Anal. Chem. **2009,** *81,* 5990–5998

Paper Microzone Plates

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This paper describes 96- and 384-microzone plates fabricated in paper as alternatives to conventional multiwell plates fabricated in molded polymers. Paper-based plates are functionally related to plastic well plates, but they offer new capabilities. For example, paper-microzone plates are thin (~180 μm), require small volumes of sample (5 μL per zone), and can be manufactured from inexpensive materials ($0.05 per plate). The paper-based plates are fabricated by patterning sheets of paper, using photolithography, into hydrophilic zones surrounded by hydrophobic polymeric barriers. This photolithography used an inexpensive formulation photoresist that allows rapid (~15 min) prototyping of paper-based plates. These plates are compatible with conventional microplate readers for quantitative absorbance and fluorescence measurements. The limit of detection per zone loaded for fluorescence was 125 fmol for fluorescein isothiocyanate-labeled bovine serum albumin, and this level corresponds to 0.02 the quantity of analyte per well used to achieve comparable signal-to-noise in a 96-well plastic plate (using a solution of 25 nM labeled protein). The limits of detection for absorbance on paper was aproximately 50 pmol per zone for both Coomassie Brilliant Blue and Amaranth dyes; these values were 0.4 that required for the plastic plate. Demonstration of quantitative colorimetric correlations using a scanner or camera to image the zones and to measure the intensity of color, makes it possible to conduct assays without a microplate reader.

This paper describes 96- and 384-microzone plates fabricated in paper as a new platform for bioassays; these plates are functionally related to conventional 96- and 384-well plates fabricated in plastic. We summarize methods for fabricating paper-based plates using photolithographic methods expanded from microelectronics,[1] and we introduce two low-cost formulations for photoresists that are tailored for this application. We characterize the properties of the paper plates, including volume requirements, solvent and reagent compatibility. We demonstrate that paper-based plates are compatible with microplate readers for quantitative absorbance and fluorescence measurements. Finally, we describe quantitative colorimetric assays using plate readers, scanners, and cameras (both cellphone and digital). This work establishes that paper-based multizone plates have the potential to be widely used in quantitative microscale bioanalysis, especially where cost, space, weight, cost-effective archiving, and easy disposal are important.

Our primary objective in this work was to develop paper microzone plates as a low-cost alternative to plastic plates. Plastic plates are widely and routinely used for high-throughput measurements of analytes. They are optimized for use in well-equipped research facilities (with freezers, multichannel pipettes, and disposable tips, and established procedures for disposal of used plates). We developed paper microzone plates primarily for use in resource-limited laboratories and for circumstances where the limited availability of freezers makes low-temperature storage impractical. We believe however, that these plates will be also useful in more developed laboratories when cost is a primary concern and where disposal of used plates is difficult. Paper-based plates are also particularly suited for innovative developing countries (IDCs),[2] which have the capabilities, resources, and motivation to develop new assays at low cost. Paper plates have, however, a number of characteristics, minimal requirement for space, the ability to support thin layers of hydrophilic gels, easy and safe disposal by incineration, and others, that should make them useful in developed countries as well.

Paper Assays. Paper has served as a medium for chemical analysis and chemical reactions for many decades, even centuries. Historically, paper had a key role as (i) a support for qualitative spot-tests for organic and inorganic analytical chemistry,[3] (ii) a medium for chromatography[4] and electrophoresis,[5] (iii) a platform for reactive indicators such as litmus paper,[6] and (iv) a medium for lateral flow analysis, such as dipstick (lateral flow) immunoassays.[7]

Spot-test analysis was the principal analytical test in the early 20th century, because of the lack of analytical instrumentation. The method relied on the selectivity and sensitivity of reactions

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10.1021/ac900847g CCC: $40.75
Published on Web 07/02/2009

and was carried out by mixing drops of a sample with a specific reagent in a test tube or on a piece of paper. Formation of colored products was the result of the reaction, and visual inspection generated the qualitative information. The diversity of spot test assays is impressive, with applications covering clinical diagnostics, environmental and geochemical analysis, and problems in pharmaceutical and food chemistry.[8]

Paper is a good medium for spot tests because it is white and provides a strong contrast with colored tests, and because it has a high surface-to-volume ratio (which facilitates accelerated evaporation of solutions and results in concentrated analytes). An array of distinct test zones (organized into 96- or 384-zone formats) takes advantage of both characteristics.

We recently introduced a paper-based platform for conducting analytical assays.[9] Using conventional techniques for photolithography, we created hydrophobic barriers that extended through the thickness of chromatography paper and that defined hydrophilic channels that wicked liquids and directed them to reaction zones for detection of analytes. Paper was surprisingly compatible with these microfabrication techniques because the photoresist readily wets the paper, and the paper remains hydrophilic after removal of excess photoresist. In microfluidic paper-based analytical devices (μPADs), capillary action wicks liquid samples without the need for pumps.

Paper-based assays and μPADs have again become the subject of active interest. Two recent publications were perspective articles highlighting the potential of μPADs.[10,11] Several recent research articles explored new procedures for making simple and inexpensive paper-based assays (most of them μPADs). One publication evaluated the role of hydrophobicity and hydrophilicity of papers as a means to promote colorimetric DNA assays with conjugated gold nanoparticles.[12] Other articles described new ways to make μPADs using (i) a refurbished $x-y$ plotter for direct writing of the hydrophobic layout with poly(dimethylsiloxane) (PDMS),[13] (ii) photolithography to activate sheets of paper embedded with photoresist,[14] (iii) the same photolithographic patterning of paper in conjunction with cellphones, as a system for telemedicine for use in developing countries,[9] (iv) a combination of paper and tape to create three-dimensional devices to enable multiplexing of analysis,[15] (v) an inkjet printer to dissolve the hydrophobic polymer and dispense the chemicals for colorimetric analysis,[16] (vi) a plasma cleaner to etch a hydrophobic layer from the paper through metallic stencils,[17] and (vii) a knife plotter to define the functional elements for assays on paper.[18]

In all publications but one, the analytical platform consisted of small devices intended for individual processing of samples and later disposal of the device. The work of Zhao et al.[12] was the only one that demonstrated an *array* format, capable of several analyses in parallel, but was not fabricated in a 96-zone format.

The recent interest in paper-based diagnostics and analytical systems suggests the potential of this technology in the analytical sciences, particularly in IDCs. Such development opens new areas for scientific investigation. For example, our group and others addressed questions regarding the biocompatibility of paper, long-term storage of reagents on paper, and the effect of printing enzymes and other biomolecules on paper.[9,19,20] As this field evolves, more fundamental studies are needed for the complete design of functional bioassays on μPADs. Here, we introduce a platform for parallel and multiplexed analysis of samples by means of a multizone (96- or 384-) paper plate.

EXPERIMENTAL DESIGN

Choice of Substrate. Paper is a versatile and technologically highly developed class of material that is biocompatible and hydrophilic and can be used for both aqueous and nonaqueous assays. Paper and its derivatives are inexpensive and widely available. In this research, we used primarily Whatman grade 1 Chr chromatography paper because it is uniform in structure and free of hydrophobic binders or coatings. In principle, any hydrophilic paper could be used if it provides enough mechanical strength to handle the stress of production and analysis. We tested a variety of papers and tissues in studies designed to decrease the cost of the multizone plates and to demonstrate their versatility. We note that "paper", in the context of this work, can include any of a wide variety of thin, nonwoven, and woven sheets based on cellulose, synthetic fibers, or mixtures; paper, as a generic type of structure, can thus have a very wide range of properties. Hydrophobic fibers can often be made sufficiently hydrophilic to wick water by brief treatment with a plasma or corona.

Design of the Paper Plates. A recent technical survey indicated that 90% of microplate users in the U.S. used the 96-well format exclusively (within a population of 82 700 users); 9.6% of users used both the 96- and 384-well plates, and only 0.7% used higher well-density plates.[21] We designed the paper plate to match the dimensions of a standard plastic 96- or 384-well plate for convenient reading using standard plate readers. In this design, every zone was independent and no fluid flowed between them: the hydrophobic barrier constrained the movement of fluids within each zone, and assays were accomplished by adding chemicals

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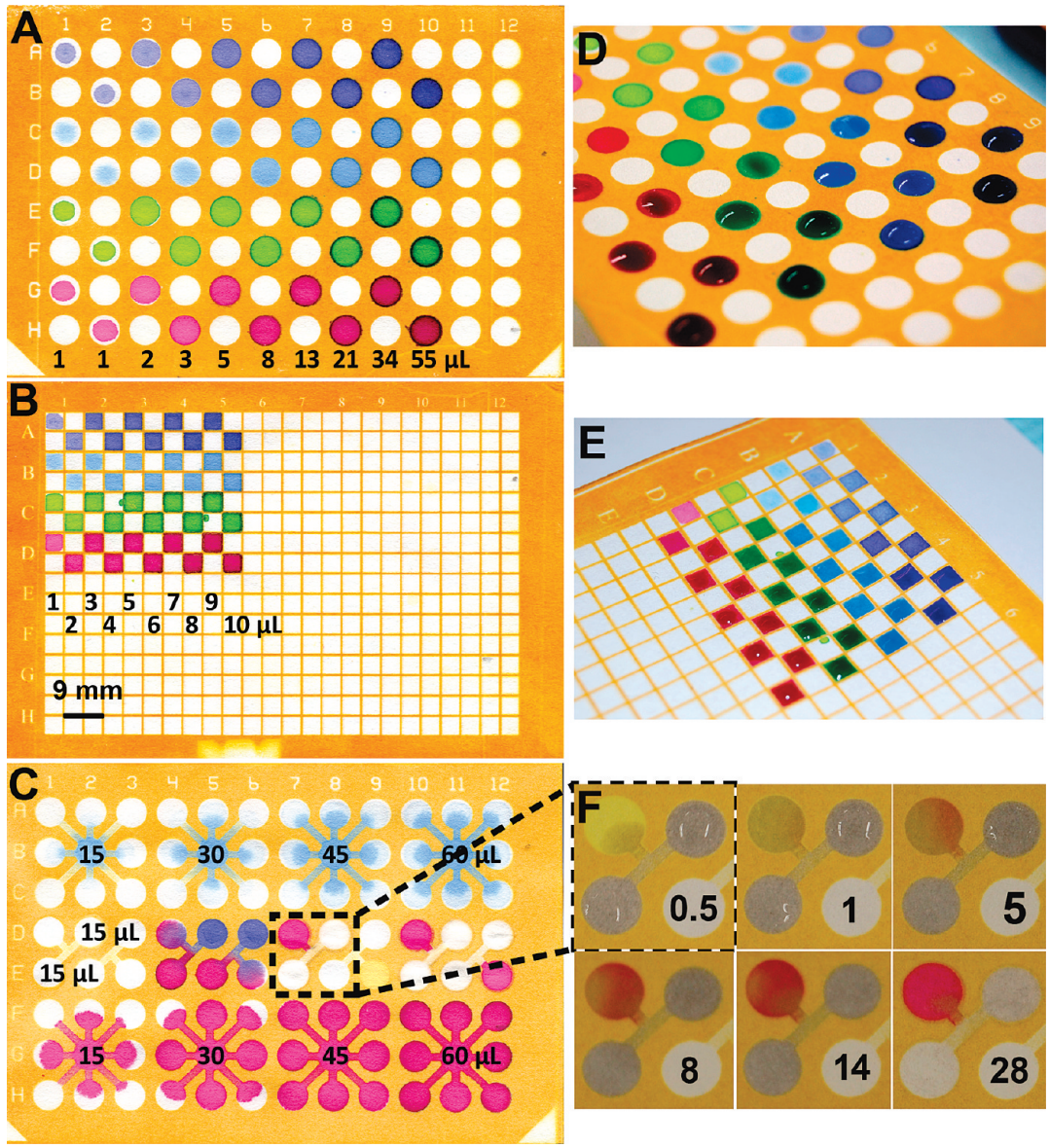

Figure 1. Paper plates for multizone assays produced using photolithography. (A) Image of a 96-zone plate after application of a range of volumes (from 1 to 55 μL) of solutions of different dyes (Coomassie Brilliant Blue R250, rows A and B, Coomassie Brilliant Blue G250, rows C and D, mixture 1:1 of erioglaucine and tartrazine, rows E and F, and Amaranth, rows G and H) in alternating zones. This image demonstrates the fluidic isolation of the zones. (B) Image of a 384-zone plate after application of 1−10 μL of the same solutions as in part A. Two zones in the fifth and sixth rows show small breaches in the hydrophobic walls. (C) Alternative design of a 96-zone paper plate incorporating distribution or connection channels between zones. In the top and bottom rows, every nine zones were connected to the central zone with 1.5 mm wide channels. Solutions of Coomassie Brilliant Blue G250 and Amaranth were applied to the top and bottom row, respectively. Coomassie G250 interacts more to the paper and does not spread as well as Amaranth. In the central row, two zones were interconnected, allowing reagents to mix and react in a third zone. In this example, solutions of Amaranth and Coomassie Brilliant Blue R250 from different zones meet at the center junction and wick side-by-side in laminar flow into the third zone where mixing occurs (see zones D-4 and E-6). In zone D-7, we show an example of a colorimetric reaction mixing two colorless solutions (30 mM sodium borate from E-7 and 30% sulfuric acid from D-8) to mix and react with a yellow reagent (curcumin 0.1% in ethanol, 3 μL, dried) previously spotted in D-7 to develop into a colored product. (D) Image showing the 96-zone plate with volumes of liquid up to 55 μL that were completely restrained from spreading over the paper by the hydrophobic barrier. (E) Similar experiment demonstrating that the smaller zones in the 384-zone plates held at least 10 μL of liquid. (F) Time-lapse images of the detail shown in part C illustrating the mixing of two solutions and reaction. The color development is apparent within minutes after application of the two solutions. All the volumes were applied as indicated in parts A−C. All plates measured 13 mm × 8.5 mm.

sequentially. A micropipet dispensed the reagents or samples onto the zones; precise alignment of the pipet in the center of the zone was unnecessary since the reagents spread evenly in the zone by capillary action.

The paper-based systems have the attractive feature that the zones can be easily patterned in more complex geometries that enable more complex function than the commonly used 96-well plates. Although we designed the layout of the paper plates to be compatible with automated plate readers, other designs can be prototyped rapidly since the photolithographic procedure is exceptionally flexible. Figure 1 shows three paper plates that (a) mimic a conventional 96-well plate, (b) mimic a 384-well plate, and (c) show an example of a functional design that incorporates channels within the zones in the paper for application and

distribution of the sample to run replicates of assays (or to run many assays simultaneously), and to perform mixing of two reagents to develop an assay. For the 96-zone paper plate (Figure 1A), the zones, arranged in an array of 12 columns by 8 rows, have a diameter of 6.8 mm with a center-to-center distance of 9.0 mm. A circular wall of photoresist (1.2 mm thick) defines these zones. For the 384-zone paper plate (Figure 1B), the length of the sides of the square zone is 4 mm, and the zones are spaced by barriers that are 0.5 mm wide. Figure 1C shows the same array of a 96-zone paper plate with two examples of channels to add function to the paper plate that (i) distribute the sample (rows A−C and F−H) and (ii) combine samples and reagents to react in a different zone (rows D and E). All connecting channels in Figure 1C are 1.5 mm wide. Parts D and E of Figure 1 show details of the 96-and 384-zone plates, respectively, while holding excess volume of liquids. Figure 1F shows the time course of two liquids mixing in the channels and reacting in a third zone.

Choice of Patterning Process. We patterned paper using photolithography.[1,9,14] Photolithography on paper is flexible, convenient for small scale prototyping, and surprisingly inexpensive; using photoresists prepared for this application, we estimate the cost of materials to be ~$0.03 per 96-zone paper plate at the laboratory level. Ultimately, the method of patterning will not affect the function of paper-based plates, and we intend to use technologies developed for large-area printing (e.g., for print media and fabrics) for scale up.

We evaluated two types of photoresists for fabricating paper-based plates: SU-8 (a photoresist base on bisphenol A diglycidyl ether resin) and SC (cyclized poly(isoprene) derivative) photoresist. We developed the protocol for patterning by adjusting parameters to reduce the total time required to fabricate the device and parameters to reduce the total cost of the final paper plate.

SU-8 is a commonly used, epoxy-based, negative photoresist[22] that we showed previously to be useful for patterning paper.[1] The commercial, small-volume price of SU-8 is approximately $800/L. We use 2.5 mL of SU-8 photoresist to prepare each paper plate, which is equivalent to $2.00 of photoresist per plate. This cost is too high to be useful for our purposes. Although SU-8 is a relatively simple mixture, its cost as a commercial photoresist reflects the very high standards of purity and homogeneity required for applications in high-resolution photolithography. These standards are unnecessary for the large features of the paper-based plates. To reduce the cost of fabrication, we produce a photoresist with similar properties in-house.[14] This laboratory-formulated photoresist costs ~$80/L, and the cost of photoresist per plate decreases to ~$0.2.

We chose to work with SC photoresist because it is inexpensive (~$100/L) and convenient to use (no pre- and postbaking steps required, see Supporting Information). Diluting commercial SC resist with xylenes lowered the cost further. We chose to work with xylenes for two reasons: (i) it is the primary solvent in the commercial formulation and the solvent used for development of the image, and (ii) it is one of the least expensive industrial solvents. Xylenes have the disadvantage of absorbing UV light, and although we dry the substrate before exposure, it may contribute to the lack of polymerization through the thickness of the paper. Decalin could be a good substitute but is more expensive. A 10% w/w solution of SC photoresist in xylenes gave results similar to those obtained with the commercial formulation. The 10% SC photoresist is approximately 5-fold less expensive (~$20/L) than the undiluted resist, and the cost of this photoresist needed to fabricate a paper plate is ~$0.03 (corresponding to 1.5 mL of 10% w/w solution). The cost of photoresist could be reduced further by buying the components of the resist (isoprene or poly(isoprene) and xylenes) in bulk and preparing the photoresist in-house.

A precise comparison of cost between plastic and paper plates is not trivial and requires the inclusion of many issues such as (i) raw material costs,[23] polymer vs paper, (ii) process cost for production, injection or replica molding vs photopatterning (or for large volume, printing), (iii) capital expense and amortization for production, molder, photolithographic tools, and mono (or multicolor) press, and (iv) other chemicals and additives needed in each case. The price of a 96-well plate (which of course includes a variety of other costs, such as distribution, advertising, and profit) is that of a large-volume research supply and varies from $1 to $5.

Choice of Detection Method. We chose to work with two types of detection devices: microplate readers and scanners. Microplate readers, fully automated spectrophotometers and spectrofluorimeters, are commonly used for measuring the results of assays performed in plastic well plates and are ubiquitous in life science laboratories and clinics.[21] To make a strong case for paper-based well plates as a useful alternative to plastic well plates, we wanted to show that paper-based plates are compatible with microplate readers. According to a recent survey, the capability of multimode detection for microplates on plate readers is more important than the density of the microplate (96-, 384-, and higher number of wells).[21,24] Such a preference indicates that the paper plate must be compatible with multimode detection. The problem with microplate readers is that they are relatively expensive (from $20 000 to $60 000) and not necessarily available in rural clinics. As a less expensive alternative to microplate readers, we demonstrate that desktop scanners ($200) can be used as reflectance detectors to measure colored substances on paper-based well plates. The limitation of scanners is that they can only measure visible colors, and they are not useful for measuring fluorescence.

Choice of Samples and Assays. The objective of this initial work was to determine the sensitivity of paper-based well plates for measuring analytes using microplate readers and scanners. We tested the platform using common biological stains (e.g., Coomassie Brilliant Blue and Amaranth) for absorption spectrophotometry (microplate reader) and reflectance detection (scanner); we used fluorescein-labeled bovine serum albumin (BSA-FITC) for fluorescence measurements (microplate reader).

RESULTS AND DISCUSSION

Fabrication of Paper 96- or 384-Zone Plates. Paper-based plates were fabricated by photolithography[14] following the pro-

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(23) Typically, plastic plates are made of polypropylene (PP), polystyrene (PS), polycarbonate (PC), polyvinyl chloride (PVC), or polyethylene (PE). The average price per metric ton is ~$2000 for PE, $2200 for PP, and ~$600 for pulp and paper.

(24) Multimode plate readers corresponded to 62% of the equipment available in the U.S. Equipment that reads only in the absorption mode corresponded to 32%, and only 6% are exclusively used in luminescence modes.

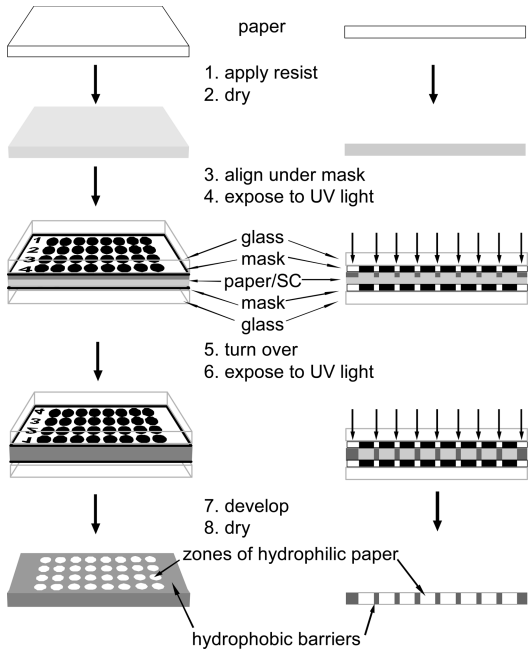

Figure 2. Procedure for patterning paper using SC photoresist. Note partial polymerization indicated as dark gray in steps 4−6.

cedure illustrated in Figure 2 and as detailed in the Supporting Information. Sheets of paper, cut to the size of the plate, were impregnated with photoresist (SU-8 or 10% SC), dried, and then exposed to UV light through a transparency mask. After exposure, the paper was baked (for SU-8 only) and then developed in an appropriate solvent. To dry the photoresist after applying it to the paper, SU-8 required a baking step on a hot plate set at 95 °C for 5 min. SC photoresist could be dried at 25 °C inside a fume hood for 1−2 min. Although heat could shorten this period of drying, a prebake step resulted in a certain level of cross-linking of the SC photoresist, even in the absence of light. This uncontrolled cross-linking of the SC photoresist decreased the hydrophilicity of the paper and, consequently, decreased the flow rate of aqueous fluids within the zones and their wettability.

SU-8 and SC required slightly different exposure steps. SU-8 photoresist is transparent and requires a single UV exposure (15 s under a 600 W metal halide UV lamp) to polymerize the photoresist through the thickness of the paper. SC photoresist is light yellow and has a maximum absorbance of UV light near 350 nm. During photopolymerization, SC photoresist becomes opaque and does not polymerize through the thickness of the paper (i.e., no reaction occurs on the side of paper opposite to the UV lamp). To overcome this problem, we exposed both the front and the backside of the paper (in two sequential exposures) to UV light for 30 s through the same transparency mask on both sides.

After exposure to UV light, SU-8 required a baking step while SC was ready for development. SU-8 was developed in acetone, followed by a rinse in 70% isopropyl alcohol. SC was developed in xylenes and rinsed in methanol. After development, the paper plates were dried under ambient conditions (on a paper towel inside a fume hood), or under vacuum; once dry, the paper plates were ready to use. The paper plates required no special environment for storage. Liquid samples were applied directly onto the hydrophilic zones. A short exposure to oxygen plasma (less than 10 s) enhanced the wettability of the paper patterned with SU-8 while the paper patterned with SC did not need any additional treatments. The volume of solution required to wet each zone depended on the size of the zone. Typically, as observed in parts A and B of Figure 1, 5 μL of solution was adequate to completely wet one zone on a 96-zone plate, and 2 μL of solution was sufficient to completely wet one zone of a 384-zone paper plate, regardless of the analyte. For the interconnected channels, the spreading of the sample was sensitive to the nature of the analyte and how it interacted with cellulose, but in general, 5 μL per zone was sufficient to fill all zones (for example, 45 μL of Amaranth completely filled nine zones in Figure 1C, zone G-8).

The experiments shown in parts A and B of Figure 1 also demonstrated that paper is a suitable platform for fast concentration of analytes by evaporation of the solvent due to its high surface area. For example, 5, 10, and 50 μL of water evaporated completely from the zones in less than 5 min, in about 25 min, and just over 90 min, respectively, while in plastic wells, just 5 μL required over 100 min to evaporate at room temperature (22 °C). The rate of evaporation in the paper zone is ~20 times faster than in the plastic well and allows concentration of analytes directly on the test zone. Evaporation of water from paper takes place from both sides of the zones, convection across the surface of the paper is more efficient in moving air and vapor, and the paper is a porous medium, which greatly enhances evaporation in the regime below saturation (<10 μL).

In order to minimize the cost of the photoresist, we diluted commercial SC photoresist, which we call "100% SC", with xylenes on a mass-to-mass basis to prepare 50, 25, 10, and 1% SC. We patterned a 96-well plate using 2.5 mL of each photoresist. With the exception of 1% SC, all the dilutions of SC photoresist yielded well-defined patterns. Figure 3A shows images of all the plates from 100 to 1% SC photoresist with aqueous dyes spotted in alternating zones. Despite the obvious differences in the yellow color of the plate, due to the total load of SC photoresist, there were no differences in the definition or quality of the zones, except for the 1% SC plate, which failed to contain the solution in the zones (Figure 3B). The load of SC resist on the cellulose fibers at 1% w/w was not enough to create a hydrophobic barrier to contain the liquid.

We also optimized the volume of 10% SC photoresist required to pattern a plate and found that only 1.5 mL of 10% SC was sufficient to pattern thinner papers. The diluted photoresist was conveniently applied to the paper on a Petri dish using a Pasteur pipet. Flipping the paper over several times in a fume hood evaporated the xylenes. This procedure also helped to homogeneously distribute the photoresist over the entire area of the paper and minimized losses of the photoresist to the container (a Petri dish or a piece of aluminum foil), which are extensive when using the commercial formulation (100% SC).

Alternative Papers as Substrates for Multizone Plates. Using 10% SC photoresist, we successfully patterned (i) household paper filters for coffee, (ii) laboratory wipes for cleaning (Kimwipes), and (iii) special laboratory wipes for cleaning (TechniCloth, a blend of cellulose and polyester fibers). In these experiments, we analyzed four features of the paper substrates: (i) the hydrophobicity of the barriers, i.e., the efficiency of retention of liquids inside the zones, (ii) the homogeneity of the spreading of the sample, i.e., the hydrophilicity of the cellulose fibers in the

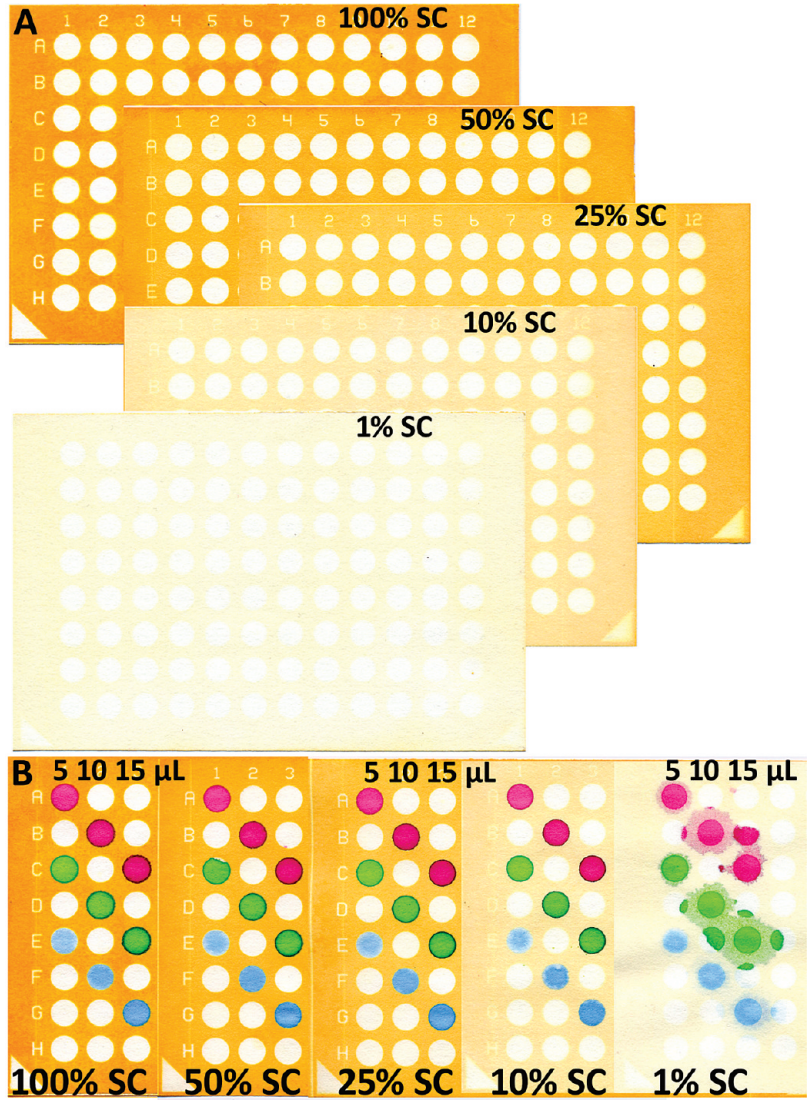

Figure 3. (A) Paper microzone plates (96-zone) made using different concentrations of SC photoresist. (B) Detail of each plate after application of 5, 10, and 15 μL of Amaranth, erioglaucine, and tartrazine mixture and Coomassie Blue Brilliant G250.

zones after contact with the resist, (iii) the capacity of the zones, i.e., the combination of the both features plus the thickness of the paper, and (iv) the effect of plasma oxidation. The results showed that the thickness of the paper defined the volume of sample (or capacity of the hydrophilic zone), while the plasma oxidation improved the hydrophilicity of the zones, yielding more homogeneous spreading of the dyes. (For particular cases, see relevant data and discussion for this section and Figure SI-1 in the Supporting Information). We believe that most types of paper will be suitable for patterning by photolithography. The limiting factor will likely be the thickness of the paper. For very thick papers, it may not be possible to photopolymerize SC photoresist through the entire thickness of the paper.

Reading 96-Zone Paper Plates in Multiple Modes. We demonstrated that a conventional plate reader could measure fluorescence and absorbance of analytes on paper plates. Comparing the figures-of-merit of paper plates and plastic plates in both reading modes provides a measure of the capability of the new platform, paper, relative to the validated platform, plastic.

Fluorescence Detection Using a Microplate Reader. The paper-based system showed greater sensitivity than plastic plates (about 40 times) for fluorescence detection as demonstrated by the slopes in Figure SI-2 in the Supporting Information. For example, 12.5 pmol of FITC-BSA produced a relative fluorescence of 2700 ± 850 and 70 ± 33 RFU on the paper and in solution, respectively (Figure 4). The mass limit of detection (LOD) (defined as the lowest detectable quantity of analytes in moles with a relative standard deviation (RSD) <15%) for the paper plate was 0.125 pmol of FITC-BSA, while the LOD for the plastic plate was 6.25 pmol of FITC-BSA. In both systems, the LOD was obtained for the same concentration of protein, i.e., 25 nM. The difference in the mass LOD was proportional to the volumes used in each case (5 μL adsorbed on the paper versus 250 μL in the plastic plate). The *concentration* sensitivity was, therefore, similar for the two methods, but the *mass* sensitivity was higher for the paper.

The average RSD for the replicates of all concentrations was 2% (ranging from 0.7% to 5.8%) for the plastic plate and 10% (ranging from 1.2% to 25%) for the paper plate. We hypothesized that the higher RSDs in the paper plate were due to the scattering of light on the cellulose fibers in paper caused by the difference in index of refraction between cellulose (n_D = 1.55) and air (n_D = 1.00). Adding mineral oil (n_D = 1.46), glycerol (n_D =

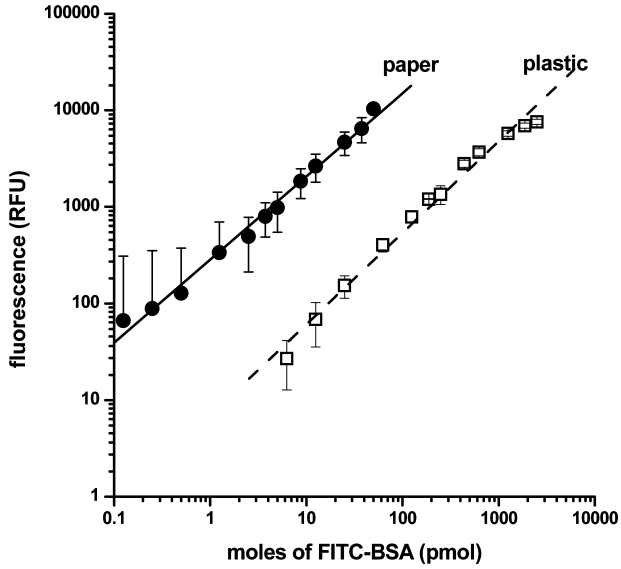

Figure 4. Comparison of fluorescence measurements for samples of FITC-BSA read in a microplate reader using either a paper 96-zone plate (●) or a plastic 96-well plate (□). The RSD for 0 nM FITC-BSA (blank) was 3% for the plastic plate, and 11% for the paper plate. We plotted all the data from the three data sets in a log−log graph and fit the data using least-squares linear regressions. For the paper plate (solid line), the equation was $\log(y) = 2.45 + 0.86 \log(x)$ with $r = 0.995$, while for the plastic plate (broken line) the equation was $\log(y) = 0.84 + 0.95 \log(x)$ with $r = 0.993$. The negative error bars for the lowest amounts of labeled proteins were suppressed due to incompatibility with the graphing program. The log−log scale was chosen for clarity. The linear scale graphs are shown in the Supporting Information, Figure SI-2.

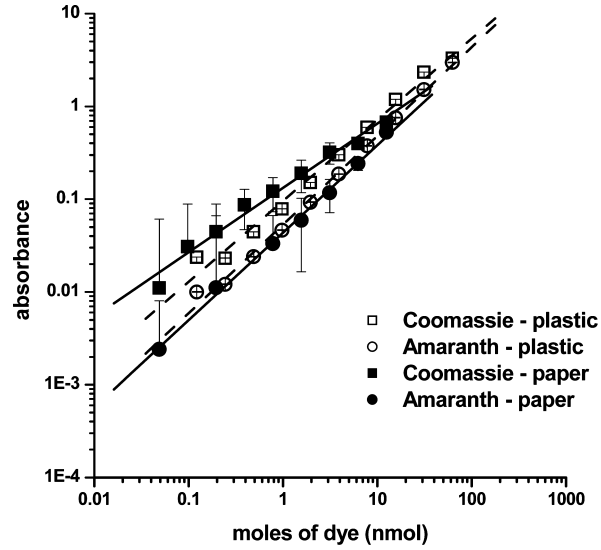

Figure 5. Analytical calibration plots for Coomassie Brilliant Blue and Amaranth red in paper plates, with a layer of mineral oil applied to the zones, and plastic plates. Apparent linear regression lines (solid lines) for both dyes in the paper plates yielded the following equations: (■) Coomassie blue, $\log(y) = -0.88 + 0.69 \log(x)$, $r = 0.989$, and (●) Amaranth red, $\log(y) = -1.36 + 0.94 \log(x)$, $r = 0.998$. Apparent linear regression line (broken lines) yielded the following equations for both dyes in plastic plates: (□) Coomassie blue, $\log(y) = -1.02 + 0.87 \log(x)$, $r = 0.994$, and (○) Amaranth red, $\log(y) = -1.27 + 0.96 \log(x)$, $r = 0.998$. The log−log scale was chosen for clarity. The linear scale graphs are shown in the Supporting Information, Figure SI-3.

1.47), or water ($n_D = 1.33$) to the paper to displace air from the pores in the paper did not improve the precision of the measurements (data not shown). We conclude that other unidentified factors, perhaps the heterogeneity of the paper itself, contribute to the increased RSD for paper compared to plastic. An additional factor is that scattering in paper takes place for both excitation and emission light. A photon may be scattered before reaching an analyte molecule on the optical path with the same likelihood that an emitted photon may not reach the detector. Such processes could explain the deviation from linearity at low levels of analytes.

We can see in Figure 4 (and in Figure SI-2 in the Supporting Information) that the RFU values, at high quantities of FITC-labeled BSA, start to deviate from linearity. The deviation could be due to inner-filter effects for the 96-well plates. We also cannot rule out saturation of the photomultiplier tube as an explanation for the deviation, since we did not adjust instrumental parameters on the plate reader. Nevertheless, the quantities detected span almost 4 orders of magnitude (a range we consider remarkable for a system of this simplicity). By contrast, the fluorescence values for paper plates appear to deviate from linearity at low quantities of analytes, and we believe that inner-filter effects can be ruled out in this case. The relative error due to the Poisson noise can be influenced by the number of fibers (and their distribution of sizes and densities) in the sampling zone and also by the number of molecules in the optical path. We have, in fact, used a range of diameters and densities of fibers but have not analyzed the effects due to these differences in this work.

Absorbance Detection Using a Microplate Reader. We assessed the spectrophotometric behavior of the paper 96-zone plates by measuring absorbance using two dyes, Coomassie Brilliant Blue R250 and Amaranth red, both in solution when in plastic plates and in solid phase when adsorbed on the fibers of cellulose in the paper plates. Figure 5 shows analytical calibration curves for both dyes. The average intraday standard deviation (σ) for replicates of all concentrations was 0.014 ($\sigma^2 = 0.0002$) for Coomassie and 0.006 ($\sigma^2 = 0.00003$) for Amaranth. The interday standard deviation for the three sets of replicates was 0.025 ($\sigma_{total}^2 = 0.0007$) for Coomassie, and 0.010 ($\sigma_{total}^2 = 0.0001$) for Amaranth red. The mass limits of detection are approximately 50 pmol for both dyes; this value is surprising, given the short path length of the system (~180 μm). Again, scattering of the light on the paper plays an important role on the values of LOD and RSD. Even though mineral oil helped to decrease the difference in the refractive index of the medium (n_D mineral oil = 1.46), it is still significantly different from that of cellulose ($n_D = 1.55$). We have already reported a more detailed study of the "best match" of the refractive index of cellulose.[25] Using poly(methylphenylsiloxane) (the best match), the transmittance improved approximately 10% over that with mineral oil. In any event, there is no "perfect" match, since cellulose is itself heterogeneous, and the index of refraction varies with local composition, structure, and crystallinity. Application of mineral oil or any other liquid that matches the refraction index of cellulose is essential to make absorbance measurements.

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Although it is one additional step in the analysis, it is a straightforward process at the laboratory level. For applications in the field, the presence of wind and dust/dirt in the air might require extra care in the handling of the paper plate.

Reflectance Detection or Analysis of Concentration Using Digital Images. Although scattering of the light on the surface of the paper limits the analytical performance of paper-based plates on the microplate reader, it enables the measurement of analytes by reflected (backscattered) light. Diffusive reflectance is the basis of analytical reflectometry, which has a well-described theory[26] and works with simple instrumentation.[27] The underlying principles of reflectance and image acquisition on a scanner are essentially the same. The reflectance on any surface at any given wavelength defines the image, and the colors, acquired on the scanner, and such images are analytically related to the chemical composition defining the colors in the image.[28,29] Analysis of the digital image through the analysis of the color, and color matching systems, are very well established in the paint, coatings, and printing industries. Color spaces CMYK, RGB, $L^*a^*b^*$, and HLS are the best-known systems for numeric representation of color. (CMYK stands for cyan, magenta, yellow, and black; RGB stands for red, green, and blue; L in L^*a^*b is luminosity, while a and b are coordinates for colors; HLS stands for hue, luminosity, saturation.) Detailed descriptions of such systems are available on the Internet.[30]

Analysis of color developed in paper-based microfluidic devices was the basis for quantitation of analytes by Martinez et al.[9] and by Abe et al.[16] In the work of Martinez, a glucose assay correlated better with gray scale counts in a digital format, while a protein assay correlated better when read in the cyan channel of the CMYK color code.[9] Abe et al. used each component of the $L^*a^*b^*$ color system to correlate with concentration of glucose, concentration of protein, and the pH, respectively.[16] Any of the color spaces is suitable for the colorimetric analysis on paper, and the choice will depend mostly on the particular assay. When an assay changes from colorless to color, gray scale is the most straightforward selection. When an assay changes from one color to another (for example from yellow to blue, as in the assay for protein using tetrabromophenol blue), a particular color channel, the cyan in this case, yields better quantitative correlations.[9]

Figure 6 shows that a scanner or digital camera can correlate well with a plate reader. We chose to convert the image of the paper plate to a gray scale image, because it best suited the simple experiment we designed to measure the quantities of dyes on the paper. In this analysis, the color changed from white to blue (for Coomassie) and from white to red (for Amaranth). The average intensity of the gray color in the zone correlated well with the total quantity of dye that was added to the zone.

The absorbance values of dyes on 96-zone paper plates measured on a microplate reader at the maximum wavelength for each dye correlated adequately with the amount of dye applied in each zone (Figure 6A). We then scanned the same plate using a desktop scanner, obtained the digital image of the plate, flattened the color image to gray scale, and read the average count within

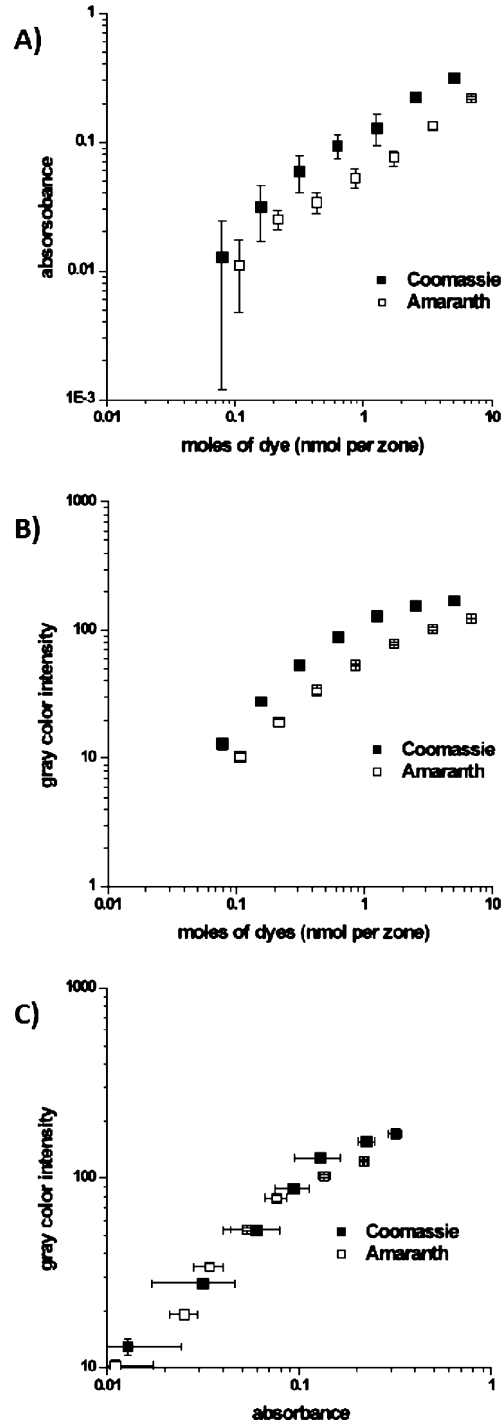

Figure 6. Quantitative relationship between quantities of dyes adsorbed on the zones of paper in the 96-zone paper plate and the signal of different read-out modes. (A) Absorbance measured in the microplate reader at the maximum wavelength for each dye after background subtraction. (B) Gray scale intensity measured from the digital image for each dye. (C) Correlation between absorbance and gray scale color intensity for each quantity of dye applied on the wells of paper. Note the error bars ($n = 6$) in the gray scale read-out are smaller than the symbol size. The log−log scale was chosen for clarity. Figure SI-4 in the Supporting Information shows the linear scale graphs.

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each zone (Figure 6B). Figure 6C shows the direct correlation of the absorbance values (measured with a plate reader) with the reflectance values (measured with a desktop scanner) for a serial dilution of analytes adsorbed on paper.

The conversions used in computer programs for handling images are acceptable for the human eye; they add, however, noise (and, possibly, spectral interferences) to scientific data. Use of flatbed scanners to read colorimetric assays is well established.[31−33] Teasdale et al.[31] correlated gray scale images (8-bit) with quantity of analytes with a nonlinear function ($y = a \ln(bx)$). The authors discussed the nonlinearity in terms of particularities of their chemical system and not in terms of color saturation, which could be the case as well. We observed the same nonlinearity at elevated quantities of analytes, but we did not manipulate the image to any extent other than converting it to gray scale. Having pure white and black regions on the image could increase the dynamic range and improve linearity.[33] Control of light conditions is less of an issue when using scanners, but it is critical when obtaining images using cellphones or digital cameras.[9]

CONCLUSIONS

We envision the immediate value of the paper-based 96- and 384-zone plates to be in applications where the cost and portability of the plates are major considerations. Paper-based plates may also be suited for familiar, high-technology applications that require high throughput and low cost. Because paper-based plates are lightweight, they could be easily taken into the field, and the results could be measured on-site using a camera phone and transmitted to a central laboratory;[9] alternatively, the plates could be transported back to a central laboratory for analysis using a microplate reader. In either case, the paper plate can be stored as a physical record of the results. If the paper plate is no longer needed, disposal of the plate by incineration is straightforward. Because the paper plates are thin and flexible, the results from large numbers of paper plates could be recorded and quantified automatically using a computer and a flatbed scanner equipped with a sheet feeder.

We anticipate that other common read-out modes from microplates readers, for example, time-resolved fluorescence, will be feasible and will expand the scope of this method. Although the sensitivity of microplate readers in reading transmittance through the paper is limited by light scattering caused by the differences in index of refraction between the fibers of the paper and air, this limitation can be reduced with mineral oil or other fluids having a refractive index matching that of paper (albeit with a decrease in convenience). For applications in the field, use of oils can be cumbersome, and reflectance read-out (without oil) may be preferable to absorbance read-out (with oil). Under the controllable conditions available in central laboratories, the use of automated plate readers yields analyte concentration directly and is preferable to scanners, even if it requires mineral oil.

There are many other properties of paper that transfer to paper-based plates and may allow applications that are not practical using conventional 96-well plates. For example, thanks to the high surface-to-volume ratio of paper, analytes in solution can be concentrated rapidly in the zones of a paper-based plate by evaporation. While conventional 96-well plates facilitate serial dilutions, paper-based plates facilitate serial concentrations by adding and then evaporating incremental volumes of a solution to the zones. Paper-based zones are also well suited for work with small volumes of sample: 5 μL of solution is sufficient to fill a zone in the 96-zone format and as little as 2 μL for the 384-zone array, while a plastic plate requires a minimum of 15 μL to fill the bottom of the well. Paper can be used for storing reagents and samples in a dry form, without refrigeration, for long periods, and this capability translates directly to paper-based plates. The paper matrix can also support gels and hydrogels, and paper plates can thus be developed for sample processing, analysis, and storage in gels.

Development of a variety of assays is now required in order to establish analytical protocols, to learn how suspended cells or other sample matrix constituents interfere with the signal, and to define the limits of such applications. One can imagine that adding untreated sample from one side of the paper, while reading fluorescence from the other side, might eliminate or reduce interference from solids by filtration. For absorbance detection, because the light must cross both sides of the paper, the procedure will rely on examining the specific systems on a case-by-case basis. Interferences will depend on the wavelength, the types of cells, buffers, etc. For example, red blood cells would be a serious problem in some assays due both to absorbance and scattering.

We foresee that paper-based plates can benefit resource-poor laboratories by making 96-zone analyses affordable and by enabling the use of microplate readers for simple diagnostics based on fluorescent or colorimetric assays. Laboratories in the developed world might reduce the costs of consumables, particularly that of plates in appropriate assays, while maintaining throughput and decreasing the quantities of waste. The characteristics of low volume and easy archiving may also be useful when it is required to archive samples or assays. The fabrication of such multizone paper plates on an industrial scale, besides reducing the cost/plate, can also incorporate a step in the fabrication that dispenses and incorporates the chemicals needed for bioanalytical assays.

ACKNOWLEDGMENT

This work was funded in part by the Bill & Melinda Gates Foundation under Award Number 51308 and in part by support from the Micro-Nano Fluidics Fundamentals Focus Center (MF3) at the University of California, Irvine. The authors acknowledge a visiting scholar fellowship from the Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Brazil (E.C.), a postdoctoral fellowship from the National Science and Engineering Research Council (NSERC) of Canada (S.J.V.), and a postdoctoral fellowship from the Damon Runyon Cancer Research Foundation (Grant DRG-1805-04) (S.T.P.).

SUPPORTING INFORMATION AVAILABLE

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

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Received for review April 20, 2009. Accepted June 18, 2009.

AC900847G