concentrations of cholesterol were then prepared by diluting this stock solution with plasma solution. All solutions were kept stirring with magnetic stirring bars to obtain a homogeneous solution before each measurement.

Assays for Ethanol. Chemical reagents required for the analysis of glucose were stored on EμPADs. Briefly, we spotted 2 μL of a solution of 250-mM potassium ferricyanide in water on the paper channel. The solution was allowed to dry for 20 minutes at room temperature in the dark. We then spotted 2 μL of a PBS buffer solution (pH 7.0) containing a mixture of 3400 units/mL alcohol dehydrogenase and 5 mM β-NAD^+ on the paper channel. The device was ready for use after the solution dried for 20 minutes at room temperature in the dark. A stock solution of ethanol in water with a concentration of 200 mM was prepared by adding ethanol in Millipore-purified water in a 20 mL-glass vial. Aqueous solutions containing the desired concentrations of ethanol were prepared by diluting the stock solution with water. All aqueous solutions of ethanol were freshly prepared before use. Each measurement was performed by dipping the inlet of a dry EμPAD into a drop of an aqueous solution of ethanol.

Glucometer and Code Chip

For this model of glucometer, each batch of commercial test strips is accompanied with a code chip for calibration. By inserting the code chip into the

glucometer from its side port, a specific code will be input into this glucometer, and the glucometer will be calibrated to that batch of test strips (Figure SI2).

Electrochemical Behavior of EμPADs

We compared the electrochemical behavior of EμPADs treated with APDES with that of non-treated EμPADs using ferrocene carboxylic acid as a model electroactive compound (Figure SI3). The peak shape of the cyclic voltammograms obtained in EμPADs treated with APDES shows a typical reversible electrochemical reaction in which the rate of reaction is governed by the diffusion of the electroactive species to the surface of a planar electrode (Figure SI3A). The difference in potential between the reduction peak and oxidation peak is about 0.061 V, and the ratio of anodic peak current to cathodic peak current is close to 1.0. The anodic peak current, I_p, is linearly proportional to the square root of the scan rate ($\nu^{1/2}$) (Figure SI3B). In contrast, the non-treated EμPADs show irreversible electrochemical characteristics, and poor reproducibility (Figure SI3C). Moreover, the current signals obtained in treated EμPADs are about 40-fold higher than those obtained in non-treated EμPADs. We explain that the surface of electrodes treated by APDES is much more hydrophilic than that of non-treated electrodes, thus substantially increasing their effective surface area of the electrodes.

Figure SI2. Calibration of the glucometer with a code stored in a code chip.

Figure SI2.

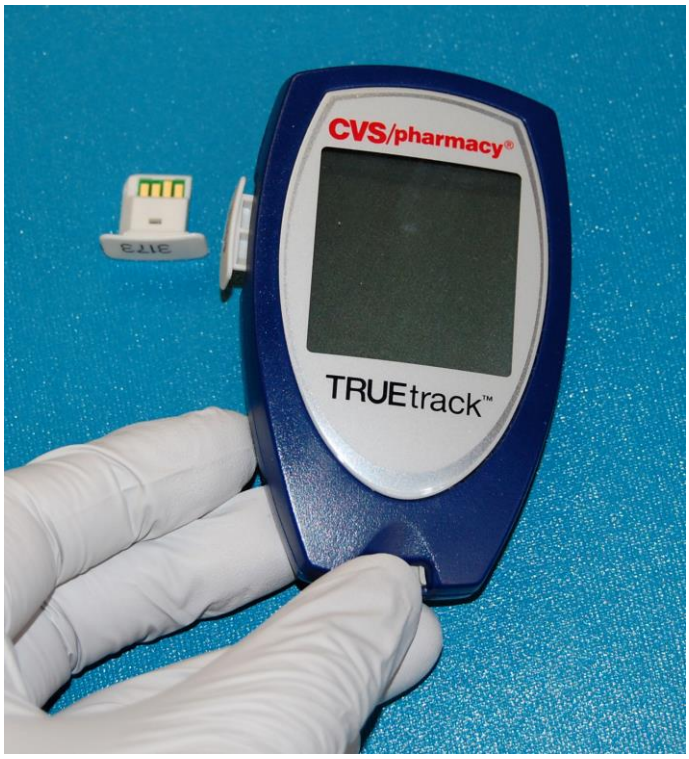
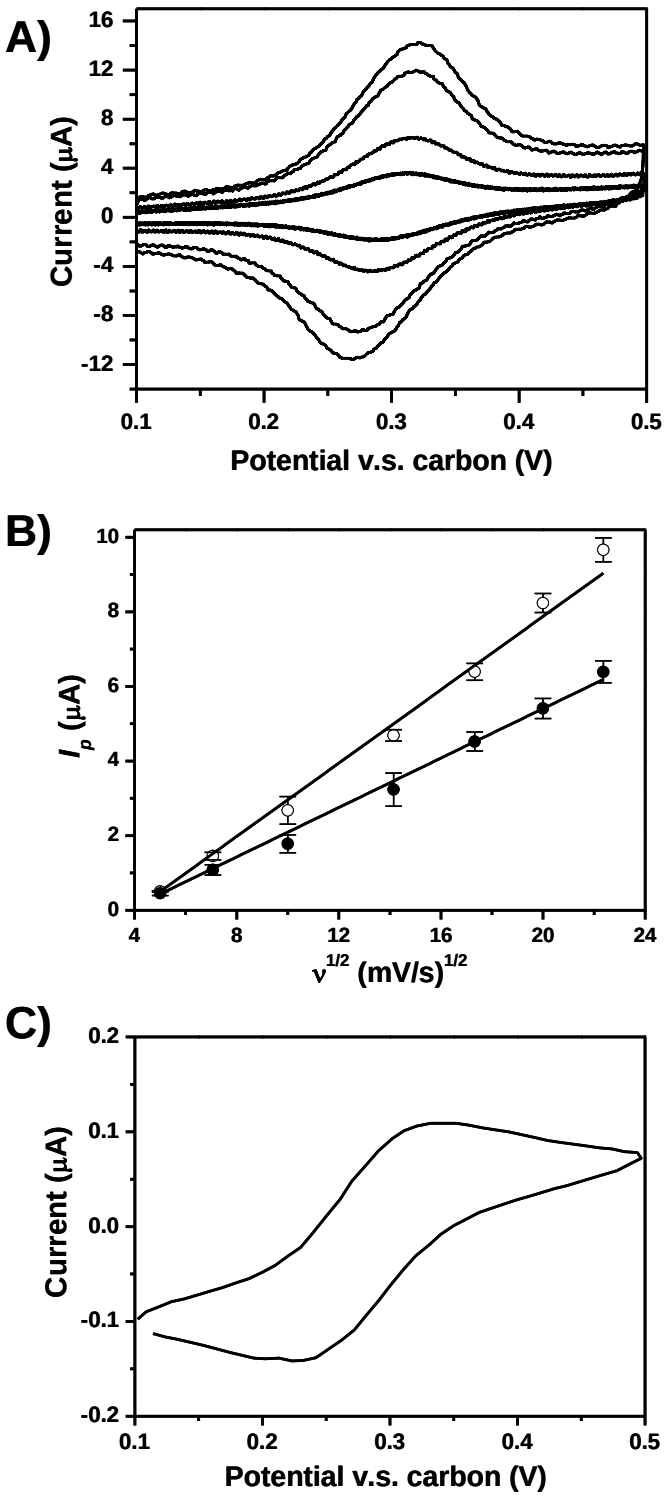


Figure SI3. Electrochemical behavior of single-layer EμPADs treated with APDES, and without being treated with APDES. (A) Cyclic voltammograms of 1.0 mM ferrocene carboxylic acid in PBS buffer solution (pH 7.0) in a single-layer EμPAD treated twice with APDES at various scan rates (ascending along y-axis): 100, 200, 400 and 500 mV/s. (B) The plot of anodic peak current *v.s.* the square root of the scan rate ($\nu^{1/2}$) for cyclic voltammetric experiments conducted on an EμPAD treated twice with APDES (○) and treated once with APDES (●). The solid lines represent linear fits to experimental data with regression equations: y=-1.95+0.49x (R^2=0.996, n=8) (○), and y=-1.22+0.33x (R^2=0.996, n=8) (●). (C) Cyclic voltammogram of 1.0 mM ferrocene carboxylic acid in PBS buffer solution (pH 7.0) at a scan rate of 200 mV/s in a single-layer EμPAD without being treated with APDES.

Figure SI3.


A)
Current (μA)
Potential v.s. carbon (V)
B)
I_p (μA)
$\nu^{1/2}$ (mV/s)$^{1/2}$
C)
Current (μA)
Potential v.s. carbon (V)

The Performance of Two-layer EμPADs

We also explored the use of two-layer configuration for EμPADs, in which another layer of paper channel was placed on top of the detection zone by folding the paper channel (Figure SI4A). The fabrication of the microfluidic channels and electrodes is described in the text. Folding the paper channel along the dashed line achieved conformal contact with the electrodes; and the folded device was held in place by transparent tape (Figure SI4A). The detection zone had dimensions of 4.5 mm × 5.5 mm, and the working and counter electrodes had 2.5 mm^2 area located in the detection zone. The two-layer EμPADs were used *without* being treated with APDES. We prepared a solution containing 1000 units/mL glucose oxidase in PBS buffer (pH 7.0) and 500 mM potassium ferricyanide in water. We took 10 μL of each of these two solutions, and mixed them thoroughly in a 0.6-mL centrifuge tube. The resulting solution was then mixed with 20 μL of glucose solutions with concentrations ranging from 0 to 400 mg/dL. The reaction was allowed to proceed for 60 s in the dark before being applied to the EμPADs. We then directly spotted 2 μL of the solution on the paper channel above the electrodes.

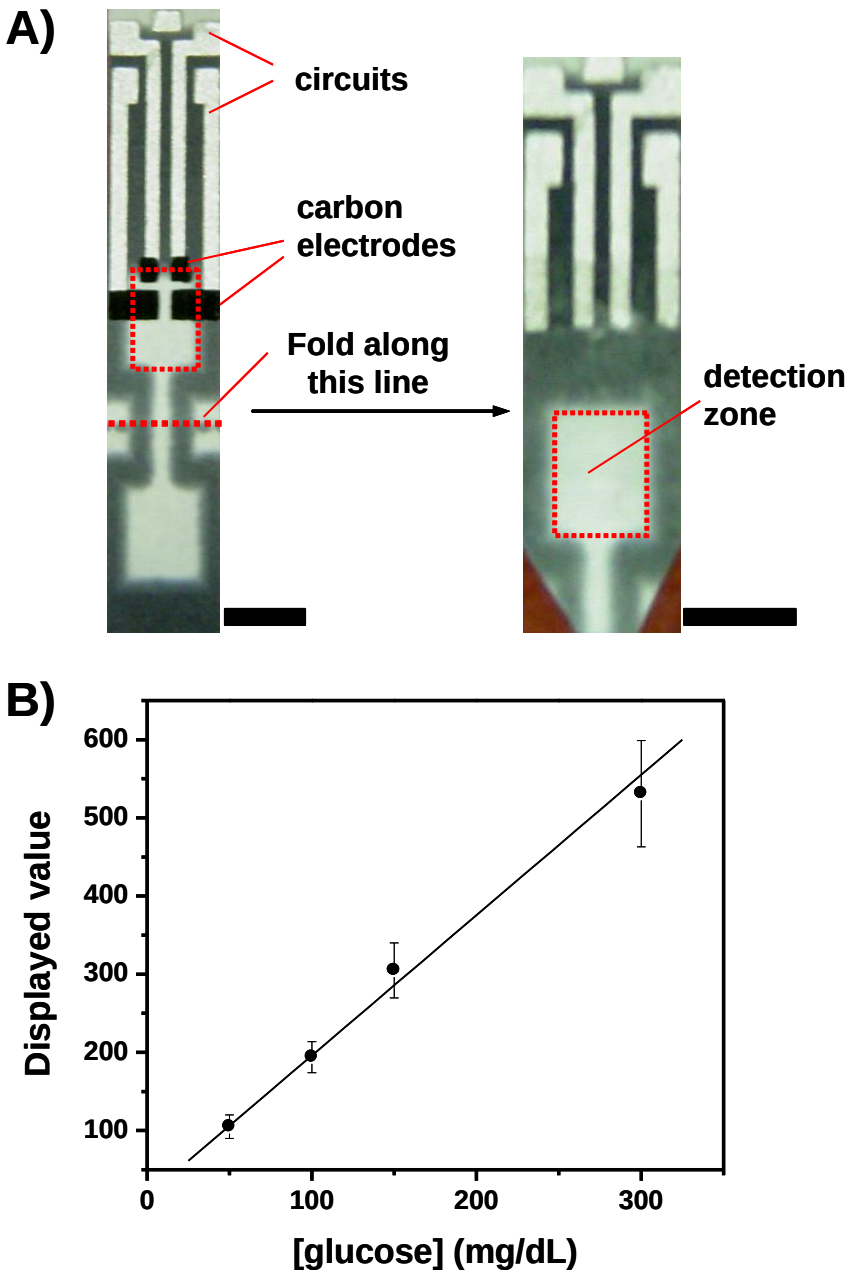
In single-layer EμPADs *without* being treated with APDES, fluids only wet the bottom surface of the electrode. In contrast, the two-layer EμPADs brought the solution of analytes to wet the whole surface of electrodes sandwiched in between two layers of the paper channels, thus increasing the effective surface area of electrodes. The two-layer EμPAD is capable of quantifying the concentration of analytes. Figure SI3B shows a calibration curve for the measurement of glucose in the double-layer EμPAD using a glucometer as an electrochemical reader. The wicking rate of the solution of analytes

along the paper channel is, however, very slow, and causes a time lapse between the wetting of different electrodes. We, therefore, have to spot the solution of analytes directly from the top of the paper channel above the electrodes; otherwise, the glucometer displays an error message.

The failure rate of the two-layer EμPADs was up to ~15 %; we presume that these failures were due to the failure of contact between the paper channel with the electrodes, and to the misalignment of the top layer of paper channel with the electrodes. Both problems could certainly be resolved by improved engineering. The two-layer EμPADs may be useful for certain detections, particularly those where multiple steps of fluid handling are required.

Figure SI4. The use of double-layer EμPAD in quantifying the concentration of glucose. A) Folding the paper channel along the red dashed line achieves conformal contact with the electrodes; and the folded device was held in place by transparent tape. The scale bars are 5 mm. B) Calibration plot for the analysis of glucose in double layer EμPADs using commercial glucometers. The solid line represents a linear fit to experimental data with a regression equation: y=16.5+1.8x (R^2 = 0.993, n=7).

Figure SI4.


A)
circuits
carbon
electrodes
Fold along
this line
detection
zone
B)
Displayed value
600
500
400
300
200
100
0
100
200
300
[glucose] (mg/dL)

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