DOI: 10.1002/ange.201001005

Clinical Analytics

Paper-Based ELISA**

*Chao-Min Cheng, Andres W. Martinez, Jinlong Gong, Charles R. Mace, Scott T. Phillips, Emanuel Carrilho, Katherine A. Mirica, and George M. Whitesides**

This paper describes enzyme-linked immunosorbent assays (ELISA) performed in a 96-microzone plate fabricated in paper (paper-based ELISA, or P-ELISA). ELISA is widely used in biochemical analyses; these assays are typically carried out in microtiter plates or small vials.[1,2] ELISA combines the specificity of antibodies with high-turnover catalysis by enzymes to provide specificity and sensitivity.[1,2] We have recently described a 96-microzone paper plate—fabricated by patterning hydrophobic polymer in hydrophilic paper—as a platform for biochemical analysis.[3,4] Although microfluidic paper-based analytical devices (μPADs) were designed primarily to provide analytical capability at low cost in developing countries,[5–7] we expect that they will also be useful in applications such as point-of-care clinical analysis, military and humanitarian aid field operations, and others where high throughput, low volumes of sample, low cost, and robustness are important.[6,7] These devices have so far been prototyped using analyses of simple analytes: glucose, total protein, and certain enzymes. P-ELISA combines the sensitivity and specificity of ELISA with the convenience, low cost and ease-of-use of paper-based platforms; P-ELISA (at it current state of development) is faster and less expensive than conventional ELISA, but somewhat less sensitive.

Porous membranes, including nitrocellulose and filter paper, have been used for decades in dot-immunobinding assays (DIA).[8–13] Though DIAs are the simplest form of immunoassays on paper, they typically require one piece of nitrocellulose for each assay; the pieces of nitrocellulose have to be processed individually in Petri dishes, and the assays take several hours to complete.[9] Quantitative DIAs have been reported,[14] but DIAs are typically qualitative, and provide only "yes/no" results.[15] Conventional ELISA, usually performed in 96-well plates (fabricated by injection molding in plastic), is quantitative and well-suited for high-throughput assays, but each assay requires large volumes (ca. 20–200 μL) of analyte and reagents, the time required for incubation and blocking steps are long ($\geq$1 h per step, because the reagents must diffuse to the surface of the wells), and the results are usually quantified using a plate reader, typically a $20 000 instrument.[9,16]

Paper microzone plates for ELISA can have the same layout as plastic 96-well plates, but each test zone requires only about 3 μL of sample, and the results can be measured using a desktop scanner, typically a $100 instrument. In addition, an entire P-ELISA can be completed in less than one hour. The ease of fabrication of paper microzone plates also opens opportunities for a wide range of non-standard formats, and customized connections to carry reagents between zones.[4] To evaluate the feasibility of P-ELISA, and the potential advantages and disadvantages of P-ELISA and 96-well-plate-based ELISA, we adapted a standard procedure to our format and then demonstrated an indirect P-ELISA using rabbit IgG as a model analyte. We also established that P-ELISA can be used to detect and quantify antibodies to the HIV-1 envelope antigen gp41 in human serum using an anti-human IgG antibody conjugated to alkaline phosphatase (ALP) to produce a colorimetric readout.

We used a 96-microzone paper plate with an array (12×8) of circular test zones for running multiple P-ELISAs in parallel (Figure 1 A);[17] the Supporting Information describes the details. The array was designed to have the same layout and dimensions as a standard plastic 96-well plate, so that it would be compatible with existing microanalytical infrastructure (eight- or twelve-channel pipettes and plate readers). Each test zone was 5 mm in diameter and required 3 μL of solution to fill (e.g., to wet completely with fluid); this design was a good compromise between convenience and conservation of reagents, as it reduced the amount of reagents and sample required for the assay but ensured accurate distribution of fluids when using a manual pipette. We also examined smaller test zones, with the smallest test zone requiring 0.5 μL of solution to fill (e.g., to wet completely). This size is similar to that required in a 384-well plate format.

The top and bottom faces of the test zones in paper-microzone plates are open to atmosphere.[4] The advantage of this configuration is that the zones can be washed by adding a washing buffer to the top of the zone while pressing the bottom of the zone against a piece of blotting paper. The washing buffer goes through the test zone vertically and into

[*] Dr. C.-M. Cheng,[+] Dr. A. W. Martinez,[+] Dr. J. Gong, Dr. C. R. Mace, Prof. S. T. Phillips, Prof. E. Carrilho, K. A. Mirica, Prof. G. M. Whitesides
Department of Chemistry and Chemical Biology
Harvard University
Cambridge, MA 02138 (USA)
E-mail: gwhitesides@gmwgroup.harvard.edu
Homepage: http://gmwgroup.harvard.edu
Prof. E. Carrilho
Instituto de Química de São Carlos, Universidade de São Paulo
13566-590 São Carlos—SP (Brazil)
[+] These authors contributed equally to this work.

[**] This work was funded by the Bill & Melinda Gates Foundation under award number 51308, by the Micro-Nano Fluidics Fundamentals Focus Center (MF3) at the University of California, Irvine, and by a visiting scholar fellowship from the Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Brazil (E.C.).

Supporting information for this article, including details of experimental methods, is available on the WWW under http://dx.doi.org/10.1002/anie.201001005.

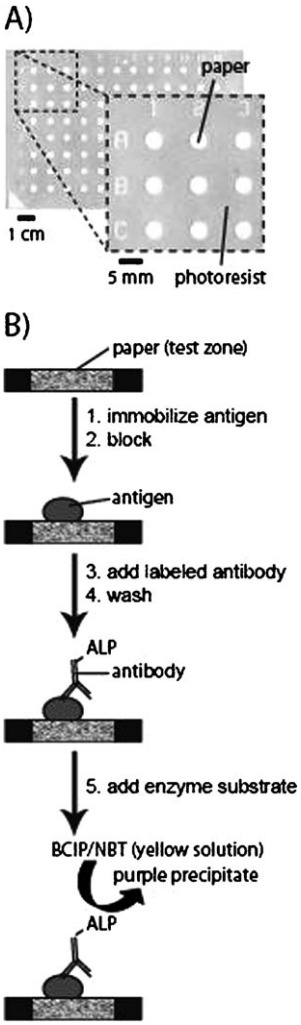

Figure 1. Prototype demonstration of an indirect P-ELISA. A) A paper 96-microzone plate for P-ELISA designed with the equivalent dimensions of a 96-well microtiter plate. Each circular test zone of hydrophilic paper is bounded by hydrophobic photoresist. B) Schematic diagram of a general paper-based indirect ELISA utilizing antigen/antibody recognition (ALP: alkaline phosphatase; BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium).

the blotting paper, and carries unbound reagents with it. The disadvantage of this configuration of the paper-microzone plate is that when reagent solutions are added to the test zones, the paper plate must be suspended in air to prevent the reagent solutions from wicking through the test zones. Empty pipette-tip boxes provided a simple platform for supporting 96-microzone paper plates such that the test zones are completely suspended and isolated.

We chose a colorimetric readout so that the results of the P-ELISA could be interpreted without the need for a specialized detector and was compatible with visualization by a cell phone.[6] Colorimetric assays are well-suited for use in settings lacking plate readers or fluorescence scanners. Many enzyme/substrate pairs have been used in conventional ELISA to produce colored products;[18,19] we chose alkaline phosphatase as the enzyme and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium)[19] as the substrate because they produce a color change from light yellow to dark purple, and thus have excellent contrast with the white background provided by paper. This system has been extensively characterized; a range of ALP-conjugated antibodies are commercially available.[20] In its simplest form, indirect P-ELISA comprises five steps: 1) immobilization of the antigen on paper, 2) blocking the paper to prevent nonspecific adsorption of proteins, 3) labeling the immobilized antigens with an enzyme-conjugated detection antibody, 4) washing away unbound antibody, and 5) adding a solution of a substrate for the enzyme (Figure 1B). The Supporting Information describes details.

We carried out P-ELISA for the detection of rabbit IgG in 10-fold dilutions (67 mM to 670 nM per zone, corresponding to 67 pmol to 670 amol of IgG per zone spotted on the paper) (Figure 2). The intensity of the purple color that developed in

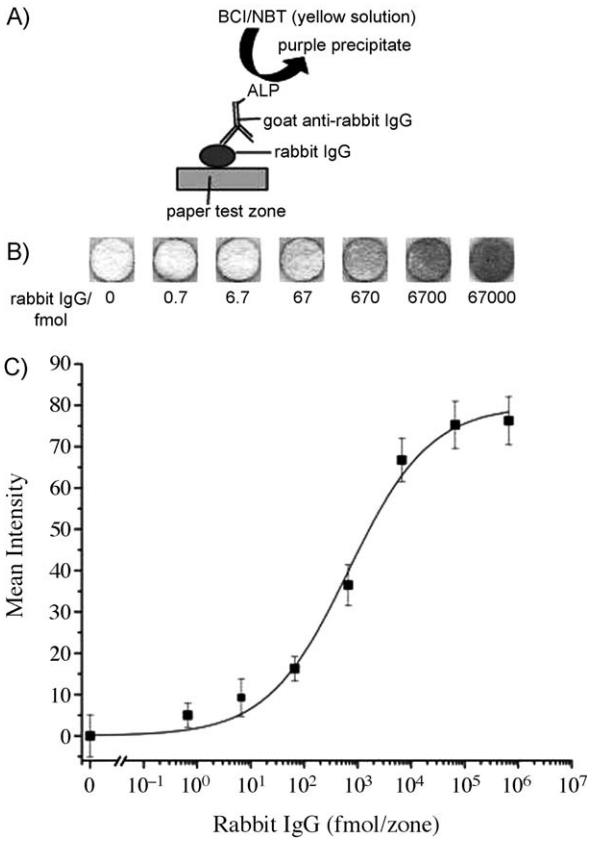

Figure 2. Paper-based ELISA for rabbit IgG. A) Schematic of the paper-based indirect ELISA for rabbit IgG. B) Images of the indirect ELISA results from 670 amol to 67 pmol of rabbit IgG per zone. C) Calibration plot for the mean intensity I of the color produced by the enzymatic reaction of alkaline phosphatase (ALP) in the indirect ELISA assay versus the amount of rabbit IgG adsorbed to each microzone. Each datum is the mean of eight replicates ($N=8$), and the error bars represent the standard deviations of the measurements. We used Image J[21] to analyze the intensity of the color of each test zone after scanning the paper 96-microzone plate. The R^2 value of the curve fit to the data using the Hill Equation is 0.992. In addition, we can approximate the curve as linear between the concentrations of 10^1–10^4 fmol/zone. The fitting parameters calculate to $I_{max}=79.9\pm3.5$ a.u., $[L_{50}]=671\pm208$ fmol/zone, $n=0.57\pm0.08$ [see Eqs. (1)–(3)].

each test zone was proportional to the amount of rabbit IgG adsorbed on the paper. We analyzed the data from the dilution series by nonlinear regression using the Hill Equation [Eq. (1)] to generate a sigmoidal curve fit. In this equation, θ is the fraction of occupied binding sites, $[L]$ is the ligand concentration (in mol), $[L_{50}]$ is the ligand concentration corresponding to half of the binding sites occupied (in mol),and n is the Hill coefficient.

$$\theta = \frac{[L]^n}{[L]^n + [L_{50}]^n} \quad (1)$$

The intensity, I, of the observed signal, developed colorimetrically by the ALP conjugate, is proportional to the number of detected antigens [Eq. (2)], such that the fraction of occupied binding sites can be represented by the ratio of the observed intensity to the maximum intensity [Eq. (3)].

$$\theta = \frac{I}{I_{max}} \quad (2)$$

$$I = \frac{I_{max}[L]^n}{[L]^n + [L_{50}]^n} \quad (3)$$

We determined the limit of detection of rabbit IgG using an indirect P-ELISA to be 54 fmol/zone, as defined by the concentration that results in a signal that is three times the standard deviation of the control. This sensitivity is approximately ten times lower than that obtained by ELISA experiments in 96-well plates for the same antigen/antibody pair (4 fmol/zone, as detected by absorbance).[16] The difference in the detection limit for P-ELISA may be a consequence of the abbreviated antibody–antigen incubation period (lower loading) or non-specific interactions between antibodies and the cellulose fibers (high deviations in the background signal).

Paper-based ELISA has three advantages over conventional ELISA for routine use (Table 1): 1) An entire paper-based ELISA (from antigen immobilization to final quantitative result) can be completed in less than one hour; 96-well-plate ELISA requires at least one hour of incubation for immobilizing the antigen on the surface of the plate, 2) paper-based ELISA requires less than 20 μL of reagents to perform the full assay, and 3) the results of paper-based ELISA can be quantified with a desktop scanner rather than the more expensive microtiter plate reader.

The small volumes reduced in P-ELISA can be attributed to the small volume of the test zone. A paper test zone has a volume of 3.5 μL (circular test zone with a diameter of 5 mm and a height of 0.18 mm; with approximately 30% of this volume occupied by the paper fibers). The short incubation times for P-ELISA can be attributed to the high surface-to-volume ratio of the paper fibers in the test zone; the antigens, antibodies, and reagents have only short distances over which they must diffuse to reach the surface of a paper fiber.

Table 1: Comparison of paper-based ELISA and conventional ELISA.[16]

	Paper-based ELISA		Conventional ELISA	
antigen/antibody	rabbit IgG/goat IgG		rabbit IgG/goat IgG	
enzyme/substrate	ALP/BCIP/NBT		ALP/nitrophenyl phosphate	
detection device	desktop scanner ($100)		plate reader ($20000)	
sensitivity	54 fmol/zone		4 fmol/zone	
Time and reagents	Volume [μL]	Time [min]	Volume [μL]	Time [min]
1) antigen immobilization	3	10	70	120
2) blocking	3	10	100	30
3) antibody complexing	3	1	30	60
4) signal amplification	3	30	100	3
total per zone	12	51	300	213

Furthermore, as the solutions dry in the test zones, the concentration of each reagent increases; this concentration further enhances the kinetics of antibody–antigen binding. One disadvantage of working with test zones that are open to the atmosphere is that the rate of evaporation of water from the test zones is dependent on the ambient relative humidity and temperature, and these environmental conditions may affect the results of the P-ELISA.

We also demonstrated that P-ELISA could be used to detect specific antibodies in human serum using the HIV-1 envelope antigen gp41 as a model antigen. Figure 3 A outlines

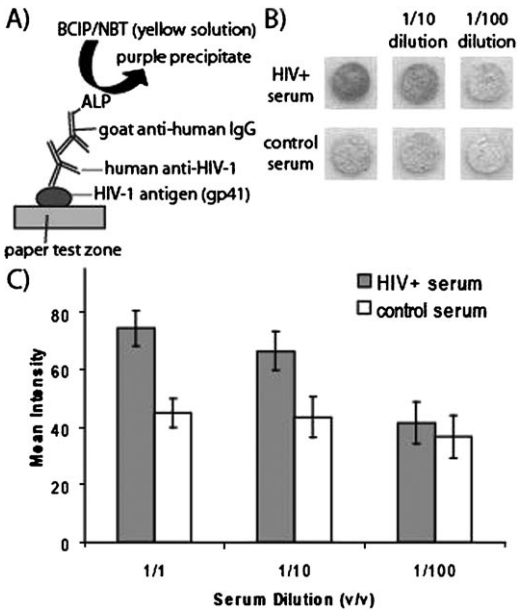

Figure 3. Paper-based ELISA for the detection of antibodies to the HIV-1 envelope antigen gp41. A) Schematic diagram of the antibodies and antigens in the assay. HIV-1 envelope antigens were applied to the paper microzones from purified solutions, while antibodies to gp41 came from the serum samples. B) Results of assays for serum from HIV-1 positive patients and control patients. The positive result could be distinguished even for a tenfold dilution of human serum. C) Graph of the mean intensity of the color developed in the test zones for the assays. The height of the bars represent the average of eight independent measurements ($N = 8$), the error bars represent one standard deviation from the average.

the detection process; this procedure is the same as that we developed to detect rabbit IgG. We examined a number of dilutions of serum samples from HIV-1 positive patients, as well as control samples (human serum without anti-gp41). These results indicated that colorimetric signal intensities of both samples decrease upon two sets of ten-fold dilutions of serum (Figure 3B,C). The positive result could be distinguished even for a ten-fold dilution of the human serum. This result demonstrates that it is practical to use quantitative paper-based ELISA to measure antibodies to HIV-1 envelope antigen gp41 in a complex mixture such as human serum.

We believe that the combination of ELISA and patterned paper will provide a useful new protocol for performing immunoassays. P-ELISA offers three principal advantages over conventional ELISA in plastic 96-well plates: 1) it is more rapid, 2) it requires only small volumes (1–10 μL) of samples and reagents, and 3) it utilizes simple equipment: a pipette, a refrigerator for storing the reagents, and a scanner. P-ELISA thus extends the range of application of ELISA, especially to small laboratories, and to developing countries.

The most obvious current disadvantage of P-ELISA is that it is less sensitive than conventional ELISA by approximately an order of magnitude. It may be possible to decrease the limit of detection for the P-ELISA by optimizing the assay to suppress the background signal.

Received: February 17, 2010
Revised: April 9, 2010
Published online: May 28, 2010

Keywords: analytical methods · antigens · bioassays · clinical chemistry · ELISA

[1] R. Edwards, *Immunodiagnostics*, Oxford University Press, Oxford, **1999**.
[2] E. P. Diamandis, *Immunoassay*, Academic Press, St. Louis, **1996**.
[3] A. W. Martinez, S. T. Phillips, M. J. Butte, G. M. Whitesides, *Angew. Chem.* **2007**, *119*, 1340 – 1342; *Angew. Chem. Int. Ed.* **2007**, *46*, 1318 – 1320.
[4] E. Carrilho, S. T. Phillips, S. J. Vella, A. W. Martinez, G. M. Whitesides, *Anal. Chem.* **2009**, *81*, 5990 – 5998.
[5] W. Zhao, A. van der Berg, *Lab Chip* **2008**, *8*, 1988 – 1991.
[6] A. W. Martinez, S. T. Phillips, E. Carrilho, S. W. Thomas, H. Sindi, G. M. Whitesides, *Anal. Chem.* **2008**, *80*, 3699 – 3707.
[7] A. W. Martinez, S. T. Phillips, G. M. Whitesides, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 19606 – 19611.
[8] R. Hawkes, *Methods Enzymol.* **1986**, *121*, 484 – 491.
[9] S. Sumi, A. Mathai, V. V. Radhakrishnan, *Methods Mol. Biol.* **2009**, *536*, 89 – 93.
[10] J. Fletcher, *Appl. Environ. Microbiol.* **1987**, *53*, 183 – 184.
[11] C. F. Beyer, *J. Immunol. Methods* **1984**, *67*, 79 – 87.
[12] R. L. Heberling, S. S. Kalter, *Dev. Biol. Stand.* **1986**, *64*, 199 – 203.
[13] R. Hawkes, E. Niday, J. Gordon, *Anal. Biochem.* **1982**, *119*, 142 – 147.
[14] R. Jahn, W. Schiebler, P. Greengard, *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 1684 – 1687.
[15] S. S. Kalter, R. L. Heberling, J. D. Barry, I. K. Kuramoto, P. V. Holland, K. Sazama, *J. Clin. Microbiol.* **1992**, *30*, 993 – 995.
[16] S. K. Sia, V. Linder, B. A. Parviz, A. Siegel, G. M. Whitesides, *Angew. Chem.* **2004**, *116*, 504 – 508; *Angew. Chem. Int. Ed.* **2004**, *43*, 498 – 502.
[17] A. W. Martinez, S. T. Phillips, B. J. Wiley, M. Gupta, G. M. Whitesides, *Lab Chip* **2008**, *8*, 2146 – 2150.
[18] E. D. Hilborn, W. W. Carmichael, M. Yuan, S. Azevedo, *Toxicon* **2005**, *46*, 218 – 221.
[19] N. van Poperin, D. E. Lopatin, *J. Clin. Microbiol.* **1991**, *29*, 2554 – 2558.
[20] J. J. Leary, D. J. Brigati, D. C. Ward, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 4045 – 4049.
[21] Public software from National Institutes of Health; http://rsbweb.nih.gov/ij/.

