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Paper-Based Analytical Device for Electrochemical Flow-Injection Analysis of Glucose in Urine

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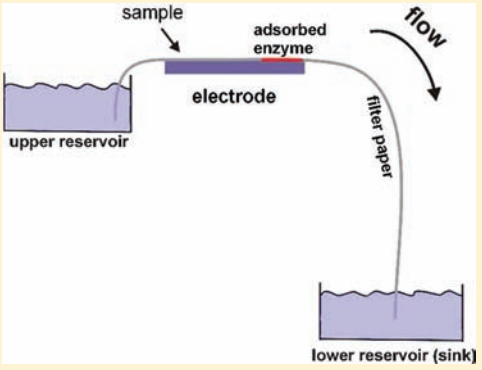
Supporting Information

ABSTRACT: This article describes a new design for a paper-based electrochemical system for flow-injection analysis. Capillary wicking facilitates a gravity-driven flow of buffer solution continuously through paper and nitrocellulose, from a buffer reservoir at one end of the device to a sink at the other. A difference in height between the reservoir and the sink leads to a continuous and constant flow. The nitrocellulose lies horizontally on a working electrode, which consists of a thin platinum layer deposited on a solid support. The counter and reference electrodes are strategically positioned upstream in the buffer reservoir. A simple pipetting device was developed for reliable application of (sub)microliter volumes of sample without the need of commercial micropipets; this device did not damage the nitrocellulose membrane. Demonstration of the system for the determination of the concentration of glucose in urine resulted in a noninvasive, quantitative assay that could be used for diagnosis and monitoring of diabetes. This method does not require disposable test strips, with enzyme and electrodes, that are thrown away after each measurement. Because of its low cost, this system could be used in medical environments that are resource-limited.



This article describes an electrochemical system for quantifying dynamic electrochemical signals generated by a working electrode operating at a fixed potential. This system comprises a channel (a strip of nitrocellulose sheet) with one end in contact with a buffer reservoir and the other end in contact with a long strip of filter paper that is dipped in a lower reservoir that acts as a sink. The system, illustrated in Figure 1, has three electrodes (a large working electrode of platinum sputtered on glassy carbon or glass and smaller counter and reference electrodes of stainless steel and silver, respectively). A plastic Petri dish was used to support the electrodes; other containers could be used for the same purpose. When in operation, a difference in height between the buffer reservoir and the sink drove the flow of buffer; the system thus was operated at a flow rate determined by its geometry, rather than by relative humidity or temperature.

The part of the flow path in contact with the working electrode consists of a nitrocellulose pad on which glucose oxidase is immobilized, locally, near the downstream end of the pad. Immediately after a sample is introduced at the beginning of the pad, upstream of the glucose oxidase (Figure 1B), oxidation of unidentified components in the sample takes place and generates an oxidation current. This current decays, but this is followed later by a glucose-specific current. When glucose in the flowing sample is in contact with the immobilized glucose oxidase, it is oxidized to gluconic acid in a reaction that generates hydrogen peroxide; this species is subsequently detected by oxidation at the working electrode. This system achieved a temporal and spatial separation between glucose and potentially interfering, redox-active, components of urine.

This work demonstrates the functionality of the system by the determination of glucose in urine; the system, however, is also potentially applicable to other species for which oxidase activities exist or can be developed. Urine is the biological fluid most readily available noninvasively; it is still only partially exploited in healthcare, and its analysis can provide biomedically useful information. A measuring system that can be easily built and used for multiple analyses without employing disposable strips could be useful in resource-limited environments, where costs limit the options for biomedical analysis, and in research.

The system we describe is based on amperometry, and the measured current is proportional to the glucose concentration. The maximum height of the signal can be directly related to

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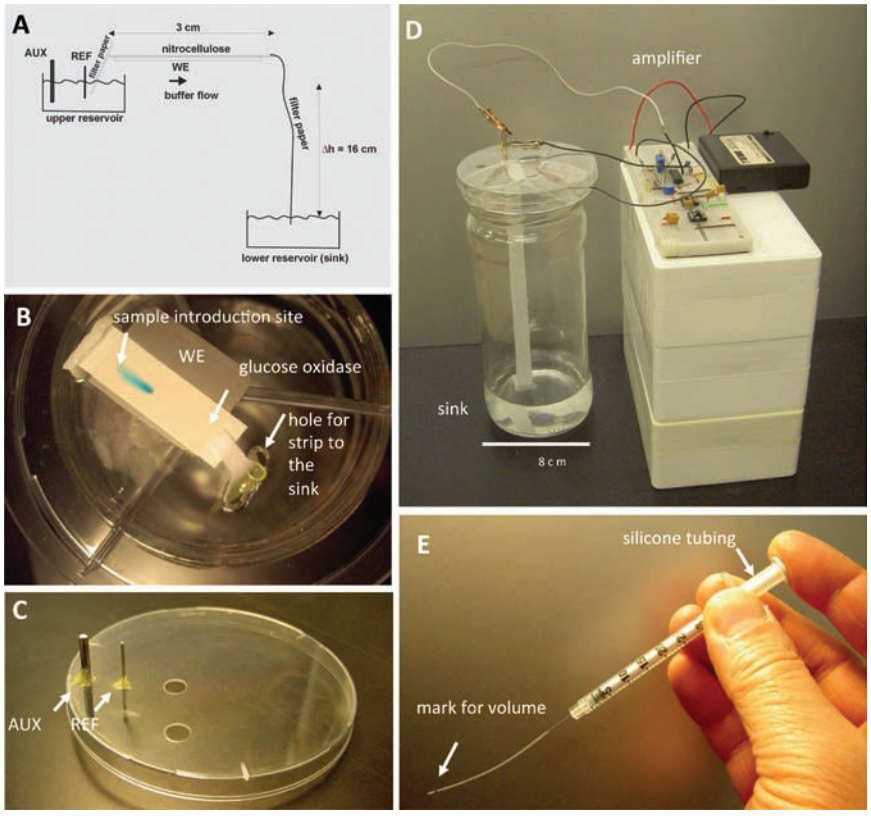

Figure 1. Schematic representation of the electrochemical detection in a paper-based flow-system. (A) Sketch of a side view of the experimental setup (for a larger version, see Figure S-1 in the Supporting Information), (B) top view showing a closeup of the paper and working electrode (Pt on glass) assembly, (C) covering lid of the Petri dish with holes for the introduction of sample and buffer, (D) photograph of the entire system, and (E) adaptation of an insulin syringe to dispense (sub)microliter-sized samples with good reproducibility (CV = 2.7%, n = 10). The working electrode (WE) of platinum deposited on glass (or glassy carbon) was covered with nitrocellulose (8 mm × 30 mm, thickness = 140 μm). After being wetted, the porous membrane (nitrocellulose or filter paper) automatically flattened and adhered to the surface and made conformal contact with it. This working electrode was fixed with double-sided Tesa tape on the dividers of a standard three-compartment Petri dish (B). One of the compartments is the buffer (upper) reservoir, in which the auxiliary (AUX) and reference (REF) electrodes were positioned (A,C). Another compartment has a cut for the strip of paper to connect with the sink. The third compartment was not used in this setup. After introduction of 0.1 μL of a dye (Dr. Oetker food dye) with the modified syringe (E) onto the surface of the membrane, a plume was produced (B) that moved toward the sink. This dye was used to visualize the trajectory of the sample and to help in understanding the dynamics of the flow. The upper part of the plunger was surrounded by a piece of silicone tubing (length = 1.5 cm, i.d. = 2 mm, o.d. = 6 mm). Because of its elasticity, the tubing will act as a spring (E). After the plunger is pressed, the tubing is dipped into the sample solution; release of the tension raises the plunger and draws the sample into the tubing. A mark on the tubing indicates the position of the meniscus for precise adjustment of the volume. The sample liquid column was brought onto the nitrocellulose by pressing again. For a mean volume (n = 10) of 0.27 μL of urine, a standard deviation of 0.007 μL was measured. The syringe can be used for hundreds of samples. Between samplings, the tubing can simply be rinsed with buffer or tap water. The working electrode (WE) was operated at +0.7 V vs a Ag/AgCl reference (REF) electrode. Both the reference electrode (a fine silver wire) and the counter electrode (auxiliary, AUX; made of stainless steel) were located in the upper reservoir, where they were in contact with the buffer, ca. 1 cm below the WE. The nitrocellulose on the WE is connected with both buffer reservoirs by 8-mm wide strips of polyester−cellulose blend paper (VWRSpec-Wipe). The lower reservoir (sink) was tap water with acetic acid (final concentration = 0.1−1%) added to inhibit microbial growth. For electrical connections, we used micro alligator clips (Radio Shack). The Supporting Information describes the circuitry of the amplifier (Figure S-2).

that of a glucose standard. This system can be constructed rapidly, and it is inexpensive. The amplifier that reads out the current from the working electrode costs less than $30 for off-the-shelf components. In combination with a data-logging system, we visualized the dynamic signals on a computer monitor. Data logging can also be accomplished with a low-cost hand-held multimeter. We believe that, in addition to being appropriate for use in the clinical laboratory, the system has characteristics (simplicity; ruggedness; low cost; adaptability for battery operation, if required) needed for certain diagnostic applications in developing countries; for home healthcare settings; and for applications in, for example, water and food quality, veterinary medicine, and environmental monitoring. This system has the potential to be less expensive in repetitive analyses than many other currently used systems because the electrode surface is cleaned after each use by the continuous flow of buffer. Disposable components are therefore not necessary. The ease of construction and low cost also make the system attractive for use in education and training in analytical chemistry.

We and others have developed microfluidic paper-based analytical devices (μPADs) by patterning hydrophobic barriers of wax or photoresist (or other hydrophobic polymers) in hydrophilic paper.[1−10] When the end of a μPAD is dipped into a fluid (e.g., urine), capillary wicking transports the fluid through the paper. The cost and physical characteristics of these systems make them appropriate for a range of bioanalyses. Paper-based systems are, of course, widely used

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in bioanalysis. Examples range from pH paper to lateral-flow immunoassays and blood glucose analysis. Extending electrochemical methodologies increases the scope of this flexible, low-cost technology; electrochemistry is, of course, essential to most commercial "glucometers" for use in testing blood glucose.

This work describes a unique combination of μPADs, continuous gravity-driven flow, and amperometry. We and others have demonstrated simple μPADs and electrochemical sensing using amperometry, chronoamperometry, cyclic voltammetry, and anodic stripping.[5,11,12] For example, amperometric detection on paper has been reported after a separation with paper chromatography, using a gold electrochemical cell on polyester.[13] In such demonstrations, the working electrode is generally close to the counter electrode. As a consequence, this configuration of electrodes makes the calculation of concentrations in reversible oxidation/reduction reactions complicated or ambiguous [because the ions to be analyzed, such as Fe(II)/Fe(III), can shuttle between electrodes at rates that are a priori unknown]. Analyses in which electrodes are positioned in sequence in a flowing stream of buffer are easier to interpret, as the products of the electrochemical reaction at one electrode are transported irreversibly to a second electrode or to a sink.

Preoxidation of interferences before the measurement of glucose using lead oxide on disposable strips has been reported before.[14] In a continuous flow system, the oxidative capacity of lead oxide would gradually decrease. Moreover, an assay based on deposition and use of lead oxide would not be a "green" process. Our system separates the signal of the interfering oxidizable species from the glucose signal by oxidation at the same electrode, sequentially in time.

We have devoted special attention to sample introduction, to ensure that accurate pipetting can be achieved simply and reproducibly. Simple methods of sample introduction are a requirement for this methodology to be practical in environments where users have little or no laboratory training. Normal pipetting of the sample onto the nitrocellulose pad using disposable polypropylene tips could easily damage the nitrocellulose. We used thin polyethylene tubing (i.d. = 0.28 mm) attached to the needle of a low-cost disposable insulin syringe. This tubing was flexible and thus did not damage the nitrocellulose. A sleeve of elastic silicone tubing was placed around the plunger, which acted as a spring after bringing the slightly pressed syringe into contact with the sample. The sample volume can easily be calculated from the length of the fluid column in the polyethylene tubing. A typical injection volume was 0.3 μL (coefficient of variation = 2.7%, n = 10).

The system can also be used to measure glucose in other fluids, such as cell culture medium. Use of enzymes other than glucose oxidase (e.g., cholesterol oxidase, lactate oxidase, or D-3-hydroxybutyrate dehydrogenase[15]) will enable the measurement of other metabolites, (e.g., cholesterol, lactate, or D-3-hydroxybutyrate, respectively), making this system versatile for general clinical applications. It also has the potential for multiplexed analyses. The analytical platform can be adapted for checking water quality using anodic stripping voltammetry, as well.

RESULTS

Figure 2 shows a typical signal after introduction of a urine sample spiked with glucose at the beginning of the nitrocellulose strip. This concentration lies in a range of

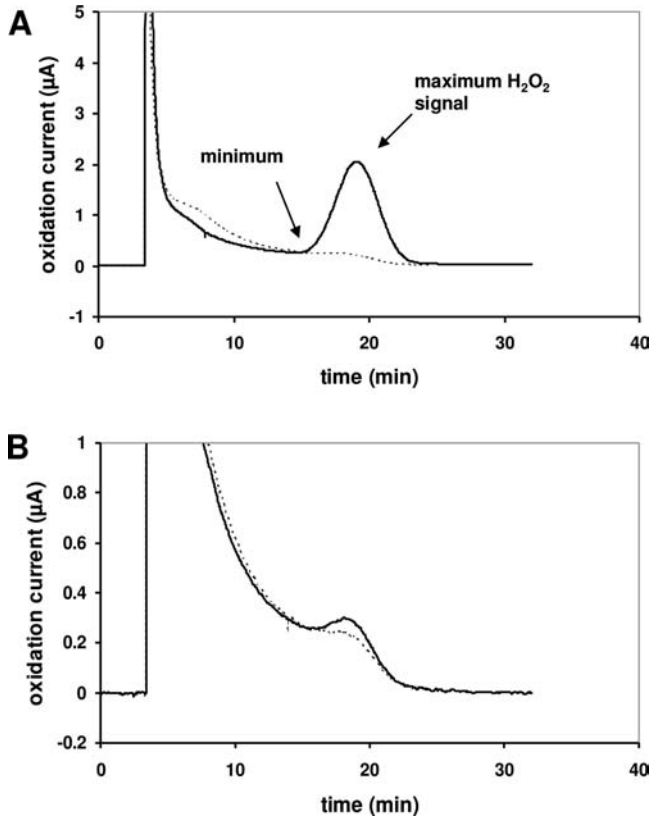

Figure 2. (A) Oxidation current after introduction at 3.5 min of 0.25 μL of urine spiked with glucose (final concentration = 26 mM) and a reference signal of blank urine without glucose (dotted line) using the system described. The voltage of the working electrode was 0.7 V vs Ag/AgCl, the area of the nitrocellulose strip (thickness = 140 μm) was 30 mm × 8 mm, and the distance between the site of sample introduction and the site of glucose oxidase was 20 mm. (B) Comparable experiment using a sample with a urinary glucose concentration of 0.8 mM. This sample represents the limit of detection for urinary glucose, with an absolute amount of only 200 pmol of glucose. Between these concentrations, the difference between the minimum before and the maximum of the glucose-related H_2O_2 signal (indicated in A) was linear in the glucose concentration. This difference in signal can be measured easily with a multimeter.

concentrations measured in the urine from diabetes patients. A new sample could be introduced every 20 min. Based on the time required to empty the upper buffer reservoir, the flow rate of buffer, driven by gravity and facilitated by wicking, was calculated to be approximately 2 μL/min. The filter paper never dried, as long as there was fluid in the reservoirs (even only in the sink); the ability to keep the nitrocellulose hydrated helped to preserve the activity of the enzyme. The identity of the urinary components causing the first oxidation signal is unknown, although uric acid might be an important contributor. The oxidation peak that appeared after the introduction of uric acid was at the same position as the first peak in urine. After consulting textbooks on laboratory medicine and after introducing a uric acid standard solution, we concluded that uric acid accounted for 20−100% of the first oxidation peak (data not shown).

Over time, the signal decreased, for a constant concentration of glucose, as a result of degradation of the enzyme and perhaps loss of enzyme by elution from the nitrocellulose, but only very slowly. Periodic recalibration using a known standard compensated for this loss. We measured the decrease in

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response at two buffer conditions. The data could be fitted using exponential fitting. At pH 5.5 (50 mM acetate buffer, 1 M potassium chloride), the decrease was 2.1% per hour (34 data points over 9 days, R^2 = 0.98). At pH 7.4 (100 mM phosphate buffer, 0.14 M sodium chloride), the decrease was only 0.5% per hour (9 data points over 3 days, R^2 = 0.98). Although pH 5.5 is the optimum pH for glucose oxidase, at pH 7.4, the amplitude of the signal was nearly the same, and the decline of the enzyme activity over time was approximately four times lower, indicating better stability or stronger adsorption of the enzyme. Except for the pH, the difference might have been caused by a difference in chloride concentrations as well. We chose the pH 7.4 buffer for further testing. The amplitude of the glucose signal depended on the site where glucose oxidase was spotted. Spotting downstream at the end of the nitrocellulose (near the sink) was most favorable, as the background signal was low and changing only slightly with time; this signal shape allowed measurements of the "peak height" above background (see Figure 2A). This peak height can be measured easily with a multimeter and was proportional to the glucose concentration up to a concentration of at least 26 mM (measured at seven concentrations, R^2 = 0.9995). At 26 mM, the coefficient of variation was 4%, and at 0.8 mM (see Figure 2B), it was 15% (n = 4); these data thus show a linear dynamic range of at least 32. Urine of four different individuals with no detectable glucose were subsequently spiked with glucose (final concentration = 10 mM). We measured an average concentration of 10.2 mM with a standard deviation of 0.11 mM, indicating that differences in urine composition had no influence on the amplitude of the glucose-related signal under these conditions. Introduction of a urine sample more downstream or spotting of glucose oxidase more upstream decreased the analysis time, but also introduced a systematic error due to difficulties in correcting for the background signal, especially for lower concentrations of glucose. When the sample was introduced at the same location where the enzyme had been spotted, the spatial resolution between the glucose signal and the signal from interferences was obviously lost (not shown).

Although the decrease of sensitivity could be estimated, it is advised that a glucose standard such as urine spiked with 25 mM glucose (with sodium azide at a final concentration of 0.05% w/w to prevent microbial degradation) be included at regular intervals to check the sensitivity. It is good practice to replace the nitrocellulose and glucose oxidase after approximately 3 days of use. This interval would correspond to approximately 100−200 samples, depending on the intensity of use.

A more elaborate configuration of the apparatus with a salt bridge, made of 2% agar in incubation buffer, allowed conductive contact with the nitrocellulose near the spot of glucose oxidase and the buffer in the upper reservoir and increased the linear range. This salt bridge was easily damaged by drying, however, when the system was temporarily unused. Moreover, for diabetes, most informative urinary glucose concentrations lie in the linear range of the method we present here. Accurate quantitative information for higher concentrations is usually not needed in practice, and these concentrations are labeled simply as "very high" (in the event that it is useful to measure higher concentrations, an extra dilution step could be added to enable better quantitation). We note that, for monitoring diabetes, low concentrations in urine (in the range of 0−2 mM) are sometimes not informative, because they can be caused by mixing urine with a small volume of urine with a high glucose concentration already in the bladder (that had remained in the bladder after emptying). In buffer, we estimated the lower limit of detection to be about 0.2 mM. This lower limit was enabled by a lower background in buffer relative to urine.

We compared glucose concentrations of 15 urine samples, tested positive for glucose during routine analysis in the clinical laboratory (measured on a "Roche automat") with the concentration measured by our method. Samples with concentrations of >30 mM were first diluted 10-fold. Figure 3 shows the correlation between the two methods. Before analyses, the samples were kept at −20 °C and homogenized after thawing. The two methods showed good agreement. The difference of the slope from unity might have arisen from differences in preparation of the standards.

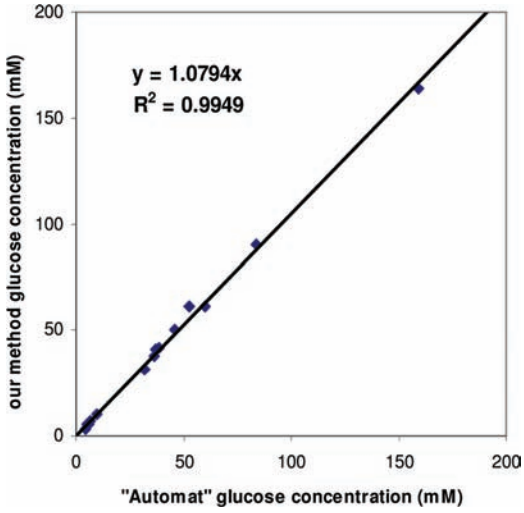

Figure 3. Comparison of methods for determining glucose concentrations in 15 urine samples, measured with an automat in the clinical laboratory setting and with the method described in this article. For two samples with glucose concentrations of 5.9 and 31.7 mM (measured with the automat), we measured average glucose concentrations (based on three separate measurements each) of 5.8 and 31.3 mM, respectively; the standard deviations were 0.5 and 0.05 mM, respectively.

■ DISCUSSION AND CONCLUSIONS

This work demonstrates that it is feasible to measure urinary glucose concentrations using a flow-injection method with gravitational pumping; in this method, a constant flow of liquid is generated by siphoning and facilitated by capillary wicking. The selectivity of a glucose assay in a complex matrix such as urine—containing other oxidizable components that might interfere with the glucose signal (e.g., uric acid or acetaminophen)—is obtained by a spatial separation of signals, as the sample moves from the site where sample introduction takes place to the site where glucose oxidase is adsorbed. It is remarkable that the absolute amount of glucose in urine at the detection level (Figure 2B) was only 200 pmol. The basic principles of this system can be tailored in several ways: The flow rate generated by wicking can be adjusted by (i) varying the thickness, length, or width of the paper or (ii) altering the hydrostatic pressure by changing the height difference between the buffer source and the sink. More materials to facilitate

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wicking and increase enzyme adsorption can be found. Pipetting the same volume of sample is the basis for comparison of the glucose-related signals in the method we describe. Accurate pipetting as described herein should be feasible after some practice, even for a minimally trained person.

The signal will become higher when more glucose oxidase is available on the nitrocellulose. Theoretically, every glucose molecule could deliver two electrons to the working electrode through glucose oxidase-mediated conversion to hydrogen peroxide, followed by electrochemical oxidation of H_2O_2 to O_2.[16,17] Complete conversion would make the use of a standard unnecessary, because the peak area could be related directly to the amount of glucose using Faraday's laws. Of all stereoisomers of glucose present in the sample, only the β-D-glucose is a substrate for glucose oxidase.[16,18] The β-D-isomer constitutes 64% of the total glucose at equilibrium. After removal of β-D-glucose, the other 36%, α-D-glucose, will convert to additional β-D-glucose by mutarotation.[19−21] Mutarotation kinetics is not negligible during the enzymatic conversion, which requires 10 min in our assay. Mutarotation should, however, not cause a problem for the assay presented here, as it also happens in the standard spiked with glucose. A lower flow rate would result in a higher yield of interconversion, but it would increase the analysis time to unpractical levels. A 100% conversion (so-called coulometric conversion) can be approached only asymptotically. The more closely this limit is approached, the more stable the signal is over time, as almost all glucose molecules yield electrons independently of electrochemical or transport variations. We calculated the fraction of total glucose oxidized by integrating the current over time (the "peak area") and dividing the charge obtained (in Coulombs) by that calculated using Faraday's law. Under our conditions, approximately 50% of all β-D-glucose was converted. We believe that the capacity of nitrocellulose to bind glucose oxidase limits this percentage. Theoretically, the readout of the electrochemical signal is linearly proportional to the amount of bound glucose oxidase at low glucose oxidase amounts, whereas it becomes nonlinear at higher glucose oxidase amounts that exceed the 100% conversion limit. This limit could be reached by adding more layers of nitrocellulose for extra binding capacity. We chose not to do so, however, because the increase in diffusion distance would result in longer analysis times and worse dynamic behavior.

It is uncertain how high the yield is for commercial glucose meters. The FreeStyle commercial system claims to be based on coulometry,[17] mentioning the advantage of greater stability, but we cannot find the percentage conversions for this system (or other devices) in the literature.

Most analyses of glucose concentrations for control of blood sugar in diabetes use blood as the sample. Using blood allows rapid feedback and control. Glucose is, however, also excreted in urine when the blood concentration exceeds roughly 10 mM. Measurements of glucose in urine can add information complementary to measurement of blood glucose, especially in the first morning urine, in cases in which patients have not taken blood samples during the night. Urine measurement is noninvasive. In addition to monitoring diabetes during therapy, the method could be used for inexpensive detection of the disease.

The system could also be used in metabolic studies in which the glucose concentration in cell culture medium must be measured, for example, in D-MEM medium (Dulbecco's modified Eagle's medium) used to culture cancer cells. The background signal of electrochemically active interferences was much lower than in urine; the signal-to-noise ratio was therefore more favorable.

Other applications of this methodology, for example, measuring urinary creatinine or lactate concentrations with appropriate enzymes, can be envisaged in the field of metabolomics. The system also has the potential for multiplexing, measuring metabolites in series when the sample passes locations where different enzymes have been spotted on the nitrocellulose. It can also be used as a low-cost detector for micro high-performance liquid chromatography for urine, food, or environmental analysis.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as mentioned in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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