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Quantifying Colorimetric Assays in Paper-Based Microfluidic Devices by Measuring the Transmission of Light through Paper

Audrey K. Ellerbee,[†] Scott T. Phillips,[†] Adam C. Siegel,[†,‡] Katherine A. Mirica,[†] Andres W. Martinez,[†] Pierre Striehl,[§] Nina Jain,[†] Mara Prentiss,[§] and George M. Whitesides*[,†]

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, School of Engineering and Applied Sciences, Harvard University Cambridge, Massachusetts 02138, and Department of Physics, Harvard University, Cambridge, Massachusetts 02138

This article describes a point-of-care (POC) system—comprising a microfluidic, paper-based analytical device (μ-PAD) and a hand-held optical colorimeter—for quantifying the concentration of analytes in biological fluids. The μ-PAD runs colorimetric assays, and consists of paper that has been (i) patterned to expose isolated regions of hydrophilic zones and (ii) wet with an index-matching fluid (e.g., vegetable oil) that is applied using a disposable, plastic sleeve encasement. Measuring transmittance through paper represents a new method of quantitative detection that expands the potential functionality of μ-PADs. This prototype transmittance colorimeter is inexpensive, rugged, and fully self-contained, and thus potentially attractive for use in resource-limited environments and developing countries.

Accurate medical diagnosis—required for effective medical treatment—often calls for quantitative measurements of metabolites, enzymes, and other biomarkers. For urban and rural populations in so-called "innovative developing countries" (IDCs),[1] the need for such assessments is as pressing as in the developed world, and related assays for water and food safety are even more important. Practical methods for detecting and quantifying analytes in the developing world differ greatly from those in the developed world: appropriate detection technologies must be robust, lightweight, simple to operate, and above all, low-cost.[2−6] The most useful methods should also be quantitative.[2] This report proposes a combination of paper-based assays and a portable detector that offers a cost-sensitive and effective solution to some of these needs.[7,8]

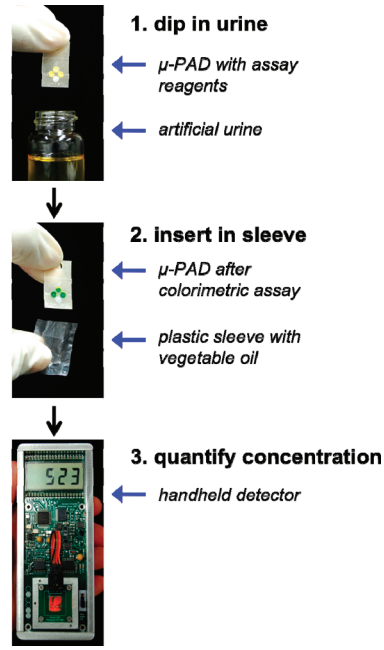

Figure 1. General strategy for performing quantitative, inexpensive, point-of-care assays in resource-limited environments using transmission colorimetry in paper; demonstration of a urinalysis assay using a microfluidic paper device spotted with reagents for detecting protein in urine. The yellow color is tetrabromophenol blue, and the green/blue color is tetrabromophenol blue when exposed to protein.

We demonstrate a point-of-care (POC) system—comprising a microfluidic paper-based analytical device (μ-PAD) and a hand-held optical colorimeter—for quantifying the concentration of analytes in biological fluids (Figure 1). The μ-PAD runs colorimetric assays, and the colorimeter quantifies the concentration of analytes based on the optical absorbance of colors produced in the assays. The system has several characteristics relevant to POC applications that are appropriate for resource-limited environments: (i) it requires low volumes of fluid (~2 μL), and is thus appropriate for use with adults (where large quantities of fluid

* To whom correspondence should be addressed.
† Department of Chemistry and Chemical Biology.
‡ School of Engineering and Applied Sciences.
§ Department of Physics.

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may be available), children (including neonates, where only quite small quantities of fluid may be available) and small animals, for which available sample volumes limit the usefulness of existing tests; (ii) it is low in cost (paper is inexpensive and readily available, and the colorimeter is fabricated with off-the-shelf components that cost ~$50 and are available from common electronics suppliers); (iii) it provides an on-site quantitative readout, facilitating POC interpretation of the results; (iv) it is designed to be relatively insensitive to fluctuations in signal caused by dust or variable lighting conditions, unlike typical light scattering measurements; (v) it is fully self-contained, portable, and powered by a single 9 V battery. Finally, the sensitivity of the method to different ranges of concentrations of analytes can be easily adjusted by using papers of various thicknesses.

EXPERIMENTAL DESIGN

Choice of the Assay for Demonstration of Clinical Sensitivity. Colorimetric assays are particularly appropriate for detecting analytes in low-resource environments for six reasons: (i) the results can be observed easily; (ii) many of the reagents are readily available; (iii) they perform reliably in a variety of environmental conditions; (iv) appropriate assays provide sensitivity to specific analytes; (v) they typically generate results quickly; and (vi) they lend themselves to easy interpretation by semiskilled technicians.[2,9] The wide variety of reagents that have already been developed and tested for colorimetric assays make it possible to use this system for measuring many different analytes. Thus, the combination of technologies presented here may also be useful for detecting analytes in urine, tears, sweat, saliva, amniotic fluid, wound exudant, and other fluids and should also be adaptable to detection of pollution in water, spoilage of food, and biochemical markers of stress in plants. Moreover, the opportunity (and need) to develop new colorimetric tests is still high, and thus an important target for analytical chemistry.

Previous work has shown that paper-based microfluidic devices can be effectively designed to run colorimetric assays.[10−13] In this work, we validate the concept of transmittance colorimetry in μ-PADs for a colorimetric assay (total protein urinalysis) for which clinically relevant measurements previously have been obtained using μ-PADs with other detection schemes.[14] Here we measure the level of protein in artificial urine[15] using bovine serum albumin (BSA) as a model protein, although we expect that the general method we have developed will be compatible with more complex assays than urinalysis. Albumin is the primary protein excreted in urine; since other proteins are in low abundance relative to albumin, the levels of albumin are used clinically to diagnose disease, where the concentration of protein in urine is indicative of the type of disease.[16−19]

Choice of the Detection Method. Currently, analytes can be detected using colorimetric assays in POC applications by visually observing changes in color, intensity, or brightness caused by the presence of the analyte (e.g., dipsticks),[12] using digital aids (e.g., camera phones or scanners) to quantify changes in color (i.e., telemedicine)[14,20] or by measuring the amount of light reflected from a surface where an assay has occurred.[21] Visually based measurements are, however, usually qualitative, rather than quantitative, telemedicine-based approaches rely on the accessibility of highly skilled technicians or clinicians to interpret the results (albeit remotely), and reflectance-based measurements detect only the quantity of surface-bound analytes.

Although reflectance-based measurements are common in detection schemes employed in POC applications,[22,23] these measurements require high (e.g., millimolar) concentrations of analytes, and are relatively insensitive to trace quantities of analytes. In contrast, the analytes of greatest interest for clinical applications (e.g., markers of infectious and metabolic diseases, hormones and drugs, and metabolites and enzymes that reveal the effects of therapeutics) are frequently present in biological fluids (e.g., blood, urine) in micro- to picomolar concentrations, and thus require alternative and much more sensitive methods for quantification, particularly when large quantities of biological fluids are not available. We pursued a transmittance-based detection scheme to allow for on-site, quantitative analysis of colorimetric assays through the entire thickness of paper and, thereby, to enable detection of lower concentrations of analytes in smaller volumes of fluid (microliter volumes of sample)[12,14,24] than can typically be detected with reflectance-based methods. Quantitative transmission colorimetry in paper is accomplished by wetting paper with a fluid that approaches the index of refraction of the materials in the paper:[25] index-matching significantly reduces the scattering in wet compared to dry paper, and the optical absorption depends on the concentration of analytes present in the paper.

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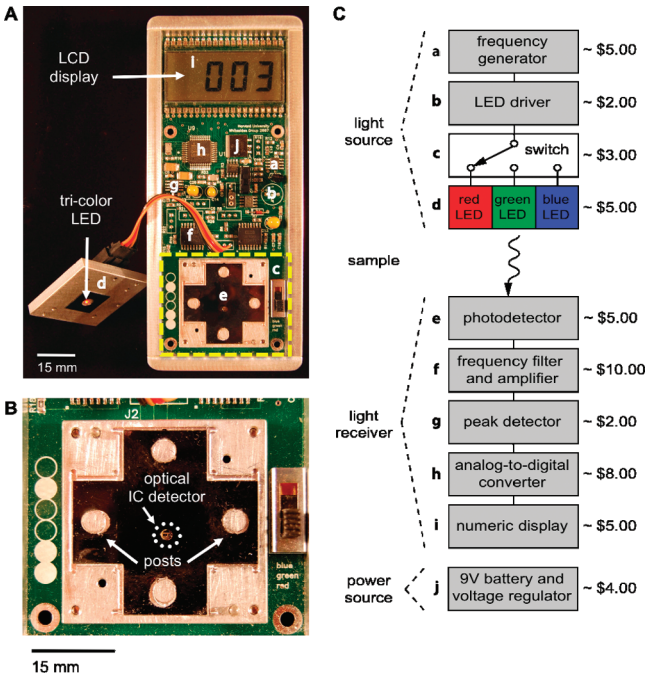

Figure 2. Design of a point-of-care transmittance colorimeter. (A) Photograph of the colorimeter. (B) Expanded view of the bottom of the holder (the dashed area from A) for the μ-PAD. (C) A block diagram of the components of the optical detector with a breakdown of costs; block components correspond with their lettered counterparts in A. The entire system is designed to run off a single, 9 V battery.

Design of the Transmittance Colorimeter. We designed a transmittance colorimeter to measure the absorbance of light as it passes through a region in a μ-PAD where color develops during an assay (Figure 2). Modeled after an earlier prototype ("POCKET") that we designed for quantifying assays in poly(dimethylsiloxane)-based microfluidic devices,[5] this colorimeter improves on this earlier device in three ways. First, it incorporates a tricolor LED and an integrated switch to allow the user to select one of three illumination wavelengths; it can, therefore, detect color,[26] whereas the former detector could only detect monochromatic (e.g., black and white) changes. Second, it includes a manifold adapted for paper substrates that facilitates alignment of the LED, the detector, and the μ-PAD. Third, it is battery-operated and consists of a single, integrated piece (battery, detector, manifold, substrate) that has been housed in aluminum ($6.5 \times 14.7 \times 3.4$ cm) and is easily held in the hand. The light is pulsed, and detected, by kHz modulation and narrow bandpass, frequency-sensitive detection, to enable sensitive measurements in a variety of lighting conditions. A complete description of the design of the colorimeter, including a schematic illustration of the electronic circuitry, is included as Supporting Information (SI).

Choice of Materials for the μ-PAD. The μ-PAD consists of photoresist-patterned paper that is wet with an index-matching fluid and encased in a plastic sleeve. The selection of materials and overall design of the μ-PAD represent what we believed to be a good balance of cost, reliability, sensitivity, disposability, and availability in resource-limited environments.

Paper is an appealing material for portable analytical applications: it is inexpensive, available in all regions, lightweight, easy to store and transport, can be burned at the conclusion of an assay to dispose of biohazardous waste, and can be easily functionalized. For the device described here, we used cellulose-based Whatman chromatography paper No. 1 because it is of medium thickness (190 μm) relative to other common papers (10−250 μm) (thickness affects the optical path length, scattering, assay sensitivity, and volume of fluid required for an assay) and it adsorbs and retains reagents better than other papers and cloths (e.g., polyester) we tested. Moreover, the homogeneity of the cellulose ($n = 1.54-1.62$),[27] as demonstrated by the low variability in the measured transmittance[28] for different regions of paper subjected to the same wetting conditions, makes this a suitable paper choice.

For the index-matching fluid, we chose to use commercial vegetable (soybean) oil because it is inexpensive, readily and locally available, nontoxic (or of low toxicity), and colorless (or nearly so). Particularly important for low-resource environments, vegetable oil is available widely and used nearly everywhere as cooking oil and fuel, although the specific type of vegetable oil available may depend on the geographic region where the device is being used.

To apply the vegetable oil, we designed a disposable plastic sleeve to encase the patterned paper that served two functions: (i) it provided a mechanism for applying index-matching fluids without using tools (e.g., pipettes, cotton swabs, cups for dipping) and (ii) it constrained the index-matching fluid to the microfluidic device, thereby minimizing transfer of the fluid (as well as biological contaminants embedded in the paper) into the components of the colorimeter. In this demonstration, the vegetable oil was added to the sleeve using a micropipet, before inserting the paper into a sleeve; however, we envision that for POC applications, sleeves preloaded with fluid can be mass-produced and cut open (using scissors or by tearing along perforated lines) just prior to use.

Design of the μ-PAD. The microfluidic portion of the system is small (~12.5 mm × 13.7 mm × 0.11 mm) and lightweight (~36 mg) (Figure 3A). Microfluidic features were patterned into the paper using photolithography,[24,29] as photolithography is the best method currently available for patterning reproducible hydrophobic features in paper with well-defined and uniform boundaries. The paper-based device includes a photoresist tab that serves as a handle for manipulating the device and provides a wicking barrier to prevent human contact with biological fluids. The device also has a 4.2 mm-diameter hole to match the diameter of the posts in the transmittance colorimeter and facilitate alignment (Figure 3D).

We designed the hydrophilic detection zones of the paper as 2 mm-diameter circles to minimize the quantity of reagents and volume of sample needed for an assay. The four circular hydrophilic regions are arranged in a square pattern in the center of the device and can be individually read by mounting the paper in the holder and aligning the hole with the post corresponding to the assay of interest (Figure 3C). We reserved one of the circles to perform a control measurement. Thus, the device can perform

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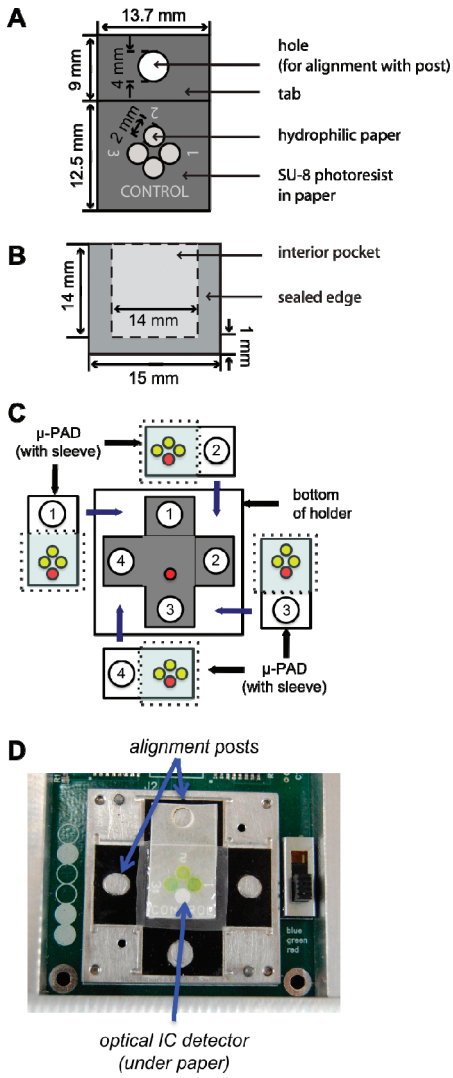

Figure 3. Design of a μ-PAD that measures the level of protein in urine quantitatively using transmittance colorimetry. The device allows for running an assay in triplicate along with a control zone. Schematic representation of A) the microfluidic device, B) the plastic sleeve used for application of the vegetable oil, and C) the process for aligning the sleeve-encased device with the photodetector of the colorimeter. Only the transmittance for a single assay region (red) is detected at any given time; the absorbance in a particular detection zone can be measured by aligning the μ-PAD tab with the appropriate post of the holder (here, numbered 1−4). Simple rotation of the device enables measurement from each of three sample detection zones (labeled 1−3) and one control zone (used to correct for the color of the urine sample and for scattering/absorption of light due to the properties of the paper). D) Photograph showing the alignment of the μ-PAD with post 1 of the holder of the spectrometer.

multiple assays simultaneously or run a single assay in triplicate. The plastic sleeves were designed such that the interior dimensions of the sleeves were slightly larger than the dimensions of the paper microfluidic devices, and the exterior dimensions fit into the aluminum holder (Figure 3B).

RESULTS AND DISCUSSION

Effects of Paper Type and Environmental Conditions on Transmittance Through Index-Matched Paper. When absorbed by the paper, an index-matching fluid replaces the air gaps between individual paper fibers. This substitution decreases the scattering that occurs within the detection volume by reducing the refractive index mismatch between the paper fibers—which scatter light—and the surrounding environment. The change in index mismatch, in turn, changes the effective refractive index of the paper−fluid matrix. The overall transmittance for paper treated with an index-matching fluid thus depends both on the refractive index of the fluid relative to that of the paper and on the completeness of the permeation of the paper (i.e., how much air has been displaced within the detection volume). This latter factor is affected by wetting time, paper thickness, and fluid viscosity (which is a function of interaction temperature). In general, the total transmittance (T) should vary inversely with any one of these factors, all others being held constant (eq 1). Here, I (W/cm^2) is the intensity transmitted to the detector after a colorimetric assay, I_0 (W/cm^2) is the source intensity, α_{samp} (cm^{-1}) accounts for attenuation (both scattering and absorption) caused by the sample (e.g., urine), and z is the thickness of the paper. The molar extinction coefficient and concentration of the analyte, ε (M^{-1} cm^{-1}) and c (M), respectively, also contribute to the absorbance measured. We call the variable T_c (unitless) the characteristic transmittance of the paper: it represents the transmittance of the paper in the absence of an applied sample or colorimetric test, and takes into account scattering and absorption due to the paper fibers and attenuation at the air-paper interfaces (see SI for a more thorough description of the transmittance of light through paper).

$$T = \frac{I}{I_0} = T_c 10^{-\alpha_{samp} z} 10^{-\varepsilon c z} \quad (1)$$

We examined several combinations of fluids and papers to determine pairs that form transparent matrices (SI Table S-1), including common household papers such as Scott Roll Paper Towels. All of the papers tested became transparent to some extent. Thin paper became more translucent than thick paper (e.g., Whatman chromatography paper No. 1 vs No. 3) when wet with the same volume of the same fluid, probably reflecting better permeation of the fluid. The relative porosity of the paper should also affect transmittance: more porous papers should scatter less because they contain fewer scattering particles. Finally, the amount of light transmitted through stacks of paper of identical types (simulating thicker paper) was inversely proportional to the height of the stack, as expected by Beer's law (SI Figure S-7).

The effect of wetting time and environmental temperature and humidity (a probe for fluid viscosity) were investigated by measuring T over time until the transmission reached steady-state, in a room with controllable temperature and humidity (SI Figures S-6). When the vegetable oil was applied to the paper, the intensity of light transmitted through the paper increased instantaneously by more than 150% for all humidity and temperature conditions probed (relative humidities of ~21, 36, and 66% and temperatures of 26, 31, and 36 °C). The transmittance after 30 s exceeded 90% of the steady state value obtained after 10 min in all cases, suggesting that this technique can be used in cases where rapid-response times are critical. In general, the time to reach steady-state transmittance appears slightly shorter for higher humidity conditions or temperature conditions.

Measuring the Concentration of Protein in Artificial Urine in Analytically Challenging Environments. We used the colorimeter to measure the concentration of protein in triplicate

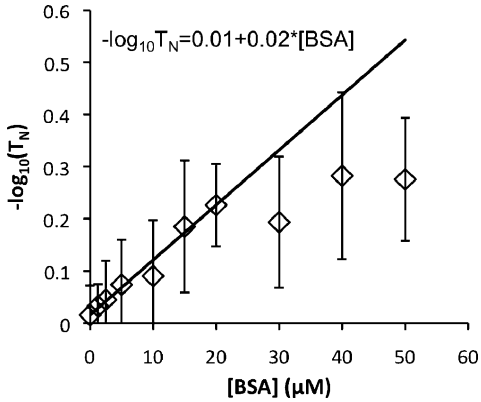

Figure 4. Calibration curve for clinically relevant levels of protein (0−40 μM BSA, a model protein for human albumin) in artificial urine derived from normalized transmittance values. Transmittance values were obtained using the hand-held transmittance colorimeter after wetting the circular detection zones with vegetable oil; normalized transmittance (T_N) was determined by dividing the output intensity for the colorimetric assay zones by the output intensity associated with the on-device control zone. Values represent the averages and standard deviations from nine measurements. The solid line is a linear best fit for low concentrations of BSA (0−20 μM).

(detection zones 1−3), and to correct for background attenuation associated with the color and chemical composition of different samples of urine (detection zone labeled "CONTROL"). The control circle also corrects for the effects of scattering by the paper when the output is reported as the normalized fraction T_N (eq 2).

$$T_N = I_{\mathrm{ASSAY}}/I_{\mathrm{CONTROL}} = 10^{-\varepsilon c z} \qquad (2)$$

We generated a calibration curve (Figure 4) that relates the concentration of protein in artificial urine to the transmission readout of our colorimeter by plotting $\log_{10}(T_N)$ vs [BSA], and obtained the best fit using linear regression analysis. The calibration curve has a linear range and a nonlinear range; concentrations below 20 μM fall in the linear range of the device. The nonlinear range may be due either to saturation of the colorimetric assay, low resolution of the detector at high concentrations (for concentrations above 20 μM, the transmittance was in the lower 15% of the instrument detection range), or both. The concentration of an arbitrary urine sample that falls within the linear range of detection can be determined by using the value of T_N obtained to interpolate the corresponding concentration value on the calibration curve. In principle, the calibration curve can be replaced with a look-up table that would obviate the need for the user to perform a division operation, which may too difficult under real conditions in IDCs. Because the transmittance through paper can depend on the environmental conditions (e.g., temperature, humidity), certain parameters of the assay protocol (e.g., wetting time) and the relevant calibration curves may need to be adapted to local conditions.

We measured the concentration of BSA in artificial urine under a variety of lighting conditions and under simulated wind to test the ability of the system to determine accurately the level of protein in analytically challenging environments. Data were collected indoors at room temperature under three lighting conditions: overhead fluorescent lighting, natural lighting (dark), and direct exposure to sunlight. As expected, samples falling within the linear range of the device correlate well with the expected results (SI Table S-2). The overlap of the values across lighting conditions suggests that the combination of a light-blocking manifold and kHz lock-in detection make the colorimeter insensitive to lighting conditions. This insensitivity to ambient lighting is a clear advantage over telemedicine-based approaches, and would allow for analysis during the day and at night. Also, the rugged system casing can withstand harsh environmental conditions (or can be made to), and creation of a new calibration curve for any unique environment would require only slight modifications to the protocol. Samples falling in the nonlinear range of the device (e.g., 40 μM) did not correlate well with the calibration data. Thus the detection range of the device overlaps—but does not fully span—the range of clinically relevant values for this assay.

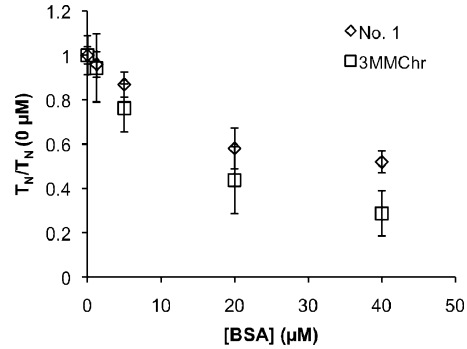

Figure 5. Increasing sensitivity to lower concentrations by using thicker paper. Transmittance as a function of the concentration of BSA in artificial urine for μ-PADs made from two types of Whatman chromatography paper differing only in thickness (No.1: ~180 μm; 3MMChr: ~340 μm). Plotted data are normalized transmittance values (T_N) that have been further normalized relative to the maximum transmittance for each data set (T_N at 0 μM). Values represent the averages and standard deviations from nine measurements.

Since the main source of error in the results is likely due to alignment, a future prototype that improves the alignment of the μ-PAD within the POCKET detector should also decrease the measurement error and improve the sensitivity of this instrument. Furthermore, if the nonlinearity in the calibration data is due to the resolution of the detector, the dynamic range of the system can be tuned so that the transmittance from the control sample corresponds with the highest possible readout (in these experiments, $I_{\mathrm{CONTROL}}/I_0 \sim 0.3$), as described in SI.

Changing the Sensitivity of Detection Using Papers of Different Thicknesses. The sensitivity of detection may be varied by using μ-PADs of different thickness. We recorded the transmittance as a function of the concentration of BSA in artificial urine for μ-PADs made from two types of Whatman chromatography paper that differed only in their thickness (Figure 5). The normalized transmittance T_N for the 3MMChr paper (~340 μm) decreased faster than that for the No. 1 paper (~180 μm) (T_N(1.25 μM)/T_N(0 μM) = 0.94 and 0.96, respectively; T_N(40 μM)/T_N(0 μM) = 0.29 and 0.52, respectively), confirming that a thicker device produces a larger change in transmittance for a given analyte concentration than a thinner device, as expected by Beer's Law. Thus, using a thicker device will enable better discrimination of the change in transmittance at lower concen-

trations of analyte. The trade-offs for using a thicker device, however, include decreasing the range of detectable concentrations, and possibly requiring higher volumes of sample fluids to ensure that the paper is saturated sufficiently. In this experiment, the thicker device also had higher variability at each concentration.

CONCLUSIONS

As presented here, implementation of the clinical assay itself requires few technical skills: the paper is dipped into a sample of urine, the paper is slipped into a sleeve to isolate it from the detector electronics and to apply an index-matching fluid, and a value associated with the concentration of the analyte is displayed on the LCD screen. The present system is cost-effective, although use of cheaper or more widely available commercial products (the availability of which may depend on the geographic region), such as common tissue paper for the μ-PAD or a plastic case for the colorimeter, could help to drive the costs down further. While the general method of measuring transmittance through paper is promising, we recognize that the current components of the prototype system have some disadvantages that make it practically useful only in more restricted conditions.

First, although the use of an index-matching fluid (e.g., vegetable oil) is a necessary step to make the paper translucent, it is messy, and our solution to use a disposable plastic sleeve for fluid application is a nuisance that would be, in practice, particularly difficult in harsh conditions (e.g., cold weather, wind) or for those with unsteady hands. Despite the use of a sleeve, there remains a need to wipe clean the surface of the detector (after ~30 measurements) in order to preserve the functional capacity of the colorimeter. A more highly engineered system could include rollers that deposit a thin film of a reproducible volume of oil on the paper as it is inserted through the rollers and into the transmittance colorimeter, or some other alternative like an aerosol fixant that obviates the need for the sleeve.

Second, the colorimeter readout is meaningless on its own, as determining the analyte concentration requires an additional step to interpolate the value from a calibration curve or table. Not only does interpolation limit the precision of the output (being subject to human error), the simple arithmetic required to compute the ratio T_N may be difficult for some users of the system. In the future, the colorimeter prototype could include separate LEDs for the control and assay so that the division is done in hardware using a comparator or subtractor circuit; a dual-LED system would also reduce the amount of necessary handling during the assay.

Third, the current colorimeter can probe a limited number of wavelengths (three), which may be inappropriate for some assays. An improved device could employ a white light source and various filter wheels to select for the wavelength of interest. For a sufficiently broad and powerful light source, this implementation could allow for ratiometric measurements and the instrument could be easily modified to enable fluorescence detection.

Finally, as with any machinery with complicated electronics, repairing and recalibrating the detector may necessitate off-site, skilled technicians.

That said, this paper suggests a new approach for inexpensive transmittance colorimetry with clinically relevant accuracy. The ability to make paper translucent provides new opportunities for developing quantitative, paper-based diagnostic assays. Moreover, the ability to tune the sensitivity of the method easily by varying the thickness of the paper (i.e., the path length) provides a simple and low-cost approach for changing the method detection range of an assay. This combination of disposable microfluidics and inexpensive, but quantitative (or semiquantitative), optical detection provides a potentially useful means to detect analytes in resource-limited settings, and may also be useful for quantifying analytes in such settings as emergency situations, military field assignments, home healthcare (as a disposable and inexpensive method for monitoring chronic diseases), and "innovative developing countries."

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SUPPORTING INFORMATION AVAILABLE

Additional discussion and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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